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Biological Responses of Osteocytic Connexin 43 Hemichannels to Simulated Microgravity

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Abstract

Connexin 43 (Cx43) hemichannels and gap junctions in osteocytes are responsive to mechanical loading, which is important for bone formation and remodeling. However, the mechanism of these Cx43-forming channels in the process of mechanical unloading is still not very clear. In this study, unloading caused by weightlessness was simulated by using a random position machine (RPM). Osteocytic MLO-Y4 cells were subjected to 2 h of RPM treatment, and levels of Cx43 mRNA and total and cell surface expressed protein were determined by quantitative real-time PCR, western blotting, and biotinylation analysis. Although mRNA was elevated by RPM, total protein level of Cx43 was not altered; however, surface biotinylated Cx43 was significantly reduced. Interestingly, RPM promoted the retention of Cx43 in the Golgi apparatus detected by co-immunofluorescence with antibodies against Cx43 and 58 K Golgi marker protein. Dye uptake assay showed that hemichannels were induced open after RPM for 2 h. Consistently, prostaglandin E_2 release was increased and this increase was completely attenuated with the treatment of a Cx43 hemichannel blocking antibody. Together, this study demonstrates increased activity of Cx43 hemichannels to RPM, and active Cx43 hemichannels with prostaglandin E_2 release are likely to module biological function under simulated weightless conditions.

Keywords

connexin43; hemichannel; osteocyte; random position machine

Bone tissue modeling and remodeling are greatly impacted by mechanical loading. Bone cells, especially osteocytes are highly responsive to mechanical stimulation. Osteocytes, the most abundant cell type embedded in bone mineral matrix, are well known as a major

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AUTHORS' CONTRIBUTIONS

Research design: HX, JXJ, and PS; Study conduct: RL, DN, JZ, RY, and JL; Data acquisition and analysis: HX, JXJ, RL, DN, JZ, and MAR; Drafting and revising manuscript: HX, JXJ, and PS. All authors have read and approved the final submitted manuscript.

coordinator to orchestrate the responses of bone cells to mechanical loading. In osteocytes, one of the important mechanical sensing molecules is connexin 43 (Cx43), which forms gap junctions and hemichannels[1,2] and is involved in the transduction of mechanical signals between adjacent cells or between the cell and extracellular environment, respectively.[3,4] Fluid flow shear stress promotes the opening of mechanosensitive hemichannels in osteocytic MLO-Y4 cells,[5] leading to the release of regulatory factors, such as prostaglandin E_2 (PGE_2)[6] and ATP.[7] These factors act in an autocrine/paracrine fashion to regulate the functions of other bone cells, including osteoblasts and osteoclasts.[8,9,10]

Bone loss is a serious physiological problem for astronauts. Weightlessness causes 1–2% decrease of bone mass during space flight. Fourteen-day space flight leads to increased numbers of empty osteocytic lacunae and destruction of osteocytes in bone tissue.[11] Also 12.5-day space mission reduces the osteocytes in rat cortical bone periosteum.[12] The involvement of Cx43 in response to the unloading regimes in vivo has been reported using Cx43 knockout mouse models. Lloyd et al. investigated the response of osteoblast/osteocyte-specific deletion of Cx43 mice to three weeks of mechanical unloading using hindlimb suspension (HLS), a model used to simulate the effects of weightlessness on the ground.[13] They found that Cx43 deficiency desensitized bone to the effects of mechanical unloading.[14] Another study by Grimston et al. showed similar observation in osteoblast/osteocyte-specific Cx43 knockout mice using a muscle paralysis unloading model with the treatment of botulinum toxin A (BtxA).[15] However, the cellular mechanism with regards of the role of Cx43 in weightless conditions is largely unknown. We showed earlier that gravity changes decreased the expression of Cx43 in MLO-Y4 cells after 24 h of parabolic flight.[16] In this study, we investigated the effects of mechanical unloading induced by random position machine (RPM) on the expression and cellular distribution of Cx43, the activity of hemichannels and secretion of PGE_2 in MLO-Y4 cells. RPM was used to simulate the effects of weightlessness, in which the direction of gravity vector varied continuously so that cells could not respond to gravity. RPM has been reported to generate similar biological responses as weightlessness conditions.[17,18]

MATERIALS AND METHODS

Cell Culture

The MLO-Y4 cell line was generously provided by Dr. Lynda Bonewald (University of Missouri-Kansas City).[1] The cells were cultured in a-MEM (Life Technologies, CA) with 2.5% fetal bovine serum (FBS) (Life Technologies) and 2.5% calf serum (CS) (Life Technologies) including 1% penicillin/streptomycin.

Random Position Machine (RPM)

RPM was manufactured by the Center for Space Science and Applied Research of the Chinese Academy of Sciences.[19] This device is composed of an inner and an outer frame, and the rotation speed and direction of each frame are automatically regulated by a program (Fig. 1). Cells were cultured on glass slides ($2.0\times6.0\ cm^2$) at the density of $1.0\times10^4/cm^2$ for 24 h. The slides were then placed in the flasks specially designed for RPM. The dimension of the flask is 4 cm (L)×2.5 cm (W)×1.8 cm (H), with a total volume of 20 ml. The flasks

were filled completely with medium and tightly capped to avoid bubbles and subsequent fluid shear stress (without air or CO_2 permeation), and then randomly divided into control and RPM-treated groups. For RPM-treated group, the flasks were placed in inner frame of the RPM. The random mode of speed (0.1–10 rpm) and direction (three dimensions) were chosen for the inner and outer frames.[17,20] The time averaged gravitational vector acting on these samples was reduced to 2×10^{-3} g at the center of the frame.[21] The static control cells were cultured in the same incubator with RPM-treated groups at 37°C. After 2 h, the cells or the conditioned medium were collected for the next experiments.

Haematoxylin and Eosin (H&E) Staining

Cells on slides were washed twice with phosphate-buffered saline (PBS), pH 7.4 and fixed in 0.5% glutaraldehyde solution in PBS for 10 min. After incubated in 0.5% hematoxylin for 10 min and 0.5% eosin for 7 min, respectively, the cells were dehydrated by a series of ethanol solutions with increasing concentrations, and then made transparent with dimethylbenzene. The slides were sealed and observed under phase microscope.

PGE_2 Measurement

MLO-Y4 cells were preincubated with Cx43(E2) antibodies (1:50 dilutions) specific to block the hemichannels[22] for 30 min and were then subjected to RPM for 2 h. The flasks were removed from RPM and the conditioned medium was collected. The extracellular PGE_2 released into the medium was measured using PGE_2 EIA kit (Cayman, Ann Arbor, MI) according to the manufacturer's instruction.

Quantitative Real-Time PCR Analysis

Total RNA was extracted using Trizol (Invitrogen, CA). Reverse transcription experiment was performed based on the protocol provided with the kit (TaKaRa, Dalian, China). Primers were designed and synthesized (Sangon, Shanghai, China) based on the sequence information available in GenBank. These primer sequences are shown in Table 1. SYBR Green real-time relative quantitative RT-PCR analysis was performed with SYBR Premix Ex TaqTM based on the manufacturer's protocol (TaKaRa). Each sample was measured in triplicate and normalized to 18S rRNA. The Livak method was used to estimate the relative change in gene expression.[23]

Immunofluorescence Labeling

The cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT), rinsed three times with PBS for 5 min each time and permeabilized with 0.25% TX-100 (in PBS) for 30 min. After rinsed twice with PBS, cells were blocked with 3% BSA in PBS for 30 min at RT. The cells were then incubated overnight at 4°C with affinity-purified antibodies against Cx43 (E2, 1:300 dilution) and Golgi 58 K protein (1:50) (Abcam, Cambridge, MA), followed by rinsing four times with PBS and incubating 1.5 h with fluorescein isothiocyanate (FITC) or rhodamine labeled secondary antibody (1:50) (EarthOx, CA). Hoechst dye (5 μg/ml) (Beyotime, Nantong, China) was used for nucleus staining. The slides were sealed and observed under confocal laser fluorescence microscope (Leica TCS SP5, Germany). The extent of colocalization of Cx43 and Golgi bodies was

quantified using the Pearson's correlation coefficient (Rr) and Overlap coefficient (R) with NIH Image J software.

Dye Uptake Assay

Dye uptake assay was performed according to previous protocol.[24] MLO-Y4 cells were cultured at density of $7.0\times10^3/cm^2$ for 24 h (most of cells were not in contact with each other), then subjected to 2 h of RPM or Cx43(E2) antibodies (1:300 dilution) treatment, and followed by washing three times, 5 min each with recording solution (154 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). The cells were exposed to ethidium bromide (Etd^+) (TaKaRa) for 5 min and followed by rinsing three times with PBS. Cells were fixed with 2% paraformaldehyde in PBS for 30 min and observed under fluorescence microscope. Image J software was used to analyze dye uptake. In each visual field, integrated optical density was calculated, and the extent of dye uptake was determined as a ratio of integrated optical density (IOD) to total cell number.

Biotinylation Analysis

Biotinylation analysis was performed according to previous protocol.[6] MLO-Y4 cells were labeled with or without 1 mg/ml EZ-link Sulfo-NHS-LC Biotin (Thermo Scientific, NH) in PBS at 4°C for 20 min and the biotinylation analysis was performed according to the manufacturer's instructions (Pierce Chemical, IL). The cell lysates were immunoblotted with Cx43(E2) (1:300 dilution) or GAPDH antibody. The cell surface expression of Cx43 was determined as a ratio of biotinylated Cx43 to total Cx43 protein.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 statistics software (San Diego, CA). All data were obtained from at least three replicates and presented as mean value standard deviation (SD). One-way analysis of variance (ANOVA) and Student Newman–Keuls test were used to compare groups. Asterisks indicate the degree of significant differences compared with the controls (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

RESULTS

RPM Decreased the Surface Expression of Cx43, But Had No Effect on Total Cx43 Protein

Osteocytic MLO-Y4 cells were subjected to RPM treatment for 2 h and no detectable change of cellular morphology was observed (Fig. 2). Quantitative real-time PCR and western blotting were used to determine levels of Cx43 mRNA and protein. Cx43 mRNA expression was significantly increased (Fig. 3A), while total protein level of Cx43 was not affected by RPM treatment (Fig. 3B). To assess the level of Cx43 on the cell surface, biotinylation assay was performed, and biotinylated and total control samples were analyzed by western blotting. Compared to total Cx43, the biotinylated Cx43 protein was significantly reduced by RPM (Fig. 3C and D). Therefore, RPM reduced the cell surface expression of Cx43, but not the total Cx43.

RPM Reduced the Delivering of Cx43 From the Golgi to Plasma Membrane

Cx43 hemichannels (connexons) are reported to be assembled in trans-Golgi network before migrating to the plasma membrane.[25] Confocal microscopy detection showed that after the treatment with RPM for 2 h, more Cx43 protein was accumulated in the Golgi bodies and less on the cell surface compared to control, as shown by co-localization of Cx43 and Golgi 58 K marker protein (Fig. 4A). Quantification analysis using Pearson's correlation co-efficient (Rr-value) (Fig. 4B, left panel) or overlap coefficient (R-value) (Fig. 4B, right panel) further confirmed the significant increase of Cx43 protein accumulated in the Golgi.

RPM Promoted the Opening of Hemichannels and PGE_2 Release

The activities of Cx43 hemichannels in response to RPM were studied using Etd^+ dye uptake assay. The quantification analysis using integrated optical density showed that opening of hemichannels detected by the uptake of Etd^+ dye was significantly enhanced by RPM as compared to non-treated control in MLO-Y4 cells (Fig. 5). The uptake assay showed that Cx43 (E2) antibody significantly blocked the opening of hemichannels (Fig. 6A and B). RPM also significantly increased the release of PGE_2 and this release was abrogated by Cx43(E2) antibodies (Fig. 6C). However, the mRNA expression of cyclooxygenase 2 (Cox-2), an inducible enzyme required for PGE_2 synthesis, was not influenced by RPM treatment (Fig. 6D).

DISCUSSION

Mechanical loading plays a critical role in skeletal tissues to maintain normal bone structure and function. In last decade or so, osteocytes are being recognized as a major coordinator in modulating biological responses of other bone cells to mechanical loading.[26,27] Osteocytes sense and translate the mechanical stimulation to chemical signals, and transmit to other bone cells through gap junctions between adjacent cells, or hemichannels between cells and extracellular environment.[28,29] In osteocytes, Cx43 is the most abundant connexins responsible for the formation of gap junctions and hemichannels, therefore plays a key role in mediating mechanotransduction. Mechanical stimuli are known to affect the expression and cellular distribution of Cx43 in osteocytes. Cheng et al. reported that 2 h of FFSS caused a redistribution of Cx43 from a location around the nucleus to punctate spots in the cytoplasm and in the dendritic processes in MLO-Y4 cells.[30] Fluid flow shear stress (FFSS) enhances the cell surface expression of Cx43,[6,22] and this enhancement sustains up to 24-h after FFSS.[31] In contrast to all other studies, Thi et al. have shown that in MLO-Y4 cells membrane-bound Cx43 downregulates at both 5 and 20 dyn/cm^2 of flow shear stress, whereas cytosolic Cx43 noticeably increases in response to both levels of shear stress.[32] Several papers have reported the graviresponses of Cx43 expression in osteocytes. The study from our laboratory shows a reduction of Cx43 expression after hypo- and hyper-gravity treatment provided by parabolic flight.[16] Matsuda et al. have shown that gap junction communication is significantly decreased after 24 h of multi-dimensional gravity in MLO-Y4 cells.[33] Cx43 deficiency mouse model shows desensitization of the bone to simulated weightlessness modeled by HLS.[14] Grimston et al. also showed similar results after BtxA injection to simulate mechanical unloading.[15] These two studies have shown that conditional deletion of Cx43 in osteoblasts and osteocytes are partially resistant to the cortical bone loss

induced by mechanical unloading, indicating that Cx43 is involved in the bone responses to weightlessness conditions. Here, we showed that the total Cx43 protein expression was not changed by 2 h of RPM treatment, though a slight increase of Cx43 mRNA was observed. It is unlikely that RPM causes a general effect on protein expression, because in our previous studies the changes of protein expression pattern were not homogenous, some proteins increased, while the others decreased.[19,34] Moreover, we showed that RPM had no effect on cell morphology, cell viability and apoptosis. The increased Cx43 mRNA without changes in protein level can be explained by delayed protein translation or/and increased degradation. Further studies will be required to sort out these possibilities.

Cx43 is reported to be localized in the trans-Golgi network where it assembled from monomers to hexameric connexons and then transported to plasma membrane to form hemichannels or gap junctions.[35] Mechanical simulation promotes the migration of Cx43 from Golgi to the cell surface to form mechanosensitive hemichannels.[36] Contrary to mechanical stimulation, we showed here that lesser amount of Cx43 protein was present on the cell surface after RPM treatment, while the total amount of Cx43 was not changed compared with the control. The results suggest that RPM treatment increases the retention of Cx43 in Golgi bodies, and reduces the transport of Cx43 from Golgi to plasma membrane. A previous study reports that the interaction with 14-3-3 assists the delivery of Cx43 to plasma membrane to form hemichannels, and knocking down 14-3-3 expression reduces the surface expression of Cx43 by over 50%.[36] It is plausible that the decrease of membrane transport of Cx43 by RPM could be caused by the downregulation of 14-3-3.

In general, hemichannel activity is correlated with cell surface expression level of connexins. However, we observed inverse responses to RPM by which surface expressed Cx43 was reduced while hemichannel opening was enhanced. A previous study in HeLa cells also showed that enhanced activity of hemichannels was inversely correlated with cell surface levels of Cx46 protein.[24] The results indicated that increased Cx43 hemichannels is not directly regulated through the enhancement of Cx43 expression. Firstly, only small percentage of Cx43 on cell surface forms hemichannels.[37] In addition to protein level, connexin channels are regulated through multiple factors including protein phosphorylation, intracellular trafficking, assembly and direct protein– protein interaction.[38,39] Second, increased activity may actually reflect altered permeability (gating) of functioning hemichannels.[24]

We showed that RPM treatment increased the release of PGE_2 and this release was attenuated with the inhibition of Cx43 hemichannels. This result suggests that hemichannels mediate PGE_2 release in response to RPM. Our previous work also shows that PGE_2 production increases after subjecting MLO-Y4 cells to 48 h of two-dimensional (2D) clinostat treatment.[40] Simulated weightlessness by RPM and 2D clinostat both promote the release of PGE_2. Moreover, previous studies have shown that osteocytes release PGE_2 by Cx43 hemichannels in response to mechanical stress.[6,28,31,41,42] Both mechanical loading and simulated weightlessness cause PGE_2 release, which may indicate a common response of osteocytes to changed physical environment,. As a key regulatory factor of bone metabolism, PGE_2 could modulate bone remodeling via the regulation of both bone-forming osteoblasts and bone-resorbing osteoclasts.[8,43–45] Furthermore, intermittent treatment with

PGE_2 is anabolic, while continuous PGE_2 treatment is catabolic with a negative balance on bone.[46] Cox-2 mediates the biosynthesis of PGE_2 and its mRNA expression is increased in response to mechanical stimulation.[47] But in our study, RPM had no effect on the level of Cox-2 mRNA. Therefore, the increased release of PGE_2 is unlikely caused by its biosynthesis.

Together, our results suggest that short-term RPM treatment reduces surface expression of Cx43, which may be caused by the inefficient transport of Cx43 from Golgi bodies to plasma membrane. RPM promotes the opening of hemichannels with increased release of PGE_2. This study suggests that Cx43 hemichannel is likely to be a key factor in mediating biological function of bone cells in response to weightlessness.

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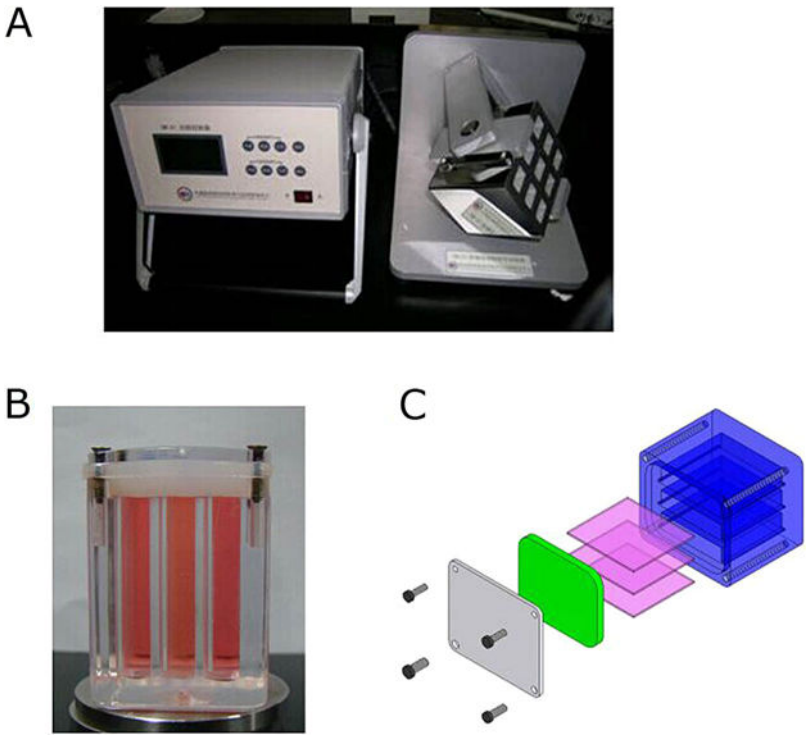

Figure 1.

Random position machine (RPM) was used to simulate the effects of weightlessness. (A) RPM set up. Left panel is the controller to control the rotation speed and direction; right panel shows the inner and outer frames. (B) The flask specially designed for RPM. (C) The internal structure of the flask, which is tightly capped by silicone pad and screws, and three glass slides are placed into the flask at a time.

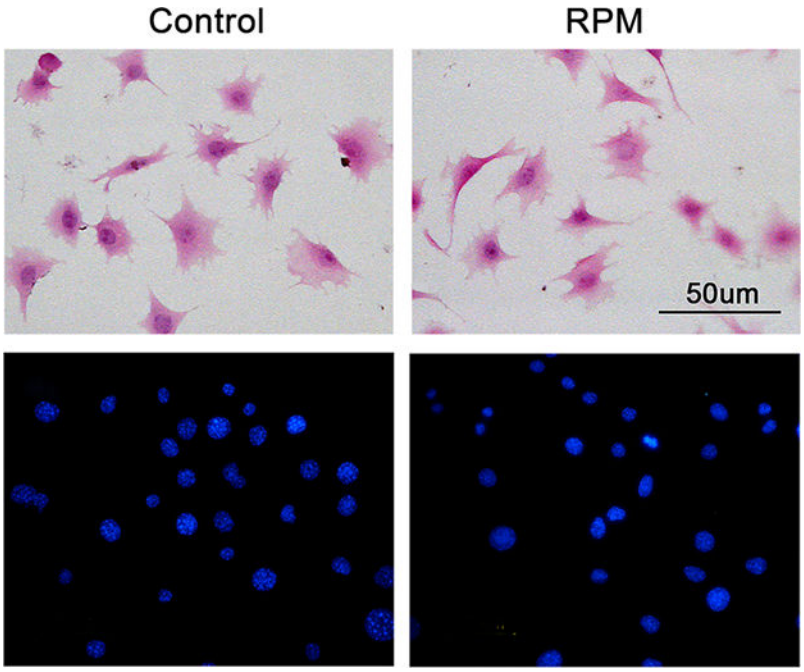

Figure 2.

Cell morphology of MLO-Y4 cells was not altered after 2 h of RPM treatment. Cells were stained with hematoxylin and eosin (upper panels) or Hoechst dye (5 μg/ml) (lower panels) and observed under phase or fluorescence microscope. Bar, 50 μm.

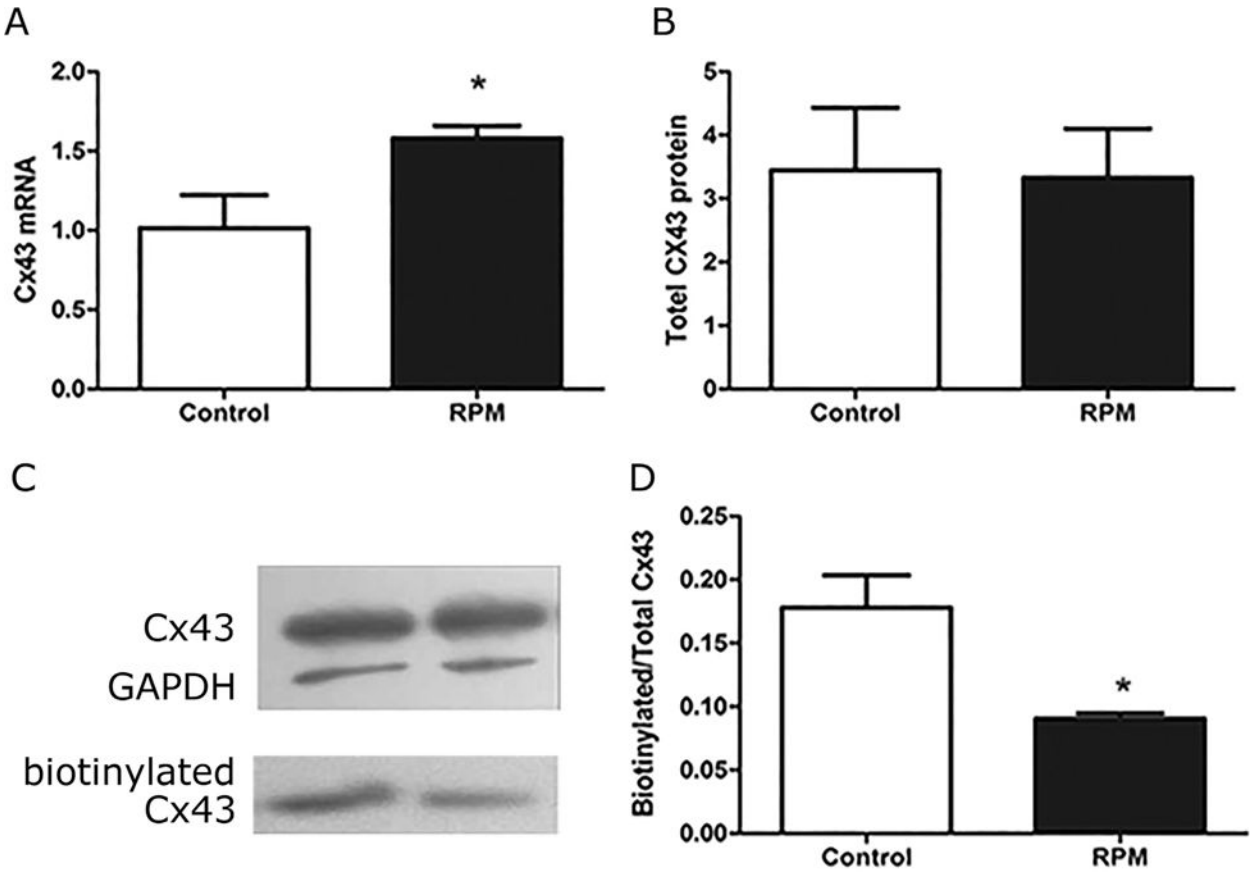

Figure 3.

Two hours of RPM treatment had no effect on total protein level of Cx43, but decreased the amount of Cx43 on cell surface. Quantitative real time PCR and western blotting were performed to determine the expression levels of Cx43 mRNA (A) and protein (B), respectively. The surface expression of Cx43 was determined by biotinylation assay. Cell extracts bound to avidin beads were immunoblotted with Cx43(E2) (1:300 dilution) or GAPDH (1:2,000) antibody. (C) The intensity of protein bands was quantified by densitomery (D). *$p < 0.05$.

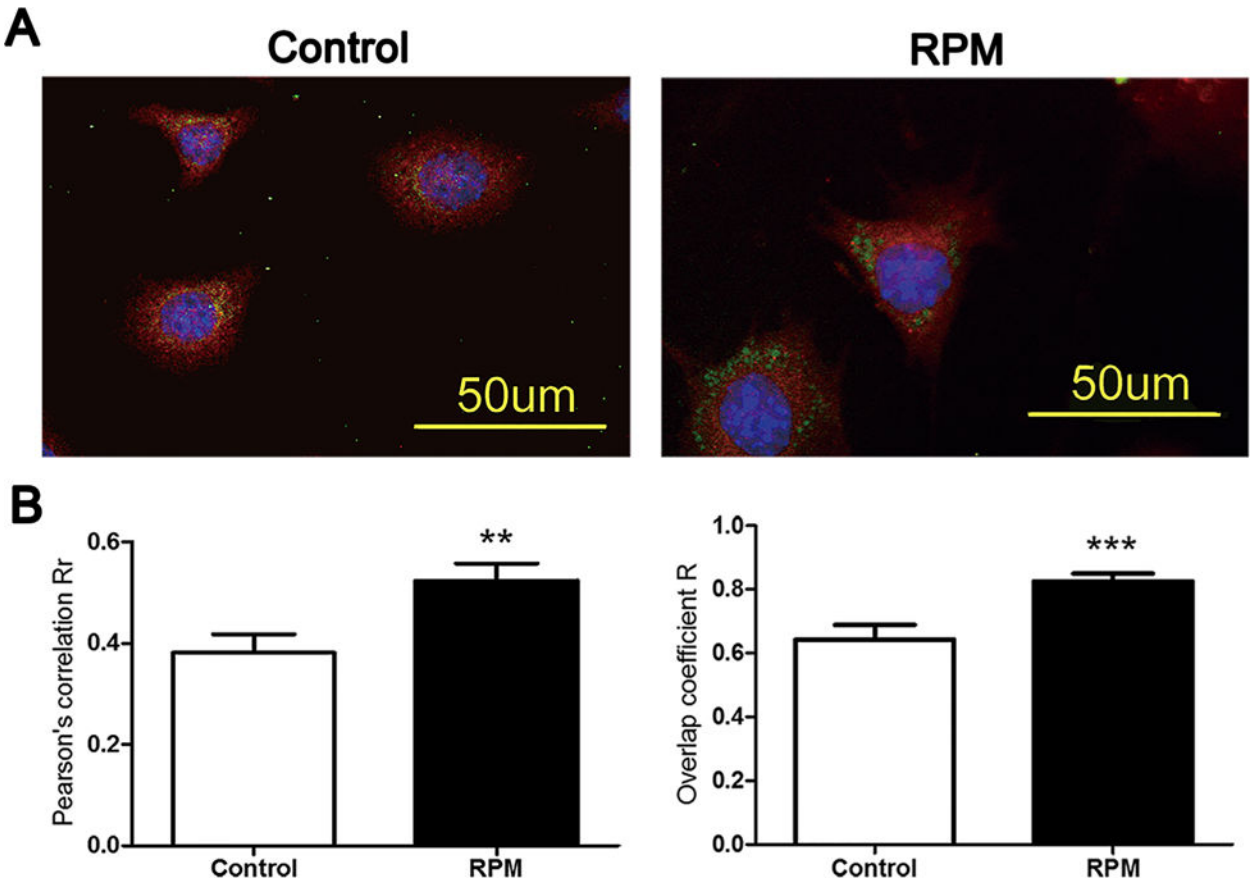

Figure 4.

Cx43 was retained in the Golgi bodies after 2 h of RPM. MLO-Y4 cells were double labeled with antibodies against Cx43 (red, 1:300) and 58 K Golgi marker (Green, 1:50) antibodies and, followed by rhodamine and FITC-conjugated secondary antibodies, respectively (A). The degree of colocalization of Cx43 and 58 K was analyzed using the Pearson's correlation coefficient (Rr) (left panel) and Overlap coefficient (R) (right panel) using the Image J software (B). $^{**}p < 0.01$; $^{***}p < 0.001$.

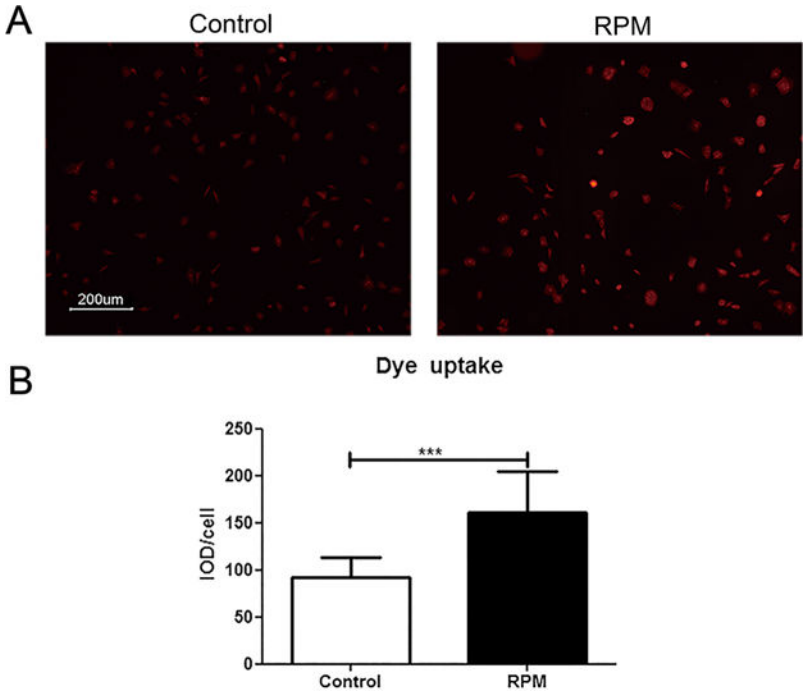

Figure 5.

The opening of hemichannels was promoted by 2 h of RPM treatment. Etd^+ dye uptake was performed after cells were subjected to RPM for 2 h. Etd^+ dye uptake was observed under fluorescence microscope (A) and quantified and presented as a ratio of integrated optical density (IOD) to total cell number using Image J (B). ***$p < 0.001$.

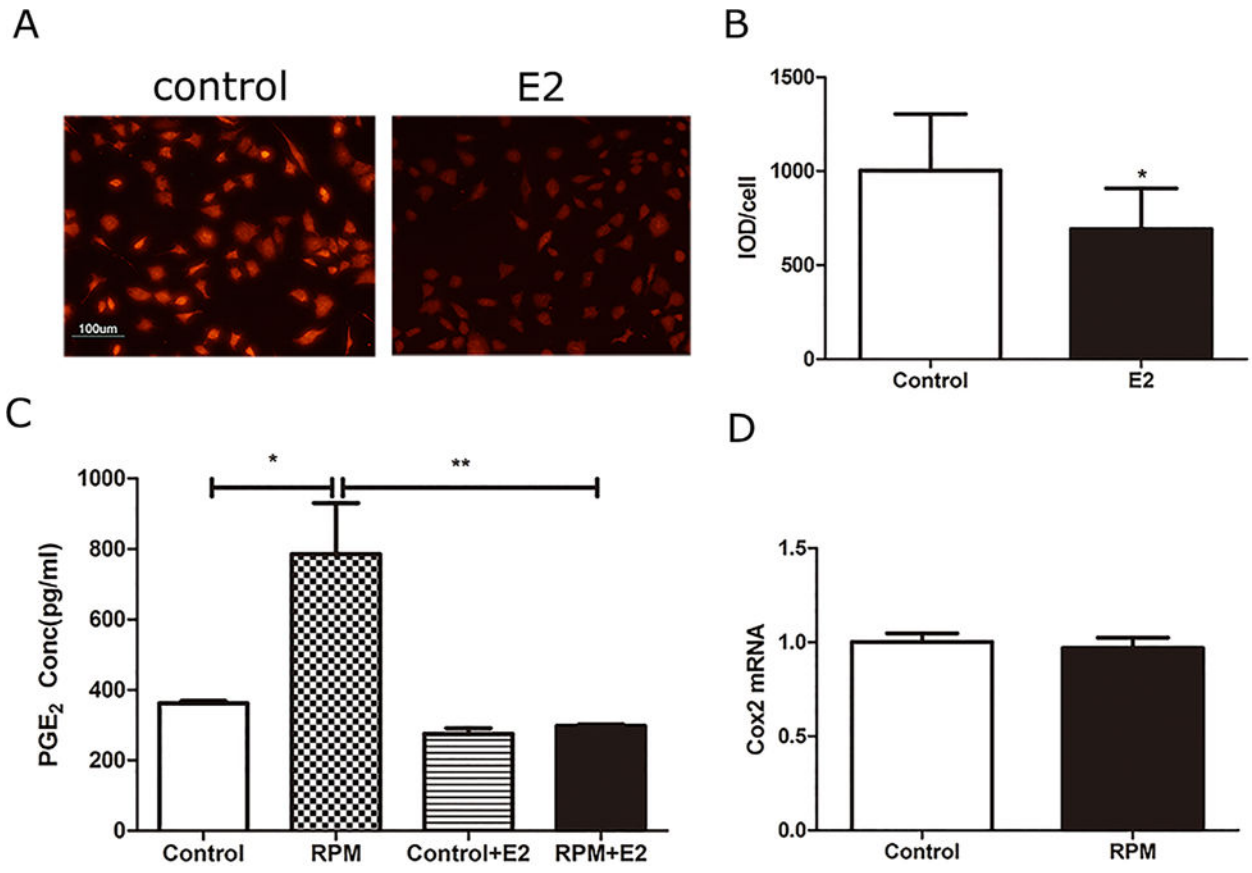

Figure 6.

The increase of PGE_2 release in response to RPM treatment was mediated by active Cx43 hemichannels, but not by the increase of Cox-2 expression. Two hour-RPM treatment increased Etd^+ dye uptake in MLO-Y4 cells and this uptake was significantly inhibited by Cx43(E2) antibody (E2) (A and B). RPM treatment promoted the release of PGE_2, which was inhibited by Cx43(E2) (E2) antibody (C). Cox-2 mRNA expression was not altered by 2 h of RPM treatment in MLO-Y4 cells (D). *$p < 0.05$; **$p < 0.01$.

Table 1

PCR Primer Sequence

Gene Name	Primer Sequences	Annealing Temperature (°C)
cox-2	F5GGTCTGGTGCCTGGTCTGATGAT3 R5CTGCTGGTTTGGAATAGTTGCTC3	55
18S	F5AATCAGGGTTCGATTCCGGA3 R5 CCAAGATCCAACTACGAGCT3	55