

Mechanical Strain Opens Connexin 43 Hemichannels in Osteocytes: A Novel Mechanism for the Release of Prostaglandin[□]

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Mechanosensing bone osteocytes express large amounts of connexin (Cx)43, the component of gap junctions; yet, gap junctions are only active at the small tips of their dendritic processes, suggesting another function for Cx43. Both primary osteocytes and the osteocyte-like MLO-Y4 cells respond to fluid flow shear stress by releasing intracellular prostaglandin E₂ (PGE₂). Cells plated at lower densities release more PGE₂ than cells plated at higher densities. This response was significantly reduced by antisense to Cx43 and by the gap junction and hemichannel inhibitors 18 β -glycyrrhetic acid and carbenoxolone, even in cells without physical contact, suggesting the involvement of Cx43-hemichannels. Inhibitors of other channels, such as the purinergic receptor P2X₇, and the prostaglandin transporter PGT, had no effect on PGE₂ release. Cell surface biotinylation analysis showed that surface expression of Cx43 was increased by shear stress. Together, these results suggest fluid flow shear stress induces the translocation of Cx43 to the membrane surface and that unapposed hemichannels formed by Cx43 serve as a novel portal for the release of PGE₂ in response to mechanical strain.

INTRODUCTION

Prostaglandins are important extracellular mediators that have a physiological and pathophysiological impact on a variety of systems, such as the immune, cardiovascular, gastrointestinal, respiratory, reproductive, and skeletal systems. Prostaglandin (PG) is a skeletal anabolic agent that can increase bone mass in animals (Jee *et al.*, 1985; Keller *et al.*, 1993; Baylink *et al.*, 1995, 1996; Harada *et al.*, 1995), and long-term release of PGE₂ is significantly reduced in cultured osteoporotic bone cells compared with age-matched control cultures (Sterck *et al.*, 1998). Prostaglandin seems to be essential for new bone formation in response to mechanical strain (Forwood, 1996); however, mice lacking the genes for enzymes required for prostaglandin synthesis, cyclooxygenase (COX)-1 and COX-2, do not seem to have any major bone developmental defects (Dinchuk *et al.*, 1995; Langenbach *et al.*, 1995). Therefore, prostaglandins seem to be more important in the mature skeleton that is exposed to greater mechanical strain than the developing skeleton.

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Abbreviations used: β -GA, 18 β -glycyrrhetic acid; Cx43, connexin 43; DIDS, 4,4'-diisothiocyanatostilbene 2,2'-disulfonate; LY, Lucifer yellow; oATP, oxidized ATP; ODN, oligodeoxynucleotide; PGE₂, prostaglandin E₂; RD, rhodamine dextran.

Osteocytes are the major mechanosensory cells in bone (Aarden *et al.*, 1994). These cells are ideally located in the bone to sense shear stress induced by fluid flowing in the canaliculi surrounding their dendritic processes. They have been shown to be more sensitive than other types of bone cells with respect to release of prostaglandins in response to fluid flow shear stress (Klein-Nulend *et al.*, 1995). In our previous studies, we have reported that fluid flow increases the release of PGE₂ and that the released PGE₂ functions in an autocrine manner to stimulate gap junction function and connexin (Cx)43 expression (Cheng *et al.*, 2001a).

A most intriguing question is how prostaglandin is rapidly released by the cell, especially by osteocytes in response to mechanical strain. As a charged organic anion at physiological pH, the diffusion rate of prostaglandin across plasma membranes is at least a magnitude lower than the response of PGE₂ release observed in most cells (Schuster, 2002). A prostaglandin transporter, PGT, has been identified from rat and bovine cells (Kanai *et al.*, 1995; Banu *et al.*, 2003); however, this transporter mainly mediates the uptake of PGE₂ into the cell, not its release (Chan *et al.*, 1998). Therefore, the means by which PGE₂ is rapidly released is not known.

In addition to being the major component of gap junction channels, connexins have recently been shown to exist and function in the form of unapposed halves of gap junction channels called hemichannels. These channels are localized at the cell surface, independent of physical contact with adjacent cells (Goodenough and Paul, 2003). These hemichannels, like gap junction channels, display low substrate selectivity and permit molecules with molecular masses <1 kDa to pass through. Hemichannels are regulated by extracellular Ca²⁺, and these Ca²⁺ regulated hemichannels control the osmotic volume of the cell (Quist

et al., 2000; Gomez-Hernandez *et al.*, 2003). The existence of functional hemichannels formed by Cx43 has been reported in several cell types, including neural progenitors, neurons, astrocytes, cardiomyocytes, osteoblasts, and osteocytes (Goodenough and Paul, 2003). Mechanical stimulation has been shown to open hemichannels in astrocytes (Stout *et al.*, 2002) and osteoblasts (Romanello *et al.*, 2003), although none of these studies used specific, physiologically relevant, defined forms of mechanical strain, such as fluid flow shear stress. In general, the regulation of the opening of hemichannels under normal physiological conditions remains uncharacterized.

MATERIALS AND METHODS

Materials

Tissue culture medium and protein standards were purchased from Invitrogen (Carlsbad, CA); fetal bovine serum (FBS) and calf serum (CS) were from Hyclone Laboratories (Logan, UT); rhodamine dextran (RD) (M_r of 10 kDa) and Lucifer yellow (LY) (M_r of 547 Da) were from Molecular Probes (Eugene, OR); paraformaldehyde (16% stock solution) was from Electron Microscopy Science (Fort Washington, PA); nitrocellulose membrane was from Schleicher & Schuell (Keene, NH); rat tail collagen type I (99% pure) was from BD Biosciences (Bedford, MA); polyester sheets were from Regal Plastics (San Antonio, TX); PGE₂ enzyme immunoassay (EIA) kit was from Cayman Chemical (Ann Arbor, MI); EZ-link Sulfo-NHS-LC-Biotin, avidin beads, and bicinchoninic acid microprotein assay kit were from Pierce Chemical (Rockford, IL); enhanced chemiluminescence (ECL) kit was from Amersham Biosciences (Piscataway, NJ); X-OMAT AR film was from Eastman Kodak (Rochester, NY); and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cell Culture and PGE₂ Measurement

MLO-Y4 cells were cultured at various densities on collagen-coated (rat tail collagen type I; 0.15 mg/ml) surfaces, including plastic plates, polyester sheets, and glass slides. Cells were grown in α -modified essential medium (MEM) supplemented with 2.5% FBS and 2.5% CS and incubated in 5% CO₂ incubator at 37°C as described previously (Cheng *et al.*, 2001a).

After the fluid flow treatment for 30 min or 2 h at 16 dynes/cm², the conditioned medium was collected, and the extracellular PGE₂ released into the medium was measured using a PGE₂ EIA kit according to the manufacturer's instructions, whereas intracellular PGE₂ was measured after the cells were thoroughly washed three times with phosphate-buffered saline (PBS) and lysed.

Cell Shear Stress Induced by Fluid Flow

Fluid flow experiments were performed as described previously (Cheng *et al.*, 2001b). MLO-Y4 cells were cultured on collagen-coated surfaces for protein assay, PGE₂ measurement and microscopic assay. Flow rate was continually monitored by an in-line flowmeter (Cole-Parmer Instrument, Vernon Hills, IL). Using this flow system, wall shear stress levels caused by steady laminar flow of 16 dynes/cm² were generated by adjusting the channel height (using spacers) and medium flow rate (1–1.8 ml/s). All experiments were repeated at least three times.

Antisense Cx43 Oligodeoxynucleotide (ODN) Approach

Based on previous studies (Becker *et al.*, 1999), Cx43 sense and antisense ODNs derived from nt 954 to 983 were synthesized at the DNA Core Laboratory of the University of Texas Health Science Center at San Antonio. MLO-Y4 cells were treated every 8 h in the absence or presence of fresh 50 μ M sense or antisense ODN for a total of 24 h, and the expression of Cx43 was analyzed using immunofluorescence and Western blots.

Dye Uptake Assay

MLO-Y4 cells were grown at low initial plating density to ensure that the majority of the cells were not physically in contact. LY (M_r of 547 Da) was used as a tracer for hemichannel activity, and RD (M_r of 10 kDa) was used as a negative control. Cells maintained in Ca²⁺-free MEM were exposed to vehicle, 5 mM EGTA, 100 mM β -GA, or glycyrrhetic acid (GA) for 10 min. For fluid flow treatment, cells were treated in the absence or presence of 100 mM β -glycyrrhetic acid (β -GA) or glycyrrhetic acid (GA) under fluid flow shear stress at 16 dynes/cm² for 10 min. After treatment, dye uptake experiments were conducted in the presence of 0.4% LY and 0.4% RD for 5 min, and cells were washed with medium containing 1.8 mM Ca²⁺ to close the hemichannels and then fixed with 1% paraformaldehyde. Similar fields were observed under the fluorescence microscope. Dye uptake was presented as a percentage of fluorescent cells.

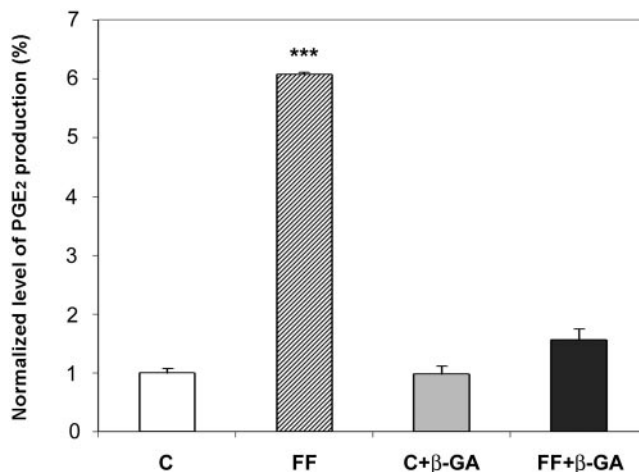


Figure 1. PGE₂ release in response to mechanical stress is blocked by the gap junction inhibitor β -GA. MLO-Y4 cells were treated with or without 100 μ M β -GA under FF at 16 dynes/cm² for 2 h. The conditioned medium was collected and PGE₂ was measured. The relative level of PGE₂ compared with control is shown in the y -axis. Controls (C) are cells not subjected to fluid flow. C, C + β -GA or FF + β -GA versus FF: *** p < 0.001. The data are presented as mean \pm SD and n = 3.

Isolation of Chick Primary Osteocytes

Preparation of primary osteocytes from chick calvaria was based on previously published procedures (Tanaka *et al.*, 1995) with some modifications. Briefly, calvarial bone was dissected from 16-d-old embryonic chicks and minced. The soft tissues and osteoid were removed by collagenase treatment followed by decalcification by using EDTA. The final particles were treated with collagenase and vigorously agitated to release osteocytes, followed by filtration through an 8- μ m membrane filter to eliminate most of the larger sized osteoblast/fibroblast cells.

Cell Surface Biotinylation

Biotinylation of monolayered cells was performed based on a modification of a procedure that was described previously (Daniels and Amara, 1998). MLO-Y4 cells were labeled with or without 1 mg/ml EZ-link Sulfo-NHS-LC-Biotin in PBS at 4°C for 20 min. The cells were washed three times with PBS containing Ca²⁺, Mg²⁺, and glycine and lysed in lysis buffer (133 mM NaCl, 5 mM KCl, 1% dextrose, and 20 mM HEPES) plus 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and proteases inhibitors. Cell lysates were mixed with equal volumes of monomeric avidin beads and incubated for 60 min at room temperature. The beads were then washed five times with PBS until no proteins could be detected by measurement of spectrometric absorbance at 280 nm. The biotinylated proteins were eluted by boiling for 5 min in sample loading buffer containing 1% SDS and 2% 2-mercaptoethanol, and equal volumes of each sample were loaded on SDS-PAGE and analyzed by Western blotting by using affinity-purified anti-Cx43 or anti- β -actin antibody.

Immunofluorescence Labeling and Fluorescence Microscopy

The cells cultured on the microscopic slides were washed three times with PBS, each wash of 5-min duration. This was followed by fixation in 2% paraformaldehyde in PBS for 30 min at room temperature after which the cells were washed three times with PBS of 5 min each, followed by incubation for 30 min in blocking solution containing 2% normal goat serum, 2% fish skin gelatin, and 1% bovine serum albumin in PBS, and then incubated overnight at 4°C with an affinity-purified anti-Cx43 antibody diluted 1:250 in blocking solution. Cells were washed three times; 5 min for each wash with PBS and then incubated for 1 h with fluorescein-conjugated goat anti-rabbit IgG diluted 1:250 in blocking solution. Fluorescence microscopy was performed using an Olympus B-MAX microscope (Olympus, Tokyo, Japan) and recorded on a "Spot II" digital camera (Diagnostic Instruments, Tokyo, Japan). For dye uptake and dye transfer results, LY was detected using the filter set for fluorescein and RD by using the filter set for rhodamine.

Western Blotting

The protein concentration of crude membrane samples of MLO-Y4 cells was determined using the MicroBCA assay according to the manufacturer's in-

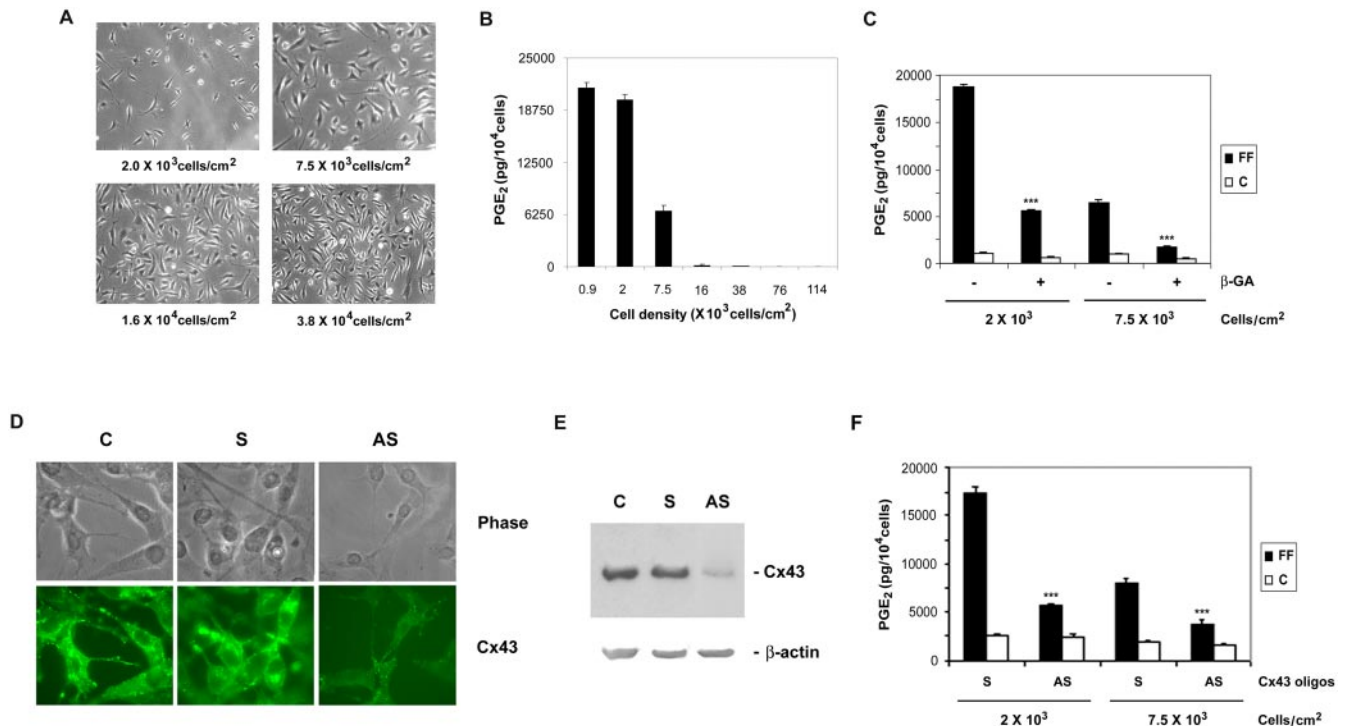


Figure 2. PGE₂ release due to fluid flow shear stress is blocked by β -GA and Cx43 antisense ODN in cells lacking physical contact. (A) MLO-Y4 cells shown were plated at cell densities of 2.0×10^3 , 7.5×10^3 , 1.6×10^4 , and 3.8×10^4 cells/cm². At the cell density of 2.0×10^3 cells/cm², the majority of cells do not make physical contact. (B) MLO-Y4 cells were subjected to fluid flow at the stress level of 16 dynes/cm² for 2 h. In response to fluid flow, MLO-Y4 cells at lower densities released more PGE₂ per cell than cells at higher densities. (C) MLO-Y4 cells at the densities of 2 and 7.5×10^3 /cm² were treated with or without 100 μ M β -GA in absence (control [C]) or presence of FF at 16 dynes/cm² for 2 h. PGE₂ release in response to fluid flow was inhibited even at a low cell density of 2×10^3 /cm² where gap junctions barely exist. Untreated versus β -GA treated at 2×10^3 or 7.5×10^3 cells/cm²: *** $p < 0.001$. (D) MLO-Y4 cells were treated with 50 μ M Cx43 sense (S), antisense (AS) ODNs, or not treated (C). The cells were labeled with affinity-purified Cx43 antibody. The Cx43 protein was detected using fluorescein-conjugated anti-rabbit secondary antibody. Cx43 antisense ODN reduced Cx43 protein expression. (E) Immunoblots of membranes isolated from cells treated with Cx43 S, AS, or not treated (C) were labeled with 1:300 dilution of affinity-purified anti-Cx43 antibody. (F) MLO-Y4 cells at densities of 2 and 7.5×10^3 /cm² were pretreated with 50 μ M Cx43 S or AS ODNs before FF at 16 dynes/cm² for 2 h. The release of PGE₂ was inhibited by Cx43 antisense ODN at both densities tested. S versus AS treated at 2 or 7.5×10^3 cells/cm²: *** $p < 0.001$. In B, C, and F, the conditioned medium was collected, and the release of PGE₂ was measured using a PGE₂ EIA kit. The data are presented as mean \pm SD and $n = 3$.

structions (Pierce Chemical). Western blotting was performed as described previously (Cheng *et al.*, 2001b), and membranes were incubated with a 1:250 dilution of affinity-purified Cx43 antibody or a 1:5000 dilution of monoclonal anti- β -actin antibody. The primary antibody was detected using peroxidase-conjugated secondary anti-rabbit or anti-mouse antiserum followed by use of a chemiluminescence reagent kit (ECL) according to the manufacturer's instructions. The intensity of Cx43 bands was quantified by densitometry (NIH Image).

Statistical Analysis

Data were analyzed using the one-way analysis of variance and Bonferroni's multiple comparison tests with the Prism biostatistic program (GraphPad Software, San Diego, CA) and presented as the mean \pm SD of three determinations. In the figures, asterisks indicate the degree of significant differences (** $p < 0.01$ and *** $p < 0.001$).

RESULTS

As we have reported previously, fluid flow shear stress promotes the release of intracellular PGE₂ from osteocytes (Cheng *et al.*, 2001a). To determine whether gap junction channels and hemichannels play a role in this process, we applied β -GA, an inhibitor known to block gap junction channels as well as unapposed hemichannels, to cells exposed to fluid flow shear stress (Figure 1). The release of

PGE₂ stimulated by fluid flow was significantly suppressed by this inhibitor. We found that β -GA or GA at a concentration of 100 μ M does not interfere with PGE₂ quantitation (our unpublished data). These results suggest that the release of PGE₂ is likely to occur through the function of either gap junctions, hemichannels, or both.

Almost all chemical inhibitors for gap junctions are known to block gap junction channels as well as unapposed hemichannels. To distinguish the relative contributions of these two different types of channels, cells were plated at decreasing densities to the point where few cells were in contact, 2.0×10^3 cells/cm² or lower (Figure 2A). Interestingly, cultures at lower cell densities (0.9×10^3 and 2×10^3 cells/cm²) released significantly more PGE₂ per cell than cells cultured at normal and higher densities (7.5×10^3 cells/cm² and higher) (Figure 2B), suggesting that at high cell density, i.e., more gap junctions, the release of PGE₂ is suppressed, although the increase in response to fluid flow shear stress is significant. The inhibitor β -GA significantly blocked PGE₂ release at both lower (2×10^3 cells/cm²) and normal (7.5×10^3 cells/cm²) cell densities in response to fluid flow shear stress (Figure 2C). The observation that the blockage of the release of PGE₂ at a density where few gap junction channels could physically exist suggests that

hemichannels are the likely mechanism responsible for the release of PGE₂ in response to fluid flow shear stress.

Because the precise blocking mechanism for all of the chemical inhibitors of gap junctions and hemichannels is not well understood (Goodenough and Paul, 2003), we used a Cx43 antisense approach to further verify that release of PGE₂ is a connexin-mediated event. An antisense ODN was derived from nt 954 to 983 of the Cx43 coding sequence (Becker *et al.*, 1999). Immunofluorescent staining and Western blots showed that expression of Cx43 was greatly reduced after antisense ODN treatment compared with non-treated and sense ODN control (Figure 2, D and E). The increased release of PGE₂ induced by fluid flow shear stress was also significantly attenuated upon treatment of cultures with antisense Cx43 ODN at both lower and normal cell densities (Figure 2F). The observation of a blockade of PGE₂ release by antisense Cx43 ODN in cells without gap junctions strongly suggests that hemichannels formed by Cx43 are responsible for the release of PGE₂ from osteocytes.

Another effective channel blocker, carbenoxolone (Plotkin *et al.*, 2002), was used to further confirm the role of hemichannels in the release of PGE₂. Similar to the effects by β -GA and Cx43 antisense, the fluid flow-induced release of PGE₂ from cells without physical contact was significantly inhibited by carbenoxolone (Figure 3A). The amount of intracellular PGE₂ was also increased in response to fluid flow; however, there was no discernible increase in the intracellular PGE₂ levels in carbenoxolone-treated cells (Figure 3B), which implies a potential feedback inhibition of biosynthesis by the substrate. Alternatively, metabolic instability of intracellular PGE₂ due to oxidation (Schuster, 1998) may contribute to the lack of increase in intracellular PGE₂ in the presence of a hemichannel inhibitor. Together, these results suggest that fluid flow induced the opening of hemichannels, which leads to a release of intracellular stores of PGE₂.

In addition to connexin-forming hemichannels, two other types of transport mechanisms, the purinergic receptor P2X₇ (North, 2002) and a prostaglandin transporter, PGT (Chan *et al.*, 1998), could potentially provide alternative or additional pathways for the exit of PGE₂ from the cell. To determine whether the release of PGE₂ by fluid flow could be mediated by either of these two mechanisms, two commonly used antagonists, oxidized ATP (oATP), specific for P2X₇ (North, 2002), and 4,4'-diisothiocyanatostilbene 2,2'-disulfonate (DIDS), specific for PGT (Chan *et al.*, 1998), were applied, and the intracellular as well as extracellular levels of released PGE₂ were determined (Figure 4). Only the hemichannel inhibitor, not the inhibitors for either P2X₇ or PGT, blocked the release of PGE₂ (Figure 4A). Similar to the observation in Figure 3B, the elevated intracellular PGE₂ level induced by fluid flow was not altered by β -GA or by the other inhibitors for P2X₇ receptor and PGT transporter (Figure 4B).

To verify that hemichannels are being induced to open in response to mechanical stimulation, dye uptake experiments were conducted in MLO-Y4 cells as well as in primary osteocytes subjected to fluid flow (Figure 5). EGTA, which removes extracellular Ca²⁺, is known to induce the opening of hemichannels and was used as a positive control (Quist *et al.*, 2000). Compared with unstressed (Figure 5, A and C) and EGTA-treated (Figure 5A, EGTA) cells, fluid flow shear-stressed cells (Figure 5A, FF) increased the uptake of LY. The number of cells taking up the dye upon fluid flow treatment is even higher than those treated by EGTA. The induction of the opening of hemichannels by fluid flow was blocked by β -GA (Figure 5A, FF + β -GA). Quantification of dye uptake showed a significant increase in the number of cells that took up the dye when subjected to fluid flow compared with

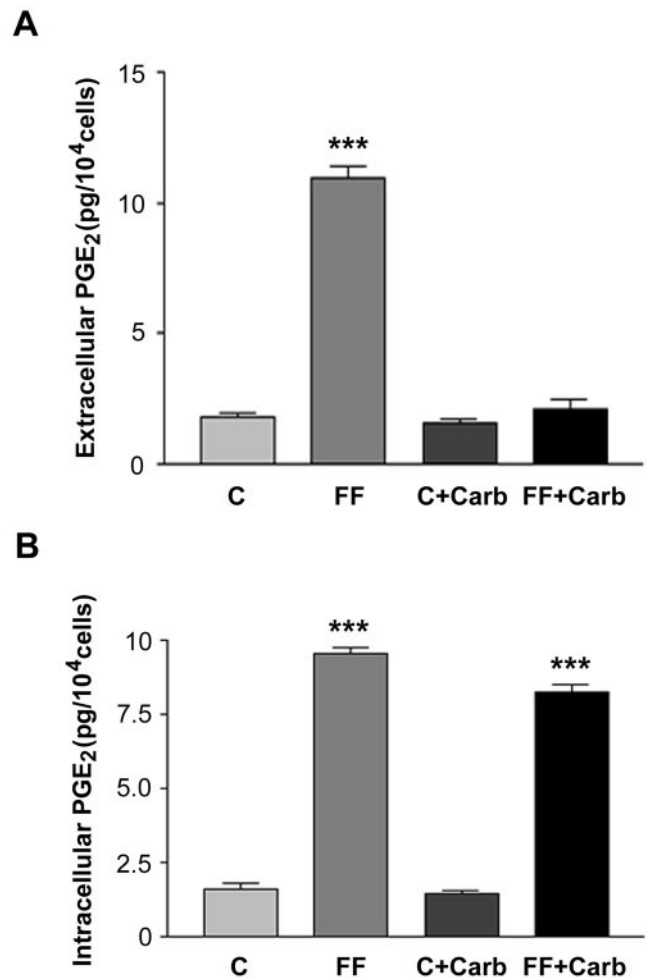


Figure 3. Fluid flow-induced release of PGE₂ is blocked by carbenoxolone in cells lacking physical contact. MLO-Y4 cells plated at $2 \times 10^3/\text{cm}^2$ were treated with or without 100 μM carbenoxolone (Carb) in the absence (control [C]) or presence of FF at 16 dynes/ cm^2 for 2 h. The media and cells were collected. The amount of extracellular (A) and intracellular (B) PGE₂ was measured using a PGE₂ EIA kit. For extracellular PGE₂, C, C + Carb, or FF + Carb versus FF: *** $p < 0.001$. For intracellular PGE₂, C or C + Carb versus FF or FF + Carb: *** $p < 0.001$. All data are presented as mean \pm SD and $n = 3$.

unstressed control cells, and this increase was significantly inhibited by β -GA (Figure 5B). Hemichannel activity in primary osteocytes also was observed using the dye uptake assay (Figure 5C). Similar to MLO-Y4 cells, fluid flow induced the uptake of LY in primary osteocytes. These results suggest that fluid flow shear stress significantly stimulates the opening of functional hemichannels in osteocytes.

Because fluid flow shear stress increased the activity of hemichannels to allow the release of PGE₂, it would be expected that the expression level of the hemichannel-forming Cx43 protein would be increased on the cell surface as well. Therefore, cell surface expression of Cx43 was assessed by determining the amount of Cx43 that can be biotinylated due to exposure on the cell surface (Figure 6A) ($n = 3$). More biotinylated Cx43 protein was observed from lysates of cells subjected to fluid flow shear stress compared with the control sample (Figure 6A, lanes 3 and 4), even though the total amount of Cx43 being subjected to precipitation by avidin

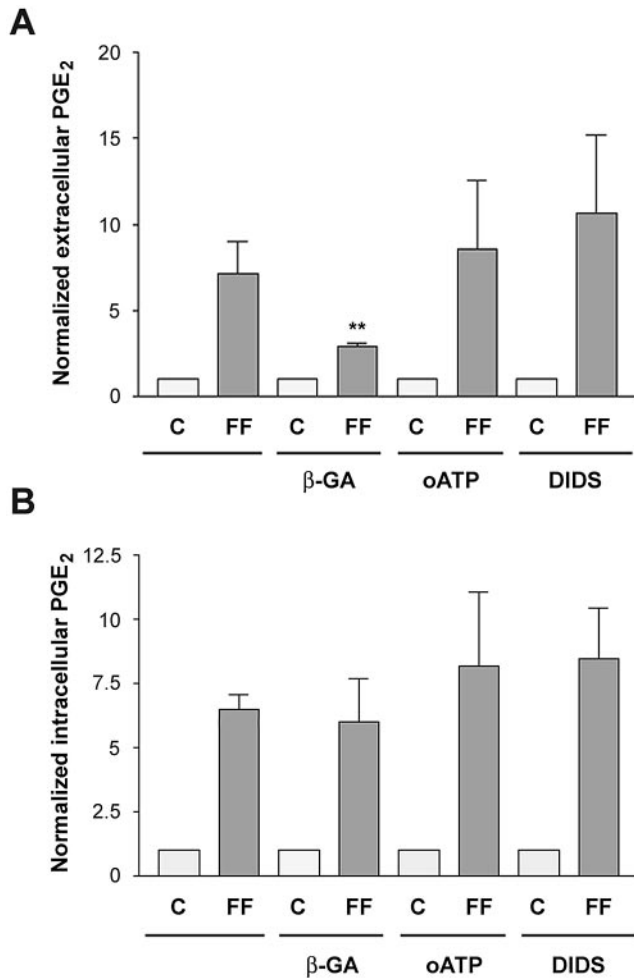


Figure 4. Hemichannels, not the purinergic receptor P2X₇, nor the prostaglandin transporter PGT, mediate the release of PGE₂. MLO-Y4 cells plated at $2 \times 10^3/\text{cm}^2$ were treated with or without 100 μM β -GA, 10 μM oATP, or 100 μM DIDS in the absence (control [C]) or presence of FF at 16 dynes/cm² for 2 h. The media and cells were collected. The amount of extracellular (A) and intracellular (B) PGE₂ was measured using a PGE₂ EIA kit. For extracellular PGE₂, untreated, oATP, or DIDS treated versus β -GA treated: ** $p < 0.01$. All data are presented as mean \pm SD and $n = 3$.

beads in the fluid flow sample was less than that of its control (Figure 6A, lanes 1 and 2). In addition, the highly biotinylated form of Cx43 (top band) seems to be more dominant in samples subjected to fluid flow, implying greater extracellular exposure of Cx43 in response to shear stress. The relative ratio of biotinylated to total Cx43 was determined and showed an increase in the amount of surface-biotinylated Cx43 in cells subjected to fluid flow shear stress compared with controls (Figure 6A, right). Lysates of the cells without biotin treatment were applied to avidin beads to control for nonspecific binding (Figure 6B). Nonbiotinylated Cx43 was not precipitated by the beads (Figure 6B, lane 2). To eliminate the possibility that intracellular domains of Cx43, due to incomplete quenching, became accessible to the biotinylating reagent after the cells were lysed, a parallel control biotinylation assay was conducted for β -actin, a protein only expressed intracellularly (Figure 6C). No biotinylation of β -actin was detected (Figure 6C, lane 2). Together, the data show an approximate doubling in surface Cx43 expression in response to fluid flow shear stress.

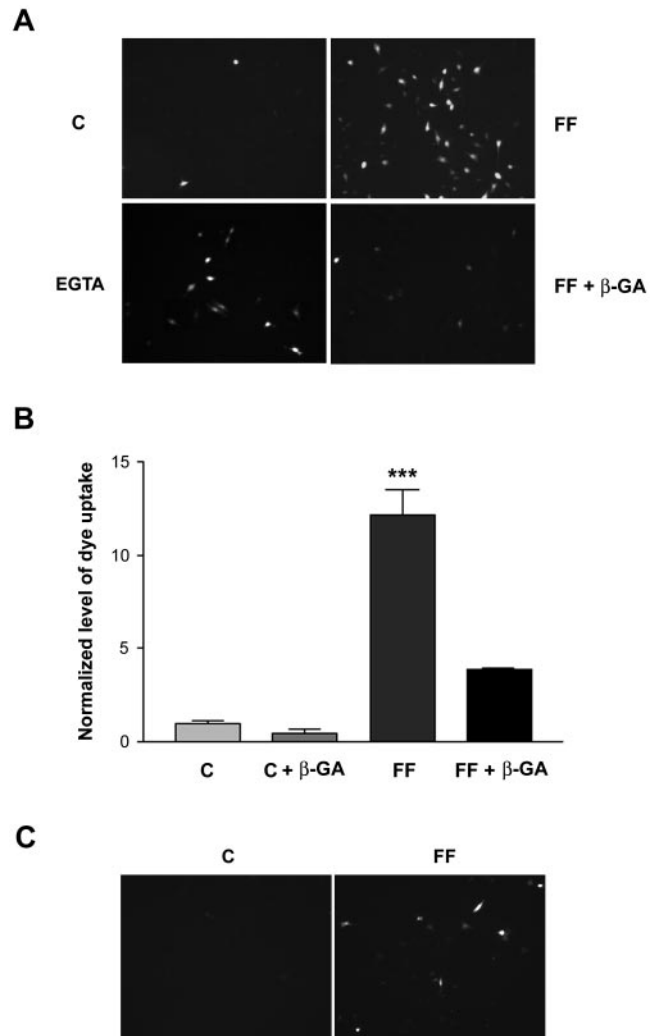
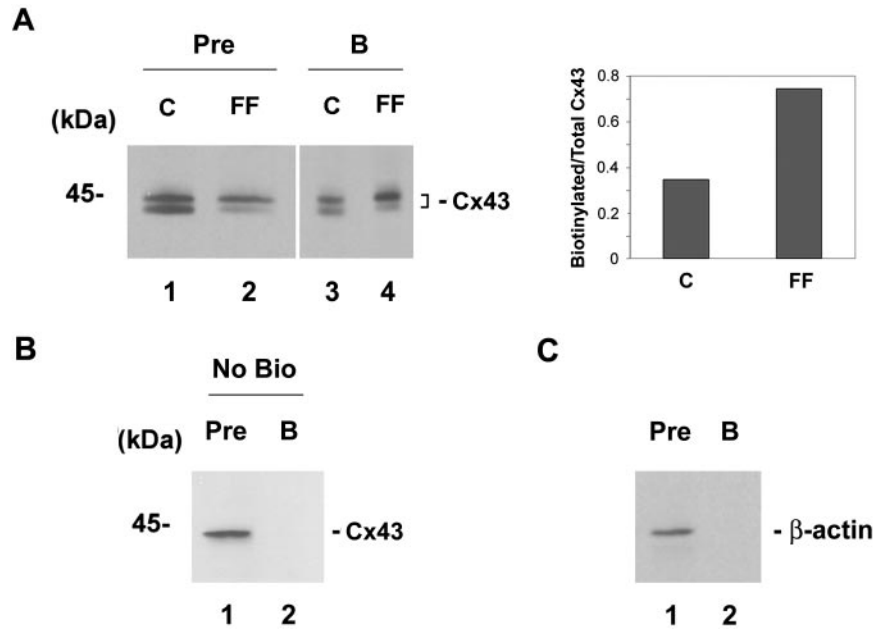


Figure 5. Fluid flow shear stress increases dye uptake in MLO-Y4 cells and primary osteocytes. (A) Compared with untreated control (C) and EGTA-treated (EGTA) cells, FF shear stress stimulated the uptake of the dye in MLO-Y4 cells, in which the degree of uptake is even more than EGTA-treated cells. This increase is blocked by β -GA (FF + β -GA). (B) The number of MLO-Y4 cells with dye uptake with and without fluid flow treatment or β -GA were counted and quantified. C, C + β -GA or FF + β -GA versus FF: *** $p < 0.001$. The data are presented as mean \pm SD and $n = 3$. (C) Compared with untreated control (C), FF shear stress similarly stimulated the uptake of the dye in primary osteocytes.

DISCUSSION

We demonstrate that Cx43-forming hemichannels seem to serve as a direct portal for the exit of intracellular PGE₂ in osteocytes induced by fluid flow shear stress. Chemical inhibitors for gap junctions and hemichannels, β -GA and carbenoxolone, can block the release of PGE₂ even at low cell densities with minimal cell contact, which prevents any gap junction formation. The release of PGE₂ was only inhibited by β -GA and carbenoxolone, but not by inhibitors of other channels such as the purinergic receptor P2X₇ or the prostaglandin transporter PGT. A Cx43 antisense ODN also inhibited the release of PGE₂ at low cell density. Dye uptake analysis, indeed, showed that hemichannels were induced to open by fluid flow shear stress (Jiang and Cherian, 2003). Moreover, we found that fluid flow stimulated the increased

Figure 6. Fluid flow shear stress increased the surface expression of Cx43. (A) The MLO-Y4 cells with (FF) or without (C) 2-h treatment of fluid flow at 16 dynes/cm² were treated with biotin. The cells were lysed, and equal volumes of total protein of untreated (C) and FF-treated samples were applied to avidin-conjugated beads. The biotin-labeled samples were isolated by binding to avidin-conjugated beads. The preloaded (Pre) and biotinylated Cx43 bound (B) to avidin beads was detected by Western blots by using anti-Cx43 antibody. The relative ratio of biotinylated to total Cx43 was quantified using densitometric measurements of the band intensity (right). (B) To control for nonspecific binding, lysates of cells without biotin treatment (No Bio) were applied to avidin-conjugated beads, and preloaded (Pre) and bound (B) fractions were detected by Western blotting with anti-Cx43 antibody. (C) The Pre and biotinylated B samples were analyzed by Western blots by using anti- β -actin antibody.



expression of Cx43 on the cell surface. Together, these results suggest that fluid flow shear stress stimulates the movement of Cx43 to the cell membrane and induces the opening of Cx43-hemichannels likely to be involved in the exit of intracellular PGE₂ from the cell.

Osteocytes and osteocyte-like MLO-Y4 cells express large amounts of Cx43, and this protein is located on the plasma membrane and predominantly, in the cytoplasm. Our previous study showed that in the presence of fluid flow shear stress, Cx43 seems to migrate toward the plasma membrane (Cheng *et al.*, 2001b). In the present study, biotinylation analysis revealed that Cx43 expression on the cell surface is doubled, most likely to accommodate an increased demand for hemichannel function in response to fluid flow shear stress. Because gap junction-forming Cx43 is not accessible to the cross-linking reagent biotin (Lampe, 1994), and thus cannot be surface-biotinylated, the biotinylated Cx43 is likely the component of hemichannels on the cell surface.

Osteocyte dendritic processes are surrounded by canaliculi, which form an extensive three-dimensional network or syncytium in the bone matrix. The release of PGE₂ from mechanically stimulated osteocytes has been reported in previous publications by us and others (Klein-Nulend *et al.*, 1995; Ajubi *et al.*, 1996; Cheng *et al.*, 2001a). We also have shown that increases in release of PGE₂ are correlated with increases in magnitude of fluid flow shear stress (Cheng *et al.*, 2001a). PGE₂ is an anabolic factor that can increase bone mass in animals (Jee *et al.*, 1985; Keller *et al.*, 1993; Baylink *et al.*, 1995; Harada *et al.*, 1995; Baylink *et al.*, 1996). The use of indomethacin, the inhibitor of prostaglandin synthase (COX-1 and COX-2), blocks bone formation in response to mechanical strain (Forwood, 1996). Conversely, prostaglandin also stimulates osteoclast formation and activation (Collins and Chambers, 1991, 1992; Kaji *et al.*, 1996). It is possible that extracellular release of PGE₂ into the bone fluid could reach the bone surface and have effects not only on osteoclasts but also on osteoblasts or any other cell on the bone surface. Osteoclastic bone resorption is coupled to bone formation and is responsible for bone remodeling. Bone remodeling in response to mechanical strain has been shown to be due to prostaglandin synthesis (Reich *et al.*, 1990; Reich and Frangos, 1991). In our

previous studies (Cheng *et al.*, 2001a), we showed that the PGE₂ released in response to fluid flow shear stress also acted in an autocrine/paracrine manner to stimulate gap junction-mediated communication. Therefore, not only could prostaglandin be having an effect on cells on the surface of bone but also on osteocytes downstream of the released prostaglandin.

Prostaglandin signaling mechanisms through their EP receptors is well known (Breyer *et al.*, 2001), and a new function and signaling pathway has recently been identified—that of spinal PGE₂ acting as an inflammatory pain-sensitizing molecule through the glycine receptor to boost neuronal activity (Harvey *et al.*, 2004). However, the pathway(s) for the exiting of prostaglandins from the cell remains largely unknown. Prostaglandins are charged anions that diffuse poorly across membrane bilayers (Schuster, 2002). Even though the human prostaglandin transporter gene hPGT is up-regulated by fluid flow mechanical stimuli in endothelial cells (Topper *et al.*, 1998), studies exclude the possible involvement of PGT in the release of PGE₂ (Chan *et al.*, 1998; Schuster, 2002). PGT, instead, is suggested to play a role in metabolic oxidation clearance through the reuptake of the released PGE₂ from the extracellular milieu and matrix. It has been suggested that PGE₂ exits the cell by a channel-like diffusion process, not through a specific transporter-facilitated process (Chan *et al.*, 1998). A potential channel that could be responsible for the efflux of PGE₂ is the purinergic receptor P2X₇, the only type of purinergic receptor that acts as a channel and permits passage of molecules up to M_r of 800 Da (North, 2002). However, specific inhibitors for P2X₇ failed to block the release of intracellular PGE₂ in the present study, which excludes this possibility. Moreover, opening of P2X₇ channels requires a high concentration of ATP (close to 1 mM) (North, 2002). We found that conditioned medium collected from fluid flow-stimulated cultured cells failed to induce dye uptake (our unpublished data), indicating that the amount of ATP released is not sufficient to activate P2X₇ channels. Additionally, P2X₇ channels are less permeable for anionic molecules such as PGE₂ because these channels selectively allow passage of cationic molecules over anionic molecules. Hemichannels, like gap junction channels, seem to be passive, energy independent, and less selective with regard to the charge of the substrate (Goodenough and Paul, 2003), thus

probably permitting rapid release of anionic PGE₂ in response to mechanical stimulation.

The present study provides evidence that hemichannels play an important role in osteocyte function and response to mechanical strain under physiological conditions. It provides a solution to a long-standing, important issue of how the highly charged, anionic prostaglandins traverse the plasma membrane and exit the cell under physiological conditions. The function of hemichannels is likely to offer a general mechanism for the rapid exit of not just prostaglandins, but presumably other extracellular regulatory factors, when cells are activated and challenged by physiological stimuli, such as mechanical stimulation.

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