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OSCILLATING FLUID FLOW ACTIVATION OF GAP JUNCTION HEMICHANNELS INDUCES ATP RELEASE FROM MLO-Y4 OSTEOCYTES

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Abstract

Mechanical loads are required for optimal bone mass. One mechanism whereby mechanical loads are transduced into localized cellular signals is strain-induced fluid flow through lacunae and canaliculi of bone. Gap junctions (GJ) between osteocytes and osteoblasts provides a mechanism whereby flow-induced signals are detected by osteocytes and transduced to osteoblasts. We have demonstrated the importance of GJ and gap junctional intercellular communication (GJIC) in intracellular calcium and prostaglandin E₂ (PGE₂) increases in response to flow. Unapposed connexons, or hemichannels, are themselves functional and may constitute a novel mechanotransduction mechanism. Using MC3T3-E1 osteoblasts and MLO-Y4 osteocytes, we examined the time course and mechanism of hemichannel activation in response to fluid flow, the composition of the hemichannels, and the role of hemichannels in flow-induced ATP release. We demonstrate that fluid flow activates hemichannels in MLO-Y4, but not MC3T3-E1, through a mechanism involving protein kinase C, which induces ATP and PGE₂ release.

Keywords

ATP; hemichannel; connexin 43; mechanotransduction; bone; osteocyte; protein kinase C; osteoblast

INTRODUCTION

Maintenance of appropriate skeletal integrity requires biophysical signals that are induced by physical activity (Rubin, 1984; Rubin and Lanyon, 1984). Numerous studies have demonstrated that increased loads promote the activation of osteoblasts (Buckley et al., 1988) and osteocytes (Pead et al., 1988) and can increase skeletal mass through bone formation, whereas decreased loads, as occur during limb immobilization (Minaire et al., 1974; Uthoff and Jaworski, 1978; Wronski and Morey, 1983), promote bone resorption and loss of skeletal mass. One mechanism whereby an external mechanical load can be translated into a localized cellular signal is through load-induced fluid flow through the canaliculi and lacunae of bone

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(Cowin et al., 1991; Kufahl and Saha, 1990; Weinbaum et al., 1994). Accumulating evidence suggests that osteocytes, the most abundant cell type in bone, are best situated to detect load-induced fluid flow (Burger et al., 1995; Cowin et al., 1991; Duncan and Turner, 1995; Klein-Nulend et al., 1995). However, there is little evidence that osteocytes can either form or resorb bone. Therefore, it has been suggested that osteocytes communicate load-induced signals, such as fluid flow, to effector cells, *i.e.* osteoblasts and osteoclasts (Burger et al., 1995; Donahue, 2000; Duncan and Turner, 1995). The mechanism by which this occurs is poorly understood. One mechanism by which osteocytes may communicate with other bone cells is via gap junctional intercellular communication (GJIC) (Donahue, 2000; Taylor et al., 2006).

Gap junctions (GJ) are membrane-spanning channels, composed of connexin (Cx) subunits, that allow rapid and direct diffusion of small molecules (<1kDa), such as ionized calcium (Ca^{2+}), inositol phosphates and cyclic nucleotides, from the cytosol of one cell to the cytosol of another cell. A functional gap junction is composed of a connexon, or hemichannel, in each membrane of adjacent cells that pair and fuse to form a functional gap junction. Both *in vivo* and *in vitro* studies have demonstrated that gap junctions exist between osteocytes and osteoblasts (Doty, 1981; Duncan and Turner, 1995; Jeansonne et al., 1979; Palumbo et al., 1990; Yellowley et al., 2000). It has been demonstrated that GJIC contributes to bone cell responsiveness to a diverse array of extracellular signals, including parathyroid hormone (Vander Molen et al., 1996), electromagnetic fields (Vander Molen et al., 2000), and fluid flow (Alford et al., 2003; Jorgensen et al., 1997; Saunders et al., 2001; Saunders et al., 2003).

Typically, studies pertaining to gap junctions and their constituent connexin composition have focused on GJIC. Recently, however, data from other cell lineages has demonstrated surface expression and functional activity of nonjunctional, or unapposed, connexon hemichannels (Contreras et al., 2002; Hofer and Dermietzel, 1998; Saez et al., 2003; Stout et al., 2002). Studies examining whether hemichannels exist in cells of the osteoblastic lineage, and whether they function in a similar manner, have produced inconsistent results. Jorgensen *et al.* were unable to detect functional hemichannels in osteoblastic UMR 106-01 cells over-expressing Cx43 or in ROS 17/2.8, and only a small percentage of primary human osteoblastic cells expressed functional hemichannels under low extracellular Ca^{2+} conditions (Jorgensen et al., 2003). In contrast, Romanello *et al.* demonstrated that transformed human osteoblastic (HOBIT) cells express hemichannels that allow the cellular uptake of Lucifer Yellow and inositol 1,4,5-triphosphate from the extracellular environment (Romanello and D'Andrea, 2001), and Plotkin *et al.* reported that alendronate activates hemichannels in osteoblastic ROS 17/2.8 cells (Plotkin et al., 2002).

Unlike the conflicting evidence of hemichannel activity in osteoblastic cells, studies examining hemichannels in osteocytic cells have produced more consistent results. Plotkin *et al.* demonstrated alendronate activation of hemichannels in osteocytic MLO-Y4 cells (Plotkin and Bellido, 2001; Plotkin et al., 2002). Additionally, it has recently been shown that fluid flow activates hemichannels in MLO-Y4 osteocytes by increasing the insertion of Cx43 hemichannels in the plasmalemma, and that these hemichannels are responsible for flow-induced increases in PGE_2 release (Jiang and Cherian, 2003). However, in these studies, cells were exposed to steady fluid flow, which osteocytes are unlikely to experience *in vivo* in response to mechanical load (Jacobs et al., 1998). Therefore, we examined whether osteocytic MLO-Y4 express functional hemichannels that are activated by oscillating fluid flow, a more physiologically relevant mechanical signal that osteocytes are more likely to experience *in vivo*, and the mechanism underlying this activation. We also examined whether osteotropic molecules other than PGE_2 , such as ATP, are released by MLO-Y4 osteocytes in response to oscillating fluid flow.

Our results demonstrate that physiologically relevant oscillating fluid flow rapidly activates hemichannels through a mechanism involving protein kinase C, and this promotes ATP release;. Additionally, ablation of Cx43 using siRNA strategies abrogated flow-induced hemichannel activation and ATP release, implicating Cx43 as a major component of osteocytic hemichannels. The addition of ATP to osteocytes treated with the hemichannel inhibitor AGA demonstrated PGE₂ release, suggesting that PGE₂ is not directly released through hemichannels but is instead under the control of an upstream factor, possibly ATP, that is itself released through hemichannels. These data demonstrate that hemichannels formed by Cx43 are activated in response to oscillatory fluid flow and are responsible for ATP release in MLO-Y4 osteocytic cells.

MATERIALS AND METHODS

Cell culture

Osteocytic MLO-Y4 cells (kindly provided by Dr. Lynda F. Bonewald, Department of Oral Biology, University of Missouri at Kansas City School of Dentistry, Kansas City, MO) were cultured on 75×38mm glass slides coated with rat tail type I collagen (150µg/mL in 0.02N acetic acid; Becton-Dickson) in alpha-modified essential medium (α-MEM; Gibco BRL) containing 5% fetal bovine serum (FBS; Hyclone), 5% calf serum (CS), and 1% penicillin and streptomycin (P/S; Gibco BRL). MC3T3-E1 cells were purchased from ATCC and maintained in α-MEM, 10% FBS, and 1% P/S. Cells were plated at a low density of 900 cells/cm² to ensure minimal cell-cell contact on the day of the experiment (2 days post-seeding). At all times, cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Oscillating fluid flow

Cells were exposed to oscillating fluid flow (20 dynes/cm², 1Hz) for 5 or 15 minutes as described previously (Jacobs et al., 1998). Flow media consisted of α-MEM with 1% FBS, 1% CS, and 1% P/S for MLO-Y4 cells, and 2% FBS and 1% P/S for MC3T3-E1 cells. Control slides were similarly placed in parallel plate flow chambers, but not exposed to oscillating fluid flow. The flow rate was monitored with an ultrasonic flow meter (Transonic systems, Ithaca, NY) during all experiments. PGE₂ release studies were performed in reduced serum media (0.2% each of FBS and CS) to minimize the effects of serum, and were performed for 30 minutes.

Dye Uptake Assay

Hemichannel activity was monitored by Lucifer Yellow (LY; 1mg/mL; Sigma-Aldrich) dye uptake, as described previously (Jiang and Cherian, 2003; Plotkin and Bellido, 2001). Briefly, Lucifer Yellow is plasmalemma-impermeant but small enough (M_r, 547 Da) to traverse GJs and GJ hemichannels. Rhodamine dextran 10kDa (1mg/mL; Sigma-Aldrich) was used as a negative control, as its larger molecular weight precludes passage through hemichannels under physiologic conditions; Hoechst 33258 (5µg/mL from 10mg/mL stock in PBS) was added as a nuclear stain to label all cells. Dye solutions were dissolved in flow media and added at the start of the assay. Experiments were performed in the presence of the gap junction and hemichannel antagonist 18α-glycyrrhetinic acid (AGA; 30µM; Sigma-Aldrich) or vehicle control (DMSO; 0.1% v/v). Further experiments were performed in the presence of specific antagonists of PKA (2µM H-89; Calbiochem), PKC (1µM GF 109203X; Biomol), and MEK1/2 (10µM U0126; Cell Signaling Technologies) (Cherian et al., 2003; Datta et al., 2005; Toullec et al., 1991), or appropriate vehicle control. At the conclusion of the experiment, cells were removed from their flow chamber, washed briefly in HBSS, and fixed in 4% paraformaldehyde. Fluorescence was monitored using a Nikon fluorescent microscope (Nikon EFD-3; Optical Apparatus) and visualized using appropriate filters. Data from dye uptake experiments are presented as the number of LY-positive cells divided by the total number of cells within a

randomly chosen field of view. Cells staining positively for rhodamine dextran uptake were not analysed. A minimum of 16 fields of view were imaged for dye uptake experiments.

Quantification of ATP release

ATP levels in conditioned media from cells exposed to oscillating fluid flow or chamber controls were quantified using a luciferin-luciferase reaction (ATP Bioluminescence Assay Kit HS II; Roche) as described previously (Genetos et al., 2005; Ostrom et al., 2000). After exposure to oscillating fluid flow or static control, 1 mL of conditioned media was collected from the inlet and outlet ports of the flow chamber and centrifuged at 10,000 rpm for 1 minute to pellet any cellular debris. The supernatant was then transferred to a new tube and stored at -80°C. After isolation of conditioned media, the slide was briefly washed twice in ice-cold PBS, lysed in 50µL of lysis buffer, and frozen at -80°C until protein concentrations were determined. ATP levels were normalized to total cellular protein for each slide.

PGE₂ release

PGE₂ levels in conditioned media from cells exposed to oscillating fluid flow or chamber controls were quantified using a commercially available ELISA (Amersham Biosciences) as described previously (Genetos et al., 2005). After exposure to oscillating fluid flow or static control, 1 mL of conditioned media was collected from the inlet and outlet ports of the flow chamber and centrifuged at 10,000 rpm for 1 minute to pellet any cellular debris. The supernatant was then transferred to a new tube and stored at -80°C. After isolation of conditioned media, the slide was briefly washed twice in ice-cold PBS, lysed in 25µL of lysis buffer, and frozen at -80°C until protein concentrations were determined. ATP levels were normalized to total cellular protein for each slide.

siRNA-mediated ablation of Cx43

An siRNA construct (AAGTGTGTAAGCGTGTGTTTT) against murine Cx43 (GenBank™ accession no. **NM_010288**) was generated using the Qiagen siRNA Design Tool (www.qiagen.com); the target construct was searched with NCBI BlastN to confirm specificity to Cx43. A pre-designed non-silencing (scrambled) siRNA construct was used as a negative control for transfection studies. Delivery of scrambled or Cx43 siRNA into cells was performed using RNAiFect Reagent (Qiagen). 24 hours later, cells were sub-cultured onto 75×38mm slides and used 48 hours later for Western blotting or flow experiments.

Western immunoblotting

Cells were washed twice in phosphate-buffered saline (PBS) and lysed in 0.1% Triton X-100, 10 mM Tris pH 8, 1 mM EDTA, 200 nM Na₃VO₄, and protease inhibitor cocktail (Calbiochem). 25µg of total cellular protein was separated on 10% SDS gel (Gradipore) and transferred to PVDF membrane (Bio-rad). Cx43 protein expression in scrambled siRNA-transfected, and Cx43 siRNA-transfected cells was examined with a monoclonal anti-Cx43 antibody from Chemicon and visualized using enhanced chemiluminescent detection (Amersham Biosciences); membranes were then stripped and re-probed for GAPDH (Accurate Scientific & Chemical Corp.) to confirm equal loading of all lanes.

Statistical analysis

All data are presented as mean ± SE. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons tests were used to compare groups using Prism (GraphPad Software). As there was no significant difference in the percentage of MLO-Y4 demonstrating dye uptake between 5 and 15 minutes of oscillating fluid flow, these data were averaged. P < 0.05 was considered statistically significant.

RESULTS

Oscillating fluid flow activates hemichannels in MLO-Y4 osteocytes but not MC3T3-E1 osteoblasts

Initial experiments were performed to examine hemichannel activation in response to oscillating fluid flow in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes. Hemichannel activation was monitored by cellular uptake of the hemichannel-permeant dye Lucifer Yellow (LY). Exposure to oscillating fluid flow for 5 minutes increased LY uptake compared to static controls in MLO-Y4 osteocytes but not in MC3T3-E1 osteoblasts (Figure 1A). There was no significant difference in the number of LY-positive MLO-Y4 cells between 5 minute and 15 minute flow bouts (data not shown). The addition of the gap junctional and hemichannel antagonist 18 α -glycyrrhetic acid (AGA; 30 μ M) significantly attenuated LY uptake in response to flow (Figure 1A), compared to cells flowed in the presence of vehicle control. Experiments performed in the presence of the ATP-degrading enzyme apyrase similarly demonstrated LY uptake in response to 5 minutes of fluid flow, indicating that activation of P2 receptors is not involved in LY uptake at the time points examined (data not shown).

We next examined whether a reduction in extracellular calcium concentration, a known, non-physiologic activator of hemichannels (Li et al., 1996), produced similar results. The addition of 5mM EGTA to the experimental media resulted in LY uptake in MLO-Y4 osteocytic cells but not in MC3T3-E1 osteoblastic cells (Figure 1B). As in response to oscillating fluid flow, the addition of 30 μ M AGA to EGTA-treated osteocytes significantly impaired LY uptake, indicating that hemichannel activation is responsible for the staining patterns observed (Figure 1B). For this reason, we used MLO-Y4 osteocytes exclusively for the remaining experiments.

PKC activity is required for flow-induced hemichannel activity

Whereas connexon phosphorylation regulates gap junction localization and conductance (Alford et al., 2003; Lampe and Lau, 2000; Lampe et al., 2000), pharmacologic inhibitors were used to examine the effect of connexin phosphorylation on hemichannel activation. Treatment with GF 109203X, an inhibitor of protein kinase C (Toullec et al., 1991), significantly attenuated flow-induced dye uptake (Figure 2), whereas inhibitors of protein kinase A (H-89) (Cherian et al., 2003) and MEK1/2 (U0126) (Datta et al., 2005) had no effect on dye uptake. In a subset of experiments, the addition of the phorbol ester PMA (100nM or 1 μ M for 10 minutes) failed to increase LY uptake in static cells (data not shown), suggesting that PKC activation alone is not sufficient for hemichannel opening. Further, there was no additive effect on dye uptake for MLO-Y4 cells exposed to fluid flow in the presence of 100nM PMA, suggesting maximal activation of hemichannels by fluid flow (data not shown).

siRNA-directed decreases in Cx43 attenuate flow-induced hemichannel activation

Introduction of siRNA directed against Cx43 lowered Cx43 protein levels compared to scrambled siRNA-transfected osteocytes (Figure 3A), demonstrating the efficacy of the siRNA. Similar to pharmacologic inhibition of hemichannel activity with AGA (Figure 1A) and GF 109203X (Figure 2), Cx43 siRNA transfection decreased dye uptake in response to flow compared to scrambled siRNA-transfected osteocytes (Figure 3B).

Hemichannel activity is required for flow-induced ATP release

Conditioned media from MLO-Y4 osteocytes exposed to oscillating fluid flow contained more ATP than conditioned media samples from static controls (Figure 4A). ATP levels in fresh flow media were below the detectable limits of the assay (<10pM), demonstrating that flow media does not contribute significantly to the measured ATP from the conditioned media (data not shown). Inhibition of hemichannel activity through the use of pharmacological agents AGA

or PKC antagonist GF109203X significantly attenuated flow-induced ATP release compared to appropriate controls (Figure 4B). Similarly Cx43 siRNA-treated cells demonstrated no significant increase in ATP release in response to oscillating fluid flow, whereas scrambled siRNA-treated cells did (Figure 4C).

Oscillating fluid flow-induced PGE₂ release in osteocytes involves P2 receptor activation

The addition of ATP to static osteocytes dose-dependently increased PGE₂ release, implicating P2 purinoceptor activation in this process (Figure 5A). The addition of AGA significantly impaired oscillating fluid flow-induced PGE₂ release compared to cells flowed in the presence of vehicle (Figure 5B), yet PGE₂ release was restored with the addition of exogenous ATP, suggesting that PGE₂ release can occur in the presence of hemichannel impairment.

DISCUSSION

Appropriate skeletal architecture is maintained, in part, by mechanical loading. Loading induces multiple mechanical signals at both the tissue and cellular level, among them substrate strain, streaming potentials, and fluid shear. The distribution of osteocytes within canaliculi and lacunae suggests a role for osteocytes as mechanosensory cells that translate physical signals into appropriate biochemical responses in osteoblasts. Communication from osteocyte to osteoblast could involve the release of paracrine factors such as ATP, NO, or PGE₂, or the direct transmission of a chemical or electrical signal through gap junctions and GJIC. Indeed, we (Alford et al., 2003; Saunders et al., 2001; Yellowley et al., 2000), and others (Cheng et al., 2001; Jiang and Cherian, 2003; Jorgensen et al., 1997; Thi et al., 2003; Xia and Ferrier, 1992), have demonstrated the importance of gap junctions in mediating cellular responsiveness to fluid flow. The discovery that unapposed gap junctions, or hemichannels, are biologically active (Bruzzone et al., 2001; Coco et al., 2003; Contreras et al., 2003; Contreras et al., 2002; Jiang and Cherian, 2003; Plotkin et al., 2002) presents two possible mechanisms wherein connexons mediate cellular responsiveness: functioning either as hemichannels that provide a direct link from the cytosol to the extracellular environment, or by increasing GJIC between coupled cells. Unfortunately, glycyrrhetic acid derivatives inhibit both GJIC (Davidson and Baumgarten, 1988; Davidson et al., 1986) and hemichannels (Contreras et al., 2003), making it challenging to detect the contribution of GJIC versus hemichannels towards a given response in nearly-confluent cell monolayers. In order to differentiate between the two possible roles of a connexon (gap junction or hemichannel), our experiments were performed at extremely sub-confluent levels, thereby allowing us to minimize the role of GJIC independently of hemichannel activity.

Our results demonstrate that exposure of MLO-Y4 osteocytes, but not MC3T3-E1 osteoblasts, to oscillating fluid flow promotes uptake of Lucifer Yellow (Figure 1A), a fluorophore whose small molecular weight allows for passage through a connexon. Addition of AGA, an inhibitor of both GJIC and hemichannels, significantly attenuated dye uptake in response to flow (Figure 1A). The culture conditions used in this study, which prevented significant cell-cell contact and therefore GJIC, allow us to conclude that connexons functioning as hemichannels are mediating dye uptake. Additionally, the absence of rhodamine dextran uptake further confirms dye uptake through connexons and not an unrelated cellular process, such as endocytosis or plasmalemmal disruption, in response to flow. The effect of flow on dye uptake in MLO-Y4 osteocytes is consistent with other reports on fluid flow and hemichannels in osteocytes (Cherian et al., 2005; Jiang and Cherian, 2003). Further, we found no significant difference in the percentage of LY-positive cells for flow durations of 5 and 15 minutes, suggesting that, within this time frame, the effect of flow on hemichannel function was independent of the duration of exposure. In contrast to our results reported herein, Li *et al.* demonstrated dye uptake through the P2X₇ receptor in response to fluid shear stress that was inhibited by apyrase

(Li et al., 2005), whereas we report that apyrase did not inhibit fluid flow-induced Lucifer Yellow uptake. One potential explanation for this difference is that their study utilized unidirectional fluid flow whereas our study used oscillatory fluid flow, and that these different flow regimes may differentially regulate signaling cascades (You et al., 2001). Alternately, the differences could be explained by the duration of exposure to fluid shear, with hemichannels involved in early (5-15 minutes) dye uptake, and P2X₇ receptors involved in later dye uptake. That we have implicated hemichannels in ATP release supports this hypothesis.

To date, over 20 connexons have been described and a subset of these—Cx43 (Schirrmacher et al., 1992), 45 (Steinberg et al., 1994), and 46 (Koval et al., 1997)—are expressed in cells of the osteoblastic lineage. We sought to define the connexin composition of hemichannels by using small interfering RNA (siRNA) to selectively ablate connexin expression. We postulated that the presence of flow-activated hemichannels in osteocytes but not osteoblasts (Figure 1A) was mediated by Cx43, as it has been reported that osteocytes express significantly higher Cx43 mRNA (Yellowley et al., 2000) and protein (Bonewald, 1999; Kato et al., 1997) than do osteoblasts (Bonewald, 1999; Kato et al., 1997; Yellowley et al., 2000), including MC3T3-E1 cells (Yellowley et al., 2000). Furthermore, we found that neither MLO-Y4 nor MC3T3-E1 cells express detectable Cx45 or Cx46 (unpublished data) and previous studies demonstrate that Cx46 is retained as a monomer in osteoblastic cells and does not form functional gap junctions (Koval et al., 1997). Therefore in these studies we focused on Cx43. Using siRNA strategies, we found impaired dye uptake in Cx43 siRNA-transfected osteocytes relative to scrambled siRNA-transfected osteocytes (Figure 3B), suggesting that, at the minimum, at least some of the hemichannels activated by oscillating fluid flow are composed of Cx43.

Contrary to our findings in an osteoblast cell line, one report demonstrates the presence of hemichannels in osteoblast-like cells exposed to low extracellular calcium conditions (Romanello and D'Andrea, 2001). Our results are, however, consistent with those from Jorgensen *et al.*, wherein neither UMR-106.01, ROS 17/2.8, nor primary osteoblasts demonstrated EGTA-activated hemichannels (Jorgensen et al., 2002). Further, the absence of flow-activated hemichannels in MC3T3-E1 osteoblasts is consistent with our previously published report on ATP release in MC3T3-E1 osteoblasts, wherein AGA had no effect on ATP release in response to flow (Genetos et al., 2005).

The carboxy terminal tail of Cx43 contains several consensus phosphorylation sites for PKA, PKC, and MAPK (reviewed in (Lampe and Lau, 2000)). Because oscillating fluid flow-induced dye uptake was inhibited by the GJ and hemichannel antagonist AGA, and because we have previously demonstrated that oscillating fluid flow increases GJIC through ERK1/2 (Alford et al., 2003), we investigated the hypothesis that hemichannel opening requires Cx43 phosphorylation. Thus, the effects of PKA, PKC, and MEK1/2 (immediate upstream activator of MAP kinases) antagonists on flow-induced dye uptake were determined. Inhibition of PKC activity with GF 109203X, but not PKA or MEK1/2 antagonism, prevented dye uptake in response to flow (Figure 2). These results were surprising, since many reports have correlated PKC activation by phorbol esters with a decrease in GJIC. *In vitro*, PKC has been shown to phosphorylate two Cx43 serine residues, Ser368 and Ser372. Phosphorylation of Ser368 decreases the frequency of a ~100pS channel population while concomitantly increasing the frequency of a ~50pS channel population; cells expressing a Ser368Ala Cx43 mutant demonstrate no change in the frequency of the ~100pS population in response to TPA addition (Lampe et al., 2000). However, there are also reports of PKC increasing gap junctional conductance (Kwak et al., 1995). Additionally, these, and other, studies have examined the effect of Cx43 phosphorylation on GJIC, and not hemichannel activation. Insofar as a connexon may function as a gap junction or as a hemichannel, it is possible that phosphorylation of a given residue could differentially regulate GJIC and hemichannel activity. Finally, it is possible

that different kinases are required for acute (PKC) versus sustained (MEK1/2) changes in connexon conductance.

There is a growing recognition that ATP functions as an extracellular signaling molecule, by activating P2 purinoceptors and mediating numerous cellular processes (Brambilla and Abbracchio, 2001; Dezaki et al., 2000; Gerasimovskaya et al., 2002). We have previously demonstrated that fluid flow promotes ATP release in MC3T3-E1 osteoblasts (Genetos et al., 2005) and that P2 receptor activation is required for oscillating fluid flow-induced transients in cytosolic calcium (You et al., 2002). Based on the size and charge of ATP, one candidate pathway wherein ATP may be released from the cytosol to the pericellular space is through connexon hemichannels formed by Cx43. Indeed, C6 glioma cells transfected with Cx43 demonstrated increased ATP permselectivity compared to cells transfected with Cx32 (Goldberg et al., 1999; Goldberg et al., 1998). Additionally, ATP release in response to mechanical load in chondron pellets was inhibited by AGA (Graff et al., 2000), implicating connexons in load-induced ATP release. We found that oscillating fluid flow significantly increased ATP levels in conditioned media approximately three-fold compared to conditioned media from static cells (Figure 4A), and this increase was significantly attenuated in the presence of pharmacologic inhibitors of hemichannel activation (Figure 4B). Additionally, osteocytes transfected with Cx43 siRNA released less ATP than did scrambled siRNA-transfected osteocytes. Interestingly, there was a trend, albeit not statistically significant, for AGA to decrease ATP release in MLO-Y4 cells under static conditions. This would suggest that there may be active hemichannels and ATP release in the absence of flow. Interestingly, these data indicate that the mechanism for ATP release is dependent upon differentiation state, with osteoblasts releasing ATP from vesicles and osteocytes utilizing hemichannels. From these data, one may hypothesize that a given response (*i.e.*, an increase in Ca^{2+} or ATP release) is engendered via distinct signaling cascades. This hypothesis is supported by data indicating that osteoblasts, but not osteocytes, express the L-type voltage-sensitive calcium channel, whereas osteocytes express higher levels of connexin 43 than do osteoblasts (Bonewald, 1999; Kato et al., 1997; Yellowley et al., 2000).

A previous report on flow-activated hemichannels in osteocytes demonstrated that AGA or Cx43 siRNA attenuated PGE_2 release in response to flow (Cherian et al., 2005). Addition of DIDS, a non-specific anion channel inhibitor, or oxidized ATP, a P2X_7 receptor inhibitor, had no effect on flow-induced PGE_2 release. From these data, the authors concluded that PGE_2 is directly released through Cx43 hemichannels. In contrast, our data indicate that exogenous ATP increases PGE_2 release in static osteocytes (Figure 5A). These data suggest that PGE_2 release is mediated by activation of P2 receptors, as in MC3T3-E1 osteoblasts (Genetos et al., 2005), and that PGE_2 release can occur independently of hemichannel formation. Secondly, we report that the addition of exogenous ATP to AGA-treated (*i.e.*, hemichannel-inhibited) osteocytes rescued PGE_2 release from the inhibitory effects of AGA alone. We posit that the inhibition of PGE_2 release with AGA or Cx43 siRNA, as reported by Jiang *et al.* (Jiang and Cherian, 2003) and Cherian *et al.* (Cherian et al., 2003) is not entirely due to the inhibition of direct PGE_2 flux through a hemichannel, but, rather, is partially due to inhibition of an upstream signaling factor, such as ATP.

In conclusion, our data demonstrate that physiologically relevant fluid flow rapidly induces the opening of hemichannels that are responsible for ATP release in osteocytes. Reduction in Cx43 expression through siRNA strategies indicate that Cx43 is the predominant connexin composing osteocytic hemichannels, as Cx43 siRNA decreased both LY uptake and ATP release in response to oscillatory fluid flow. As a whole, these data suggest that oscillating fluid flow promotes Cx43 hemichannel activation and ATP efflux from the cytosol to the pericellular environment. Provided the expanding knowledge of ATP as an osteotropic factor

(Genetos et al., 2005; Ke et al., 2003; Li et al., 2004), these data support the emerging importance of Cx43 hemichannels in bone cell mechanotransduction.

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Figure 1.

Lucifer Yellow dye uptake through hemichannels occurs in MLO-Y4 osteocytes but not MC3T3-E1 osteoblasts. (A) Quantitation of Lucifer Yellow dye uptake in response to oscillatory fluid flow in MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. MLO-Y4 osteocytes exposed to oscillatory fluid flow take up extracellular Lucifer Yellow that is inhibited in the presence of the hemichannel antagonist AGA. In contrast, MC3T3-E1 osteoblasts exposed to oscillatory fluid flow do not take up extracellular Lucifer Yellow, and the hemichannel antagonist AGA has no effect on dye uptake. Data are presented as the mean percentage of

cells staining positively for Lucifer Yellow divided by the total number of cells, visualized by Hoechst 33258, within a field of view. Data were obtained from 4 independent experiments. (**a**: $p < 0.01$ compared to static MLO-Y4; **b**: $p < 0.01$ compared to MLO-Y4 exposed to oscillatory fluid flow). (B) Quantitation of Lucifer Yellow dye uptake in response to 5mM EGTA treatment in static MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. Lucifer yellow dye uptake and hemichannel activation in response to oscillatory fluid flow is mimicked in static cells treated with 5mM EGTA. MLO-Y4 osteocytes treated with 5mM EGTA take up extracellular Lucifer Yellow that is inhibited in the presence of the hemichannel antagonist AGA. MC3T3-E1 osteoblasts treated with 5mM EGTA do not take up extracellular Lucifer Yellow, and the hemichannel antagonist AGA has no effect on dye uptake. Data are presented as the mean percentage of cells staining positively for Lucifer Yellow divided by the total number of cells, visualized by brightfield imaging. Data were obtained from three independent experiments. (**a**: $p < 0.01$ compared to static MLO-Y4; **b**: $p < 0.01$ compared to MLO-Y4 +EGTA).

Figure 2.

Oscillating fluid flow-induced dye uptake is mediated by PKC activity. MLO-Y4 osteocytes were incubated in the presence of inhibitors of PKA (H-89, 2 μ M), PKC (GF 109203X, 1 μ M), or MEK1/2 (U0126, 10 μ M) and exposed to oscillatory fluid flow as in Figure 1. Lucifer Yellow dye uptake in response to oscillatory fluid flow was significantly attenuated for cells pre-treated with the PKC inhibitor GF 109203X. Neither inhibitors of PKA nor MEK1/2 affected static or oscillatory fluid flow-induced Lucifer Yellow uptake. Data were obtained from 3 independent experiments. (a: $p < 0.001$ compared to appropriate static condition).

Cx43 **GAPDH** **Scrambled****Cx43 siRNA**

Figure 3.

Cx43 siRNA decreases Cx43 protein expression and inhibits oscillatory fluid flow-induced dye uptake. (A) Western blotting of Cx43 protein levels demonstrated a reduction in Cx43 expression in MLO-Y4 cells treated with Cx43 siRNA, but not scrambled, non-silencing siRNA. Membranes were stripped and re-probed for GAPDH as a loading control. (B) Hemichannel activation, assessed by LY dye uptake, was significantly attenuated in response to flow in osteocytes transfected with Cx43 siRNA but not in scrambled siRNA-transfected osteocytes. Data were obtained from 3 independent experiments. (a: $p < 0.05$ to static scrambled siRNA).

Figure 4.

ATP release in MLO-Y4 osteocytes in response to oscillatory fluid flow requires hemichannel activation and Cx43 expression. (A) Oscillating fluid flow significantly increased ATP content in conditioned media relative to static controls. Data were obtained from 4 independent experiments. (a: $p < 0.05$ compared to static). (B) Pharmacologic inhibitors of hemichannel activation prevented significant increases in ATP release in response to oscillating fluid flow. Data were obtained from 3 and 2 independent experiments for AGA and GF109203X treatment, respectively. (a: $p < 0.05$ compared to static). (C) Transfection of MLO-Y4 osteocytes with Cx43 siRNA significantly attenuated ATP release in response to oscillating fluid flow compared to cells transfected with scrambled siRNA. Data were obtained from 4 independent experiments. (a: $p < 0.05$ compared to static).

Figure 5. Purinoceptor activation increases PGE₂ release independent of hemichannel formation. (A) Static osteocytes dose-dependently release PGE₂ in response to treatment with exogenous ATP. Data were obtained from 3 independent experiments (**a**: $p < 0.05$ compared to control; **b**: $p < 0.01$ compared to control). (B) Addition of exogenous ATP rescues the inhibitory effect of AGA on oscillatory fluid flow-induced PGE₂ release. Data were from 5 independent experiments and are presented as fold-change in PGE₂ release compared to static control. (**a**: $p < 0.05$ compared to static control).