Published in final edited form as:

Exp Cell Res. 2011 April 1; 317(6): 737–744. doi:10.1016/j.yexcr.2011.01.007.

PURINERGIC SIGNALING IS REQUIRED FOR FLUID SHEAR STRESS-INDUCED NF- κ B TRANSLOCATION IN OSTEOBLASTS

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Abstract

Fluid shear stress regulates gene expression in osteoblasts, in part by activation of the transcription factor NF- κ B. We examined whether this process was under control of purinoceptor activation. MC3T3-E1 osteoblasts under static conditions expressed the NF- κ B inhibitory protein I κ B α and exhibited cytosolic localization of NF- κ B. Under fluid shear stress, I κ B α levels decreased, and concomitant nuclear localization of NF- κ B was observed. Cells exposed to fluid shear stress in ATP-depleted medium exhibited no significant reduction in I κ B α , and NF- κ B remained within the cytosol. Similar results were found using oxidized ATP or Brilliant Blue G, P2X₇ receptor antagonists, indicating that the P2X₇ receptor is responsible for fluid shear-stress-induced I κ B α degradation and nuclear accumulation of NF- κ B. Pharmacologic blockage of the P2Y₆ receptor also prevented shear-induced I κ B α degradation. These phenomena involved neither ERK1/2 signaling nor autocrine activation by P2X₇-generated lysophosphatidic acid. Our results suggest that fluid shear stress regulates NF- κ B activity through the P2Y₆ and P2X₇ receptor.

Keywords

Osteoblast; Mechanotransduction; Purinergic; NF- κ B; P2Y₆; P2X₇; Lysophosphatidic acid; ERK1/2

INTRODUCTION

The mammalian skeleton demonstrates a tremendous capacity for functional adaptation to mechanical forces. For example, conditions of reduced skeletal loads promote bone resorption to minimize unnecessary energy expenditure [1], while increased skeletal loading promotes bone formation to minimize stress and/or strain [2]. Fluid shear stress (FSS) has

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been presented as a localized biophysical signal generated in response to dynamic loading [3-5], and *in vitro* data demonstrate that FSS has robust effects on osteoblast proliferation, differentiation, and survival (reviewed in [6]).

The influence of FSS on alterations in osteoblast metabolism involve activation of a variety of mechanosensory protein complexes, such as integrins and focal adhesions [7-9], primary cilia [10], gap junctions and/or gap junction hemichannels [11-13], and mechanosensitive ion channels [14-16]. We have demonstrated that much of the capacity for osteoblastic cells to respond to FSS requires release of ATP and subsequent activation of ATP-binding P2 purinoceptors [13,17,18]. P2 receptors are present in nearly all cell types, and induce cellular responses *via* G-protein activation (in the case of metabotropic P2Y) or ion flux (ionotropic P2X). Within osteoblastic cells, P2Y₂ receptors have been implicated in the intracellular Ca²⁺_i response of osteoblastic cells to FSS [17], and mice deficient in P2X₇ demonstrate a markedly reduced anabolic response to mechanical loading [19] and impaired fracture healing [20].

We have demonstrated that prostaglandin E₂ (PGE₂) synthesis or release requires purinergic signaling [21], and that the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is required for maximal cyclooxygenase-2 (COX-2) induction in response to FSS [22]. Cytosolic sequestration of NF-κB is achieved by its binding to the inhibitory protein, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), which masks the nuclear localization signal of NF-κB. Targeted degradation of IκBα by the 26S proteasome allows for nuclear accumulation and transcriptional activity of NF-κB. In this study, we determined whether NF-κB signaling in osteoblastic cells under FSS is under purinergic control and, if so, which purinoceptors are responsible. As the mechanism of P2X₇ action in osteoblasts is partially mediated by the synthesis and autocrine function lysophosphatidic acid (LPA) [23], we also sought the role of LPA in FSS-induced NF-κB activation.

MATERIALS AND METHODS

Cell culture

MC3T3-E1 osteoblasts were cultured as described previously [24]. Because these cells exist as subclones with varying differentiation potential *in vitro* [25], experiments were performed upon three different subclones; similar results were obtained with each subclone. Cells were seeded at density of 2800 cells/cm² upon 75x38mm glass slides coated with fibronectin (10μg/mL; Becton Dickson). Two days thereafter, when cells were 75–90% confluent, medium was removed and replaced with reduced serum medium consisting of αMEM, 1% penicillin/streptomycin, 0.5% FBS, and 10mM HEPES, pH 7.2. Fluid shear stress experiments were performed on the following day in the same medium. For static experiments, cells were cultured in 60mm dishes but were otherwise managed in an identical fashion.

Fluid shear stress and static experiments

FSS was applied to cells in a parallel plate flow chamber using a closed flow loop, as described previously [26]. Flow was applied for 45 minutes, after which slides were fixed for immunocytochemical analysis or lysed in 0.1% Triton X-100, 10mM Tris pH 8, 1mM EDTA, 0.2mM Na₃VO₄, supplemented with a protease inhibitor cocktail (Calbiochem) for Western immunoblotting. For static experiments, cells were collected in lysis buffer after 15 or 45 minutes of agonist treatment.

Pharmacologic agents

The P2X₇ antagonists oxidized ATP (oATP, adenosine 5'-triphosphate-2',3'-dialdehyde; Sigma) [27], which irreversibly inhibits P2X₇ function through the formation of a Schiff base with P2X₇ after prolonged incubation (>30 minutes), was dissolved in distilled water and added to cells at 300μM for three hours prior to FSS. Another P2X₇ antagonist, Brilliant Blue G (Sigma) [28,29], was added at 30μM for 45 minutes prior to FSS. The selective P2Y₆ antagonist MRS2578 (Tocris) [30] was added at 1 or 10μM to cells for 45 minutes prior to FSS. The ATP-degrading enzyme, apyrase (grade III, Sigma), was dissolved in water to a working concentration of 10U/mL and added to cells 10 minutes prior to FSS. The LPA₁ and LPA₃ receptor antagonist Ki16425 (Cayman Chemicals) was dissolved in DMSO and added at a working concentration of 5μM (0.025% DMSO v/v) to cells 15 minutes prior to FSS. The MEK1/2 antagonist U0126 was dissolved in DMSO and added at a working concentration of 10μM (0.1% DMSO v/v) to cells 15 minutes prior to FSS. All inhibitors were also present within media used during FSS. In a related set of experiments, static cultures were treated with 20ng/mL TNF-α, 1μM LPA, , 100μM of ATP, UTP, or UDP, 300μM 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) (all from Sigma), or 100μM MRS2578 (Tocris), or appropriate vehicle control for 15 or 45 minutes.

Western immunoblotting

Western immunoblotting was performed as described previously using 10μg of whole cell lysates [13]. Primary antibodies were used at the following ratios: IκBα (1:1000, Santa Cruz Biotechnologies), pERK1/2 (1:1000, Santa Cruz Biotechnologies), ERK1/2 (1:1000, Cell Signaling Technologies), pan-actin (1:3000, Sigma) or α-tubulin (1:2000, Cell Signaling).

Immunocytochemistry

The p65 subunit of NF-κB was imaged as described previously [22]. Briefly, cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and treated with 3% donkey serum to reduce non-specific binding. A primary antibody against the p65 subunit of NF-κB was applied for 1 h, followed by incubation with rhodamine-conjugated secondary antibody. Images were recorded using a Nikon Optiphot II microscope through a 60x objective (1.4 numerical aperture).

Statistical analysis

Western blots were scanned into the open-source software GNU Image Manipulation Program, and mean pixel intensities for immunoreactive bands were determined using the Histogram tool. Band intensity for IκBα was normalized to band intensity for α-tubulin or pan-actin, in order to compensate for any inconsistencies in protein loading from lane to lane. Data are presented as fold change±SEM in immunoreactivity compared to static, no-flow samples. ANOVA or Student's *t*-test were used where appropriate. *P* values less than 0.05 were considered statistically significant.

RESULTS

P2 receptor activation is required for IκBα degradation NF-κB translocation

In order to determine whether extracellular ATP and P2 receptors are implicated in NF-κB nuclear translocation in response to fluid shear stress, MC3T3-E1 cells were exposed to 12 dynes/cm² of laminar flow for 45min in the absence and presence of the ATP disphosphohydrolase, apyrase (5 or 10U/mL). The expression of IκBα relative to pan-actin in cells exposed to fluid shear stress was significantly lower compared to static controls (Fig 1A, B). In contrast, cells exposed to fluid shear stress in the presence of apyrase demonstrated no significant reduction in IκBα levels compared to static controls (Fig 1A,

B), indicating that purinergic P2 receptor activation is required for flow-mediated I κ B α degradation. Reduced degradation of I κ B α in the presence of apyrase correlated with attenuated nuclear translocation of NF- κ B: in the absence of FSS, the p65 subunit of NF- κ B demonstrated cytosolic localization (Fig 1C, top-left panel), whereas exposure to FSS induced strong nuclear localization of the p65 NF- κ B subunit (Fig 1C, top-right panel). Cells treated with apyrase (10U/mL), in the absence (Fig 1C, bottom-left panel) or presence (Fig 1C, bottom-right panel) of FSS, demonstrated cytosolic NF- κ B localization. These data indicate that purinergic receptor activation is required for FSS-induced I κ B α degradation and subsequent nuclear translocation of NF- κ B.

P2X₇R and P2Y₆R are responsible for fluid shear stress-induced NF- κ B translocation

We have previously shown that osteoblasts derived from that *P2rx7*^{-/-} mice revealed diminished COX-2 induction in response to fluid shear stress compared to osteoblasts from wild-type mice [19]. We have also demonstrated that NF- κ B translocation is required for maximal COX-2 induction in response to fluid shear stress [22]. These data, combined with the findings reported above, suggest that P2X₇R may be a pivotal P2 receptor involved in flow-mediated NF- κ B activation. To test this hypothesis, cells were treated with P2X₇R antagonists oxidized ATP (oATP) or Brilliant Blue G (BBG) [28,29,31–33]. Similar to results using apyrase, both P2X₇R antagonists oATP (300 μ M) and BBG (30 μ M) prevented flow-induced reductions in I κ B α levels (Figure 2A and 2B).

In addition to the P2X₇ receptor, the P2Y₆ receptor has also been shown to mediate NF- κ B activation in rabbit osteoclasts [34]. We have previously demonstrated that MC3T3-E1 osteoblasts express the P2Y₆ receptor, the protein expression of which did not significantly change over the course of 21-day osteogenic differentiation [35]. Cells exposed to FSS in the presence of the P2Y₆ antagonist MRS 2578 (10 μ M) also prevented shear-induced I κ B α degradation (Figure 2C), indicating that this receptor also plays a role in FSS-induced activation of NF- κ B.

We next examined whether addition of ATP or specific purinoceptor agonists to static osteoblasts was sufficient to induce nuclear translocation of NF- κ B. Cells were treated for 15 minutes with 20ng/mL TNF- α (positive control for I κ B α degradation), 100 μ M ATP (pan-purinoceptor agonist), 100 μ M UTP (P2Y agonist), 100 μ M UDP (P2Y₆ agonist), 10 μ M MRS2693 (P2Y₆ agonist), or 300 μ M BzATP (P2X₇ agonist), after which protein lysates were probed for I κ B α levels. Whereas significantly TNF- α decreased I κ B α expression, no other agonist did (Figure 2D). Similar results were found after 45 minutes, although the decrease in I κ B α in response to TNF- α was not as great as at 15 minutes (data not shown), most likely due to NF- κ B-induced I κ B α expression. Similarly, ATP at 10, 100, or 1000 μ M revealed no influence upon nuclear localization of the p65 subunit of NF- κ B after 0.5, 1, or 2 hours of treatment (data not shown). These data indicate that purinergic signaling is required, but not sufficient for nuclear translocation of NF- κ B.

P2X₇R-mediated activation of NF- κ B occurs independently of LPA signaling

Activation of the P2X₇ receptor in osteoblasts generates lysophosphatidic acid (LPA) [23], a potent hydrophilic growth factor that exerts mitogenic and chemotactic effects upon osteoblastic cells [26,36,37]. LPA also is linked to NF- κ B activation in other cellular phenotypes including fibroblasts [38], endothelial cells [39] and cardiac myocytes [40]. LPA signaling increases Ca²⁺_i within MC3T3-E1 osteoblastic cells through either receptor LPA₁ or LPA₃ [36]. Because we have previously shown that fluid shear stress-induced NF- κ B translocation requires Ca²⁺_i signaling, we hypothesized that LPA₁ or LPA₃ could be involved in fluid shear stress-induced I κ B α degradation. Inhibition of LPA₁ and LPA₃ receptor with Ki16425 revealed no change in I κ B α degradation under conditions of fluid

shear stress (Figure 3A and 3B). We next examined ERK1/2 phosphorylation under static conditions to confirm the inhibitory effect of these compounds upon LPA receptor activation (Figure 3C). Consistent with its ability to inhibit LPA-induced Ca^{2+}_i transients, 5 μM Ki16425 fully inhibited LPA-induced ERK1/2 phosphorylation. These data indicate that signaling *via* LPA₁ or LPA₃ is not involved in FSS-induced I κ B α degradation and NF- κ B signaling.

MEK1/2 is not involved in fluid shear-induced I κ B α degradation

Activation of the P2X₇ and an undefined P2Y receptor mediate fluid shear stress-induced ERK1/2 phosphorylation in osteoblasts [41], and ERK1/2 has been implicated in NF- κ B activation in macrophage [42], endothelial [43], and epidermal cells [44]. Therefore, we next examined the role of ERK1/2 in I κ B α degradation using an antagonist of MEK1/2, a kinase immediately upstream of ERK1/2 [45]. Similar to vehicle controls, cells exposed to FSS in the presence of the MEK1/2 antagonist U0126 (10 μM) demonstrated significant reductions in I κ B α (Figure 4A and 4B), indicating that fluid shear stress-induced I κ B α degradation does not require ERK1/2.

DISCUSSION

While the benefit of mechanical loading upon maintenance of skeletal architecture and bone mineral content is well accepted, the cellular mechanisms whereby application of exogenous loads translates into appropriate skeletal homeostasis or adaptation remain under vigorous study. Multiple cell types release ATP in response to mechanical load or mechanical deformation, underscoring the potential for purinergic receptor signaling to mediate a plethora of cellular responses [46]. In osteoblastic cells, purinergic signaling enhances the ability of parathyroid hormone to stimulate gene expression [47], and is required for PGE₂ release [18] and ERK1/2 phosphorylation [41] in response to FSS. *In vivo*, P2rx7 deletion markedly reduces the relative bone formation rate in response to axial loading of the mouse ulna [19]. These results are similar to data generated from the low-density lipoprotein-related receptor 5 (*Lrp5*) knockout mouse, although whether there is interaction between the purinergic and Wnt signaling pathways remains to be demonstrated.

Cells within bone, including mesenchymal stem cells [48,49], osteoblasts [50–54], and osteoclasts [53–57] express purinergic receptors. MC3T3-E1 cells have been shown to express P2X₂, P2X₄, P2X₆ and P2X₇ (reviewed in [58]), and P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₄ [59]. There are no data to date that reveal which P2 receptor isoforms are expressed by osteocytes or osteocyte-like cells, but we have previously demonstrated that ATP induces PGE₂ release in the MLO-Y4 cell line [13], indicating that these cells express functional P2 receptors. Similarly, the P2X₇ agonist BzATP has been shown to increase PGE₂ release from MLO-Y4 osteocytes [19], indicating that these cells express P2X₇ receptor.

Within this work, we demonstrate a requirement for purinergic signaling for FSS-induced I κ B α degradation and nuclear localization of NF- κ B. This was not dependent upon a single class of P2 receptors, as inhibition of either the ionotropic P2X₇ receptor or the metabotropic P2Y₆ receptor prevented shear-induced decreases in I κ B α . The influence of the P2Y₆ receptor upon I κ B α levels is likely to be mediated through G_q/phospholipase C-mediated intracellular calcium release, as we previously demonstrated the requirement of such in FSS-induced NF- κ B activation [22]. How the P2X₇ receptor is involved is less clear. It is unlikely to involve calcium entry from the pericellular space, as we showed that FSS is able to decrease I κ B α levels in the presence or absence of extracellular calcium. P2X₇ activation is linked to ERK1/2 phosphorylation [41], although ERK1/2 is not required for FSS-induced I κ B α degradation (Figure 4A and 4B). Another possible mechanism is by P2X₇ activation of Akt, as has been shown in to occur in astrocytes [60]. Alternately, P2X₇ activation causes

formation of arachidonic acid, which is metabolized by COX enzymes to form prostaglandins. Indeed, the increase in markers of osteogenesis in response to BzATP was abrogated when cells were co-cultured with the general COX antagonist indomethacin [23]. Thus, it is possible that FSS-induced I κ B α degradation is mediated directly by prostaglandins, which are themselves synthesized due to activation of the P2X₇ receptor.

P2X₇ receptor activation leads to the formation of LPA *via* phospholipase D [23]. LPA has diverse effects upon osteoblast and osteocyte activity, and induces chemotaxis [36,37], dendrite outgrowth [61], and differentiation [62]. Panupinthu *et al.* observed that the stimulatory effect of P2X₇ upon the osteogenic differentiation of calvarial cells is indeed mediated by LPA working in an autocrine fashion upon LPA₁ or LPA₃ receptors. Having demonstrated the role of P2X₇ in FSS-induced NF- κ B activation, we determined whether this involved LPA function. Osteoblasts exposed to FSS in the presence of the LPA₁/LPA₃ receptor antagonist Ki16425 revealed a similar magnitude of I κ B α degradation compared to vehicle-treated cells (Figure 3A and 3B), suggesting that LPA autocrine activity does not mediate NF- κ B activation in osteoblastic cells.

Whereas antagonists of P2X₇ and P2Y₆ prevented FSS-induced I κ B α degradation, it is interesting to note that addition of ATP (general P2 agonist), UTP (P2Y agonist), UDP or MRS2693 (P2Y₆ agonists), or BzATP (P2X₇ agonist) did not promote I κ B α degradation in static cells. This suggests that activation of P2X₇ or P2Y₆ is required, but not sufficient, to induce NF- κ B translocation, and that another signaling cascade that is induced by FSS is also involved. A possible candidate for this additional signaling cascade is the integrin/focal adhesion kinase (FAK) pathway. Integrins have been identified as mechanosensory molecules in osteoblasts and osteocytes [7,63–65]. Similarly, FAK is required for FSS-induced COX-2 and osteopontin expression [66]. Deletion of FAK prevented FSS-induced NF- κ B activation in osteoblasts [67], suggesting that purinergic signaling may interplay with integrin/FAK in regulation of NF- κ B translocation in osteoblasts.

Figure 5 represents an integrative model for the work described within and our previous efforts. Fluid shear stress promotes the vesicular release of ATP from pre-osteoblastic cells, where it subsequently functions in an autocrine fashion to modulate prostaglandin synthesis and release [18]. We have further demonstrated that both P2X₇ and an undefined P2Y (although not P2Y₂) receptor are required for maximal ERK1/2 phosphorylation in response to FSS [41]. Data presented in Figure 1 demonstrate that purinoceptor activation is required for I κ B α degradation and NF- κ B nuclear localization, while use of the P2X₇ antagonists oATP and BBG confirm that this is an obligate receptor for FSS-induced NF- κ B activation (Figure 2) in a process that does not ERK1/2 (Figure 4). Although P2X₇ receptor activation promotes synthesis of the bioactive lipid LPA [23], use of an LPA receptor antagonist did not modulate the suppressive influence of FSS upon I κ B α degradation (Figure 3).

CONCLUSION

Our data show that FSS induces NF- κ B degradation in osteoblastic cells by a mechanism involving P2X₇ and P2Y₆ receptors but not ERK1/2 or autocrine LPA signaling in osteoblastic cells. These results expand the framework for purinergic regulation of mechanotransduction in osteoblasts, yet also suggest that another signaling pathway activated by shear stress is necessary to interact with P2 receptors to elicit cellular responses.

Acknowledgments

NIH NIAMS AR051901 (RLD), NIA AG13087 (HJD), and NIAMS AR057547 (DCG) supported the work described within.

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Figure 1A

Figure 1B

Figure 1C

FIGURE 1. IκBα degradation and NF-κB activation requires extracellular nucleotides
 (A) Immunoblotting shows a decrease in IκBα expression in cells exposed to fluid shear stress, whereas the addition of apyrase (10U/mL) to flow media prevented FSS-induced IκBα degradation. (B) Quantitation of IκBα levels in osteoblasts, normalized to fold change compared to appropriate control static cells. Bars represent mean ± SEM, n=3-4. **a**: indicates $p<0.05$ compared to static control; **b**: indicates $p<0.05$ compared to FSS vehicle. (C)

Immunostaining of the p65 subunit of NF- κ B demonstrates nuclear accumulation of p65 in response to FSS, whereas degradation of extracellular nucleotides with apyrase prevents FSS-induced p65 nuclear translocation. Representative immunocytochemical images are shown from 3 replicates. The white scale bar indicates 10 μ m.

Figure 2A

Figure 2B

Figure 2C

Figure 2D

FIGURE 2. The P2X₇ and P2Y₆ receptors mediate FSS-induced IκBα degradation

(A) IκBα expression was monitored in MC3T3-E1 under static and FSS conditions in the absence or presence of the P2X₇ antagonists oATP (300μM) or BBG (30μM). (B) Quantitation of IκBα levels in osteoblasts, normalized to fold change compared to appropriate control static cells. Bars represent mean±SEM, n= 3–4. **a**: indicates $p<0.05$ compared to static control; **b**: indicates $p<0.05$ compared to FSS vehicle. (C) Quantification of IκBα levels in MC3T3-E1 osteoblasts exposed to FSS in the absence or presence of the P2Y₆ antagonist MRS2578 (10μM). (D) NF-κB p65 immunostaining in MC3T3-E1 osteoblasts maintained under static conditions and treated with 0, 10, or 1000μM ATP for 45 minutes reveals cytosolic localization of NF-κB. Representative immunocytochemical images are shown from 6 replicates.

Figure 3A

Figure 3B

Figure 3C

FIGURE 3. I κ B α degradation in response to FSS does not require LPA signaling

(A) I κ B α expression was monitored in MC3T3-E1 under static and FSS conditions in the absence or presence of the LPA₁ and LPA₃ receptor antagonist Ki16425 (5 μ M). B) Quantitation of I κ B α levels in osteoblasts, normalized to fold change compared to appropriate control static cells. Bars represent mean $\pm$ SEM, n= 3–4. **a**: indicates $p<0.05$ compared to static control. (C) Static MC3T3-E1 cells reveal 1 μ M LPA-induced ERK1/2 phosphorylation, and this was inhibited only in cells treated with Ki16425. Representative Western blots are shown from 2 replicates.

Figure 4A

Figure 4B

FIGURE 4. MEK signaling is not required for FSS-induced IκBα degradation

(A) IκBα expression was monitored in MC3T3-E1 under static and FSS conditions in the absence or presence of the MEK1/2 antagonist U0126 (10μM). B) Quantitation of IκBα levels in osteoblasts, normalized to fold change compared to appropriate control static cells. Bars represent mean±SEM, n=6. **a**: indicates $p<0.05$ compared to static control. C)

FIGURE 5. Mechanistic model for P2X₇ and P2Y₆ signaling under FSS in osteoblastic cells
 FSS promotes the vesicular release of ATP, where it binds to metabotropic P2Y or ionotropic P2X receptors; activation of P2X₇ causes ERK1/2 phosphorylation, although this is not involved in IκBα degradation, and nuclear localization of NF-κB. Activation of P2X₇ promotes LPA formation through PLA₂ or PLD, but subsequent activation of LPA₁ or LPA₃ does not promote IκBα degradation. Activation of P2Y₆ promotes the release of intracellular calcium from the endoplasmic reticulum. Purinoceptor activation alone is not sufficient for NF-κB translocation, suggesting that another FSS-activated signaling pathway (indicated by ?) is involved in this process.