ATP RELEASE AND PURINERGIC MECHANOSENSATION IN BONE CELLS

A Thesis in
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by

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ABSTRACT

Osteoporosis is a disease of the skeleton that is characterized by fragile bones, which increases the risk of skeletal fracture. According to the latest report from the Surgeon General of the United States, 10 million Americans over the age of 50 have osteoporosis, and 34 million are at risk [1]. Osteoporosis causes 1.5 million skeletal fractures per year at an annual cost of $12.2–17.9 billion per year [2]. As the population continues to age [3], this annual expenditure will surely rise as well. Consequently, osteoporosis prevention is at the forefront of orthopaedic research. One method to prevent osteoporosis is to increase the formation of new bone while preventing the resorption of older bone. A wealth of reports have demonstrated that mechanical loads can regulate skeletal architecture by increasing the activity of bone-forming osteoblasts and inhibiting the activity of bone-resorbing osteoclasts. This would strengthen the skeleton and thereby reduce the risk of osteoporosis. What continues to perplex orthopaedic investigators are the cellular mechanisms whereby external mechanical forces are perceived by cells of the osteoblastic lineage and transduced into an anabolic response (i.e., formation of new bone by osteoblasts). Movement of pericellular fluid, or fluid flow, induced by mechanical loads appears to be the most likely localized signal perceived by osteoblasts and osteocytes. The application of fluid flow to these cell lineages induces a rapid, yet transient increase in cytosolic calcium (Ca\textsuperscript{2+}) that requires both calcium entry into the cell and calcium release from cytoplasmic stores [4]; changes in Ca\textsuperscript{2+} are implicated in the regulation of a multitude of cellular responses ranging from acute (such as kinase activation [5]) to trophic (i.e., proliferation [6-8]). One signaling molecule that is able to induce both calcium entry and calcium release in osteogenic cells is adenosine triphosphate (ATP) which can activate both ionotropic P2X and metabotropic P2Y receptors [9]. Indeed, the importance of P2 receptors in the calcium response of osteoblasts to fluid flow was recently demonstrated by You et al. [10]. However, the mechanism(s) whereby cytosolic ATP is released from an osteoblast or osteocyte in response to fluid flow has not been described. The
overall aims of this thesis were to address how ATP is released from osteoblasts and osteocytes and to further elucidate the importance of P2 receptor activation by ATP in bone cell mechanotransduction.

We first examined whether osteoblasts, as the cells directly responsible for the formation of new bone, release ATP in response to fluid flow. Using a steady, laminar flow system with a flow rate that induced a shear rate of 12 dynes/cm², we found that conditioned media from osteoblasts exposed to fluid flow for 5 minutes contained approximately 10-fold more ATP than did conditioned media from static osteoblasts (59.8±15.7 vs 6.2±1.8 nM). We next used a Harvard pump one-pass system, which perfused fresh media across the osteoblasts, to examine the time course of ATP release in response to fluid flow. We found that fluid flow induced a rapid release of ATP within one minute of the onset of flow that returned to pre-flow levels with prolonged fluid flow. That ionomycin, a calcium ionophore, increased ATP release in static osteoblasts not exposed to fluid flow, suggested that changes in Ca²⁺ regulated ATP release. Because the L-type VSCC (Ca,1.2) and the MSCC have been implicated in the Ca²⁺ response to mechanical load, we hypothesized that these channels may be involved in flow-induced ATP release. We found that inhibition of the L-type VSCC with nifedipine or verapamil significantly inhibited flow-induced ATP release, whereas inhibition of the MSCC had no effect on ATP release. Inhibition of gap junctional intercellular communication (GJIC) or hemichannel activation with either 18α-glycyrrhetinic acid or 18β-glycyrrhetinic acid failed to inhibit flow-induced ATP release, suggesting that neither GJIC nor hemichannels are involved in osteoblastic ATP release. Immunolocalization of cytosolic ATP revealed punctate, granular stores of ATP, possibly within cytosolic vesicles. As exocytosis of such ATP-filled vesicles has been implicated in endothelial cells exposed to fluid flow, we examined the effect of pharmacologic antagonists of vesicle formation, release, and fusion on flow-induced ATP release. Inhibition of each of these exocytotic steps significantly attenuated flow-induced ATP release. Taken as a whole, these data
suggest that ATP is localized within vesicles in osteoblasts, and that fluid flow promotes vesicle
exocytosis through a mechanism requiring calcium entry through the Ca$_{1.2}$ calcium channel.
Wherein load-induced osteogenesis requires prostaglandin synthesis, we demonstrated that
activation of P2 purinoceptors by ATP mediates prostaglandin E$_2$ release.

Having reported that fluid flow induces ATP secretion that regulates PGE$_2$ release, we sought
to refine the signaling between P2 receptor activation and PGE$_2$ release. Flow-induced PGE$_2$
synthesis has been shown to be due solely to cyclooxygenase-2 (COX-2), which is itself induced
in response to flow. Further, we have previously demonstrated that maximal COX-2 induction in
response to flow requires the activation and translocation of the transcription factor NF-κB. As
such, we sought whether the effect of purinoceptor activation on PGE$_2$ release involved NF-κB
activation and translocation. Wherein fluid flow for 1 hour at a shear stress of 12 dynes/cm$^2$
induced robust nuclear staining for the p65 subunit of NF-κB, osteoblasts flowed in the presence
of an ATPase, apyrase, demonstrated cytosolic NF-κB localization. Similarly, flow-induced
degradation of IκBα was impaired in the presence of apyrase, suggesting that purinoceptor
activation occurs proximal to IκBα phosphorylation. Intriguingly, pharmacologic inhibition of
the P2X$_7$-R also demonstrated impaired IκBα degradation, implicating this purinoceptor in flow-
induced NF-κB translocation.

Although many investigators examine the effect of fluid flow on osteoblast
mechanotransduction, there is disagreement whether osteoblasts in vivo experience magnitudes of
fluid flow used in vitro. As such, we chose to confirm our results using osteocytes which, because
of their localization in lacunae and canaliculi in vivo, are speculated to be the primary
mechanosensor in bone. As with osteoblasts, the conditioned media from osteocytes exposed to
fluid flow contained significantly higher levels of ATP than did the conditioned media from static
osteocytes. In contrast to osteoblasts, however, osteocytic ATP release was inhibited in the
presence of 18α-glycyrrhetinic acid, suggesting that either GJIC or connexon hemicannels were
responsible for this effect. Because of the culture conditions used, GJIC was physically impaired and these results instead implicate connexon hemichannels. Using the uptake of fluorescent dyes as a marker for hemicannel activation, we reported that fluid flow increased hemicannel activity in MLO-Y4 osteocytes, but not in MC3T3-E1 osteoblasts. Pharmacologic inhibition of protein kinase C inhibited both dye uptake and ATP release in response to flow, strengthening the hypothesis that flow-induced ATP release occurs via connexon hemichannels. The addition of siRNA directed against connexin43 similarly impaired both dye uptake and ATP release, suggesting that hemichannels formed by Cx43 are the primary mechanism for flow-induced ATP release in MLO-Y4 osteocytes. Given the surprisingly different mechanism for ATP release between osteoblasts and osteocytes, we speculated whether purinoceptor activation differentially mediated PGE_2 release among the two cell types. As with osteoblasts, the addition of ATP to static osteocytes significantly increased PGE_2 release, suggesting that, despite being released by two different mechanisms, ATP activates P2 purinoceptors in both osteoblasts and osteocytes to increase PGE_2 release.

In summary, these data suggest that mechanical load can directly regulate skeletal architecture by increasing the release of a local factor (ATP) that directly affects osteoblasts and osteoclasts, and indirectly through a mechanism involving increased prostaglandin synthesis and release.
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For Clare.
1. **BONE**

Bone is a highly specialized form of connective tissue that performs multiple functions. Calcification, or mineralization, of the extracellular matrix endows strength and rigidity to the tissue, thereby engendering the protective and supportive function of the skeleton. Further, the mineralized matrix, composed of calcium (Ca\(^{2+}\)) and phosphate (PO\(_4^{3-}\)), provides a reservoir that can be easily degraded or added to in order to maintain serum ion levels. Additionally, bone participates in hematopoiesis by housing bone marrow inside its internal cavities.

Bone mass is dynamic, and bone is added or lost in response to structural needs or hormonal influence. Older bone, that is weaker or whose mechanical properties are diminished, is removed and replaced by new bone in a process known as remodeling. *De novo* bone forms during embryonic growth or during fracture repair. Hormones, such as vitamin D\(_3\) and parathyroid hormone (PTH), promote bone degradation to release calcium and phosphate to the blood. Thus, the maintenance of optimal bone structure requires concerted action of bone formation and bone resorption in response to mechanical load or hormone levels. Indeed, pathological states of the skeleton arise from inappropriate imbalances between formation and resorption.

1.1. **Bone Structure**

At the macroscopic level, the shapes of bones in the mammalian skeleton are varied and intimately related to their function. Bones can be divided into three groups: long bones, such as the femur or humerus; short bones, such as the bones of the carpus or vertebrae; and flat bones, such as the skull and ribs. Long bones are long, hollow, marrow-filled shafts with rounded, sealed ends. A conical region called the metaphysis connects the central shafts of the long bone, or the diaphysis, and sealed ends, or epiphyses. In growing mammals, the epiphysis is separated from the metaphysis by the epiphyseal plate, whereas in adult mammals the epiphyseal plate has been replaced by bone, and the epiphysis and metaphysis are fused [11]. Long bones are found primarily in the limbs, where they carry compressive loads and serve as levers for muscle
contraction and locomotion. In contrast to long bones, short bones are generally cuboidal and are grouped together for complex movement. Flat bones, such as the scapula, are thin in one dimension and longer in the remaining two dimensions.

In vivo, the external and internal surfaces of bones are covered by a sheet of connective tissue, known as the periosteum and endosteum, respectively. The periosteum is composed of two layers: an outer fibrous layer and an inner osteogenic layer immediately apposed to the bone surface. The outer fibrous layer is composed of dense, irregular connective tissue perforated by blood vessels and nerves, while the inner osteogenic layer primarily contains osteoblasts and osteoprogenitors. The inner osteogenic layer is attached to the bone surface by bundles of collagen, known as Sharpey’s fibers, trapped in the mineralized matrix. The periosteum is absent at sites of tendon and ligament insertion, as well as the epiphyses, which are instead capped by articular cartilage. The endosteum, lining the medullary cavity of bone, forms a continuous layer on the inner bone surface and functionally separates the bone surface from the marrow stroma. Similar to the periosteum, the endosteum is composed of an outer fibrous layer and an inner osteogenic layer.

Structurally, bone tissue is either cortical (compact) or cancellous (trabecular or spongy). Axial bones are mainly cancellous, whereas appendicular bones are mainly compact in structure. While cancellous and cortical bones are made by the same cells and of the same collagenous components, there are discrete functional and structural differences between the two types of bone. The primary functions of cortical bone are mechanical and protective, and its structure belies these functions. The majority (80% to 90%) of the volume fraction of cortical bone is calcified, thereby generating a dense, solid mass of bone. The bulk of cortical bone is in the diaphysis of long bones, and approximately 80% of the skeletal mass in the adult human skeleton is cortical bone [12]. The remaining volume fraction is composed of Haversian and Volkmann’s canals that contain capillaries and blood vessels for gaseous exchange and nutrient delivery to
osteoblasts and osteocytes within bone. In contrast to highly dense cortical bone, only 15% to 25% of cancellous bone tissue is calcified [13].

Cortical and cancellous bone can be further divided into two types, woven and lamellar bone, based upon microarchitecture. Woven bone is quickly formed during embryonic development or in response to fracture, and is composed of randomly oriented collagen fibers. Woven bone provides temporary support during skeletal repair or disease but, due to its random, haphazard deposition, is mechanically inferior to lamellar bone. In order to compensate for this, woven bone is more highly mineralized than lamellar bone. Over time, woven bone is resorbed and replaced by lamellar bone that is formed more slowly and is much more organized than woven bone. Lamellar bone is composed of parallel layers of type I collagen, called lamellae. Four types of lamellae organizations are found in cortical bone [11]: 1) Haversian systems, wherein concentric circles of lamellae surround a central longitudinal cavity containing blood vessels and nerves; 2) outer circumferential lamellae, near the periosteum; 3) inner circumferential lamellae, near the endosteum; and 4) interstitial lamellae, in between osteons and the inner or outer circumferential lamellae. Volkmann’s canals, which similarly encircle blood vessels and nerves, are transverse to the long axis of bone, perforate the lamellae, and intersect Haversian systems.

1.2. Matrix Composition

The matrix of mature, calcified bone is composed of a mineral (hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and organic (proteins, lipids and water) phase. Although the individual contribution of each component varies throughout the skeleton, the average composition of healthy, adult bone is 50-70% mineral, 20-40% organic matrix (proteins), 5-10% water, and <3% lipid [11]. The mineral phase of bone provides strength and rigidity to bone, and is primarily in the form of hydroxyapatite that is formed and secreted by the osteoblast. In contrast, the organic phase provides elasticity and flexibility to bone. The organic phase consists of extracellular matrix proteins, such as collagens, glycoproteins, and proteoglycans, of which type I collagen is
the principal component (90% of the organic phase). Collagen formation is the result of multiple sequential levels of self-aggregation of subunits synthesized and secreted by osteoblasts, from propeptide to procollagen to tropocollagen to fibril to fiber [14] (**Figure 1-1**). Prior to secretion from the osteoblast, collagen propeptides trimerize to form a pro-collagen helix; the structure of pro-collagen is stabilized by the high number of glycine and proline residues in each collagen. Once secreted into the extracellular environment, carboxy- and amino-terminal sequences are removed by extracellular procollagen peptidases, generating tropocollagen. Tropocollagen condenses to form larger, rope-like fibrils with a characteristic, staggered spacing. Fibrils further self-aggregate to form collagen fibers. Covalent bonds formed between lysine residues of the constituent collagen molecules strengthen collagen fiber formation [15]. Collagen fibril formation is staggered, generating “hole zones” between fibers. The hole zones are critical for matrix mineralization, as they function in an as-yet-unknown manner, possibly by facilitating nucleation of calcium and phosphate into hydroxyapatite. Additionally, cytokines and growth factors are often trapped in the matrix during calcification; released during osteoclastic resorption, they function as negative feedback effectors on osteoclast function.
1. Inside the osteoblast, collagen propeptides trimerize into procollagen

2. Once secreted from the osteoblast, amino and carboxy terminal residues are cleaved from the procollagen molecule, forming tropocollagen

3. Tropocollagen molecules aggregate into fibrils whose strength is due to hydrogen bonds between tropocollagen molecules. Fibrils further condense into collagen bundles (not shown).

**Figure 1-1.** Collagen fiber formation
1.3. The Cellular Components of Bone

1.3.1. Osteoblasts

Osteoblasts are directly responsible for bone formation by synthesizing the components of the organic matrix, or osteoid, described above. In accordance with the high metabolic demand for protein synthesis and secretion, osteoblasts demonstrate an abundant endoplasmic reticulum, enlarged Golgi, and vast numbers of secretory vesicles. Osteoblasts contribute to osteoid mineralization by secreting matrix vesicles that are super-saturated with calcium and phosphate, and various enzymes, such as alkaline phosphatase and other pyrophosphatases. As the vesicles rupture outside of the osteoblast, they release calcium and phosphate, thereby increasing the local mineral concentration and initiating mineralization by forming hydroxyapatite crystals that intercalate into the hole zones of fibrillar collagen. Osteoblasts secrete osteoid at a rate of 3-10µm/day (dependent upon whether woven or lamellar bone is formed), while osteoblasts mineralize osteoid at the rate of 0.5µm/day [16]. The distinct spatial and temporal lag between osteoid secretion and mineralization is known as the mineralization front or osteoid seam.

Osteoblasts are derived from mesenchymal precursors located in the marrow stroma. These mesenchymal precursors are able to differentiate into cells of skeletal tissue, such as bone and cartilage. Commitment to a determined phenotype is under the control of phenotype-specific genes, such as Sox9 for chondrocytes, MyoD for muscle, and Cbfa1 for osteoblasts [17]. Induction of Cbfa1 promotes a period of massive osteoprogenitor proliferation followed by differentiation and expression of osteoblastic markers. The importance of Cbfa1 in the commitment to the osteoblastic phenotype is demonstrated in Chfai-deficient mice [18, 19], whose skeleton is composed entirely of cartilage, and is further characterized by complete absence of osteoblasts or osteoclasts [20, 21]. Osteoblasts are able to further differentiate into osteocytes or bone lining cells (Figure 1-2 and below).
Figure 1-2. Mesenchymal stem cell differentiation.
1.3.2. **Osteocytes**

Osteocytes are terminally differentiated osteoblasts that have become entrapped in bone matrix during formation of the osteoid [22, 23]. Limited resorption of the adjacent osteoid generates a surrounding space, or lacuna, in which the osteoblast/pre-osteocyte resides. During calcification of the osteoid matrix, the osteoblast/pre-osteocyte becomes entrapped in the osteoid and loses a number of osteoblastic phenotypic markers, but acquires others, most specifically the stellate, dendritic morphology that characterizes an osteocyte. Small channels, or canaliculi, interposed within the matrix allow osteocytic processes to spread to osteoblasts and neighboring osteocytes, thereby forming a network of communicating osteocytes.

The relative isolation of osteocytes within mineralized matrix suggests that osteocytes do not directly participate in matrix formation. In contrast to the large endoplasmic reticulum found in an osteoblast, mature osteocytes possess few organelles necessary for matrix synthesis and secretion [23]. Indeed, osteocyte survival depends on localized inhibition of matrix formation and maturation, thereby allowing diffusion of oxygen, nutrients, and waste products through the lacuno-canalicular network. As will be discussed below, osteocytic distribution inside of the matrix suggests that osteocytes are the primary cells responsible for sensing changes in the mechanical environment of the skeleton.

1.3.3. **Bone Lining Cells**

Bone lining cells are terminal osteoblasts residing on the periosteal and endosteal surface of bone that are not actively involved in bone formation. In contrast to the cuboidal morphology of osteoblasts, bone lining cells are flattened, elongated cells covering quiescent bone surfaces, with fewer organelles than osteoblasts. Bone lining cells communicate with each other and osteocytes through gap junctions [24-26] in a manner similar to an osteocytic network. It is currently believed that bone lining cells are terminally differentiated osteoblasts; however, data suggests that bone-lining cells may de-differentiate into osteoblasts [27, 28] with changing demands for
bone formation, thereby providing a readily available source of osteoblasts when rapid bone deposition is needed.

Additionally, bone lining cells serve as an important barrier to the bone surface in two ways. Firstly, they maintain the differential ionic composition between the interstitial fluid and the bone fluid compartment [29], suggesting that bone lining cells mediate mineral homeostasis [30]. Secondly, bone lining cells limit osteoclast or osteoclast progenitor access to the bone surface: osteoclast activation occurs upon exposure to an acellular, mineralized bone surface, but not by an intact endosteal surface [31, 32]. For bone resorption to occur, the morphology of the bone lining cell is altered, thereby allowing osteoclast progenitor access to the bone surface. However, the mechanisms involved in bone lining cell retraction in response to nearby osteoclasts remain poorly understood.

1.3.4. Osteoclasts

Osteoclasts are the only known cells capable of resorbing bone. Resorption occurs through a highly-coordinated sequence of events involving osteoclast attachment to bone, dramatic cell polarization, generation of a proton-rich resorbing pit environment immediately apposed to the bone, and transcytotic release of matrix catabolites.

Although the exact mechanism remains unknown, it is known that bone lining cells retract from the bone surface to allow osteoclast attachment to calcified bone that is mediated by $\alpha_v \beta_3$ integrins [33, 34]. Once attached and activated, osteoclasts become highly polarized by forming two new membrane domains: the sealing (or clear) zone, a specialized cell-ECM structure, and the ruffled border, the bone-resorbing organ of the osteoclast [35]. Formation of the impermeable sealing zone is required to maintain the proton-rich environment generated by the ruffled border. The ruffled border is a characteristic deep invagination of the osteoclast plasmalemma immediately adjacent to the mineralized matrix to be resorbed. Polarization of the osteoclast plasmalemma localizes, among other proteins, the CIC-7 chloride channel [36, 37] and $H^+$...
ATPase [38] to the ruffled border. The localized action of these channels and pumps generates a closed, highly acidic environment [39] with a pH of approximately 4.5 [40]. This microenvironment demineralizes the matrix, leaving behind an organic component further degraded by cathepsin K [41, 42], matrix metalloproteinases [43], and other lysosomal proteases. Products of matrix catabolism are then endocytosed from the osteoclast resorption pit and exocytosed at the non-resorbing face of the cell [41] (Figure 1-3).

Osteoclasts are derived from mononuclear hematopoietic precursors. A significant breakthrough in understanding osteoclastogenesis came with the demonstration that co-cultures of osteoblasts and spleen cells yielded osteoclasts [44], suggesting that some osteoblast-derived factor(s) mediated osteoclast formation. Further work demonstrated that osteoblastic co-culture was necessary for the production of RANKL [45, 46] and M-CSF [47], both of which are necessary and sufficient for osteoclastogenesis.
**Figure 1-3.** An osteoclast attached to bone.
1.4. Skeletal Development

1.4.1. Ossification

Skeletal formation, or ossification, proceeds through either endochondral or intramembranous ossification. Endochondral ossification forms the majority of bones that participate in joints and weight-bearing, and begins around the sixth week of gestation [48]. Endochondral ossification proceeds as follows [11, 49]:

i. Mesenchymal cells within connective tissue, under the control of paracrine factors secreted by nearby mesodermal cells, become committed to the chondrocytic lineage.

ii. Committed mesenchymal cells condense, rapidly proliferate, and differentiate into chondroblasts; secretion of type II collagen and aggrecans forms the cartilaginous model of the future bone.

iii. Similar to the periosteum that surrounds bones, a perichondrium surrounds the cartilaginous model that serves as a source of chondroblasts.

iv. Chondroblasts further differentiate into chondrocytes, then into hypertrophic chondrocytes, which secrete type X collagen and thereby enable matrix mineralization.

v. Central hypertrophic chondrocytes mineralize the intercolumnar cartilage matrix in the long axis of the bone. After hypertrophic chondrocyte apoptosis, the remaining mineralized cartilage acts as scaffold for the deposition of metaphyseal bone.

vi. Blood vessels invade the perichondrium, promoting migration of osteoblast progenitors into the perichondrium. Intramembranous ossification produces a layer of woven bone (periosteal collar) around the central shaft of the cartilage.

vii. Blood vessels originating in the periosteal collar invade the central diaphysis, similarly promoting the migration of osteoprogenitors into the cartilage.
In contrast to endochondral ossification, intramembranous ossification does not require a cartilaginous template. Intramembranous ossification occurs in the developing embryo around the seventh week of gestation [48], and produces flat bones, such as the clavicle, mandible, and calvariae in the facial skeleton. Similar to endochondral ossification, mesenchymal cells within connective tissue rapidly proliferate. Post-proliferation, mesenchymal precursors differentiate directly into osteoblasts, which subsequently form woven bone that is later resorbed and replaced with lamellar bone.

### 1.4.2. Alterations in Skeletal Architecture

Modeling or remodeling of bone alters skeletal architecture. Modeling produces gross alterations in bone architecture, wherein remodeling internally restructures pre-existing bone. Modeling involves either osteoclast or osteoblast activity, while remodeling involves bone formation preceded by bone resorption.

Bone modeling, which occurs primarily in the first two decades of life, changes the net size and shape of bones to achieve their final architecture. Modeling occurs only at the periosteal or endosteal surface and is characterized by either osteoblast or osteoclast activity at the bone surface. Uncoupling of osteoblast and osteoclast activity, as well as increased formation relative to resorption, results in a net change in the size or shape of the bone. Additionally, modeling is a requirement of longitudinal bone growth, reshaping the diaphysis, metaphysis, and epiphysis.

Remodeling is the process wherein pre-existing bone is removed by osteoclasts and replaced with new bone by osteoblasts. In stark contrast to modeling, remodeling produces no gross alterations in bone architecture; instead, small packets of bone are removed and replaced. Remodeling requires tight orchestration, or coupling, of matrix resorption and deposition: uncoupling of resorption and formation would ultimately increase or decrease bone mass and impair the mechanical function of the skeleton. Indeed, changes in coupling of bone formation
and bone resorption provide the basis for skeletal pathology, such as osteoporosis or osteopetrosis.

Remodeling is achieved by a group of osteoblasts and osteoclasts, known as a basic multicellular unit (BMU). A BMU consists of about ten osteoclasts and hundreds of osteoblasts [12], and proceeds in a cyclical pattern (Figure 1-4):

i. Retraction of bone lining cells, thereby exposing the mineralized bone matrix.
ii. Recruitment of osteoclast precursors to the exposed matrix.
iii. Resorption of matrix by active osteoclasts.
iv. Reversal, wherein osteoclastic resorption is completed and osteoblastic formation begins.
v. Osteoid formation by osteoblasts.
vi. Osteoid mineralization by osteoblasts.

At the conclusion of the remodeling cycle, 50-70% of osteoblasts undergo apoptosis [50], while the surviving osteoblasts differentiate into bone lining cells or osteocytes, if trapped in the osteoid matrix. Osteoclasts, on the contrary, can participate in multiple cycles of remodeling before finally undergoing apoptosis [51] [52]
Figure 1-4. The stages of bone remodeling. After Parfitt, 1993 [53].

**Quiescent bone surface:**
Bone surface covered by bone lining cells

**Mineralization:**
Mineralization of osteoid

**Formation:**
Osteoid formation by osteoblasts

**Activation:**
Retraction of bone lining cells and invasion by osteoclast precursors

**Resorption:**
Osteoclasts degrade mineralized matrix

**Reversal:**
Osteoblast recruitment
Modeling is regulated principally by mechanical loads and secondarily by calcitropic hormones. Remodeling is primarily under hormonal control for mineral homeostasis and secondarily regulated by mechanical loads. While the sequence above describes remodeling of both cortical and cancellous bone, the osteotropic signals responsible for initiating remodeling are different for cortical or cancellous bone. Remodeling of trabecular bone occurs at the surface of the trabeculae; as such, a given BMU in trabecular bone is intimately apposed by marrow stromal cells that secrete high levels of osteotropic cytokines. In contrast to trabecular remodeling, cortical remodeling occurs in the Haversian systems described above. Thus, the BMU in cortical bone is sequestered from marrow stroma and associated cytokines and is regulated by systemic osteotropic factors, such as $1\alpha,25-(OH)_2$-vitamin D$_3$ or PTH [54].

1.5. Regulation of Bone Homeostasis

As briefly discussed above, numerous factors—mechanical load, hormones, and local factors—affect both osteoblast and osteoclast formation and function, thereby altering the ability of osteoblasts and osteoclasts to deposit or resorb bone matrix. Because the role of the mechanical environment on bone cells is a major component of this thesis, these data are discussed in Section 2.

1.5.1. Endocrine Regulation

Prior to the pioneering efforts of Frost in understanding the effects of mechanical load on bone, the calcitropic hormones—calcitonin, PTH, and vitamin D$_3$—were considered to be the primary regulators of skeletal architecture. Further, it is becoming increasingly evident that other, non-calcitropic hormones have profound effects on bone mass. Because it is beyond the scope of this thesis to address all hormones regulating bone mass, only a few, select hormones are reviewed below. For greater detail of those described below, as well as the other hormones
regulating bone mass, the reader is directed to the extensive reviews contained in Bilezikian et al. [55].

1.5.1.1 Calciotropic Hormones

The concentration of ionized calcium in the serum is 9.0-10.5 mg/dl. Given the important role of calcium in such physiological processes as nerve excitability, serum calcium levels are tightly regulated. Calciotropic hormones are those responsible for serum calcium homeostasis, and these effects are exerted on the GI tract, kidneys, and bone. Decreased serum calcium levels, or hypocalcemia, promote parathyroid hormone (PTH) release from the parathyroid glands. In the kidney, PTH promotes calcium reabsorption by the thick ascending limb and the distal tubule, thereby decreasing calcium loss in the urine and increasing serum calcium levels [56]. In bone, PTH activates osteoclasts to increase resorption and thereby release calcium to the body. There is ongoing debate whether rapid effects of PTH on bone mass occur. It is suggested, through a process termed osteocytic osteolysis, that PTH can induce matrix catabolism by osteocytes [56-58]. PTH further regulates serum calcium levels by promoting the synthesis of 1α,25-(OH)₂-vitamin D₃ by activating renal 1α-hydroxylase. 1α,25-(OH)₂-vitamin D₃ increases both serum calcium and phosphate levels by promoting their absorption and reabsorption in the GI tract and kidneys, respectively. Calcitonin is synthesized in the thyroidal C cells, and its secretion increases under hypercalcemic conditions. In the kidney, calcitonin decreases tubular reabsorption of both calcium and phosphorus in the kidneys, and inhibits osteoclastic activity in bone, thereby decreasing both serum calcium and phosphate levels.

Where the actions of PTH described above are catabolic to bone, PTH can also function in an anabolic manner by increasing bone density, mass and strength [59-63]. These differential effects are likely due to the duration of PTH administration, with intermittent administration associated with anabolism and continuous administration with catabolism [64].
1.5.1.2 Estrogen

The skeleton is a target tissue for the reproductive sex steroids, particularly estrogen. These effects are best demonstrated in post-menopausal women and ovariectomized (OVX) animals that are unable to produce estrogen. OVX rodents and primates demonstrate decreased skeletal mass that is due to increased bone resorption [65, 66] and osteoclast number [67]. In vitro evidence has demonstrated that estrogen inhibits bone resorption by altering the production of local factors [68-70] (whose functions are discussed below), such that estrogen withdrawal promotes the formation of these factors. Additionally, estrogen promotes osteoclast apoptosis [71, 72], and inhibits osteoblast and osteocyte apoptosis [73-76].

The primary effect of estrogen on the skeleton is through traditional genomic mechanisms, wherein estrogen diffuses into the nucleus to induce changes in gene transcription. There is additional evidence that estrogen induces rapid, non-genomic effects by binding to and activating a plasmalemmal estrogen receptor [77-85].

1.5.2 Local Regulation

A variety of local factors secreted by osteoblasts or osteoclasts affect skeletal architecture by modulating osteoblast and/or osteoclast differentiation and activity in an autocrine or paracrine manner. Among these factors are prostaglandins, cytokines, nitric oxide, BMP’s, and growth factors. As with the systemic hormones regulating bone mass, the reader is directed to Bilezikian et al. [55] for more extensive reviews of local factors not discussed below. Given their role in this thesis, however, particular attention is addressed to prostaglandins.

1.5.2.1 Prostaglandins

Prostaglandins (PG’s) are members of the eicosanoid family of hormones that have profound and yet confusing effects on skeletal remodeling. Early work demonstrated that PGE$_2$ is a potent inducer of bone resorption in vitro [86-90]. Later work demonstrated that PG’s induce bone
formation [91-96] and inhibits in vitro bone resorption [97-99]. PGE₂ has dual effects on collagen synthesis in cultured calvariae: at low concentrations, it increased collagen synthesis, whereas at high concentrations, its effects were inhibitory [100, 101]. Contrasting effects may be due to the differentiation state of the osteogenic cells: PG’s stimulate pre-osteoblast proliferation [102] but differentiation of mature osteoblasts [103-105]. While profoundly confusing at times, the seemingly contradictory effect of PG’s on bone formation and resorption could be explained by considering that proper remodeling requires the concerted action of both osteoclasts and osteoblasts. Remodeling firstly involves resorption of older bone by osteoclasts before new bone formation by osteoblasts. Thus, these apparent discrepancies could be the result of a common signal through which both are induced [106].

Prostaglandin synthesis involves multiple enzymes. The first is phospholipase A₂ (PLA₂), which hydrolyzes the sn-2 position of membrane phospholipids into the unsaturated fatty acid, arachidonic acid (AA) [107]. The conversion of AA into PGH₂ is mediated by the multifunctional cyclooxygenase (COX) family of enzymes, whose dual active sites firstly cyclooxygenate AA into PGG₂, which is then peroxidated into PGH₂ [108, 109]. PGH₂ is subsequently converted into bioactive PGE₂, PGI₂, PGD₂, PGF₂α, or thromboxane A₂ by PG-specific isomerases and isoreductases [110]. Conversion of AA into PGH₂ is the rate-limiting step in PG synthesis [111].

There are two accepted COX isozymes, COX-1 and COX-2. A third, putative isozyme, COX-3, has been suggested [112], although there is ongoing debate whether this is truly a novel COX isoform or, rather, a splice variant of COX-1. COX-1 is constitutively expressed in all cell types and is relatively unchanged in response to challenge. In contrast, COX-2 is normally expressed at low levels, if at all, but is rapidly induced in response to changes in mitogens [110], and proinflammatory agents [110, 113-115].

Prostaglandins, while lipid metabolites, are unable to cross the plasmalemma by simple diffusion because of their charged nature at physiological pH [116, 117]. Instead, they are
released through an ABC transporter, MRP4 [118], or a prostaglandin transporter (PGT) [119]. Extracellular prostaglandins mediate their functions by binding to prostanoid receptors, of which there are five types, named DP, EP, FP, IP, and TP, based on their sensitivity to the five primary prostanoids [120]. Prostanoid receptors are rhodopsin-type G protein-coupled receptors coupled to G_s, G_i, or G_q, depending upon the isoform. Termination of PG signaling involves its re-uptake into the cell by PGT [119, 121] and subsequent oxidation by PG 15 dehydrogenase [122].

Evidence suggests that AA regulates the COX-1 and -2 in an allosteric fashion [123]. At low AA concentrations (<2.5 µM), AA is oxygenated exclusively by COX-2 and not COX-1, whereas, at higher AA concentrations (>10 µM), AA is oxygenated exclusively by COX-1 and not COX-2 [124-126]. Since the concentration of AA formed by PLA_2 is on the order of 1 µM, these data suggest that the majority of PG’s formed in response to treatment is due to induced COX-2 [125]. Norvell et al. have demonstrated such in murine osteoblasts, wherein application of fluid flow induced a robust increase in PGE_2 release that was inhibited in the presence of a specific COX-2, but not COX-1, antagonist [127].

1.5.2.2 Nitric Oxide

Nitric oxide (NO) has also been implicated as a potent regulator of bone formation and resorption. NO is a free radical gas generated by the conversion of L-arginine to L-citrulline by the nitric oxide synthase (NOS) family of enzymes. Non-specific inhibition of all isoforms with L-NAME significantly inhibited load-induced bone formation [128, 129]. Targeted inactivation of eNOS in mice demonstrated that this isoform was responsible for load-induced bone formation because of impaired osteoblast proliferation and differentiation [130, 131]. In contrast, iNOS has been implicated in cytokine-induced bone resorption [132, 133], wherein IL-1 action on osteoblasts increases iNOS activation and NF-kB translocation in osteoclasts [134]. nNOS expression occurs primarily during skeletal development [135] and fracture repair [136, 137].
1.5.2.3 OPG and RANKL

Osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) function in a paracrine fashion to decrease or increase osteoclastogenesis, respectively [70]. RANKL exists in a membrane-bound (mRANKL) and soluble (sRANKL) form. Both are able to bind to receptor activator of NF-κB (RANK) on osteoclast precursors and mature osteoclasts, inducing differentiation and activation, respectively, through a pathway involving NF-κB, JNK, and c-jun [70, 138]. OPG is a soluble decoy receptor for RANKL, which inhibits RANKL binding to RANK and subsequent inhibition of osteoclast differentiation, activation, and bone resorption. RANKL administration to mice, as well as OPG deletion, to mice induces hypercalcemia and osteoporosis [46, 139], whereas RANKL deletion or OPG over-expression promotes osteopetrosis and an absence of osteoclasts [70, 140, 141].

1.5.2.4 Cytokines

Cytokines are another example of local factors affecting both osteoblast and osteoclast function. The interleukin family of cytokines has been shown to inhibit bone formation [142] and promote bone resorption in vivo [143-145]. Similar effects on resorption and formation are found with tumor necrosis factor (TNF) treatment [144, 146]. These effects are achieved by increasing osteoblast apoptosis [50, 147] and osteoclast formation [148, 149] through RANKL production [150], and inhibiting type I collagen synthesis [144, 146] and osteoblast formation from mesenchymal precursors [151]. There is considerable evidence that these effects are also regulated by other local factors, as well as systemic hormones [50, 152].
1.6. Gap Junctional Intercellular Communication in Bone

Cell-to-cell communication enables coordination of cellular activity and homeostatic control. This is especially true of the cells in bone. Intercellular communication is required for osteoblast differentiation and activation, as well as coordination of resorption and formation. **Section 1.5** discussed a few such mechanisms that have been implicated in maintenance of appropriate skeletal architecture. The factors described in **Section 1.5** are, in some form or another, extracellular factors. Another mode of cell-cell communication does not require the release of factors, but, instead, direct signal propagation from cell to cell. Gap junctions are an example of such a mechanism.

Gap junctions are specialized cell junction proteins that enable direct cell-cell communication between apposed cells. Gap junctions are composed of transmembrane proteins, termed connexins (Cx), that hexamerize to form a connexon; connexons protrude from the plasmalemma of two adjacent cells and fuse to form a functional gap junction. Once formed, a gap junction allows direct transfer of small molecules (<1 kDa) from the cytosol of one cell to the cytosol of the adjoining cell, in a process termed gap junctional intercellular communication (GJIC).

To date, over 20 connexins have been characterized in mammals, and a subset of these—connexin43 [153, 154], 45 [154, 155], and 46 [156]—are expressed in cells of the osteoblastic lineage. There is discrepancy as to the expression of gap junctions and functional GJIC in osteoclastic cells, although Cx43 expression has been demonstrated by immunofluorescence [157] and *in situ* hybridization [158]. Functional gap junctions are demonstrated between homotypic bone cells [159-162], as well as between heterotypic bone cells [25, 157, 163].

The importance of GJIC has been repeatedly demonstrated in osteoblastic cells. *In vivo*, ablation of connexin43, the most highly expressed connexin in osteoblasts and osteocytes, impairs ossification, promotes craniofacial abnormalities, and osteoblast dysfunction [164]. *In vitro* data on osteoblasts and osteocytes demonstrates a role for gap junctions in flow-induced calcium transients [165, 166], prostaglandin release [167, 168], and differentiation [169-171]. In
osteoclasts, inhibition of GJIC decreases bone resorption [172, 173] by inhibiting fusion of mononuclear osteoclast precursors into mature osteoclasts [173-175].
2. MECHANICAL LOADING AND BONE

2.1. Mechanical Loading and the Mechanostat Theory

For bone to best serve as a weight-bearing organ, its architecture must be modified to adequately address changes in structural demand [176]. Increased stresses, or loads, on bone must be compensated with bone formation, whereas decreased stresses on bone should reduce bone mass to prevent wasted energy expenditures. A plethora of data support this idea, known as Wolff’s Law. The microgravity conditions of spaceflight are one such example of a condition resulting in diminished skeletal loading. Rats flown in space demonstrate decreased mineralized tissue mass and osteoblast number in trabecular bone [177], and decreased bone formation rates of both trabecular and cortical bone [178]. Spaceflight decreases periosteal bone formation [179] and reduces longitudinal growth [180], impairing the mechanical properties of vertebrae [181], tibiae [182] and femur [183]. Increased mechanical loads in humans, such as occurs during high-impact exercise, reveals significant increases in BMD at the lower limb, lumbar spine, and upper limb sites compared to controls [184]. In contrast, those undertaking low-impact exercise, such as swimming [185] or walking [186], demonstrate no significant changes in bone formation.

This discrepancy in bone formation with certain activities but not others led Frost to propose that the magnitude of the applied mechanical strain is a key parameter of whether bone formation or resorption occurs. Strain is a measurement of deformation, defined as the change in length of an object divided by its original length, with 1% strain equal to 10,000 µε; normal and strenuous activity induce strains of 400–2000 µε [187, 188]. Frost’s mechanostat theory [189, 190] proposed that the magnitude of mechanical load must surpass a setpoint, or minimum effective strain (MES), before alterations in bone mass occur. Strains above the MES are skeletally anabolic, whereas strains below the MES are skeletally catabolic. This hypothesis is supported by both in vivo and in vitro loading experiments. Rubin and Lanyon, using the turkey ulna model, demonstrated that daily application of 100 1Hz loading cycles at strains above 1000 µε
substantially increased periosteal and endosteal bone formation in a magnitude-dependent manner, whereas strains below 1000 µε elicited endosteal resorption and increased remodeling activity [191]. Similar results were found in a study by Turner et al. on rats exposed to four-point bending, with increases in the bone formation rate occurring only at strains above 1050 µε [192]. In vivo, osteoblastic cells exposed to substrate strain demonstrated magnitude-dependent increases in osteopontin expression only above strain rates of 2mm/s; strain rates below 2mm/s demonstrated no change in osteopontin expression compared to static controls [193]. You et al. reported similar effects of strain on Ca^{2+}, wherein transients were recorded only at strains above 0.5% [194].

Although mechanical strains increase bone formation, it is necessary that the strains be applied dynamically, and not statically. Lanyon and Rubin demonstrated that the same magnitude of compressive load, when applied dynamically for only 100s a day, demonstrated significant periosteal bone formation in turkey ulnae, whereas the static load increased bone loss on endosteal and Haversian surfaces [195].

Burr and Martin furthered Frost’s mechanostat theory, incorporating the effect of sub- and supraphysiologic levels of strain on skeletal adaptation. In their mechanostat model, physiologic levels of strain are divided into four windows (Figure 2-1): 1) disuse, wherein resorption is favored over formation, thereby decreasing bone mass; 2) physiological, wherein resorption occurs at the same rate as formation, maintaining bone mass; 3) overuse, favoring bone formation over resorption, increasing bone mass; and 4) pathological, forming woven bone.
Disuse:  
- Resorption > formation  
- Increased remodeling

Physiological:  
- Resorption = formation  
- Homeostasis

Overuse:  
- Formation > resorption  
- Increased modeling

Pathological overload:  
- Woven bone formation

Figure 2-1. The four windows of mechanical usage defined by the mechanostat theory. After Burr and Martin, 1989 [196].
2.2. Mechatransduction

Copious studies have demonstrated the relationship between skeletal architecture and the mechanical environment. What remains unknown are the cellular mechanisms whereby external physical loads are transduced into signals that regulate bone homeostasis, formation, or resorption. Mechatransduction, a term coined to describe the cellular signaling processes involved in mechanical stimulation, can be divided into four phases [197, 198]. The first phase, mechanocoupling, involves the conversion of gross mechanical loads into localized mechanical signals perceived by bone cells. Mechanocoupling is followed by biochemical coupling, or the transduction of these local mechanical signals into biochemical responses in sensor cells. The third phase involves the transmission of signals from sensor cells to the effector cells in bone. The final phase is the effector cell response, in which effector cells initiate tissue-level responses (i.e., skeletal anabolism) to the original mechanical signal.

2.3. Mechanocoupling In Vivo

Before attempting to understand how the skeleton responds to physical load, one must first understand how bone cells perceive changes in their mechanical environment in vivo. That is, how are external loads transduced into a local signal to osteoblasts and osteocytes to ultimately modulate skeletal architecture? This is especially important insofar as the cellular responses to load can be initiated by multiple forms of mechanical load, among them hypotonic swelling [199-202] or direct membrane perturbation [165, 203]. While other means of mechanocoupling have been described in bone, such as piezoelectric phenomena and streaming potentials, the author will focus on the most extensively studied mechanisms, substrate strain and load-induced fluid flow.

2.3.1. Substrate Strain

Application of external mechanical loads to bone produces strain in bone through deformation. How osteoblasts and osteocytes within the bone perceive the external load remains
unknown. One possible mechanism is that bone tissue strain induced by external loads directly induces cellular deformation. Data demonstrates that osteoblasts and osteocytes are indeed responsive to mechanical strain in vitro [204, 205]. However, the magnitudes of strain used in these studies was in the supraphysiologic range of >10,000 µstrain. These data suggest that, while osteoblasts and osteocytes are indeed responsive to mechanical strains, the magnitude of strain required to induce a cellular response may be too large to assign a role to direct substrate strain as a mechanocoupling mechanism.

2.3.2. Substrate Strain-induced Fluid Flow

The results above support the hypothesis that physiologic levels of strain do not directly activate osteoblasts and osteocytes. Instead, it is currently believed that the bending of bone in response to load-bearing results in pericellular fluid within the canaliculi and lacunae being forced from areas of greater pressure (stress) to areas of lower pressure (stress) [206]. Thus, strain indirectly induces a mechanical signal (fluid flow) over the surfaces of osteoblasts and osteocytes. In agreement with this model, it has been shown that bone cells are not measurably responsive to physiologic levels of strain, but are instead responsive to fluid flow induced by physiologic levels of strain [193, 194, 207].

Mathematical models for load-induced fluid flow estimate that physiologic levels of strain can induce fluid shear stresses in the order of 8–30 dynes/cm² across an osteocyte embedded in the lacunae and canaliculi [208, 209]. Several in vitro experiments have confirmed that osteocytes are responsive to fluid flow of this magnitude, engendering changes in cytosolic Ca²⁺ [166], the release of autocrine and paracrine factors [161, 210, 211], alteration in intercellular communication [161, 212-214], and changes in protein expression [161, 215].

That dynamic, but not static, loads are anabolic could be explained by fluid flow across bone cells. Static loads would induce one bout of flow, which may not be enough to induce a cellular
response. In contrast, dynamic loads would induce multiple bouts of flow, thereby generating enough localized loading bouts to activate an osteoblast or osteocyte.

2.3.2.1 Dilemmas Involving Osteoblastic Fluid Flow

That osteoblasts are responsive to fluid flow in vitro does not indicate that they experience in vivo shear stresses on the order of 8–30 dynes/cm², as predicted for osteocytes. A significant parameter in the model for load-induced fluid flow across osteocytes [209] is the microenvironment in which osteocytes reside, whose geometry is implicit in generating shear stresses of this magnitude. In contrast, osteoblasts are enclosed by marrow sinusoids that may be too wide to generate significant levels of shear stress during loading [216]. However, where the magnitude of shear stress experienced by in vivo osteoblasts may or may not be on the order as predicted by Weinbaum et al., in vitro data demonstrate that osteoblasts are responsive to much lower magnitudes of shear stress than are traditionally used in the laboratory setting. Wadhwa et al. demonstrated COX-2 expression in osteoblasts flowed at 0.1 dynes/cm² [216], as did Ogasawara et al. in osteoblasts flowed at 0.18 dynes/cm² [217]. These data strongly suggest that osteoblasts demonstrate mechanosensory capabilities in vivo.

An alternate explanation for this dilemma recognizes osteocytes as mechanosensory cells [218] that transduce fluid flow into a signal, be it electrical or chemical, that is communicated to osteoblasts. Given osteocyte-osteoblast coupling by gap junctions in vitro and in vivo, GJIC is presented to address this dilemma. In this model, osteocytes experience load-induced fluid flow (again, predicted to be on the order of 8–30 dynes/cm²) that is perceived by such osteocytic mechanosensors as described below. This induces signaling cascades whose mediators are communicated through gap junctions to the osteoblasts, the effector cells in skeletal anabolism.
2.4. Putative Mechanosensors

To respond to a mechanical signal such as fluid flow, osteoblasts and osteocytes must have some cellular machinery that acts as a mechanosensor to convert the mechanical signal into a biochemical event within the cell. Examination of the relevant literature demonstrates the ongoing debate as to the identity of the mechanosensor in bone cells. Indeed, the vast number of mechanosensors implicated to date clearly suggests that multiple proteins and protein complexes must be responsible for mediating response to load. This section shall focus on protein and lipid mechanisms that could be directly involved in sensing the mechanical load. Numerous other proteins, such as prostaglandin receptors, mediate the responsiveness to mechanical load, but no data to date demonstrates that these proteins are themselves activated in response to flow without activation of an upstream signaling cascade by a true mechanosensor.

2.4.1. Ion Channels

Ion channels are transmembrane proteins forming a gated pore between the cytosol and the pericellular environment that, upon opening, selectively increase ion flux between the two compartments. These channels are traditionally opened by changes in electrical potential (voltage-sensitive ion channels) or by ligand binding (ligand-activated ion channels, or ionotropes). Another isoform of these channels are directly opened by changes in membrane pressure; such channels are said to be mechanosensitive (MS) or stretch-activated (SA). The existence of such a MS channel in osteoblastic cells immediately implicates it as a putative mechanosensor for load-induced fluid flow. Duncan and colleagues have demonstrated such a cation-permeable channel, named the mechanosensitive cation channel (MSCC), in osteoblasts and osteosarcomas [219]. This channel is directly activated by fluid flow [220], and MCC inhibition attenuates flow-induced calcium transients [221] and load-induced prostaglandin I₂ [222] and TGF-β1 [223] release. The molecular identity of this functional MSCC has proven elusive, although data suggests that it may be a splice variant of the L-type voltage-sensitive
calcium channel (Ca\(_{v1.2}\)) [224, 225]. Additional putative MS channels in osteoblasts that have been shown to modulate responsiveness to fluid flow include the annexins [226] and polycystins [227].

2.4.2. Integrins, Focal Adhesions, and the Cytoskeleton

Integrins are specialized transmembrane proteins that attach cells to the basement membrane. Composed of two heteromeric subunits, the cytosolic faces of integrins participate in a complex signaling environment, called a focal adhesion. This complex ultimately binds the integrin to the actin cytoskeleton, which itself is linked to the nucleus. It has been postulated that this protein complex functions as a mechanosensor that enables conversion of external mechanical signals into appropriate cellular responses [198, 228-231]. Inhibiting integrin linkage to the basement membrane and subsequent focal adhesion formation significantly attenuated osteoblastic prostaglandin release and COX-2 induction [232]. Disruption of the actin cytoskeleton has been shown to attenuate flow-induced prostaglandin release in one study [211] but not in another [127]; whether these differences are due to cell type (osteoblast vs. osteocyte) or fluid flow profile (steady vs. pulsating) remains unclear. Integrin activation with an RGD peptide-coated magnetic bead resulted in cytoskeletal filament reorientation, nuclear distortion and nucleoli redistribution [233, 234], all of which were dependent upon the three major cytoskeletal components. These data support the hypothesis that mechanical forces can be transmitted and transduced into changes in cellular activity through the direct linkage of integrins to focal adhesions to the actin cytoskeleton to the nucleus [231, 235, 236].

2.4.3. Glycocalyx

The surface of a cell is covered in carbohydrates attached to integral membrane proteins, glycoproteins, and proteoglycans in the plasmalemma. This structure has been termed the glycocalyx [237], and has been found in all eukaryotic cells. While its existence has been known
for over 40 years [238], the function of the glycocalyx remains vague. In endothelial cells, it has been shown to mediate leukocyte adhesion and rolling [239], as well as regulating blood flow [237]. Recently, it has been suggested that the glycocalyx may itself function as a mechanostransducer. It is postulated that fluid forces physically deform the glycocalyx, the force of which is transmitted to the cell surface and subsequently to the cytoskeleton [240-242], in a manner similar to integrin activation described above. Alternately, the glycocalyx could trap metabolites released by the cell; application of fluid flow could disturb this trapped layer, promoting a ‘refeeding’ effect on the cell. Several reports support the hypothesis that the glycocalyx does function as a mechanosensor. Shear-induced arteriole dilation [243], NO release [241], and actin fiber reorganization [242] were all abolished following enzymatic digestion of the endothelial glycocalyx. In osteocytes, hyaluronidase treatment significantly impaired flow-induced PGE$_2$ release but not Ca$^{2+}$ transients [244].

2.4.4. Lipid Bilayer

Limited evidence also suggests that the lipid bilayer, the main component of the plasmalemma, is itself able to transduce fluid flow to directly activate heterotrimeric G proteins. This hypothesis derives from data demonstrating that fluid flow affects plasmalemma permeability [245] and fluidity [246, 247], and that plasmalemma composition affects the function of G proteins [248, 249], which are covalently bound to the plasmalemma [250, 251]. Gudi et al. reported that heterotrimeric G protein incorporation into phospholipids vesicles increased GTPase activity in response to shear stresses of 0-30 dynes/cm$^2$ [252]. The addition of cholesterol or LPC, to decrease vesicle fluidity, attenuated both basal and flow-induced GTPase activity; in contrast, addition of benzyl alcohol to increase membrane fluidity, potentiated basal GTPase activity [249]. In a subsequent study, Butler et al. demonstrated membrane discrimination between stepped and ramped changes in the applied shear stress that differentially regulated MAPK activation [253]. Mechanistically, it is suggested that fluid flow directly
activates G proteins by disrupting the lipid packing in the bilayer. This reduces the microviscosity of the lipids surrounding the G protein, which could then increase the activity of the G protein [252, 254].

2.5. Cellular Responses to Fluid Flow

*In vitro*, cells of the osteoblastic lineage are responsive to numerous varieties of mechanical signals. For example, changes in the osmotic environment [200, 255, 256], substrate strain [204, 257], direct membrane perturbation by micropipettes [258] or atomic force microscopy [259], induce many of the same responses induced by fluid flow. It should be noted, however, that bone cells *in vivo* are highly unlikely to experience significant or consistent changes in pericellular hypotonicity, supraphysiologic levels of substrate strain, or prodding by a pipette or a cantilever. As such, the author has chosen to focus on cellular responses to fluid flow in the following section.

2.5.1. Second Messenger Activation

Calcium is a ubiquitous second messenger implicated in a far range of intracellular signaling cascades [260-262]. It is neither created nor destroyed, in contrast to other second messengers like cyclic AMP (cAMP) and inositol 1,4,5-triphosphate (IP3). Instead, calcium signaling is regulated by calcium sequestration, either outside of the cell, or in cytosolic stores. Sequestration is facilitated by ATPases and exchangers that forcibly extrude calcium from the cytosol or into the endoplasmic reticulum. As such, the \([\text{Ca}^{2+}]_{i}\) in resting cells is on the order of 100–200 nM whereas the calcium concentration outside of the cell ranges from 1.5–2.0 mM and is on the order of 5mM in the endoplasmic reticulum [263]. Stimuli inducing changes in \(\text{Ca}^{2+}\) do so by activating channels in the plasmalemma or endoplasmic reticulum, promoting calcium flux down its chemical gradient into the cytosol.
The earliest measured response of osteoblasts and osteocytes to fluid flow is a rapid and transient increase in \([\text{Ca}^{2+}]_i\) that requires action of both calcium entry through ion channels, and calcium release from the endoplasmic reticulum [4]. Both the percentage of cells responding with a \(\text{Ca}^{2+}\) increase and the amplitude of the \(\text{Ca}^{2+}\) response are dependent upon the magnitude of the shear across the cells [264, 265]. Additionally, the concentration of serum in the flow media positively correlates with the magnitude and duration of the \(\text{Ca}^{2+}\) response [266]. In an elegant set of experiments, Haut-Donahue et al. implicated flow-induced chemotransport in the osteoblastic response to flow [267]. These results indicate that calcium responsiveness is mediated not only by flow-induced shear stress across the cell monolayer, but also by mass transport.

In addition to regulating cytosolic calcium levels, fluid flow stimulates cAMP and \(\text{IP}_3\) synthesis. Reich et al. demonstrated a rapid, shear rate-dependent increase in cAMP levels from 0.1–35 dynes/cm\(^2\) that remained elevated for the duration of the flow period. Interestingly, pre-treatment with ibuprofen to prevent PG synthesis, significantly attenuated flow-induced cAMP formation, suggesting that flow-induced cAMP accumulation is mediated by PG’s [268]. In another study, Reich et al. similarly demonstrated shear rate-dependent \(\text{IP}_3\) production that was maintained during 2 hours of flow but returned to basal levels for flow periods of 4 hours or longer [269]. \(\text{IP}_3\) generation in response to flow returned to basal levels 30 minutes after the cessation of flow. Inhibition of PG synthesis with ibuprofen attenuated flow-induced \(\text{IP}_3\) production, but was still statistically different from static controls, suggesting a partial contribution by PG in \(\text{IP}_3\) synthesis.

2.5.2. Prostaglandin Release and COX-2 Induction

Section 1.5.3 reviewed the effect of prostaglandin administration on bone formation and resorption, and a wealth of data indicates the importance of prostaglandins on load-induced bone osteogenesis. Pead and Lanyon demonstrated that dynamic loading increased periosteal thickness of the rooster ulna, and this effect was attenuated in the presence of indomethacin, an inhibitor of
both COX isozymes [270]. Chow et al. found similar results in loaded rat vertebrae [271]. Using isozyme-specific antagonists, Forwood demonstrated that COX-2, not COX-1, was responsible for new bone formation [272], demonstrating that the effects of load were mediated by COX-2 induction. Similarly, strained rat ulnae explants increased the rate of PG release compared to static controls [222]. Surprisingly, the generation of COX-2 knockout mice demonstrate no overt skeletal phenotype and continue to form bone in response to external load (CH Turner, personal communication). These results, however, could be explained by the presence of endogenous COX-1 to metabolize load-induced changes in arachidonic acid levels [211].

In vitro, fluid flow that induces shear stresses from 0.1–120 dynes/cm² induces both PG release and COX-2 induction in osteoblasts and osteocytes. Reich and Frangos demonstrated a 40% increase in PGE₂ release as early as 5 minutes after the onset of flow that increased linearly with sustained flow for rates between 6–24 dynes/cm². The timing of COX-2 induction in response to fluid shear stress suggests one of two mechanisms are mediating PG release in response to flow: either flow induces the release of PG’s pre-synthesized by COX-1, or flow increases cPLA₂ activity, thereby generating higher AA levels that can be metabolized to PG by COX-1.

Various signaling pathways have been implicated in PG release and COX-2 induction in response to flow. Pavalko et al. demonstrated the requirement of an intact cytoskeleton for osteoblastic COX-2 induction [273], as did Ajubi et al. in osteocytic PG release [211]. Ponik et al. recently showed that impaired focal adhesion formation attenuated PG release and COX-2 induction [232]. However, a recent report by Norvell et al. contradicted previous findings from the same laboratory, in which disruption of the actin microfilament, intermediate filament, or microtubule network did not affect PG release or COX-2 induction [127]. In contrast, Chen et al. examined the role of Ca²⁺ in COX-2 induction and found that calcium release from the ER, but not calcium entry, was required for COX-2 induction [274]. Additionally, Chen et al. also demonstrated that changes in Ca²⁺ were required for changes in the actin microfilament,
suggesting a possible link between the cytoskeletal studies from Pavalko and the calcium signaling studies from Duncan. Additionally, Ajubi et al. implicated both calcium entry and release in osteocytic PGE$_2$ release [211]. In separate studies, Wadhwa et al. implicated both the ERK1/2 and the PKA pathways in osteoblastic COX-2 induction [216, 275], whereas Ajubi et al. implicated PKC activation [211].

Data suggest that COX-2 expression in response to flow is regulated primarily at the transcriptional level. Wadhwa et al. utilized a luciferase reporter construct fused to the murine COX-2 promoter and found that flow-induced COX-2 synthesis paralleled luciferase activity [216]. The COX-2 promoter contains consensus binding sites for the GRE, NF-IL6 (C/EBP$\beta$), NF-$\kappa$B, Sp-1, AP-1 and -2, and CRE transcription factors [217, 276, 277]. Using a series of promoter deletions in the COX-2 promoter in MC3T3-E1 osteoblasts, Ogasawara et al. [217] sought to define the transcription factors and transcription regulatory elements involved in flow-mediated COX-2 induction. For cells exposed to 2.88 dynes/cm$^2$ of flow, the C/EBP$\beta$, AP-1, and CREB sites were crucial for COX-2 expression, but no role for the NF-$\kappa$B site was implicated. In contrast, Chen et al. demonstrated that NF-$\kappa$B activation is required for maximal COX-2 induction [278]. MC3T3-E1 osteoblasts exposed to fluid shear stresses of 12 dynes/cm$^2$ demonstrated robust nuclear staining for the p65 subunit of NF-$\kappa$B. Inhibition of NF-$\kappa$B translocation, through the use of protease inhibitors or a cell-permeant peptide masking the nuclear localization signal of NF-$\kappa$B, attenuated, but did not prevent, COX-2 induction compared to osteoblasts flowed in the absence of these agents. While these two studies differentially implicate NF-$\kappa$B in flow-induced COX-2 expression, it should be noted that these studies were performed at different flow rates. Thus, it is quite possible that flow at lower magnitudes does not require NF-$\kappa$B for maximal COX-2 induction, whereas flow at higher magnitudes does.
2.5.3. Alterations in Cell-cell Communication

Whereas the importance of GJIC on normal osteoblastic and osteoclastic function is recognized, there is ongoing debate regarding the effect of fluid flow on the sub-cellular localization of Cx43 and resultant changes on GJIC. Cheng et al. have demonstrated that steady laminar flow at 16 dynes/cm$^2$ for 2 hours promoted Cx43 localization away from the nucleus towards the plasmalemma, suggesting that flow increases Cx43 insertion into the membrane. Further, Jiang et al. found that fluid flow promoted the incorporation of Cx43 into Triton X-100-resistant plaques at the plasmalemma, consistent with GJ formation [279]. From the same laboratory, Cherian et al. demonstrated that flow increased Cx43 synthesis and subsequent GJIC that was dependent upon PGE$_2$ release and subsequent EP$_2$ receptor activation. Additionally, Alford et al. showed that fluid flow doubled the extent of serine phosphorylation on Cx43 and increased the number of osteocytes involved in GJIC [214]. These data strongly suggest that fluid flow increases GJIC. In contrast, however, Thi et al. demonstrated that oscillatory fluid flow decreased plasmalemma Cx43 and Cx45 immunofluorescent staining and GJIC [213]. Whether these differential findings are the effect of varied flow profile (steady vs. oscillating) or the duration of flow exposure remains unknown.

2.5.4. Proliferation and Differentiation

Fluid flow can promote skeletal anabolism by either enhancing osteoblastic proliferation or pre-osteoblastic differentiation. The former would increase the number of cells capable of secreting osteoid matrix, whereas the latter would increase the ability of a given osteoblast to secrete matrix. Kapur et al. found a 50% increase in [$^3$H]-thymidine uptake (a measure of DNA synthesis) in primary human osteoblasts flowed at 20 dynes/cm$^2$ for only 30 minutes that involved ERK1/2 activation [280]. Additionally, Jiang et al. demonstrated a 95% increase in the proportion of cells in the S (DNA synthesis) phase of the mitotic cycle when exposed to pulsating fluid flow [281]. Fluid flow similarly affects known markers of osteoblastic differentiation. In a
number of studies, flow significantly increased osteopontin release [10], alkaline phosphatase activity [280] and matrix deposition [282].

2.6. The Role of Cytosolic Calcium in Mechanotransduction

The previous section reviewed the effect of mechanical load, both in vitro and in vivo, on factors implicated in skeletal anabolism. It is currently accepted that exogenous mechanical loads deform bone, thereby forcing interstitial fluid through the calcified matrix across osteocytes residing within, and perhaps across osteoblasts. Fluid movement is perceived by these cells and ultimately transduced into bone formation by osteoblasts. This process appears to require PG synthesis by COX-2, whose upstream mediators include ERK and PKC [211, 216]. An underlying component in the activation of these signaling pathways is their regulation by Ca\(^{2+}\). As such, this section shall focus on the role of Ca\(^{2+}\) in mechanotransduction.

Ca\(^{2+}\) entry through ion channels is one mechanism for altering [Ca\(^{2+}\)], and, to date, four such Ca\(^{2+}\)-conducting channels have been implicated in osteoblast and osteocyte mechanotransduction. The first is the Ca\(_{v}1.2\) (formerly L-type VSCC) that has been identified in primary osteoblasts [283], clonal osteoblastic cell lines [219, 284, 285], osteosarcomas [286-288], and possibly osteocytes, although there is conflicting data on its expression in this cell phenotype [200, 289]. These channels have been implicated in load-induced Ca\(^{2+}\)\(_{i}\) transients [290, 291], ERK1/2 activation [5], prostaglandin release [211, 222] and matrix production [223, 290]. Li et al. recently demonstrated that in vivo administration of nifedipine or verapamil, high-affinity Ca\(_{v}1.2\) antagonists, significantly attenuated endosteal bone formation in response to tibial bending or ulnar loading [292, 293]. One report implicates Ca\(_{v}3.2\) (formerly T-type) in flow-induced PGE\(_2\) release and COX-2 induction [294]. The other two Ca\(^{2+}\)-conducting channels implicated in osteoblastic mechanotransduction are the mechanosensitive cation channel (MSCC) and the annexin V channel, both of which were briefly described in Section 1.4.1. Inhibition of MSCC channel activity with gadolinium has been implicated in Ca\(^{2+}\)\(_{i}\) transients [221, 291], NO, PGE\(_2\),
and TGF-β1 release [211, 222, 223]. Inhibition of annexin V activity, either with a specific antagonist or an inhibitory antibody, impaired flow-induced Ca\(^{2+}\) transients and c-fos expression [226]. Calcium entry through plasmalemmal channels is not the only means of increasing Ca\(^{2+}\) that affects mediators of bone formation, as Ca\(^{2+}\) release from the ER has also been implicated in mechanotransduction. Ca\(^{2+}\) release requires the activation of phospholipase C (PLC) that cleaves the phospholipid PIP\(_2\) into IP\(_3\) and diacylglycerol (DAG). IP\(_3\) diffuses to and activates IP\(_3\) receptors in the ER, promoting calcium release from the ER. In contrast, DAG remains in the plasmalemma where it activates protein kinase C. As such, PIP\(_2\) hydrolysis induces two different signaling cascades. Chen et al. demonstrated impaired c-fos and COX-2 synthesis and NF-κB translocation in osteoblasts flowed in the presence of a PLC inhibitor [274, 278]. Further, Hung et al. and You et al. implicated IP\(_3\)R activation in flow-induced Ca\(^{2+}\) transients [4, 10], as did Ajubi et al. with prostaglandin release [211].

The data reviewed above indicate that transients in Ca\(^{2+}\) are important for activation of factors implicated in bone formation, and studies have directly demonstrated the role of calcium entry in load-induced bone formation. Transients in Ca\(^{2+}\) are clearly achieved through either calcium entry or calcium release. A ligand that is capable of inducing both is ATP [9].
3. PURINERGIC SIGNALLING

In 1929, Drury and Szent-Györgyi reported that extracellular purines were able to induce heart block and dilatation of both coronary and peripheral vessels [295]. These, and other, data suggested that ATP was able to exert biological effects outside of the cell. Data from Burnstock demonstrated that the smooth muscle of the bladder and gut was innervated in a non-adrenergic, non-cholinergic fashion [296, 297] and led to neural purinergic signaling hypothesis, wherein ATP is released from a pre-synaptic cell to activate purinergic P2 receptors in the post-synaptic membrane. As this hypothesis gained credence, it was realized that purinergic signaling was not solely limited to neuronal transmission, but, in fact, occurs among many cell types.

3.1. ATP: Structure, Synthesis, and Cellular Function

ATP, and its related compounds, is composed of three chemically different components: an adenosine ring, a ribose moiety, and a triphosphate chain. The adenosine ring is formed during purine biosynthesis, which can occur either \textit{de novo} or by purine salvage pathways. The ribose moiety is formed during the pentose phosphate pathway, and \textit{de novo} synthesis leads to the generation of IMP, which is amidated to AMP. ADP and ATP are formed from AMP by nucleoside monophosphate kinases and nucleoside diphosphate kinases, respectively.

ATP is critically important to the cell because it serves as a free-energy donor to drive chemically unfavorable reactions. In the language of thermodynamics, a reaction is either endergonic (energy-requiring; $\Delta G^\circ > 0$) or exergonic (energy-releasing; $\Delta G^\circ < 0$). Under standard conditions, endergonic reactions cannot spontaneously occur. An endergonic reaction can be made to occur if it is coupled to an exergonic reaction and if the additive reaction is itself exergonic. That is,

\[ \text{If } \quad A \rightarrow B \quad \Delta G^\circ = 4 \text{ kcal/mol (thermodynamically unfavorable as } \Delta G^\circ > 0) \]
\[ \text{And } \quad C \rightarrow D \quad \Delta G^\circ = -10 \text{ kcal/mol (thermodynamically favorable as } \Delta G^\circ < 0) \]
\[ \text{Then } \quad A + C \rightarrow B + D \quad \Delta G^\circ = -6 \text{ kcal/mol (thermodynamically favorable as } \Delta G^\circ < 0) \]
The hydrolysis of ATP into ADP is an exergonic reaction ($\Delta G^0 = -7.3$ kcal/mol) and, as such, it is used to drive the completion of endergonic reactions. The chemical component of ATP that confers this function is the triphosphate moiety that contains two phosphoanhydride bonds. In this regard, ATP is used to maintain ionic gradients (such as driving the plasmalemmal Na$^+$/K$^+$ ATPase that maintains the membrane potential or the Ca$^{2+}$ ATPase that removes calcium from the cytosol into the ER), synthesize nucleotides, carbohydrates, proteins, and fats, contract muscles, and is required for countless other metabolic reactions. Once hydrolyzed to ADP, ATP is regenerated primarily by oxidative phosphorylation in the inner mitochondrial membrane, although some ATP can also be generated from glycolysis and the citric acid cycle.
Fig 3-1. Chemical structures of adenine, adenosine, 5’-AMP (Top, L-R), ADP, and ATP (Bottom, L-R). Images from http://www.chemfinder.com
3.2. Mechanisms for ATP Release

For ATP to function as a signaling molecule, it must first be released to the extracellular environment. Its charged nature prevents simple passive diffusion across the plasmalemma; instead, some mechanism must be responsible for transporting ATP from the cytosol into the pericellular space. However, before addressing putative mechanisms for ATP release, a concern must be addressed, a concern that provided great initial resistance to the purinergic hypothesis: Given the importance of ATP for the cell, how does its release not impair cellular activity? The answer to this question involves the relative amount of ATP released from the cell compared to the amount of ATP in the cytosol. Cytosolic ATP concentration is on the order of millimolar, with values ranging from 3–10mM, depending on the cell type [298]. In contrast, extracellular steady-state levels of ATP are on the order of 50nM, and no data (in the absence of cytolysis or other cellular distress) has indicated extracellular ATP levels above 1µM [298]. Thus, the amount of cellular ATP released to the pericellular environment is on the order of 0.02–0.1% of the cytosolic stores, highly suggesting that the amount of ATP released to the pericellular environment cannot and does not compromise the metabolic activity of the cell [298].

3.2.1. Vesicular Exocytosis

A characteristic feature of nerve terminals is neurotransmitter-filled synaptic vesicles [299]. Membrane depolarization activates voltage-gated calcium channels, promoting calcium influx into the pre-synaptic terminal. This localized increase in Ca$^{2+}$, regulates vesicle fusion to the plasmalemma and subsequent neurotransmitter exocytosis into the synaptic cleft, where neurotransmitter action is exerted on appropriate receptors in the post-synaptic membrane. A variety of techniques, including electron microscopy [300] and subcellular fractionation of vesicles [301], has demonstrated vesicle fusion to the plasmalemma and nucleotide storage in vesicles, respectively.
A requirement of the neural purinergic hypothesis is ATP localization within vesicles, which has been demonstrated both directly and indirectly. One such direct mechanism involves the fluorophore, quinacrine, an anti-malarial agent that demonstrates high affinity for ATP [302]. ATP-filled vesicles reveal punctate fluorescent bodies, indicating particulate ATP storage. Such a staining pattern has been demonstrated in the endothelium [303], epithelium [304], pancreatic acini [305] and the colon [306], amongst others. Subcellular fractionation to isolate vesicles demonstrates ATP localization in synaptic vesicles [307], chromaffin granules, and dense core granules [305], wherein the intravesicular ATP concentrations may be as high as 150 mM [308]. ATP-containing vesicles have also been found in the organ of Corti from guinea pig cochlea [309] and the electric organ of the electric eel [301]. Indirectly, vesicular ATP localization is suggested because of experimental similarities between its release and the release of classical neurotransmitters. For example, membrane depolarization with potassium chloride demonstrates loss of quinacrine fluorescence [310] and increased ATP content in the extracellular fluid [311-313]. Additionally, inhibitors of exocytosis prevent ATP release in endothelial cells exposed to fluid flow [303] and in the guinea pig ureter epithelium upon distension [314 non-endothelium].

If ATP is localized into vesicles, a specific transporter must be involved, for the same reasons that ATP cannot passively diffuse across the plasmalemma. Surprisingly little investigation has focused on this issue. Biochemically, the existence of such a transporter has been demonstrated by Stadler and Fenwick [315] and putatively identified as glyceraldehyde-3-phosphpate dehydrogenase [316].

3.2.2. ABC Transporters and Anion Channels

ATP-binding cassette (ABC) proteins are a superfamily of ATPases that transport various substrates, including amino acids, ions, and sugars against concentration gradients [317]. These transporters, specifically CFTR and P-glycoprotein (Pgp), have been implicated in ATP release in epithelial and other cells. Abraham et al. transfected CHO cells with mdr (the gene encoding Pgp)
and found that conditioned media from transfected cells demonstrated 3-fold higher levels of ATP than did conditioned media from non-transfected cells [318]. A linear correlation was found between ATP levels in conditioned media and Pgp expression. Reisin et al. reported similar results in murine mammary carcinomas transfected with cftr [319]. Schwiebert et al. showed that airway epithelial cells from cystic fibrosis patients (in whom CFTR is mutated) released less ATP than did airway epithelial cells from normal patients [320]. However, electrophysiological studies demonstrated that CFTR channels do not demonstrate measurable ATP conductance [321, 322]. Instead, it was suggested that CFTR and Pgp enhance the activity of a separate channel that conducts ATP [323-325].

A variety of factors support the hypothesis that ATP can be released through a channel. The first is its overwhelming concentration gradient. Assuming cytosolic and extracellular ATP concentrations to be 10 mM and 10 nM, respectively, there is a nine-fold order of magnitude difference in its chemical gradient. Secondly, the diffusion coefficient of ATP is quite similar to that of Ca^{2+} \ (7.01 \times 10^{-6} \text{ cm}^2/\text{s} \text{ vs. } 7.54 \times 10^{-6}, \text{ respectively}) [326], indicating that ATP could easily diffuse through a channel. As the majority of ATP molecules is mainly in the form of a MgATP^{2-} anion at physiologic pH [327], it is possible that anion channels conduct ATP across the plasmalemma. One possible channel is the voltage-dependent anion channel (VDAC) that is found in the outer mitochondrial membrane, were it serves to release newly synthesized ATP to the cytosol [328-330]. Recent evidence demonstrates the existence of “VDAC-like” maxi-anion channels in the plasmalemma of astrocytes [331, 332] and the epithelium [333, 334].

3.2.3. **Connexon Hemichannels**

Connexons are multi-subunit proteins composed of six connexin isoforms. Connexons traditionally function in GJIC by directly binding to another connexon in an apposed cell, thereby promoting the exchange of chemical and electrical signals. Recent data suggest an additional role for connexons, independent of direct intercellular communication; these data demonstrate that
unpaired connexons, or hemichannels, function as do other channels and link the cytosol to the pericellular environment (Figure 3-2). Hemichannels have been shown to mediate fluxes in NAD\(^+\) [335], cyclic ADP-ribose [336] and prostaglandin [279] release, as well as regulating anti-apoptotic mechanisms [337, 338].

Several studies have demonstrated a role for gap junctions and hemichannels in ATP release. The transfection of Cx43 into C6 glioma cells increased ATP permselectivity compared to cells transfected with Cx32 [339, 340] and increased ATP release from the cytosol [341-343]. Additionally, chondrocytes exposed to cyclic compression demonstrated increased ATP levels in conditioned media that was attenuated in the presence of the gap junction uncoupler octanol [344]. The role of gap junctions and hemichannels is less clear in cells of the osteoblastic lineage. Romanello et al. demonstrated hemichannel function in a transformed human osteoblastic cell line [202]. Depletion of extracellular calcium, known to activate gap junction hemichannels [345], increased ATP release compared to cells cultured in the presence of calcium; however, addition of the gap junction and hemichannel antagonist AGA [346, 347] had no effect on the ATP release evoked by calcium-free media [202], suggesting that, in this cell line, hemichannel activation did not mediate fluxes in ATP.
Fig 3-2. Connexons function as hemichannels or in GJIC.
3.3. P2 Purinoceptors

3.3.1. Ionotropic P2X Receptors

Ionotropic P2X receptors induce rapid (< 10ms) non-selective monovalent and divalent cation flux that increases Ca\(^{2+}\)\(_i\) and depolarizes the cell. Calcium influx through a P2XR is a significant component of the Ca\(^{2+}\) source, but depolarization additionally activates voltage-activate calcium channels to enhance the Ca\(^{2+}\)\(_i\) influx [348]. P2X receptor activation has also been coupled to activation of calcium-dependent K\(^+\) and Cl\(^-\) channels [349] and has physiological roles ranging from synaptic transmission to regulation of blood clotting [350].

Seven P2X isoforms have been cloned [351]. Each P2X receptor is composed of 379–442 amino acids and contain two transmembrane domains, with much of the receptor occurring extracellularly as an intervening hydrophobic loop; both the amino and carboxy termini are located in the cytosol. A single P2X receptor is not functional as an ion channel. Instead, multimerization is required to form a channel [352] with a stoichiometry of three subunits per channel [353, 354]. Subunit trimerization can occur in both homo- and heterotypic fashions, with the phenotype of a heterotrimer ranging between those of the individual subunits. The extracellular loop contains 2-5 consensus sites for N-linked glycosylation, which is required for ATP-induced currents [351]. Mutagenesis of putative ATP-binding sites in the extracellular loop differentially affects isoform responsiveness to ATP, consistent with their different properties (Table 3-1) [350].

In the continued presence of agonist, the time course of channel desensitization ranges from fast desensitization (P2X₁, P2X₃) to slowly or non-desensitizing (P2X₂, P2X₄₋₇) [351]. A few studies have addressed which residues are involved in receptor desensitization. Koshimizu et al. found that swapping six amino acids from the P2X₃ receptor into the P2X₄ receptor slowed P2X₃ receptor desensitization and increased P2X₄ receptor desensitization, suggesting that this highly-charged stretch of amino acids in the C terminus played a major role in determining the rate of receptor desensitization [355]. Boue-Grabot et al. identified a PKC phosphorylation site in the
amino terminus of the P2X2 receptor, that was not found in rapidly-desensitizing P2XR’s, the mutation of which increased the rate of receptor desensitization [356]. As the hydrophobic transmembrane domains are not well conserved among P2X receptors and these receptors display varying degrees of desensitization, it was postulated that these hydrophobic domains are involved in receptor desensitization [351]. This was confirmed by receptor mutagenesis of the fast desensitization P2X1 and the non-desensitizing P2X2 receptors. Werner et al. reported that splicing the transmembrane domains of the rapidly-desensitized P2X1 to slowly-desensitizing P2X2 confers rapid desensitization phenotype to P2X2, and vice-versa. In contrast, transfer of extracellular domain had no effect on rate of desensitization [357]. Two isoforms demonstrate vastly altered properties in the sustained presence of ATP. The P2X2 channel becomes permeable to large organic cations, such as NMDA, whereas, when the presence of divalent cations is low, the P2X7 receptor channel becomes a non-selective pore that is permeable to small molecules as well as ions.

Similar experiments have sought which residues are critical for ligand binding. The mutation of aromatic amino acid residues decreased ATP potency at both P2X1 and P2X2 receptors, suggesting the involvement of N-F-(R/T) motif in ligand binding [350, 358]. Three molecules of ATP are required to activate the receptor channel, suggesting that each extracellular loop domain binds one molecule of ATP [350, 351].
<table>
<thead>
<tr>
<th></th>
<th>Permeability(^1)</th>
<th>Conductance (pS)</th>
<th>Desensitization</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X(_1)</td>
<td>4</td>
<td>~ 18</td>
<td>Rapid and sustained</td>
<td></td>
</tr>
<tr>
<td>P2X(_2)</td>
<td>2.5</td>
<td>30</td>
<td>Non-desensitizing</td>
<td>Acidification increases current potentioned by Zn and Cu</td>
</tr>
<tr>
<td>P2X(_3)</td>
<td>4</td>
<td></td>
<td>Sustained &gt; 30 (\mu)M</td>
<td></td>
</tr>
<tr>
<td>P2X(_4)</td>
<td>4</td>
<td>~ 9</td>
<td>Intermediate</td>
<td>Acidification (His-286) decreases current Permeable to organic cations with prolonged activation</td>
</tr>
<tr>
<td>P2X(_5)</td>
<td></td>
<td></td>
<td>Slow/none</td>
<td></td>
</tr>
<tr>
<td>P2X(_6)</td>
<td></td>
<td></td>
<td>Do not form homomultimers</td>
<td></td>
</tr>
<tr>
<td>P2X(_7)</td>
<td></td>
<td>400</td>
<td>None</td>
<td>Pore formation with extended activation</td>
</tr>
</tbody>
</table>

\(^1\) Ratio of calcium permeability to sodium permeability

**Table 3-1.** Properties of P2X receptors.  
After North, 2002 [351].
3.3.2. Metabotropic P2Y Receptors

In contrast to P2X receptors, P2Y receptors are activated by both purines (ADP and ATP) and pyrimidines (UTP and UDP). Currently, there are eight members of the P2Y receptor family: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄. The gaps in P2Y numbering is reflection of recognition that certain receptors had been erroneously accepted to the family; it has since been shown that P2Y₅ and P2Y₇, P2Y₉, and P2Y₁₀ are not nucleotide receptors.

P2Y receptors are composed of 308–377 amino acids and are approximately 36-53 kDa in mass after glycosylation. Structurally, P2Y receptors belong to the rhodopsin-like G protein-coupled receptors, with a seven-pass membrane-spanning protein, cytosolic carboxyl terminus and an extracellular amino terminus [9]. In contrast to the P2X receptors, wherein the ligand binding domain is relatively unknown, the P2Y receptors demonstrate a conserved sequence of positively-charged amino acids in transmembrane domains 3, 6, and 7 that are thought to be involved in ligand binding through electrostatic interactions with the phosphate groups in ATP [359]. Site-directed mutagenesis that converted these positively-charged amino acids to neutral amino acids decreased ATP and UTP potency by 100- to 850-fold [360]. Further, all cloned P2Y receptors share the H-X-X-R/K motif in transmembrane domain 6, which is crucial for agonist activity [361].

The majority of P2Y receptor isoforms are coupled to the Gq/11 protein to activate PLC and in turn release intracellular calcium stores. There are additional isoforms coupled to Gs (to increase cAMP) or Gi (to decrease cAMP). Receptor activation is implicated in vasodilatation [362, 363], smooth muscle proliferation [364].
<table>
<thead>
<tr>
<th>Signal Transduction</th>
<th>Desensitization</th>
<th>Preferential agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y₁</td>
<td>Gq/11</td>
<td>Not apparent</td>
</tr>
<tr>
<td>P2Y₂</td>
<td>Gq/11</td>
<td>Not apparent</td>
</tr>
<tr>
<td>P2Y₄</td>
<td>Gᵢ (possibly)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Gq/11</td>
<td></td>
</tr>
<tr>
<td>P2Y₆</td>
<td>Gq/11</td>
<td>No</td>
</tr>
<tr>
<td>P2Y₁₁</td>
<td>Gq/11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gs</td>
<td></td>
</tr>
<tr>
<td>P2Y₁₂</td>
<td>Gi</td>
<td></td>
</tr>
<tr>
<td>P2Y₁₃</td>
<td>Gi</td>
<td></td>
</tr>
<tr>
<td>P2Y₁₄</td>
<td>Gi</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2. Properties of P2Y receptors. After Ralevic and Burnstock, 1998 [9].
3.4. Extracellular ATP Degradation and Re-synthesis

Termination of ATP signaling is not solely dependent upon P2 receptor. Termination of P2 signaling also involves degradation of ATP into metabolites that are unable to activate P2 receptors. Ecto-enzymes responsible for such degradation exist in both membrane-bound and soluble forms. Ecto-apyrases hydrolyze the $\gamma$- and $\beta$-phosphates of ATP, generating ADP and 5'-AMP [365-368]. Similar hydrolysis is also achieved by ecto-ATPase and ecto-ADPases [298]. Ecto-5'-nucleotidases subsequently convert 5'-AMP into adenosine (Figure 3-3), itself a high-affinity agonist for P1 receptors. Specific Na$^+$/nucleoside co-transporters are responsible for adenosine uptake into the cell and termination of P1 signaling.

The cell membrane can also express a set of kinases, ecto-nucleotide diphosphokinases (ecto-NDPKs) [368-371], that counter ATP hydrolysis by ecto-nucleotidases, and therefore prolong P2 signaling. Ecto-NDPKs catalyze the transfer of a $\gamma$-phosphate from an NTP to a NDP in the general reaction $N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$; such enzymes have recently been identified in osteoblastic cells [372].
Ecto-nucleoside diphosphokinase
Ecto-adenylate kinase
Ecto-adenylate kinase
Ecto-kinase

ATP
ADP
AMP
Adenosine

Ecto-ATPase
Ecto-apyrase
Ecto-ADPase
Ecto-apyrase
Ecto-5'-nucleotidase

Figure 3-3. Degradation and regeneration of extracellular nucleotides
3.5. P2 Receptor Activation in Osseous Cells

In the 30-plus years since Burnstock proposed the purinergic hypothesis, nearly 3500 papers have been published on the activation and action of P2 receptors. Of these, approximately 30 papers directly relate to the cells of bone, demonstrating that intensive study of these receptors in skeletal architecture has not been performed. However, these reports have detailed the mechanism of ATP-induced Ca\(^{2+}\) mobilization and implicated a vital role for P2 receptors in regulating osteoblast and osteoclast maturation.

3.5.1. P2 Receptors in Osteoblasts

P2 receptor expression has been demonstrated functionally and directly in osteoblasts, through IP\(_3\) hydrolysis and Ca\(^{2+}\) mobilization, and RT-PCR, respectively. Using the UMR-106 rat osteosarcoma cell line, Kumagai et al. first showed that addition of ATP dose-dependently induced biphasic transients in Ca\(^{2+}\)\(_i\), with the earlier response dependent upon Ca\(^{2+}\) release from the ER, and the later response requiring Ca\(^{2+}\) entry [373]. A second study by Kumagai et al. found that ADP similarly affected Ca\(^{2+}\)\(_i\), although the later response was weaker compared to the response from cells challenged with ATP [374]. Reimer and Dixon showed that UMR-106 cells failed to increase Ca\(^{2+}\)\(_i\) with subsequent ATP additions, whereas sequential addition of ADP and UTP (and vice-versa) increased Ca\(^{2+}\)\(_i\) levels to the same degree, from which they concluded that ADP and UTP are agonists at different P2Y subtypes expressed in UMR-106 cells [375]. Schofl et al. used ATP addition and the resultant Ca\(^{2+}\)\(_i\) increase in primary human osteoblasts and a human osteosarcoma cell line to demonstrate the existence of P2 receptors in human osteoblasts [376]. Interestingly, ATP failed to increase Ca\(^{2+}\)\(_i\) in another rat osteosarcoma cell line, ROS 17/2.8, suggesting that these cells do not express functional P2 receptors. The expression of P2X\(_4\), P2Y\(_1\), P2Y\(_2\), P2Y\(_4\), and P2Y\(_6\) have been confirmed by RT-PCR in human osteoblasts [8, 377].

ATP functions as a mitogen in a number of cells, including hepatocytes [378], mesangial [379] and epithelial [380] cells. Shimegi found that ATP dose-dependently increased MC3T3-E1
osteoblast DNA synthesis in a PKC- and prostaglandin-independent manner [6]. Nakamura et al. published a similar report that, through the use of purinoceptor antagonists, suggested the involvement of P2X5 receptors in the MG-63 human osteosarcoma cell line [8]. As P2X5 receptors expression is restricted to the differentiating cell layers in the epithelia and hair follicles [381], it is thought that they may also participate in the regulation of osteoblast differentiation.

Further, P2 receptor activation influences osteoblast activity. Jones et al. showed that ATP dose-dependently suppressed bone formation in dentin grooves [382], although the concentrations found to inhibit formation were rather high and the effect of UTP on nodule formation was ambiguous. A subsequent study by Hoebertz et al. found that lower concentrations of ATP and UTP, as low as 1–10 µM, strongly inhibited bone formation in rat osteoblasts in vitro [383]. Neither adenosine nor ADP demonstrated any effect on nodule formation, eliminating the possibility that P1 receptors or the P2Y1 receptor was responsible for these effects. That a P2Y2 pseudo-agonist, ATPγS, also inhibited bone formation in the study by Jones et al. lead Hoebertz et al. to conclude that activation of the osteoblast P2Y2 receptor inhibited bone formation. However, the specificity of ATPγS for the P2Y2 receptor over the P2Y4 receptor is questionable [384], clouding this implication.

P2 receptor activation has also been shown to potentiate the effects of both local and systemic factors. PTH-mediated Ca2+ signaling in rat osteoblasts was synergistic in the presence of ATP, as was PTH-induced c-fos expression, suggesting that PTH-induced bone remodeling is enhanced by localized release nucleotide triphosphates [385, 386]. Similar effects were demonstrated with localized factors PDGF and IGF-I in studies by Shimegi and Nakamura et al. [6, 8]. Provided the localized nature of bone remodeling, these data suggest a mechanism wherein localized release of ATP enhances remodeling induced by systemic and local factors [385].
3.5.1.1 Osteoblast P2 Receptors and Mechanotransduction

Of particular interest to those in the mechanotransduction field was that ATP addition to static osteoblasts induced \( \text{Ca}^{2+} \) transients to the same extent as did application of fluid flow. Utilizing the ectonucleotidase apyrase, You et al. demonstrated that flow-induced \( \text{Ca}^{2+} \) transients in MC3T3-E1 murine osteoblasts required extracellular nucleotides [10]. Based on the \( \text{Ca}^{2+} \) response to cells exposed to flow in the presence of purinoceptor agonists or antagonists, You et al. were able to conclude that either P2Y\(_2\) or P2Y\(_4\) purinoceptors were responsible for \( \text{Ca}^{2+} \) mobilization. The P2Y\(_2\) receptor was implicated when cells treated with antisense DNA against the P2Y\(_2\) receptor failed to respond to flow with a \( \text{Ca}^{2+} \) increase. However, based on a previous report by Allen et al., who demonstrated that the concentration of fetal bovine serum in the flow media modulated osteoblastic responsiveness to flow [266], You et al. surmised that the ATP required to activate the P2Y\(_2\) was present in the fetal bovine serum, and not released in response to flow.

3.5.2. P2 Receptors and Osteoclasts

Expression of P2 receptors in osteoclasts has been demonstrated in similar manners as used for osteoblasts. Yu and Ferrier reported that ATP-induced \( \text{Ca}^{2+} \) transients in rat osteoclasts persisted in the absence of extracellular calcium and were blocked by the G protein antagonist GDP\(\beta\)S [387, 388], indicating the involvement of P2Y receptors in this response. Similar results were found by Weidema et al., who used purinoceptor agonists to demonstrate the expression of multiple P2Y isoforms [389]. Additionally, the responsiveness to ATP was a function of osteoclast morphology, suggesting P2 receptor regulation by the actin cytoskeleton. Using RT-PCR, Buckley et al. showed that human osteoclasts express P2X\(_2\), P2X\(_{4-7}\), P2Y\(_1\), P2Y\(_2\), P2Y\(_4\), P2Y\(_6\), and P2Y\(_{11}\) [390].

A role for P2 receptor activation in bone resorption was first demonstrated by Bowler et al., wherein the addition of exogenous ATP to human giant osteoclastomas increased resorption that
was thought to be mediated by the P2Y$_2$ receptor [391]. A later study showed that the addition of UTP, a potent P2Y$_2$ and P2Y$_4$ agonist, failed to induce resorption, suggesting that the effects of ATP on resorption are not mediated through the P2Y$_2$ receptor [392]. Morrison et al. found that ATP at low concentrations (0.2–2 µM) increased osteoclast and resorption pit formation by 3.3-fold and 5.6-fold, respectively, whereas higher concentrations (20–200 µM) inhibited both osteoclast and resorption pit formation [393]. The effect of ATP on resorption was enhanced when cultures were grown in acidified media, suggesting the involvement of the acid-sensitive P2X$_2$ receptor. Hoebertz et al. were able to link a specific P2 receptor to bone resorption using ADP and 2-methylthioADP (2-MeSADP), both potent agonists for the P2Y$_1$ receptor [394]. Both ADP and 2-MeSADP increased resorption pit formation at concentrations from 40 nm–1 µM, whereas higher concentrations had no effect, consistent with the results of Morrison et al [393]. ADP addition in the presence of a P2Y$_1$ antagonist, MRS 2179, inhibited the stimulatory effect of ADP on resorption. Additionally, ADP and ATP at 0.2–2 µM increased osteoclast formation.

Because the effects of ADP on resorption required PG synthesis and the experiments utilized a co-culture model that may have contained osteoblasts, the authors were unable to conclude whether ADP induced its effects directly on osteoclasts or indirectly on osteoblasts, wherein P2Y$_1$ activation induces the release of pro-resorptive factors. A subsequent report by Buckley et al. indicated that the effect of ATP on resorption is indirectly mediated through upregulation of RANKL by osteoblasts through the P2Y$_1$ receptor [390].

### 3.6. The Contentious Role of the P2X$_7$ Receptor in Bone Modeling and Remodeling

Several investigators have examined the role of the P2X$_7$ receptor (P2X$_7$-R) in regulating bone formation. As osteoclasts are derived from hematopoietic cells and the P2X$_7$-R is highly expressed in such cells, several studies have examined the role of the P2X$_7$-R in osteoclast maturation and activation. Because P2X$_7$-R antagonism inhibited giant cell fusion [395], Gartland et al. examined
the role of the P2X$_7$R in osteoclast formation. Their data demonstrates that P2X$_7$R antagonism inhibited osteoclast formation 8-fold from one donor and 3-fold from another donor, impaired resorption in vitro, and promoted apoptosis of mononuclear precursors [396]. In contrast, Hiken and Steinberg recently demonstrated that ATP downregulates P2X$_7$R expression and inhibits osteoclast formation from RAW 264.7 cells [397]. Another study demonstrated that ATP induces NF-κB translocation through the P2X$_7$R in osteoclasts independent of RANKL [398], suggesting that P2X$_7$R activation increases osteoclast formation and activity.

The generation of P2X$_7$R knock-out mice has not yet clarified its role in these processes. A report by Ke et al. suggests that the P2X$_7$R is important in both bone formation and remodeling. The P2X$_7$R $^{-/-}$ mice demonstrated decreased periosteal bone formation, decreased bone content in the distal femoral metaphysis and the femoral shaft compared to matched controls, and increased osteoclast number and surface, compared to matched P2X$_7$R $^{+/+}$ controls [399]. In contrast, Gartland et al. demonstrated that these mice demonstrated no overt skeletal phenotype compared to age-matched controls and were able to form multi-nucleated osteoclasts both in vitro and in vivo [400].
| P2X<sub>2</sub> | Increased formation and resorption | [393] |
| P2X<sub>5</sub> | Increased proliferation and/or differentiation | [8] |
| P2X<sub>7</sub> | Periosteal bone formation | NF-κB activation; Increased formation | [396, 398, 399] |
| P2Y<sub>1</sub> | Increased RANKL; Enhanced PTH signaling | Increased formation | [386, 390, 394] |
| P2Y<sub>2</sub> | Inhibited bone formation; Flow-induced Ca<sup>2+</sup> transients | | [10, 401] |

**Table 3-3.** The consequences of P2 receptor activation in osteoblasts and osteoclasts.
Fluid flow, as an in vitro model for mechanical loading, induces a variety of factors implicated in skeletal anabolism. Transients in Ca$^{2+}_i$ are required for activation or release of a number of these factors. You et al. recently demonstrated that the ATP-activated P2Y$_2$ receptor is required for flow-induced Ca$^{2+}_i$ increases. Flow could mobilize ATP for P2Y$_2$ activation in two ways: increasing the rate of release of cytosolic ATP, or by promoting mass transport of ATP released constitutively. Provided the similarities between endothelial and osteoblastic responses to flow, we hypothesized that ATP is contained in cytosolic vesicles and is released through a mechanism similar to classical neurotransmitters. Additionally, we sought the role of flow-induced ATP secretion on prostaglandin release.
4. FLUID SHEAR-INDUCED ATP SECRETION MEDIATES PROSTAGLANDIN RELEASE IN MC3T3-E1 OSTEOBLASTS

4.1. Abstract

ATP is rapidly released from osteoblasts in response to mechanical load. We examined the mechanisms involved in this release and established that shear-induced ATP release was mediated through vesicular fusion and was dependent on Ca\(^{2+}\) entry into the cell via L-type voltage-sensitive Ca\(^{2+}\) channels. Degradation of secreted ATP by apyrase prevented shear-induced PGE\(_2\) release.

4.2. Introduction

Bone is a dynamic organ, with its architecture constantly changing in accordance with the mechanical usage required of it. As external forces placed upon bone decrease, as occurs in prolonged bed rest, immobilization or microgravity, the skeleton undergoes net resorption resulting in significant bone loss [402]. Conversely, increased external forces on the skeleton can produce net bone accumulation [206]. Various in vitro loading techniques, including hypotonic swelling, substrate strain, and fluid shear stress (FSS), have been developed to study the cellular responses and mechanisms involved in the perception of mechanical stimuli by bone cells. While none of these models completely replicate the stresses endured by bone, most produce osteoblastic responses that are considered anabolic in vivo. These responses include transients in intracellular calcium levels ([Ca\(^{2+}\)]\(_i\)) [4], changes in gene expression [216, 273, 291], and prostaglandin release [215, 269]. We have focused this study on the effects of fluid shear since we have shown that fluid shear, and not physiologic levels of mechanical strain, increases the expression of osteopontin, c-fos, cyclooxygenase 2, and TGF\(\beta\) [193, 291].

Osteoblasts respond to FSS with a rapid increase in intracellular Ca\(^{2+}\) that is dependent on both extracellular Ca\(^{2+}\) entry and intracellular Ca\(^{2+}\) release [4]. While we have shown that FSS-induced increases in gene expression are mediated by intracellular Ca\(^{2+}\) release in osteoblasts
[274], \( \text{Ca}^{2+} \) entry in response to shear has been shown to be required for release of prostaglandins [211], nitric oxide [403], and TGF\( \beta \) [223]. Rapid \( \text{Ca}^{2+} \) entry must occur through ion channels and two potential candidates for mediation of FSS-induced \( \text{Ca}^{2+} \) entry in osteoblasts are the mechanosensitive, cation-selective channel (MSCC) and the L-type, voltage-sensitive \( \text{Ca}^{2+} \) channel (L-VSCC) (for review, see [404]). The MSCC has been shown to be important in the release of prostaglandins [211] and TGF\( \beta \) [223], while inhibition of the L-type VSCC has been shown to inhibit nitric oxide release in bone organ cultures [222] and reduce loading-induced bone formation \textit{in vivo} [293].

There is a significant body of evidence demonstrating that ATP in the extracellular milieu induces a host of physiologic responses upon activation of ATP-binding purinergic (P2) receptors. These receptors are found in a wide variety of cell types and tissues and have been shown to alter \( \text{Ca}^{2+} \) signaling in numerous cell types. P2 receptors can be divided into two families of receptors: metabotropic P2Y receptors that induce intracellular \( \text{Ca}^{2+} \) release through activation of G proteins and ionotropic P2X receptors that are ligand-gated channels. Osteoblasts express a variety of P2Y and P2X receptors [386], and activation of these receptors have been shown to increase \( [\text{Ca}^{2+}]_i \), propagate calcium waves [165], induce \( c-fos \) [386] and increase proliferation [6, 8]. Release of ATP from the cytosol to the pericellular environment is a regulated process and its extracellular availability for P2 receptor binding is limited by the presence of membrane-bound nucleotidases [405]. The mechanism(s) of ATP release are unclear, yet chloride-conducting channels [323, 406], gap junctional hemichannels [202, 341], and vesicular mechanisms [314, 407] have been implicated in the controlled release of ATP. In this study, we examined the effects of fluid shear stress on ATP release in MC3T3-E1 osteoblasts. We demonstrate that shear transiently increases ATP and that this release is \( \text{Ca}^{2+} \)-dependent. We further show that the shear-induced release of ATP is blocked by inhibition of the L-type voltage-sensitive calcium channel (L-VSCC) mediated by vesicular fusion. Most significant is the
observation that ATP activation of P2 receptors is important for shear-induced PGE\(_2\) release.

### 4.3. Materials and Methods

#### 4.3.1. Cell Culture

MC3T3-E1 cells, a murine osteoblast-like cell line (a gift from Dr. Mary C. Farach-Carson, University of Delaware) were grown in minimal essential medium, \(\alpha\)-modification containing 10% fetal bovine serum (Gibco, New York, NY), 100 U/ml penicillin G and 100 \(\mu\)g/ml streptomycin. Cells were maintained in a humidified incubator at 37\(^\circ\)C with 5% CO\(_2\)/95% air and subcultured every 72 hours. For shear studies, 80,000 cells were seeded onto rat-tail type I collagen-coated (100 \(\mu\)g/ml; BD, Franklin Lakes, NJ) glass slides. Fluid shear experiments were performed two days later, when the cells were 80-85% confluent. Flow media consisted of minimal essential medium, \(\alpha\)-modification containing 0.5% fetal bovine serum, 100 U/ml penicillin G, 100 \(\mu\)g/ml streptomycin, and 20 mM HEPES, pH 7.4.

#### 4.3.2. Fluid Flow Experiments

Fluid flow was applied to cells in a parallel plate flow chamber using a closed flow loop, as described previously [408] (Cytodyne, San Diego, CA). This system uses a constant hydrostatic pressure head to drive media through the channel of the flow chamber to subject the cell monolayer to steady laminar flow resulting in a well-defined fluid shear stress of 12 dynes/cm\(^2\). The apparatus was maintained at 37\(^\circ\)C throughout the duration of experimentation. The correlation between shear and flow rate was calculated using the equation

\[
\tau = \frac{6Q\mu}{bh^2} \quad (\text{Equation 1})
\]

where \(Q\) is the flow rate (cm\(^3\)/s); \(\mu\) is the viscosity of the flow media (0.01 dyne\(\cdot\)s/cm\(^2\)); \(h\) is the height of the channel (0.022cm); \(b\) is the slit width (3.2cm); and \(\tau\) is the wall shear stress.
(dyne/cm²). For time course studies of ATP release, a programmable Harvard Syringe Pump (PHD programmable, Harvard Apparatus, Hollison, MA) was used to perfuse the flow chamber with fresh media at the aforementioned shear rate of 12 dynes/cm².

4.3.3. Luciferin/Luciferase-dependent Detection of ATP

An ATP bioluminescence assay containing luciferin/luciferase reagent was used to detect ATP (ATP Bioluminescence Assay kit HS II, Roche, Indianapolis, IN). This assay utilizes the conversion of D-luciferin by luciferase into oxyluciferin and light that requires ATP as a co-factor. The resultant luminescence, measured using a Monolight 3010 (BD Biosciences Pharmingen, San Diego, CA), reflects ATP concentration. Conditioned media samples were acquired in two separate protocols. To determine the time course of ATP release, media samples were taken at each time point and immediately frozen at –80 °C for further analysis. For studies using the closed flow loop, a known volume of media was added to the flow loop prior to fluid shear exposure for 5 minutes. After 5 minutes of fluid shear, 1mL of media was removed and stored as above. Controls were performed with each drug solution to ensure that the added drugs had no effect on luciferase activity. Results were normalized to cellular protein concentration, as determined by the amido black method.

4.3.4. Pharmacologic Agents

All drugs tested were purchased from Sigma Chemical (St. Louis, MO) and dissolved into distilled water unless otherwise specified. Drugs were added 45 minutes prior to the onset of flow and remained present for the duration of the experiment. The following concentrations were used: 5µM nifedipine (from 5mM stock in ethanol), 10µM verapamil, 5µM 18α-glycyrrhetinic acid (AGA; from 15mM stock in DMSO), 5µM 18β-glycyrrhetinic acid (BGA; from 15mM stock in
DMSO), 100µM monensin (from 100mM stock in MeOH), 10µM; 35µM brefeldin A (BFA; 35mM stock in EtOH), 100µM N-ethylmaleimide (NEM; from 100mM stock).

4.3.5. **Assessment of Plasma Membrane Integrity**

Cell damage was assessed quantitatively by measuring the samples of recovered media for lactate dehydrogenase (LDH). Analysis of LDH levels was performed using the CytoTox96 Non-radioactive Cytotoxicity Assay (Promega, Madison, WI). This assay uses NADH, generated by oxidation of lactate into pyruvate, with the conversion of iodonitrotetrazolium into a red formazan product in the presence of diaphorase. The absorbance at 490nm is proportional to the amount of LDH in the media sample. For a positive control, cells were disrupted by lysing in 0.1% TritonX-100. Serial dilutions of this positive control were compared to LDH levels from media samples.

4.3.6. **Gap Junctional Intercellular Communication Assay**

GJIC assays were performed using double labeling and immunofluorescence as described previously [168, 409]. In these experiments, cells were loaded with the dyes calcein-AM (Molecular Probes, Eugene, OR) and 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). Because of its small molecular weight (< 1 kDa), calcein is gap junction-permeable and able to transfer to neighboring cells in the presence of functional gap junctions, while DiI, a larger, lipophilic dye, incorporates into the membrane and is unable to pass through functional gap junctions. These double-labeled (donor) cells were dropped onto non-labeled (acceptor) cells in monolayer. If functional GJIC existed, calcein would be transferred to the acceptor cells, whereas DiI would stay in the donor cell. Donor cells can be distinguished from acceptor cells through double exposure with fluorescein and rhodamine filters: donor cells fluoresce yellow because of the presence of both calcein (green) and DiI (red), whereas acceptor cells only fluoresce green.
Two days prior to experimentation, MC3T3-E1 cells were seeded onto 35mm glass cover slips in 6-well plates at a density of 60,000 cells per well (acceptor cells). On the day of the experiments, the pre-confluent donor cells were removed from the incubator and washed with PBS followed by aspiration. The donor cells were labeled in a solution composed of 2mL HBSS, 2% BSA, 7µL DiI, 20µL calcein-AM, and 20µL pluronic acid (Molecular Probes) and incubated at 37°C for 30 minutes. After 30 minutes, the dye was aspirated and the donor cells were detached by trypsinization and resuspended in fresh growth medium. A cell pellet was obtained by 5 minutes of centrifugation at 200g. Five hundred double-labeled donor cells were then dropped onto the acceptor cells and incubated for 2 hours at 37°C. Following incubation, the cover slips were removed from the dishes, washed twice in HBSS, and mounted onto a glass slide using Fluoromount-G (Fischer Scientific). The slides were placed on a Nikon fluorescent microscope (Nikon EFD-3; Optical Apparatus, Ardmore, PA) and visualized using fluorescein and rhodamine to locate the calcein- and DiI-loaded cells, respectively. For studies using AGA or BGA to inhibit GJIC, 5µM AGA or BGA was added to acceptor cells 45 minutes prior to the addition of donor cells and maintained in the incubation media until the cover slips were mounted.

4.3.7. Quinacrine Staining of Intracellular ATP

Osteoblasts were seeded onto type I collagen-coated glass slides as described above. Two days later, when the slides were approximately 80-85% confluent, the cells were incubated in 25µM quinacrine for 30 minutes, rinsed twice in HBSS, and mounted with Fluoromount G. The slides were then immediately examined using the same Nikon fluorescent microscope as for GJIC assays.
4.3.8. Prostaglandin \( \text{E}_2 \) Measurement

For measurement of prostaglandin \( \text{E}_2 \) in sheared cells, experiments were conducted as described above but exposed to fluid shear for 60 minutes instead of 5 minutes. After the 60 minute shear treatment, slides of cells were overlaid with 1mL of fresh flow media (with or without drug, as appropriate) and incubated for 30 additional minutes at 37°C with 5% \( \text{CO}_2 \). The media was then collected and \( \text{PGE}_2 \) was measured using commercially available ELISA kits (Amersham Biosciences, Piscataway, NJ) according to the manufacturers instructions. Results were normalized to cellular protein levels. The effect of exogenous ATP addition to static cells was also addressed. Experiments were performed as above for \( \text{PGE}_2 \) release, but overlaid with 1mL of ATP (at concentrations of 100nM-1mM) in flow media for 30 minutes, after which time the media was collected and analyzed by ELISA.

4.3.9. Statistical Analysis

A minimum of two slides per treatment were examined each day on at least three different days. Two way ANOVA analyses were used to compare ATP release from MC3T3-E1 cells treated with pharmacological agents. When a significant difference was found between samples, a Fisher’s PLSD was performed to localize the significant difference. Statistical significance was considered at \( p < 0.05 \), and samples are presented as mean ± standard error of mean (SEM).

4.4. Results

4.4.1. Fluid Shear Stress Induces ATP Release

MC3T3-E1 cells exhibited a basal release of ATP (6.2±1.8 nM) that was significantly increased ~10-fold (59.8±15.7 nM; \( p<0.001 \)) when cells were subjected to 12 dynes/cm\(^2\) FSS (Figure 4-1A). Since cytosolic ATP concentrations are in the millimolar range [410], it was necessary to determine whether the changes in extracellular ATP levels resulted from an active release rather than shear-induced cell lysis. To ensure that membrane damage did not contribute
to shear-mediated ATP release, we analyzed the conditioned media from sheared cells for the presence of the cytosolic enzyme, lactate dehydrogenase (LDH): if ATP release resulted from cellular damage, LDH would be found in the conditioned media. We consistently found that fluid shear-induced ATP release occurred in the absence of significant plasmalemmal damage compared to static controls (data not shown).

ATP release in response to a variety of stimuli has been shown to be transient, occurring rapidly after the stimulus and decreasing to basal levels over a period of 30-60 minutes [411]. To determine if a similar pattern occurs in osteoblastic MC3T3-E1 cells in response to FSS, we examined ATP release at time points before and after 5 minutes using a programmable Harvard Syringe Pump to produce 12 dynes/cm² shear. We found that FSS induced a rapid release of ATP within one minute of the onset of FSS that returned to pre-flow levels with prolonged fluid shear (Figure 4-1B).

4.4.2. Shear-induced ATP Release Requires Calcium Entry

To determine whether ATP release was Ca²⁺-dependent, we exposed static MC3T3-E1 cells to the Ca²⁺ ionophore, ionomycin (1 µM), for 10 min, then removed the bathing medium for ATP analysis. Addition of ionomycin produced a 3-fold (p<0.05) increase in ATP release compared to untreated controls (Figure 4-2A). L-VSCC and MSCC channels have been implicated in the intracellular Ca²⁺ response to mechanical load [4, 221, 222, 291]. To test whether these channels are involved in shear-induced ATP release, we blocked the MSCC with GdCl₃ (10 µM) and the L-VSCC with nifedipine (5 µM) and verapamil (10 µM). Nifedipine significantly attenuated the shear-induced ATP release (Figure 4-2B) in a manner similar to verapamil inhibition (data not shown). Neither inhibitor altered basal ATP release. MSCC inhibition with GdCl₃ did not alter either basal or shear-induced ATP release.
4.4.3. Shear-induced ATP Release Does Not Require GJIC

Gap junctions and GJIC have been implicated in the mechanism through which other cell types release ATP in response to a mechanical signals [202, 341]. Because serum proteins can bind to AGA and titrate the effective AGA concentration [347], it was first necessary to demonstrate that GJIC was inhibited under the flow conditions used. GJIC was evaluated using the double-labeling technique as described previously. In Figure 4-3A, the green (calcein) fluorescence indicates the coupled cells in the monolayer while the yellow (calcein and DiI) fluorescence indicates the donor cells. We found that 5µM AGA pre-treatment in flow media (containing 0.5% FBS) effectively inhibited GJIC in MC3T3-E1 osteoblasts. Under these conditions, we found that osteoblasts released ATP in the presence of 5µM AGA when exposed to fluid shear stress (Figure 4-3B). Similar results were found when cells were sheared in the presence of another GJ inhibitor, BGA (5µM). These results suggest that shear-induced ATP release in murine osteoblasts does not require GJIC or hemichannels.

4.4.4. Localization of Intracellular ATP Stores

We examined the localization of intracellular ATP stores in MC3T3-E1 osteoblasts using quinacrine, a cell-permeant fluorophore that binds to ATP. After 30 min incubation with quinacrine, a high level of punctuated fluorescence was seen, localized primarily within the cytosol of the cells (Figure 4-4A). To assess the role of vesicular exocytosis in ATP release, we utilized three pharmacologic agents: BFA, which causes disruption of the Golgi apparatus [314]; monensin, which prevents vesicle formation from the Golgi apparatus [314, 407]; and NEM, which prevents vesicle fusion to the plasma membrane by interfering with vesicle-associated NSF proteins [314, 407]. Fluid shear in the presence of each of these antagonists significantly attenuated ATP release compared to untreated controls (Figure 4-4B through 4-4D). These data,
combined with the highly granular and punctate intracellular localization of ATP as visualized by quinacrine staining, suggest that ATP is released from murine osteoblasts in a vesicular manner.

4.4.5. Extracellular ATP is Required for Flow-induced Increases in PGE₂

Prostaglandins are rapidly released in response to shear in osteoblasts [211, 269] and their formation is required for load-induced bone formation [272]. To determine whether shear-induced PGE₂ release was mediated by extracellular ATP, we added apyrase (5U/ml), a nucleotidase that degrades nucleotide triphosphates into nucleotide monophosphates, to the flow medium. Apyrase attenuated flow-induced increases in PGE₂ release (Figure 5A). Similar results were obtained from experiments performed with the non-specific P2 antagonist PPADS, and additional experiments with heat-inactivated apyrase confirmed that the enzymatic activity of apyrase, and not some secondary, non-specific effect, was responsible for attenuating PGE₂ release (data not shown); Further, when exogenous ATP was added to static cells, we observed significant increases in PGE₂ release (Figure 4-5B) without induction of COX-2 (data not shown). These data suggest that shear-induced ATP secretion mediates the release of prostaglandins, paracrine factors that have been implicated in the anabolic response to exogenous mechanical load.

4.5. Discussion

Several mechanisms have been proposed for stimuli-induced ATP release from various cell types. These proposed mechanisms include release through a chloride conducting pathway [412], via gap junctional hemichannels [341, 413] and Ca²⁺ dependent vesicular exocytosis [314]. Our data indicate that shear-induced ATP release results from Ca²⁺-dependent vesicular release. However, we did observe that the general chloride channel blocker, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), inhibits both basal and shear-induced ATP release (data not shown). While this is a focus of another study, previous reports suggest that DIDS does not block ATP
release through inhibition of a chloride conductance, but, rather, by binding to a sulfonylurea receptor on the vesicle to prevent fusion of the vesicle to the membrane [414]. Another potential pathway, ATP conductance through gap junctional hemichannels, appears to be involved in other cell types [341, 344], but not in the MC3T3-E1 cell model. We demonstrate that gap junction inhibition with either AGA or BGA prevented dye movement through the junctional complex, but had no effect on FSS-induced ATP release. These data support a previous report that found no difference in mechanically-stimulated ATP release in human osteoblast-like cells overexpressing the Cx43 connexin compared to wild type controls [202]. Quinacrine, a ATP-binding fluorophore, demonstrated punctate, granular staining, suggesting that ATP can be localized in vesicles in MC3T3-E1 osteoblasts; further experimentation, such as density gradient fractionation, however, is required to unambiguously demonstrate vesicular ATP localization.

Similar to a previous report in epithelial cells [314], we show that FSS-induced ATP release from osteoblastic cells is the result of Ca\(^{2+}\)-dependent vesicular binding to the membrane. Three pharmacologic agents, which cause disruption of the Golgi apparatus [314] and prevent vesicle formation from the Golgi apparatus [303, 314] prevent vesicle fusion to the plasma membrane by interfering with vesicle-associated NSF proteins [303, 314], all significantly decreased FSS-induced ATP release compared to untreated controls. Interestingly, none of these agents completely blocked either basal or FSS-induced ATP release. This observation would suggest that either each of these agents does not totally block vesicular fusion to the membrane or that a secondary pathway for ATP release exists.

Whereas numerous studies have shown that ATP binding to P2 receptors results in an increase in [Ca\(^{2+}\)], that is dependent on both extracellular Ca\(^{2+}\) entry and intracellular Ca\(^{2+}\) release, few have examined the role of [Ca\(^{2+}\)], in ATP release. Osteoblasts and osteocytes respond to fluid shear with a rapid increase in intracellular Ca\(^{2+}\) [4] that is essential for shear-induced changes in actin cytoskeletal organization and gene expression [274]. While this [Ca\(^{2+}\)], response has been shown to be dependent on both extracellular Ca\(^{2+}\) entry and intracellular Ca\(^{2+}\) release, we have
shown that only IP$_3$-mediated Ca$^{2+}$ release is required for the subsequent changes in cell architecture and protein production [274]. However, others have reported that Ca$^{2+}$ entry via ion channels is important to shear-induced secretion of prostaglandins [211], TGFβ [223] and nitric oxide [222] from bone cells, leading us to postulate that Ca$^{2+}$ entry mediates signal amplification while intracellular Ca$^{2+}$ release results in changes in gene expression in osteoblasts. Katsumagi, et al. have shown that inhibition of phospholipase C / IP$_3$-mediated intracellular Ca$^{2+}$ release blocks angiotensin II-stimulated ATP release in smooth muscle cells [415]. However, in this study we found no consistent effect of inhibition of phospholipase C with U73122 on either basal or shear-induced ATP release. This lack of consistency may be due to the subsequent inhibition by U73122 of protein kinase C, which mediates a number of cellular responses, including phosphorylation of Ca$^{2+}$ channels.

Calcium entry through membrane ion channels could also mediate ATP release in response to shear. This postulate is strengthened by the observation that when extracellular Ca$^{2+}$ is removed, ATP release in response to mechanical load is attenuated [314]. Osteoblasts express a number of ion channels capable of conducting Ca$^{2+}$ [404] but, to date, only two have been shown to play a role in the [Ca$^{2+}$]$_i$ response to shear: the mechanosensitive, cation-selective channel (MSCC) [4, 221] and the dihydropyridine- and voltage-sensitive, L-type Ca$^{2+}$ channel (VSCC) [221]. Activation of the MSCC in response to loading has been associated with prostaglandin, TGFβ and NO release [211, 222, 223]. Activation of the L-type VSCC has been linked to cellular responses of osteoblasts or osteocytes to shear [222] and hormonal stimulation [221]. We have recently demonstrated that inhibition of this channel with either nifedipine or verapamil significantly reduces bone formation in mechanically loaded rat tibiae and ulnae, in vivo [293], indicating the importance of this channel in mechanotransduction in bone.

How L-type VSCC’s are activated by mechanical perturbation is unclear. We have postulated that shear induced activation of the MSCC results in a membrane depolarization that, in turn,
activates the VSCC current. However, the data reported here fails to support this premise. Inhibition of the MSCC with 10 µM GdCl₃ did not significantly block ATP release, although a reduced ATP release level in response to shear was observed. Thus, either FSS depolarizes the membrane through a separate mechanism to activate the L-VSCC, or this stimulus can directly activate the L-VSCC.

One mechanism through which the L-VSCC could be activated directly is by the autocrine/paracrine action of ATP. ATP released from the cell can bind to P2 receptors that modulate a number of second messenger pathways, including [Ca²⁺]ᵢ. There are two subtypes of P2 receptors: ionotropic (P2X) receptors that allow entry of ions through receptor-mediated channels, and metabotropic (P2Y) receptors that induce G-protein-mediated intracellular Ca²⁺ release [416]. While the P2Y receptor has been linked with IP₃-mediated intracellular Ca²⁺ release, P2X receptors have been shown to activate both K⁺ channel and Ca²⁺ channels, including the L-type VSCC [348, 417]. A number of isoforms for each of these subtypes have been described, based on the nucleotide binding selectivity of the receptor and inhibition. Osteoblasts exhibit many of these isoforms for both P2X and P2Y [386], and ATP binding to these receptors has also been associated with fast, gap junction-independent Ca²⁺ waves [203] and potentiation of the [Ca²⁺]ᵢ response to PTH in osteoblasts [418]. Here, we demonstrate that MC3T3-E1 osteoblasts respond to a defined fluid shear with release of ATP within one minute of shear onset. Since the [Ca²⁺]ᵢ response to shear exhibits a time lag from onset to intracellular response of 30-60 sec. [4, 221], it is possible that ATP initiates this event to enhance its own release.

Prostaglandin synthesis and release have been shown to occur rapidly in osteoblasts in response to shear [211, 215, 269] is essential for the anabolic response of bone to mechanical loading [272]. PGE₂ release from osteoblasts and osteocytes has been shown to be released in two stages. Upon application of shear, a brief burst of PGE₂ is observed, peaking at 5–10 min. then returning to levels near baseline [211, 215]. This is followed at 45-60 min by a large, continuous increase in release that corresponds to an increase in COX-2 production [215, 269]. The
importance of COX-2 function in bone formation was demonstrated when the COX-2 specific inhibitor, NS398, was given to rats prior to mechanical loading: the presence of NS398 completely abolished tibial bone formation in response to four-point bending compared to loaded controls [272]. While these results implicate COX-2 in bone formation in response to exogenous load, it should be noted, however, that prostaglandin synthesis and release does not directly correlate with bone formation, as prostaglandins can similarly promote bone resorption [419].

The addition of exogenous ATP to static MC3T3-E1 cells induced the release of PGE$_2$, and the hydrolysis of ATP released in response to shear blocked flow-induced increase in PGE$_2$ release, suggesting that FSS-induced ATP secretion induces PGE$_2$ release through activation of a P2 receptor. Because Reich et al. [269, 420] demonstrated that flow-induced PGE$_2$ release was mediated through a $G_q$ protein, we hypothesize that a metabotropic P2Y receptor is involved in this response.

Whereas the measured amount of secreted ATP in response to shear was on the order of 100nM (i.e., 1000-fold less than the amount required to induce PGE$_2$ release in static conditions), it should be noted that the data presented in Figures 4-1 through 4-4 represents ATP found in solution. This does not accurately demonstrate the local ATP concentration at the cell surface immediately upon its release from the cytosol, or the local concentration at P2 receptors. Further, while their expression has not yet been demonstrated in osteoblasts, other ATP-releasing cells, such as chondrocytes and endothelial cells, express membrane-bound NTP-degrading enzymes (ecto-5’-nucleotidases and exonucleotidases) that regulate the extracellular availability of ATP.

Figure 4-6 shows our working model for the action of ATP release in osteoblasts. We show that osteoblasts exhibit a basal release of ATP in static cells and respond to a well-defined fluid shear regimen with a significant increase in ATP release. This release is mediated by Ca$^{2+}$-dependent vesicular fusion to the membrane, although only the shear-induced release is sensitive to Ca$^{2+}$. We further showed that Ca$^{2+}$ entry through the L-type VSCC, but not Ca$^{2+}$ release from intracellular stores, is important to this response. PGE$_2$ release in response to shear appears to be
mediated by secreted ATP, suggesting that purinergic signaling may be an important component of the mechanotransduction response of bone.

4.6. Acknowledgements

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Figure 4-1. Effects of fluid shear stress at 12 dynes/cm² on ATP release in MC3T3-E1 osteoblasts. (A) Following 5 minutes of flow, the flow media was removed for analysis of ATP content using an ATP-dependent luciferin-luciferase reaction. Fluid shear increased ATP release approximately 5-fold compared to static controls (a: p < .01 compared to static cells). Each bar represents the mean±SE of 10 experiments. (B) Time course of ATP release in response to shear. ATP was released rapidly from MC3T3-E1 cells, peaking within 1 min of shear application. ATP release returned to near, but elevated, baseline levels with sustained shear (a: p < 0.01 vs. static cells at same time point).
Figure 4-2. ATP release is dependent on extracellular Ca\(^{2+}\) entry. (A) MC3T3-E1 cells were treated with ionomycin (1µM), a calcium ionophore, for 10 minutes. The media was then collected for ATP analysis. The addition of ionomycin significantly increased ATP release compared to control cells (a: p < 0.02 vs. vehicle control). (B) Effects of channel blockers on ATP release. MSCC inhibition with GdCl\(_3\) (10µM) had no effect on either basal or FSS-induced ATP release, while L-type VSCC inhibition with nifedipine (5µM) or verapamil (10µM) attenuated FSS-induced ATP release but not basal release (a: p < 0.05 vs. static cells in same conditions; b: p > 0.05 vs. static cells in same conditions).
Figure 4-3. The role of GJIC in ATP release. (A) GJIC was assessed in MC3T3-E1 cells by dual label dye transfer. Donor cells double labeled with the fluorescent dyes calcein and 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) were placed in contact with unloaded cells in the monolayer. Dye transfer was evaluated after 2h. In the dual-exposure photographs, calcein has transferred to acceptor cells which are green, demonstrating functional GJIC; cells fluorescing yellow contain both calcein and Dil and are the dual-labeled donor cells. Pre-incubation with 5µM 18α-glycyrrhetinic acid inhibited GJIC. (B) MC3T3-E1 cells pretreated with vehicle control (DMSO) or GJ inhibitors, 18α-glycyrrhetinic acid or 18β-glycyrrhetinic acid, demonstrated that inhibition of GJIC had no significant effect on FSS-induced ATP release (a: p < 0.01 vs. static cells in same conditions).
Figure 4-4. Localization of intracellular ATP stores. (A) Quinacrine staining of MC3T3-E1 cells demonstrates grainy, punctate localization of ATP, suggesting that ATP is contained in vesicles. (B) Brefeldin A, an agent that disrupts the Golgi and thereby prevents vesicle formation, attenuated FSS-induced increases in ATP release but had no effect on basal ATP release. (a: p < 0.01 vs. static cells in same conditions).
Figure 4-4. (C) Monensin, which maintains the Golgi structure but prevents vesicle budding from the Golgi, similarly attenuated FSS-induced ATP release without affecting static ATP release. (D) N-ethylmaleimide prevents vesicular exocytosis by inhibiting NSF proteins. Addition of NEM also inhibited FSS-induced ATP release. (a: p < 0.01 vs. static cells in same conditions).
Figure 4-5. P2 receptor-dependence of prostaglandin release. (A) Addition of apyrase (5 U/mL), which hydrolyzes extracellular ATP, significantly decreased PGE\(_2\) release from sheared osteoblasts, suggesting that FSS-induced ATP secretion mediates PGE\(_2\) release. (B) Addition of exogenous ATP to static, non-sheared cells dose-dependently increased PGE\(_2\) release (a: p < 0.01 to static control; b: p < 0.01 to shear in the absence of apyrase).
Figure 4-6. Proposed model for data presented herein. Fluid shear stress activates the L-type VSCC calcium channel through some unknown mechanism. Calcium influx through the L-type VSCC mobilizes vesicular stores of ATP to the plasmalemma, where vesicular fusion releases ATP into the extracellular environment. ATP then diffuses to P2 receptors to function in an autocrine or paracrine manner, activating either ionotropic P2X or metabotropic P2Y receptors to increase PGE$_2$ release.
Having demonstrated in Section 4 that flow-induced ATP secretion regulates PGE$_2$ release, and because of a previous study wherein we reported that maximal COX-2 induction in response to flow requires activation of the transcription factor NF-κB, we hypothesized that purinoceptor activation was required for NF-κB translocation to the nucleus.
5. P2 PURINOCEPTOR ACTIVATION IS REQUIRED FOR FLOW-INDUCED NF-κB TRANSLOCATION IN MURINE OSTEOBLASTS

5.1. Abstract

Maintenance of appropriate skeletal architecture requires exogenous mechanical forces that regulate the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Fluid flow across osteoblasts, a putative model for mechanical load in vitro, induces a number of signaling pathways associated with skeletal anabolism in vivo. One such pathway involves prostaglandin (PG) synthesis by cyclooxygenase-2 (COX-2). We have previously demonstrated that flow-induced COX-2 expression is regulated by activation of the transcription factor NF-κB that requires calcium release from the endoplasmic reticulum (ER). A putative mechanism for induction of calcium release through the ER is by activation of metabotropic P2 receptors by ATP. Additionally, we have recently demonstrated that flow induces ATP secretion that, in turn, promotes PG release through purinoceptor activation. As PG’s are the terminal product of COX-2 function, we sought whether flow-induced COX-2 expression through NF-κB activation is regulated by P2 receptors. As demonstrated previously, application of fluid flow to osteoblasts induced IκBα degradation and subsequent nuclear NF-κB translocation, both of which were inhibited in the presence of the ectonucleotidase apyrase, demonstrating the requirement for P2 receptor activation. Addition of oxidized ATP, a potent P2X7 antagonist, similarly attenuated IκBα degradation, implicating the P2X7 receptor in this response.

5.2. Introduction

Maintenance of appropriate skeletal architecture requires exogenous mechanical forces that regulate the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Sup-optimal levels of mechanical loading, as occurs in spaceflight, decreases osteoblast number and bone formation rates [177-179], whereas increased loads induce new bone formation [421]. Fluid flow
across osteoblasts, a putative model for mechanical load in vitro, induces a number of signaling pathways associated with skeletal anabolism in vivo. One such pathway involves prostaglandin (PG) synthesis by cyclooxygenase-2 (COX-2). Indeed, the importance of COX-2 in load-induced osteogenesis was demonstrated by Forwood, who reported that pharmacologic inhibition of COX-2 abrogated load-induced bone formation [272].

We have previously demonstrated that maximal COX-2 expression in response to fluid flow requires activation of the transcription factor NF-κB [278]. Additionally, we have recently demonstrated that flow promotes the secretion of ATP that, in turn, activates PG release [422]. As PG’s are the terminal product of COX-2 function, we sought whether flow-induced COX-2 expression through NF-κB activation is regulated by P2 receptors. As demonstrated previously, application of fluid flow to osteoblasts induced IκBα degradation and subsequent nuclear NF-κB translocation, which were both inhibited in the presence of the ectonucleotidase apyrase, demonstrating the requirement for P2 receptor activation in these two responses. The addition of oxidized ATP, a potent P2X7 antagonist, similarly attenuated IκBα degradation, implicating the P2X7 receptor in this response. The dose-dependent addition of ATP to static osteoblasts demonstrated no change in cytosolic NF-κB localization, suggesting that P2 receptor activation is required, but not sufficient, for nuclear NF-κB accumulation.

5.3. Materials and Methods

5.3.1. Cell Culture

MC3T3-E1 osteoblasts were kindly provided by Dr. Joseph P. Bidwell (Indiana University) and grown in minimal essential medium, α-modification, containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100μg/mL streptomycin (P/S). Cells were maintained in a humidified incubator at 37°C with 5 CO2/95% air and subcultured every 72 hours. For flow studies, 2800 cells/cm² were seeded onto 75x38mm glass slides coated with fibronectin
(10µg/mL; Becton Dickson). Two days later, when the cells were 85-90% confluent, media was removed and replaced with reduced serum media consisting of αMEM, 1% P/S, and 0.5% FBS (flow media). Fluid flow experiments were performed on the following day in reduced serum media.

5.3.2. Fluid Flow Experiments

Fluid flow was applied to cells in a parallel plate flow chamber using a closed flow loop, as described previously [422]. Flow was applied for 60 minutes at 12 dynes/cm², 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).

5.3.3. Pharmacologic Agents

Oxidized ATP (oATP; adenosine 5’-triphosphate-2’,3’-dialdehyde) was purchased from Sigma, dissolved in distilled water, and added to cells at 300 µM for three hours prior to experimentation. Apyrase (grade III; Sigma) was dissolved directly into flow media at a final concentration of 10 units per milliliter and was added at the onset of fluid flow experiments.

5.3.4. Western Analysis

20µg of cytosolic fraction was mixed with an equal volume of 2X SDS loading buffer containing 3% β-mercaptoethanol and denatured in boiling water for 5 minutes. Samples and molecular weight markers were separated on 10% SDS polyacrylamide gels and electroblotted onto PVDF membranes (Bio-Rad) using the Mini-Protean II system (Bio-Rad). Membranes were then blocked in 5% non-fat milk in TBST (150mM NaCl, 10mM Tris-HCl, pH 8.0, and 0.05% Tween-20) for 1 hour at room temperature. Primary antibodies against IκBα and actin were purchased from Santa Cruz Biotechnologies and Sigma, respectively.
5.3.5. Immunocytochemistry

After experimentation, slides were removed from their chambers, briefly washed in PBS, and fixed in 4% paraformaldehyde in PBS for 15 minutes. Cells were permeabilized with 0.1% Triton X-100 for 2 minutes, followed by two 5 minute washes in PBS. Non-specific binding of antibodies was reduced by treating the cells with 3% donkey serum in PBS for 1 hour in a humidified chamber at 37°C. Cells were then treated with a primary antibody against the p65 subunit of NF-κB (1:100 in 3% donkey serum; Santa Cruz Biotechnology, Inc.) for 1 hour in a humidified chamber at 37°C, washed twice in PBS for 15 minutes, and incubated with rhodamine-conjugated secondary antibody (1:50 in 3% donkey serum; Jackson Immunoresearch Laboratories) for 1 hour. Cells were fluorescently visualized using a Nikon Optiphot II fluorescent microscope.

5.4. Results

5.4.1. Flow-induced NF-κB Translocation Requires Activation of P2 Purinoceptors

We initially sought whether purinoceptor activation was required for flow-induced nuclear NF-κB translocation. In the absence of flow, the p65 subunit of NF-κB demonstrated cytosolic localization (Figure 5-1, top-left panel), whereas exposure to flow at 12 dynes/cm² for 1 hour induced strong nuclear localization of the p65 NF-κB subunit (Figure 5-1, top-right panel). In contrast, cells treated with apyrase (10U/mL) demonstrated cytosolic NF-κB localization in both the absence (Figure 5-1, bottom-left panel) or presence (Figure 5-1, bottom-right panel) of flow, suggesting that P2 purinoceptor activation is required for flow-induced NF-κB translocation.
5.4.2. **Purinoceptor Activation Alone is Not Sufficient for NF-κB Translocation**

Having implicated P2 receptors in NF-κB activation, we speculated whether fluid flow is required for this process, or whether P2 activation alone promotes NF-κB translocation to the nucleus. The addition of preferential agonists for various P2X and P2Y isoforms (ATP, ADP, UTP, UDP) at doses ranging from 100nM-1mM failed to induce NF-κB nuclear translocation at 30 minutes, 1 hour, or 2 hours after nucleotide addition (Figure 5-2). These data suggest that P2 receptor activation is required, but not sufficient, to induce NF-κB translocation.

5.4.3. **Flow-induced IκBα Degradation Requires Purinoceptor Activation**

Degradation of the inhibitory IκBα protein is required for nuclear NF-κB translocation. We next sought whether the effect of purinoceptor activation on NF-κB translocation occurred proximal or distal to IκBα degradation. Fluid flow induced the degradation of IκBα, as we previously reported. In contrast, flow-induced IκBα degradation was impaired in the presence of apyrase (Figure 5-3A and Figure 5-3B), suggesting that purinoceptor activation occurs proximal to IκBα phosphorylation.

5.4.4. **Pharmacologic Inhibition of the P2X₇R Prevents Flow-induced IκBα Degradation**

P2X₇R⁺/− demonstrate impaired skeletal remodeling [399] and load-induced osteogenesis [423]. In vitro, osteoblasts from these mice release less PGE₂ and induce less COX-2 in response to fluid flow than do osteoblasts from P2X₇R⁺/+ mice [423, 424]. These data suggest that the purinoceptor mediating NF-κB translocation and IκBα degradation is the P2X₇R. Although the pharmacologic antagonists for most P2R’s are non-specific, oxidized ATP is a relatively selective and irreversible antagonist of the P2X₇R. P2X₇R antagonism with oATP revealed attenuated IκBα degradation in response to fluid flow compared to osteoblasts flowed in the presence of
vehicle control (Figure 5-4A and Figure 5-4B), suggesting that the P2X7-R is indeed the purinoceptor responsible for NF-κB activation.

5.5. Discussion

It has been known for over a decade that P2 purinoceptors are expressed by cells of the osteoblastic lineage, and that their activation induces IP3 hydrolysis and Ca2+ mobilization. It is only in the past few years, however, that investigators have begun to examine the role of purinoceptors on the formation and function of both osteoblasts and osteoclasts. Specifically, in vitro studies by Morrison et al. and Buckley et al. have implicated P2 receptor activation in osteoclast formation and resorption [390, 393], whereas Jones et al. and Hoebertz et al. have implicated P2Y receptor activation in inhibiting bone formation by osteoblasts [382, 401]. In vivo, the important role of purinoceptors has been confirmed by the generation of a knockout mouse for the P2X7-R. This mouse demonstrates impaired periosteal bone formation and increased trabecular resorption [399], as well as impaired osteogenesis after mechanical load [423].

The mechanisms whereby mechanical loads induce osteogenesis remain vague, but ample data suggest that prostaglandin release is one such mechanism. Numerous investigators have that fluid flow, a model for mechanical load in vitro, increases prostaglandin synthesis and release [211, 215]. We have recently demonstrated the correlation between purinoceptor activation and PGE2 release, wherein flow-induced PGE2 release was diminished in the presence of the ATP-degrading enzyme, apyrase, that prevents purinoceptor activation [422]. Primary osteoblasts cultured from P2X7-R-/- mice release less PGE2 than do primary osteoblasts from P2X7-R +/+ mice, suggesting that the purinoceptor responsible for flow-induced PGE2 release is the P2X7-R.

Norvell et al. has demonstrated that flow-induced PGE2 release is almost entirely dependent upon induction of COX-2 [127], and we have recently demonstrated that NF-κB activation is required for maximal flow-induced COX-2 expression [278]. NF-κB is constitutively found as an
inactive complex in the cytosol bound to its inhibitory protein IκBα. Phosphorylation of IκBα at Ser32 and Ser36 targets it to the proteasome for degradation, thereby allowing for NF-κB translocation to the nucleus where it induces the expression of many pro-inflammatory proteins. The data described above demonstrate that purinoceptor activation is required for NF-κB translocation, as ATP degradation with apyrase prevented flow-induced nuclear NF-κB accumulation. Further, apyrase also prevented flow-induced IκBα degradation, suggesting that purinoceptor activation is proximal to IκBα phosphorylation. Consistent with impaired COX-2 expression and PGE₂ release in P2X₇R −/− mice reported previously [423, 424], P2X7R antagonism with oATP prevented IκBα degradation in response to flow. Similar results were reported in both primary and clonal osteoclasts by Korcok et al., wherein P2X₇-R activation increased nuclear NF-κB localization [398].
Figure 5-1. Fluid flow-induced translocation of NF-κB requires extracellular nucleotides. (A) Immunocytochemical staining of the p65 subunit of NF-κB demonstrates cytosolic localization in the absence of fluid flow (top-left panel) and strong nuclear localization in response to fluid flow (top-right panel). MC3T3-E1 osteoblasts exposed to apyrase (10U/mL), in the absence (bottom-left) or presence (bottom-right) of flow, demonstrate cytosolic NF-κB localization, suggesting that fluid flow-induced nuclear NF-κB translocation requires extracellular nucleotides.
Figure 5-2. Purinoceptor activation is not sufficient to induce NF-κB translocation to the nucleus. Extracellular nucleotides ATP, ADP, UDP, or UTP were dose-dependently added to static cultures of MC3T3-E1 osteoblasts for 30m, 1h, or 2h before fixation in 4% paraformaldehyde and immunostaining for the p65 subunit of NF-κB. At no time point nor concentration was there significant nuclear NF-κB accumulation as found in response to fluid flow. Images were obtained from addition of 10 μM ATP, and are representative of all other results.
Figure 5-3. Degradation of IκBα requires purinoceptor activation. (A) MC3T3-E1 osteoblasts were left as static controls or exposed to fluid flow in the absence of presence of the ATP-degrading enzyme apyrase (10U/mL). IκBα and actin levels were examined by immunoblotting. (B) Quantitation of IκBα levels in MC3T3-E1 osteoblasts left as static controls or exposed to fluid flow in the absence or presence of apyrase (a: p < 0.05 compared to static vehicle).
Figure 5-4. P2X$_7$R antagonist with oATP prevents IκBα degradation in response to flow. 
(A) MC3T3-E1 osteoblasts were left as static controls or exposed to fluid flow in the absence of 
presence of the P2X$_7$R antagonist oATP (300 µM). IκBα and actin levels were examined by 
immunoblotting. (B) Quantitation of IκBα levels in MC3T3-E1 osteoblasts left as static controls 
or exposed to fluid flow in the absence or presence of oATP (a: p < 0.05 compared to static 
vehicle).
Transition statement

Although osteoblasts are responsive to mechanical loads \textit{in vitro}, there is an ongoing debate whether osteoblasts \textit{in vitro} are exposed to \textit{in vivo} fluid flow of the same magnitude. In contrast, mathematical modeling has suggested that osteocytes \textit{in vivo} are exposed to fluid flow-induced shear stress on the order of 8-30 dynes/cm$^2$ [209]. As such, osteocytes are argued to be the primary mechanosensory bone cell. We next sought whether osteocytes release ATP in response to fluid flow and, if so, whether purinoceptor activation similarly regulates PGE$_2$ release.

The following section has been submitted to

\textit{Journal of Biological Chemistry}
6. OSCILLATORY FLUID FLOW ACTIVATION OF GAP JUNCTION HEMICHANNELS PROMOTES ATP RELEASE IN MLO-Y4 OSTEOCYTES

6.1. 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 the lacunae and canaliculi of bone. The distribution of osteocytes within bone suggests a role for them as the primary mechanosensor. The presence of gap junctions (GJ) between osteocytes and osteoblasts could provide a mechanism whereby flow-induced signals are detected by osteocytes are transduced to osteoblasts. We have demonstrated the importance of GJ and gap junctional intercellular communication (GJIC) in mediating changes in intracellular calcium and prostaglandin E$_2$ (PGE$_2$) release in response to flow. Unapposed connexons, or hemichannels, are themselves functional and may constitute a novel mechanotransduction mechanism. Using 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 and PGE$_2$ release. We demonstrate that fluid flow activates hemichannels, through a mechanism involving protein kinase C, and this promotes ATP release. Ablation of connexin43 using siRNA strategies attenuated flow-induced hemichannel activation and ATP release. We also report significant PGE$_2$ release in Cx43-deficient cells treated with exogenous ATP, suggesting that PGE$_2$ release does not occur directly through open hemichannels but, rather, downstream of P2 receptor activation by ATP. These data demonstrate that hemichannels formed by connexin43 are activated in response to fluid flow in MLO-Y4 osteocytic cells and are responsible for ATP release, which functions in an autocrine fashion to promote PGE$_2$ release.

6.2. Introduction

Maintenance of appropriate skeletal integrity requires biophysical factors, such as mechanical loading of bones induced by physical activity [425, 426]. Numerous studies have demonstrated
that increased load promotes activation of osteoblasts [204] and osteocytes [427] and can increase skeletal mass by new bone formation, whereas decreased loads, as occur during limb immobilization [428-430], promote bone resorption and loss of skeletal mass. These factors promote bone adaptation by optimizing mass and mechanical performance through a process known as bone remodeling [431-433]. One mechanism whereby an external mechanical load is translated into a localized cellular signal is through load-induced fluid flow through the canaliculi and lacunae of bone [208, 209, 434]. While numerous data have demonstrated that both osteoblasts and osteocytes are responsive to fluid flow in vitro [4, 161, 193, 207, 210, 214, 215, 223, 269, 273, 274, 420, 435-437], osteocytes are thought to be the primary mechanosensor in bone because of their distribution throughout the bone matrix [218, 434, 438].

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. This direct physical connection of one cell to another allows gap junctional intercellular communication (GJIC) and contributes to bone cell responsiveness to a diverse array of extracellular signals, including parathyroid hormone [439], electromagnetic fields [440], and fluid flow [165, 167, 168, 214]. Another possible mechanism whereby GJIC contributes to this responsiveness is by coupling mechanosensory osteocytes to effector cells (i.e., bone-forming osteoblasts), as we have demonstrated in vitro [409].

Traditionally, studies pertaining to gap junctions and their constituent Cx composition have focused on GJIC. Recently, however, data from other cell lineages has demonstrated surface expression and functional activity of nonjunctinal, or unapposed, connexon hemichannels [343, 441-443]. Whether hemichannels exist in cells of the osteoblastic lineage, and whether they function in a similar manner remains unclear. 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 [413]. Jorgensen et al. reported that they were unable to detect functional hemichannels in osteoblastic UMR 106-01 over-expressing connexin43 (Cx43) or in ROS 17/2.8 osteoblasts, and only a small percentage of primary culture human osteoblastic cells expressed functional hemichannels in low extracellular Ca\(^{2+}\) conditions [258]. In contrast, limited data also suggests that oscillatory 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 [279]. Therefore, we examined whether osteocytic MLO-Y4 cells express functional hemichannels, whether these hemichannels are regulated by fluid flow, and the mechanisms involved therein. We demonstrate that fluid flow rapidly activates hemichannels through a mechanism involving protein kinase C, and this promotes ATP release; ablation of Cx43 using siRNA strategies abrogated flow-induced hemichannel activation and ATP release, implicating Cx43 as a major component of osteocytic hemichannels. ATP addition to static osteocytes lacking Cx43 continued to release PGE\(_2\), suggesting that hemichannel-mediated ATP release promotes PGE\(_2\) flux. These data demonstrate that hemichannels formed by Cx43 are activated in response to fluid flow and are responsible for ATP release in MLO-Y4 osteocytic cells.

6.3. Materials and Methods

6.3.1. 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 75x38mm glass slides coated with rat tail type I collagen (150\(\mu\)g/mL in 0.02N acetic acid; Becton-Dickson) in alpha-modified essential medium (\(\alpha\)-MEM; Gibco BRL) containing 5% fetal bovine serum (FBS; Hyclone), 5% calf serum (CS), and 1% penicillin and streptomycin.
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.

### 6.3.2. Oscillating Fluid Flow

Cells were exposed to oscillating fluid flow (OFF) at a flow rate sufficient to induce a shear stress of 20 dynes/cm² at 1Hz for 5 or 15 minutes as described previously [444]. 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 OFF. The flow rate was monitored with an ultrasonic flow meter (Transonic systems, Ithaca, NY) during all experiments. Flowed cells were used for dye uptake assays, ATP release, or PGE₂ release experiments.

### 6.3.3. Dye Uptake Assay

Hemichannel activity was monitored by Lucifer Yellow (LY; 1mg/mL; Sigma-Aldrich) dye uptake, as described previously [279, 338]. Briefly, Lucifer Yellow is plasmalemma-impermeant but small enough (Mₐ, 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 18a-glycyrrhetinic acid (AGA; 30µM; Sigma-Aldrich) or vehicle control (DMSO; 0.1% v/v). Further experiments were performed in the presence of PKA (H-89; Calbiochem), PKC (GF109203X; Biomol), and MEK1/2 (U0126; Cell
Signaling Technologies) inhibitors, 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 is 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 eliminated from analysis.

6.3.4. Quantification of ATP Release

ATP levels in conditioned media from cells exposed to OFF or chamber control was quantified using a luciferin-luciferase reaction (ATP Bioluminescence Assay Kit HS II; Roche) as described previously [411, 422]. After exposure to OFF or static control, 1 mL of conditioned media was collected from the inlet and outlet ports of the flow chamber and centrifuged at 10,000g 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.

6.3.5. Quantification of PGE₂ Release

For measurement of prostaglandin E₂ release, osteocytes were seeded into 6-well plates at the same concentration used for slides. Culture media was aspirated 12 hours before experimentation and replaced with reduced serum media containing 0.2% FCS and 0.2% FBS to minimize the effect of serum on PGE₂ release. The effect of exogenous ATP (10µM to 1mM) on PGE₂ release was examined in scrambled siRNA-transfected and Cx43 siRNA-transfected static osteocytes. Media was aspirated and replaced with reduced serum media containing ATP for 30 minutes,
after which time the conditioned media was removed, centrifuged at 10,000g for 4 minutes, and the supernatant was transferred to a new tube before storing at -80 °C. Wells were washed once with PBS and lysed in lysis buffer. PGE$_2$ in conditioned media samples was quantified using commercially available ELISA kits (Amersham Biosciences) according to the manufacturers instructions. PGE$_2$ results were normalized to cellular protein content.

6.3.6. siRNA-mediated Ablation of Cx43

A siRNA construct (AAGTGTGTAAGCGTGTGTTTT) against murine Cx43 (GenBank™ accession 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 75x38mm slides and used 48 hours later for Western blotting or flow experiments.

6.3.7. 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 Na$_3$VO$_4$, 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 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.
6.3.8. **Statistical Analysis**

Each experiment was performed a minimum of three times and a maximum of five times. 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). P < 0.05 was considered statistically significant.

6.4. **Results**

6.4.1. **OFF Activates Hemichannels in MLO-Y4 Osteocytes But Not in MC3T3-E1**

**Osteoblasts**

Initial experiments were performed to examine hemichannel activation in response to OFF 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 OFF for 5 minutes increased LY uptake compared to static controls in MLO-Y4 osteocytes but not in MC3T3-E1 osteoblasts (Figure 6-1A and 6-1B). For this reason, we used MLO-Y4 osteocytes exclusively for the remaining experiments. 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 18α-glycyrrhetinic acid (AGA; 30µM) significantly attenuated LY uptake in response to flow (Figure 6-1A and 6-1B), compared to cells exposed to flow in the presence of vehicle control.

6.4.2. **PKC Activity is Required for OFF-induced Hemichannel Activation**

Whereas connexon phosphorylation regulates gap junction localization and conductance [214, 445, 446], pharmacologic inhibitors were used to examine the effect of Cx phosphorylation on hemichannel activation. Treatment with GF 109203X, an inhibitor of protein kinase C [447],
significantly attenuated flow-induced dye uptake (Figure 6-2), whereas inhibitors of protein kinase A (H-89) and MEK1/2 (U0126) had no effect on dye uptake.

6.4.3. siRNA-directed Decreases in Cx43 Attenuate Flow-induced Hemichannel Activation

Introduction of siRNA directed against Cx43 significantly lowered Cx43 expression compared to scrambled siRNA-transfected osteocytes (Figure 6-3A), demonstrating the efficacy of the siRNA construct in decreasing Cx43 protein levels. Similar to pharmacologic inhibition of hemichannel activity with AGA (Figure 6-1A and 6-1B) and GF 109203X (Figure 6-2), Cx43 siRNA transfection decreased dye uptake in response to flow compared to scrambled siRNA-transfected osteocytes (Figure 6-3B).

6.4.4. Hemichannel Activity is Required for Flow-induced ATP Release

Conditioned media from MLO-Y4 osteocytes exposed to OFF contained more ATP than conditioned media samples from static controls (Figure 6-4A). 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 6-4B). Cx43 depletion with siRNA similarly affected flow-induced ATP release compared to scrambled siRNA-transfected controls (Figure 6-4C).

6.4.5. Purinoceptor Activation Induces PGE$_2$ Release

The addition of ATP to static osteocytes dose-dependently increased PGE$_2$ release, implicating P2 purinoceptor activation in PGE$_2$ release (Figure 6-5A). Significant increases in PGE$_2$ release were also found in Cx43-depleted osteocytes, suggesting that flow-induced PGE$_2$ release can occur independently of hemichannel formation. There was, however, significantly less
PGE$_2$ released in response to ATP challenge in Cx43 siRNA-treated cells compared to scrambled siRNA-treated cells (Figure 6-5B).

6.5. 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 PGE$_2$, or the direct transmission of a chemical or electrical signal through gap junctions and GJIC. Indeed, we [166, 168, 214, 409], and others [161, 165, 213, 279, 448], 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 [279, 335, 338, 345, 442, 449] 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, glycyrrhetinic acid derivatives inhibit both GJIC [346, 347] and hemichannels [345], 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 oscillatory fluid flow promotes uptake of Lucifer Yellow (Figure 6-1A and 6-1B), 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 6-1A and 6-1B). 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 the only other report on fluid flow and hemichannels [279]. 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.

To date, over 20 connexins have been described and a subset of these—Cx43 [153], 45 [155], and 46 [156]—are expressed in cells of the osteoblastic lineage. We sought to define the Cx composition of hemichannels by using small interfering RNA (siRNA) to selectively ablate Cx expression. We postulated that the presence of flow-activated hemichannels in osteocytes but not osteoblasts (Figure 6-1A and 6-1B) was mediated by Cx43, as it has been reported that osteocytes express significantly higher Cx43 mRNA [409] and protein [450, 451] than do osteoblasts. Using siRNA strategies, we found impaired dye uptake in Cx43 siRNA-transfected osteocytes relative to scrambled siRNA-transfected osteocytes (Figure 6-3B), demonstrating that hemichannels in MLO-Y4 osteocytes are composed of Cx43.

Contrary to our findings, other authors have demonstrated the presence of hemichannels in osteoblast-like cells exposed to low extracellular calcium conditions [413]. Whether this discrepancy is due to loading conditions (fluid flow vs. low [Ca\(^{2+}\)]\(_{\text{ex}}\)) or cell lines (murine MC3T3-E1 vs. human HOBIT) is unknown. It should be noted that the absence of flow-activated hemichannels in MC3T3-E1 in 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 [422].
The carboxy terminal tail of Cx43 contains several consensus phosphorylation sites for PKA, PKC, and MAPK (reviewed in [445]). Because oscillatory fluid flow-induced dye uptake was inhibited by the GJ and hemichannel antagonist AGA, and because we have previously demonstrated that OFF increases GJIC through ERK1/2 [214], 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 OFF (Figure 6-2). 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 [446]. However, there are also reports of PKC increasing gap junctional conductance [452]. 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 [453-455]. We have previously demonstrated that fluid flow promotes ATP release in MC3T3-E1 osteoblasts [422] and that P2 receptor activation is required for OFF-induced transients in cytosolic calcium [10]. 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 [339, 340]. Additionally, ATP release in response to mechanical load in chondron pellets was inhibited by AGA [344 non-endothelium], implicating connexons in load-induced ATP release. We found that application of OFF significantly increased ATP levels in conditioned media approximately three-fold compared to conditioned media from static cells (Figure 6-4A), and this was significantly attenuated in the presence of pharmacologic inhibitors of hemichannel activation (Figure 6-4B). Additionally, osteocytes transfected with siRNA directed against Cx43 demonstrated decreased ATP release in response to flow compared to non-transfected and scrambled siRNA-transfected osteocytes. As a whole, these data demonstrate that OFF promotes Cx43 hemichannel activation to promote ATP efflux from the cytosol to the pericellular environment.

A previous report on flow-activated hemichannels in osteocytes demonstrated that Cx43 siRNA transfection or treatment with AGA significantly attenuated PGE$_2$ release with flow compared to appropriate controls; from these data, the authors concluded that PGE$_2$ is directly released through Cx43 hemichannels [279]. Because our data demonstrate that hemichannel formation is required for ATP release, and because we have previously demonstrated that P2 receptor activation is required for PGE$_2$ release in osteoblasts [422], we instead hypothesized that hemichannel activation promotes ATP release that activates P2 receptors to induce PGE$_2$ release through a non-hemichannel mechanism. We found significant increases in PGE$_2$ release in Cx43 siRNA-transfected cells treated with ATP (Figure 6-5B), suggesting firstly that PGE$_2$ release is mediated by activation of P2 receptors, and secondly that PGE$_2$ release can occur independently of Cx43 hemichannel formation. We posit that the inhibition of PGE$_2$ release with Cx43 siRNA or AGA from this previous report is not direct entirely due to inhibition of PGE$_2$ release through Cx43 hemichannels, but, rather, inhibition of an upstream activator of PGE$_2$ release, specifically, ATP.
6.6. Footnotes

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Figure 6-1. OFF induces dye uptake through hemichannels in MLO-Y4 osteocytes but not MC3T3-E1 osteoblasts. (A) Hemichannel activation was monitored by LY dye uptake as described in “Experimental Procedures”. OFF promoted dye uptake in osteocytes that was attenuated with the addition of the hemichannel antagonist, AGA; flow had no effect on dye uptake in MC3T3-E1 osteoblasts. (B) Quantitation of dye uptake in response to flow in MLO-Y4 osteocytes and MC3T3-E1 osteoblasts. Data are presented as the percentage of cells staining positively for LY relative to the total number of cells. Data were obtained from 4 independent experiments. (a: p < 0.01 to static MLO-Y4; b: p < 0.01 to flowed MLO-Y4).
Figure 6-2. OFF-induced dye uptake is mediated by PKC. To determine the mechanism of flow-induced hemichannel activation, cells were incubated in the presence of PKA (H-89, 2µM), PKC (GF 109203X, 1µM), or MEK1/2 (U0126, 1µM) inhibitors, and exposed to OFF as above. Dye uptake in response to flow was significantly attenuated for cells pre-incubated with PKC inhibitor GF 109203X. Neither PKA nor MEK1/2 inhibitors affected static or OFF-induced dye uptake. Data were obtained from 3 independent experiments. (a: p < 0.001 compared to appropriate static condition; b: p > 0.05 compared to static + GF 109203X).
Figure 6-3. siRNA depletion of Cx43 in MLO-Y4 osteocytes attenuates hemichannel activation in response to flow. (A) Custom designed siRNA directed against Cx43 functionally decreases Cx43 protein expression relative to non-silencing scrambled siRNA transfection. (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 6-4. ATP release in MLO-Y4 osteocytes requires hemichannel activation. (A) OFF significantly increased ATP content in conditioned media relative to static controls; ATP levels in flow media not exposed to cells were below the detectable limits of the assay (a: p < 0.05 compared to fresh flow media; b: p < 0.05 compared to static). (B) Pharmacologic inhibition of hemichannel activation with AGA and PKC significantly attenuate flow-induced ATP release relative to cells flowed in the presence of vehicle control. (a: p < 0.05 compared to static vehicle).
Figure 6-4. ATP release in MLO-Y4 osteocytes requires Cx43 expression. (C) siRNA-mediated decreases in Cx43 similarly attenuate ATP release in response to flow compared to non-silencing scrambled siRNA-transfected osteocytes. Data were obtained from 5 independent experiments. (a: p < 0.05 compared to scrambled static).
Figure 6-5. Exogenous ATP promotes PGE₂ release in Cx43-deficient osteocytes. (A) Addition of exogenous ATP to static osteocytes induces PGE₂ release, implicating P2 receptor activation as an upstream mediator of PGE₂ release. (a: p < 0.05 compared to control; b: p < 0.01 relative to control). (B) Exogenous ATP induces PGE₂ release in Cx43-depleted osteocytes, indicating that hemichannel formation is not required for PGE₂ release. Data were obtained from 3 independent experiments. (a: p < 0.001 compared to scrambled control; b: p < 0.005 compared to Cx43 siRNA-treated in absence of ATP; c: p < 0.01 compared to scrambled siRNA-treated in presence of ATP).
7. DISCUSSION

7.1. Overview

Although a role for ATP as an extracellular signaling molecule was initially conceived in the realm of neuroscience, it is now apparent that purinergic signaling occurs in nearly every cell in the body. Research over the last decade has reported that purinoceptor activation is able to affect both osteoblast and osteoclast function. Nearly all of these studies were performed in a similar manner, wherein exogenous ATP (or ADP, UDP, UTP, etc) was added to cell cultures and its effect on endpoint A, B, or C was examined. Few of these studies addressed a critical concept that is required for the initiation of purinergic signaling: namely, how ATP is released from bone cells. Instead, investigators referenced the mechanisms implicated in ATP release in other cells, and posited that such mechanisms are responsible in bone cells. As such, the overall goals of this thesis were to:

- Determine if fluid flow, a model for mechanical load in vitro, increased the rate of ATP release from osteoblasts and osteocytes.
- Define the mechanism(s) involved in flow-induced ATP release.
- Examine the effect of purinoceptor activation on known mechanisms implicated in the anabolic response of the skeleton to mechanical load in vivo.

7.2. Conclusions

7.2.1. Fluid Flow-induced ATP Release in Murine Osteoblasts Involves Exocytosis of ATP-filled Vesicles

In the vernacular of mechanotransduction, osteoblasts are the effector cells in skeletal anabolism, building bone by secreting type I collagen that becomes calcified. Our initial results showed that fluid flow increases the amount of ATP in conditioned media compared to static controls, suggesting that fluid flow increases the rate of ATP release. The addition of ionomycin, a calcium ionophore, increased the amount of ATP in conditioned media to the same degree as
did fluid flow, suggesting that flow-induced ATP release was mediated by changes in Ca\(^{2+}\).

Pharmacologic antagonism of calcium entry and release demonstrated that calcium entry though the Ca\(_{\text{v}}\)1.2 channel was required for flow-induced changes in ATP release. This was intriguing, insofar as Ca\(_{\text{v}}\)1.2 activation is required for neurotransmitter release [456] and other data suggest that bone cells demonstrate properties of habituation and desensitization originally described in neuronal systems [457]. Immunofluorescent staining of cytosolic ATP with the fluorophore quinacrine revealed punctate, granular localization of ATP, suggesting that ATP may be localized into vesicles, as has also been shown in endothelial cells [303]. Pharmacologic inhibitors of exocytosis, that prevented vesicle formation, release, and plasmalemmal fusion, all inhibited flow-induced ATP release. These data suggest that osteoblasts localize ATP into vesicles that exocytose in response to fluid flow, thereby releasing ATP to the pericellular environment to initiate purinergic signaling.

### 7.2.2. Flow-induced ATP Secretion from Osteoblasts Mediates Prostaglandin Release

Load-induced bone formation requires the synthesis of prostaglandins [270, 272] and fluid flow has been shown to increase the rate of PG release [207, 211, 268, 436]. As such, we sought whether purinoceptor activation mediates prostaglandin release, as has been shown in other cells [453, 458, 459]. The addition of apyrase, an ATPase, significantly decreased PGE\(_2\) release in response to flow, while ATP addition to static osteoblasts dose-dependently increased PG release. These data suggest that the effect of fluid flow on PGE\(_2\) release is mediated by proximal ATP secretion and activation of purinoceptors.
7.2.3. Flow-induced Purinoceptor Activation Mediates NF-κB Translocation in Murine Osteoblasts

We previously demonstrated that fluid flow mediates NF-κB activation, which is required for maximal COX-2 expression in response to flow [278]. Having demonstrated that fluid flow promotes ATP secretion that induces PGE$_2$ release in an autocrine fashion, we examined the effect of purinoceptor activation on NF-κB translocation. Whereas fluid flow induced robust nuclear staining for NF-κB, osteoblasts flowed in the presence of the ATPase apyrase revealed cytosolic NF-κB activation. These data indicate that P2 purinoceptor activation is required for flow-induced NF-κB translocation. Further, we found that IκBα degradation, a requirement for nuclear NF-κB accumulation, is similarly impaired in the presence of apyrase, suggesting that purinoceptor activation occurs proximal to IκBα degradation. The use of a selective antagonist against the P2X$_7$R also revealed impaired IκBα degradation in response to flow, suggesting that the P2X$_7$R is involved in this process.

7.2.4. Fluid Flow Activates Cx43 Hemichannels in Murine Osteocytes

Whereas the importance of gap junctions and gap junctional intercellular communication has been recognized in bone cell mechanotransduction, emerging data demonstrates that unapposed connexons, or hemichannels, are able to mediate fluxes of second messengers such as IP$_3$, NAD$^+$, and ATP. We sought whether osteoblasts and osteocytes express functional hemichannels, the mechanisms involved in hemichannel opening, and the connexon composition of the hemichannels. In order to examine hemichannel activation (i.e., opening), we used two fluorescent dyes that do not freely cross the plasmalemma, Lucifer Yellow (LY) and rhodamine-conjugated dextran (RD). There is an approximately 20-fold size difference between LY and RD, such that LY is small enough to permeate hemichannels, while RD is too large to do so. Application of fluid flow to osteocytes increased the number of cells retaining LY but not RD,
which was attenuated in the presence of AGA, a pharmacologic inhibitor of both GJIC and hemichannels. These data strongly suggests that LY uptake is mediated by hemichannels. Wherein Cx phosphorylation regulates permeability and conductance, we examined the effect of protein kinase A, protein kinase C, and MEK1/2 on hemichannel activation, and found that, of these kinases, only PKC is required. Finally, through the use of siRNA strategies, it was determined that Cx43 is required for hemichannel formation.

7.2.5. Flow-induced ATP Release in Murine Osteocytes Requires Activation of Cx43

Hemichannels

Although osteoblasts are responsive to mechanical loads in vitro, modeling of the expected fluid forces across osteoblasts has not been performed as it has for osteocytes. Because of their localization in canaculi and lacunae, osteocytes are speculated to be the primary mechanosensors in bone. Thus, we firstly examined whether murine osteocytes release ATP in response to flow, as did osteoblasts. Similar to our results obtained with the osteoblast model, fluid flow increased ATP levels in conditioned media approximately 4-fold compared to conditioned media obtained from static controls.

In various other cells, such as astrocytes and chondron pellets, ATP release is increased by Cx43 over-expression and impaired by pharmacologic inhibitors of gap junctions and hemichannels [344, 449]. Cx43 over-expression could increase ATP release through two mechanisms: by functioning as gap junctions to increase GJIC, and sensitizing cells to extracellular stimuli, or by functioning as hemichannels and providing a conduit for ATP release. Having demonstrated that osteocytes express functional hemichannels in response to flow, we hypothesized that osteocytic ATP release involves hemichannels. Pharmacologic antagonism of hemichannel activation, through the use of AGA and an inhibitor of PKC, significantly attenuated flow-induced ATP release. siRNA directed against Cx43, which inhibited hemichannel activation
in response to flow, inhibited ATP release in response to flow. As such, these data strongly suggest that hemichannel activation promotes ATP release.

Given the different mechanisms for ATP release between osteoblasts and osteocytes—i.e., vesicular exocytosis versus diffusion through a hemichannel—we sought whether purinoceptor activation similarly regulated osteocytic PGE\(_2\) release. Addition of ATP to static osteocytes dose-dependently increased PGE\(_2\) release, suggesting that purinoceptor activation is involved in PGE\(_2\) release in both osteoblasts and osteocytes.

### 7.3. Proposed Model of Osteoblastic and Osteocytic Purinergic Signaling

A great deal of work has already been performed in delineating the anabolic effects of flow on osteoblasts and osteocytes. The figures on the following pages inject the results of this thesis into bone cell mechanotransduction
Figure 7-1. A model for ATP release and purinergic signaling in osteoblastic mechanotransduction. Fluid flow activates the Ca\textsubscript{v}1.2 induces localized increases in Ca\textsuperscript{2+} that induces exocytosis of ATP-filled vesicles. Pericellular ATP binds to and activates P2X or P2Y purinoceptors. Purinoceptor activation is required for flow-induced increases in Ca\textsuperscript{2+}, PGE\textsubscript{2} release, ERK1/2 activation, and NF-κB translocation.
Flow

Figure 7-2. A model for ATP release and purinergic signaling in osteocyte mechanotransduction. Fluid flow activation of PKC opens Cx43 hemichannels, through which ATP moves down its electrochemical gradient. Subsequent purinoceptor activation induces PGE$_2$ release through a mechanism independent of Cx43 hemichannels.
7.4. Future Studies and Unanswered Questions

7.4.1. Are There Multiple ATP Release Pathways in Bone Cells?

In this thesis, flow-induced ATP release was significantly attenuated in the presence of pharmacologic antagonists of hemichannels or exocytosis. Basal ATP release, however, was unaffected by these agents, suggesting that ATP release from bone cells can occur via two distinct mechanisms. As discussed in Section 3, there are multiple mechanisms for ATP release from the cytosol; perhaps one of these pathways, not implicated in response to flow, mediates basal ATP flux. Interestingly, preliminary data suggest that DIDS (4,4’-diisothiocyanatostilbene-2,2’ disulfonyl acid), a putative chloride channel antagonist, inhibited basal, constitutive ATP release in MC3T3-E1 osteoblasts. However, DIDS is a non-selective antagonists of a number of biological processes, including inhibition of chloride channels, such as CFTR, and chloride/bicarbonate exchange. As such, it is premature to assign any mechanism to static ATP release in osteoblasts.

7.4.2. ATP Release and Purinoceptor Activation

The main goals of this thesis were to discern whether fluid flow induces ATP release and the mechanisms involved therein. In order to examine ATP levels in conditioned media, the author chose the luciferin-luciferase method based on its ease of use and high sample output. Having demonstrated that fluid flow increased the rate of ATP release, the implicit assumption made was that sufficient levels of ATP are released from an osteoblast or osteocyte to subsequently activate P2 purinoceptors. Whereas this appears to be true, based on experiments in which ATP was degraded with apyrase, this was not demonstrated with the luciferin-luciferase reaction. Indeed, where purinoceptors demonstrate an EC$_{50}$ of 10-50 $\mu$M (with the notable exception of the P2X$_{7}$), the luciferin-luciferase assay revealed flow-induced ATP release on the order of 10-50 nM, which is 3 orders of magnitude lower. The author feels that this is not a flaw in the thesis, but, rather, an inherent limitation of the ATP assay chosen. As described above, ATP levels were calculated
from conditioned media samples obtained during experiments, which is not the same as the localized ATP concentration at the surface of the cell. As such, a remaining goal is to be able to directly quantitate ATP concentrations at the cell surface. Such a possibility has been demonstrated by Beigi et al., who generated a chimeric luciferase construct that can be attached to cell surface proteins and thereby provide quantitative data on ATP concentrations at the plasmalemma [460].

Other experiments that could address this disparity between measured and required ATP would involve co-localization studies, wherein ATP release sites are closely associated with purinoceptors. One could immunofluorescently co-localize t-SNARE proteins (for osteoblasts) or Cx43 (for osteocytes) and purinoceptors. Further, co-localization of ecto-ATPases near purinoceptors would support rapid degradation and termination of P2 signaling. These data would provide evidence of functional compartmentation of osteoblastic or osteoclastic purinergic signaling, thereby strengthening the purinergic hypothesis in bone.

7.4.3. A Plethora of Purinoceptors

In order to implicate purinoceptor activation in mechanotransduction, these studies utilized apyrase, to degrade ATP, and a pharmacologic inhibitor of the P2X\textsubscript{7} receptor. Whereas apyrase is useful as an indicator of whether purinoceptors are involved in a given response, its use fails to inform which P2 isoform is involved. Further, the action of apyrase generates 5'-AMP, which, in the presence of ecto-5'-nucleotidases, generates adenosine, itself an agonist for P1 receptors. With the exception of the P2X\textsubscript{7} antagonist, nearly all P2 antagonists are non-selective and inhibit multiple P2 isoforms [384], making data analysis quite challenging. Instead, generation of siRNA constructs to selectively decrease P2 isoform expression, or the generation of more P2R \textsuperscript{-/-} mice, will greatly advance our understanding of which isoforms mediate osteoblastic and osteocytic mechanotransduction. Whereas antisense inhibition of the P2Y\textsubscript{2}R revealed its critical role in Ca\textsuperscript{2+}i
increases in response to flow in osteoblasts [10], we demonstrated that this receptor is not involved in flow-induced ERK1/2 activation [5].

To date, only one P2R−/− mouse, the P2X7−/− mouse, has been utilized to examine osteoblast mechanotransduction. Surprisingly, the activation of this receptor is required for flow-induced PGE_2 release [423, 424] and COX-2 induction [424], as well as load-induced osteogenesis [423]. This is surprising insofar as the P2X_7-R demonstrates two distinct permeabilities. P2X_7-R activation initially opens a channel permeant to mono- and divalent cations, but sustained activation induces the formation of a non-selective pore, which has been implicated in apoptosis [461-467]. To date, the data that have implicated P2X_7-R activation have not discerned between these two mechanisms of function.

7.4.4. Concerns Regarding Hemichannel Activation

Section 6 of this thesis examined the role of unopposed connexon hemichannels in osteocyte mechanotransduction. We report that fluid flow for 5 minutes promotes LY dye entry into osteocytes that is inhibited in the presence of the hemichannel inhibitor AGA; additionally, targeting of Cx43 with siRNA decreased flow-induced dye uptake. What remains unanswered is how rapidly hemichannels are activated and how long they remain open. Although one could conceivably examine hemichannel activation at time points earlier than 5 minutes through the method described in Section 6, a better technique involves electrophysiologic studies through whole-cell voltage clamping. This would provide such information as duration of channel opening, total conductance through a channel, and whether a given channel opens multiple times in response to flow or whether it opens, closes, and remains refractory to further opening until the load is removed. As sustained hemichannel opening would dissipate electrochemical gradients, thereby impairing cellular function, it is extremely important to discover these properties before ascribing too large a role to hemichannels in mechanotransduction.
7.4.5. How Does the P2X7-R Regulate NF-κB Translocation?

When we initially showed that fluid flow induced nuclear NF-κB accumulation, the results suggested that calcium release, and not calcium entry, was required. Antagonism of the Ca1.2 or MSCC channels failed to prevent nuclear NF-κB translocation, while inhibition of PLC with U73122 completely prevented IκBα degradation and NF-κB translocation to the nucleus. As Section 6 demonstrated the requirement for purinoceptor activation, we initially hypothesized that a metabotropic P2Y receptor was mediating this response, possible the P2Y2, given its importance in flow-induced Ca2+ transients. Instead, pharmacologic inhibition of the P2X7-R with oxidized ATP prevented IκBα degradation, suggesting that this receptor is involved in NF-κB translocation. One possible explanation for this discrepancy is that inhibition of PLC with U73122 prevents not only IP3, but DAG, formation, as well. Thus, the effect of PLC antagonism could be ascribed to inhibited DAG formation and subsequent PKC activation. In retrospect, more convincing experiments would have abstained from using U73122 and instead use either a PKC inhibitor or thapsigargin PKC inhibition would reveal whether PKC activation is required for these responses, whereas thapsigargin would prevent calcium release. If these experiments showed the same results as the U73122 studies (i.e., a requirement for calcium release from the ER), this would confirm that the mechanism of flow-induced NF-κB translocation does indeed require calcium release. Additionally, preliminary data using a pan-PKC inhibitor revealed impaired IκBα degradation in response to flow, supporting the possibility that the effects of U73122 on NF-κB translocation were the result of impaired PKC activity. With these concerns, it is worth remembering that the data in Section 6 that implicated the P2X7-R in flow-induced NF-κB translocation was obtained using pharmacologic antagonists which, while suggestive, are not necessarily ideal. As such, the use of osteoblasts derived from the P2X7-R−/− mouse should greatly clarify this confusion.
REFERENCES


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Book Chapters