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The Diminishing Returns of Mechanical Loading and Potential Mechanisms that Desensitize Osteocytes

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Abstract

Adaptation to mechanical loading is critical to maintaining bone mass and offers therapeutic potential to preventing age-related bone loss and osteoporosis. However, increasing the duration of loading is met with "diminishing returns" as the anabolic response quickly becomes saturated. As a result, the anabolic response to daily activities and repetitive bouts of loading is limited by the underlying mechanisms that desensitize and render bone unresponsive at the cellular level. Osteocytes are the primary cells that respond to skeletal loading and facilitate the overall anabolic response. Although many of osteocytes' signaling mechanisms activated in response to loading are considered anabolic in nature, several of them can also render osteocytes insensitive to further stimuli and thereby creating a negative feedback loop that limits osteocytes' overall response. The purpose of this review is to examine the potential mechanisms that may contribute to the loss of mechanosensitivity. In particular, we examined the inactivation/desensitization of ion channels and signaling molecules along with the potential role of endocytosis and cytoskeletal reorganization. The significance in defining the negative feedback loop is the potential to identify unique targets for enabling osteocytes to maintain their sensitivity. In doing so, we can begin to cultivate new strategies that capitalize on the anabolic nature of daily activities that repeatedly load the skeleton.

Keywords Osteocytes · Mechanotransduction · Diminishing returns · Negative feedback · Mechanosensitivity

Introduction

Dynamic loading of the skeleton is critical to maintaining bone health and homeostasis. The adaptive response of bone to mechanical loading was first considered in the late nineteenth century by Julius Wolff. Since then it has become well recognized that very little stimulation is required to elicit bone formation and that this response is mediated by osteocytes' sensitivity to mechanical forces [1, 2]. However, the response to loading quickly becomes saturated as osteocytes lose their sensitivity to mechanical stimuli [3, 4]. As a result, the osteogenic potential of daily activities quickly reaches a plateau, limiting their efficacy to maintain bone mass. Enabling osteocytes to maintain their mechanosensitivity has the potential to improve the efficacy of daily activities to increase bone mass and reduce fracture risk in pathological conditions. Therefore, the purpose of this review is to examine our current understanding of (1) the temporal response of bone to mechanical loading and (2) the underlying mechanisms by which osteocytes can become no longer sensitive to loading. In particular, we review signaling mechanisms that relate to the inactivation/desensitization of channels and signaling molecule along with the role of endocytosis and cytoskeletal reorganization.

Osteocyte Mechanotransduction

Osteocytes response to loading is regulated by several components that require direct contact with the extracellular matrix or mechanoreceptors that distinguish changes in pressure or fluid shear along the plasma membrane (see review by Thompson et al.) [5]. Mechanical strain of the cell membrane opens stretch-activated ion channels that initiate an intracellular calcium response [6]. The influx of calcium facilitates several down-stream events that include activation of T-type voltage-gated ion channels as well as

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hemi-channels by which osteocytes communicate neighboring cells [7]. Connexin-43 is the primary hemi-channel that facilitates the down-stream release of adenosine triphosphate (ATP) as well as the release of prostaglandin E2 (PGE2) [8]. Activation of prostaglandin receptors EP2 and EP4 are then responsible for stabilizing beta-catenin by inhibiting glycogen synthase kinase 3 beta (GSK-3B)-induced degradation through phosphatidylinositol 3′-kinase (PI3K) and protein kinase B (AKT) pathway as well as the cyclic adenosine 3′,5′-monophosphate (cAMP) and protein kinase A (PKA) pathway [9, 10]. Accumulation of beta-catenin shifts osteocyte gene expression to be more supportive of osteoblast activation by way of increasing Wnt-ligands and decreasing their inhibitors, such as Sost [11]. Beta-catenin also suppresses osteocyte potential activation of osteoclast by way of decreasing receptor activator of nuclear factor kappa-B ligand (RANKL) [12, 13]. The cAMP-PKA pathway is also likely to contribute to the increased expression of c-fos and c-jun during via cAMP-response element binding protein (CREB) [10, 14, 15]. Although extensive work has identified other signaling mechanisms that contribute to the mechanotransduction of osteocytes [16], the highlighted pathways are prone to becoming inactivated or desensitized and thereby may contribute to the diminishing returns observed during loading.

Evidence of Diminishing Returns and the Loss of Mechanotransduction

Increasing the duration or number of loading cycles without any interruption is met with "diminishing returns" as the anabolic response quickly becomes saturated. In the seminal work by Rubin and Lanyon, only 36 cycles of 2000 με in compression was needed before the anabolic response became saturated [2]. Increasing the number of cycles to 1800 did not provide any additional gains in bone formation compared to 36 cycles. A decade later similar findings were reported in rats under four-point bending and even jumping [17–19].

Very few studies have gone on to examine how the duration of loading influences the anabolic response in humans. In 1993, Martin and Notelovitz reported 30 min of exercise was sufficient to increase bone mineral content (BMC) at the hip, specifically in women less than 6 years past the onset of menopause [20]. Martin and Notelovitz went on to demonstrate that increasing the duration of exercise to 45 min had no added benefit. More recently, Marin-Puyalto reported in adolescent males that only 5 min of physical activity is needed to increase BMC and bone mineral density (BMD) [21]. Increasing the duration to 15 min did not increase BMC any further, but was only able to produce an increase in BMD. Similarly, others have reported the duration of loading is not as predictive of bone strength compared to the daily frequency of physical activity [22]. Even the use of low-intensity vibration has shown no significant gains in BMC or bone mineral density (BMD) in the spine or hip when increasing the duration of loading [23]. As a result, the efficacy of loading paradigms to reduce fracture risk is limited by the diminishing returns and desensitization at the cellular level.

A similar loss in mechanotransduction has been observed at the cellular level under continuous loading. For osteocytes, the initial response to either fluid flow, hydraulic pressure, or substrate strain is characterized by a series of "spikes" or oscillations in intracellular calcium [7, 24–26]. As loading persists, the number of responsive cells gradually declines from 95 to 25% [27]. Even those that remain responsive readily display smaller spikes in [Ca2+]i as the magnitude of loading increases [27]. The shift in magnitude, duration, and overall shape of the [Ca2+]i spikes may suggest functional changes in the ion channels or a shift in which source of calcium is contributing to the signal [28]. In particular, cell-to-cell communication through hemichannels, such as connexin43, is reported to display a significant decline in activity under continuous fluid flow for more than 4 h [29]. The subsequent release of secondary messengers, such as prostaglandin E2 (PGE2) and nitric oxide (NO), also becomes saturated as the extracellular concentrations plateau under prolonged loading [30–32]. As osteocytes release of secondary messengers becomes saturated, the capacity to recruit and activate osteoblasts to form new tissue is likely to plateau.

Defining the Refractory Period

A refractory period is the time needed before an adequate response can be induced by a second stimulus. In humans, an exact refractory period has not been established. Instead, the refractory period was first reported in the rat vertebral loading model to be 24 h given that each day of loading had an additive effect on mineralization [33]. The additive effect for each day of loading suggests a recovery in mechanosensitivity. A similar refractory period of 24 h was observed when loading the fat tibia under four-point bending for 36 cycles each day [34]. Using the same tibial loading model, Robling's group has since reported that only 8 h is needed for bone to become responsive again to loading [35]. Shorter refractor periods have been considered given that inserting a rest-period lasting 10–14 s between each loading cycle can have an additive effect compared to continuous loading conditions. In particular, a 10-s interval at low magnitudes produces comparable effects as continuous loading under magnitudes 10 times greater [36]. However, the additive effect is hypothesized to be a function of synchronization

between osteocytes' when considered as a small world network similar to that used to model the complex network of neurons [37, 38]. Overall, the degree to which inserting rest intervals increase bone formation by synchronizing cell-to-cell communication verses allowing osteocytes to recover their sensitivity remains to be established.

At the cellular level, the refractory period is far less understood. For osteocytes, a second exposure to laminar fluid flow has a similar impact on hemichannel activation compared to an initial session 24 h earlier [29]. Shorter refractory periods have been observed for osteoblasts and mesenchymal stem cells (MSCs). For osteoblasts, 30–90 min of rest between successive 2-min bouts of oscillatory fluid flow is required to restore intracellular calcium signaling back to pre-load levels [39]. Interestingly, Christopher Jacobs' group latter found osteoblasts to exhibit a shorter refractory period when exposed to only 10 s of loading followed by 10 s of rest before a second 10 s bout of loading [40]. However, the gains in calcium signaling by inserting rest periods were not accompanied by gains in the downstream release of PGE2. These findings suggest that the refractory period is dependent on the loading history and may also be specific to each signaling mechanism. Overall, the unique differences in how each cell type sense mechanical loading warrants further investigation of osteocytes' refractory period as it relates to specific signaling pathways.

Desensitization and Inactivation of Ion Channels

While extensive work has focused on how osteocytes' sense loading, less attention has been given to the underlying mechanisms that then desensitize osteocytes to loading. One way in which osteocytes become desensitized to stimuli is through the inactivation of ion channels that facilitate the mechanotransduction pathway. Osteocytes' initial response to loading is in large mediated by the rapid influx of calcium through voltage-gated ion channels, specifically the T-type voltage channel Cav3.2 [7, 24, 41]. Recently, the Cav3.2 channel was found to contain a "gating brake" that regulates or tunes the voltage range for activation [42]. Within the "gating brake" region, studies have reported calmodulin binding sites that regulate the rate at which the channel opens and the degree of depolarization required for activation [43]. Activation of calcium/calmodulin kinase II (CaMKII) occurs in osteocytes within 5 min after the onset of fluid flow [6]. Although CaMKII activation was shown to facilitate load-induced suppression of sclerostin, it is also possible for calmodulin to play a defining role in osteocytes' calcium signaling fingerprint under prolonged loading.

Inactivation or the closure of hemichannels and gap junctions is also expected to influence the degree to which osteocytes remain sensitive to loading. In particular, activation of connex-43 hemichannels are critical to mitogen-activated protein kinase (MAPK) activation as well as the release of adenosine triphosphate (ATP) and PGE2 [8, 29, 32, 44]. Under continuous loading, osteocytes' uptake of a fluorescent dyes through connexin43 peaks 2 h after loading and then gradually decreases thereafter [29]. The loss in connexin43 activity was latter attributed to a gradual closure due to phosphorylation of the channel at Ser 279/282 [44]. Sustained levels of PGE2 released under loading was found to phosphorylate connexin43 by way of activating extracellular signal regulated kinases (ERK), producing a negative feedback loop [44]. Overall, our understanding of channel activity beyond 10 min is extremely lacking and may provide valuable information regarding the diminishing returns.

Refractory Period of Signaling Molecules

Signaling molecules also exhibit a refractory in that they become inaccessible or ineffective to subsequent bouts of stimuli. One in particular is the refractory period of cAMP and its transcriptional activity. The activation of cAMP in response to loading is mediated by osteocytes' release of prostaglandins through an autocrine and paracrine manner [9, 10]. Signaling via cAMP during loading is responsible for the increased transcription of c-fos and c-jun, both of which are regulated through the CREB similar to osteoblasts [15, 45–47]. In addition, cAMP also contributes to osteocytes' nuclear accumulation of beta-catenin during loading [9]. In other cell types, transcription of cAMP-responsive genes is attenuated under subsequent stimuli due to dephosphorylation of CREB by protein phosphatase-1 (PP-1) and in some cases PP-2A [48, 49]. Inhibition of PP-1 reverses the attenuation phase, maintaining high levels of phosphorylated CREB and target gene transcription. The duration of the refractory period is largely dependent on how long the initial stimulation is applied. Interestingly, overexpressing PKA can reactivate CREB gene transcription during the refractory period [50]. As a result, strategies that increase PKA may offer a potential mechanism to improve CREB function during the refractory period.

Other signaling molecules that display a refractory period include the transcription factor beta-catenin. Stabilization of beta-catenin along the osteoblast lineage prevents bone loss due to disuse, suggesting a key role in mechanotransduction [51]. Beta-catenin is activated during fluid flow through PGE2-induced PI3K-AKT inactivation of GSK-3B [9]. Interestedly, signaling along the PI3K/AKT/GSK-3/beta-catenin axis peaks significantly declines after 30 min of fluid flow despite continued exposure to loading and elevated levels of extracellular PGE2. The exact time required before PI3K/AKT signaling can be reactivated by PGE2 remains

to be determined along with the underlying mechanisms that deactivate this pathway. The loss in PI3K/AKT activity is likely mediated by PP-2A, which dephosphorylates AKT in particular [52]. Overall, the potential to modify osteocytes sensitivity by modifying the transient nature of these signaling molecules and transcription factors remains to be seen.

Endocytosis

Desensitization can also occur through endocytosis and the internalization of activated receptors or signaling proteins. Following endocytosis, signaling is restored only when the receptor or signaling proteins are either recycled to the cell membrane or replaced entirely. To date, osteocytes' endocytosis of specific receptors or signaling molecules in response to mechanical loading has been given little attention. However, membrane proteins that regulate endocytosis of lipid rafts such as cavolin-1 has been shown to facilitate beta-catenin stabilization and activation in osteocytes under fluid flow [53]. Interestingly, osteoblast expression of caveolin-1 plays a key role in regulating purinergic receptors that are critical to mechanotransduction [54]. In osteocytes, caveolin-1 expression is likely to regulate beta-catenin levels during fluid flow by facilitating the endocytosis of the LRP5 receptor [55]. In response to loading, LRP5 is activated by various wnt ligands, such as wnt3a, and contributes to the anabolic formation of new bone [56–58]. Once LRP5 is internalized in response to loading, any subsequent bouts of loading likely have little to no additive effect until the receptor is recycled back to the membrane. Although LRP5 dynamics in osteocytes is lacking, other studies in cancer cells have found LRP5 takes at least 2 h to be recycled [59]. Increasing the recycling time of LRP6 can increase beta-catenin signaling and may prove useful to improving osteocytes' mechanotransduction that warrants further investigation [59].

Other receptors that are likely to under-go endocytosis during loading include various G-protein coupled receptors (GPCRs). In particular, the G protein-coupled E-type prostanoid 4 receptor (EP4) is likely internalized in response to osteocytes' release of PGE2 during loading [30]. Activation of the EP4 in human osteoblastic cells with a differentiated phenotype regulates sclerostin expression during loading, which is a critical component to osteocyte mechanotransduction [60]. Although the internalization process of EP4 in osteocytes has not been established, the EP4 receptor in other cell types is quickly internalized and desensitized with the aid of arrestin-2 binding independent of caveolin [61–63]. Once internalized, the EP4 receptor is recycled back to the membrane within 10 min. Although EP4 recycling also induces the externalization of additional EP4 receptors [63–65], osteocyte expression of EP4 during loading remains constant [10]. Instead, EP2 levels are increased at both the protein and gene levels, leading to an increased overall sensitivity to PGE2. As a result, PGE2 signaling has the potential to increase osteocytes' sensitivity to loading. In fact, exogenous PGE2 treatment has been shown to increase osteoblasts' mechanosensitivity [66]. However, the authors attributed the increased sensitivity to the depolymerization of the actin-cytoskeleton and decrease in cell stiffness. Regardless, further investigation is warranted to understand how changes in osteocyte mechanosensitivity are influenced through PGE2 signaling and changes in EP4 and EP2 presence at the plasma membrane.

Endocytosis is also likely to affect osteocytes' mechanosensitivity by way of removing key ion channels. Osteocyte initial response to mechanical strain is mediated by the stretch activated transient receptor potential vanilloid 4 (TRPV4) ion channel [6]. In endothelial cells, the TRPV4 ion channel is endocytosed into recycling endosomes 20 min after activation through the protein kinase C/PI3K and RhoA signaling pathway, which is also active in osteocytes under loading [67–69]. The initial calcium response is followed by the release of ATP and activation of the purinergic type 2 receptor X7 (P2X7). The P2X7 receptor is a ligand-gated ion channel that further potentiates intracellular calcium signaling and contributes to the overall anabolic response to in vivo loading [70, 71]. Although the dynamics of P2X7 are unclear in osteocytes, P2X7 stimulation in osteoblasts has been reported to induce endocytosis mediated by caveolin-1 [54]. The potential endocytosis of either P2X7 or TRPV4 during osteocyte response to loading may explain observed declines in calcium signaling as loading persists [27].

Cytoskeleton Reorganization

Robling first suggested that the recovery to mechanosensitivity may correspond with osteocytes' disassemble of actin-stress fibers [35]. Osteocytes' response to mechanical stimuli is characterized by an increase in actin-stress fiber formation (ASFF). Although ASFF in response to loading is considered to facilitate down-stream signaling and gene expression [25, 72], ASFF is also expected to reduce the degree of deformation the membrane is subjected to under loading [73]. As stress fibers accumulate and increase tension at the focal adhesions, the elasticity of the plasma membrane and potential activity of stretch-activated cation-permeable channels are both reduced. Osteocytes with a less stiff cytoskeleton and overall more elastic morphology are also more sensitive to loading [74]. Unfortunately, the degree to which ASFF in response to fluid flow impacts osteocytes' apparent stiffness has yet to be established. However, osteoblasts' response to fluid flow induced shear stress (FSS) and hydraulic pressure is characterized by a fivefold increase in

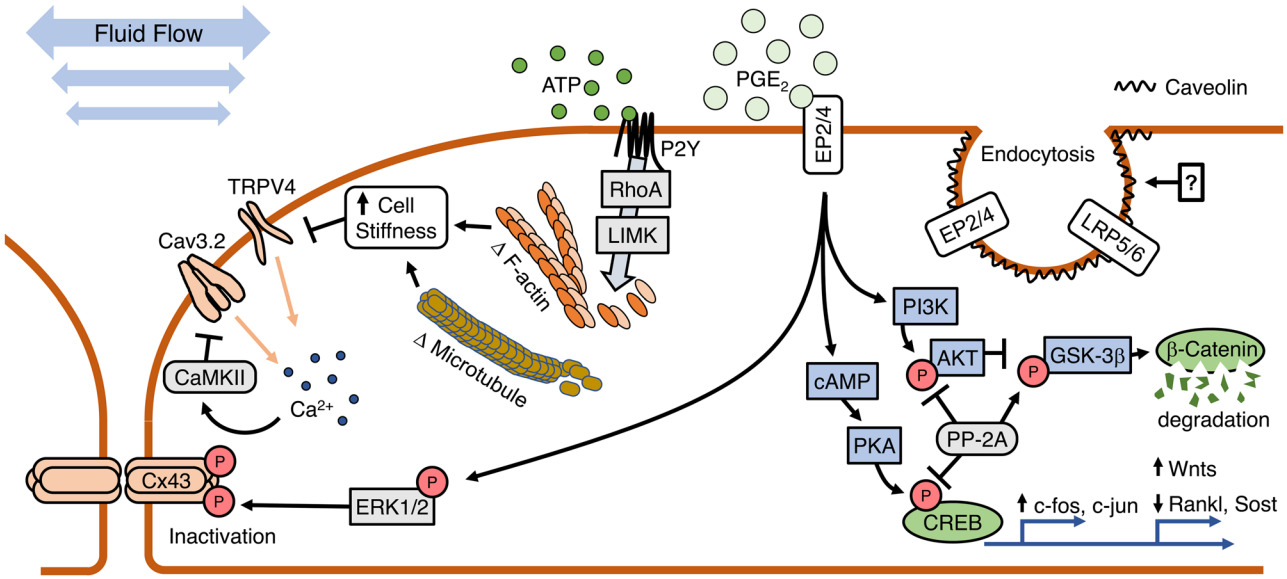

Fig. 1 Osteocyte response to loading involves several negative feedback loops that down-regualte specific components that contribute to the mechanotransduction pathway. Hemi-channels and voltage sensitive ion channels are prone to inactivation via ERK1/2 and CaMKII, respectively, while stretch activated ion channels are desensitized by changes in cell stiffness following reorganization of the cytoskeleton. Activation of transcription factors CREB and beta-catenin via prosteglandins are prone to inactivation by PP-2A as well as the loss of receptors through endocytosis

their modulus of elasticity measured by atomic force microscopy (AFM) [75]. Previous models estimated that a twofold increase in osteoblasts modulus of elasticity reduces strains along the plasma membrane by 30–50% during hydrostatic pressure and laminar FSS [76].

Since actin-stress fibers increase cell stiffness, it would stand to reason than that disrupting the stress fibers would increase osteocytes' mechanosensitivity. However, disrupting the actin-cytoskeleton pharmaceutically prior to mechanical loading results in a loss of intracellular calcium signaling, and an absence of ERK phosphorylation, NO production, and PGE2 release [30, 77–80]. The loss in sensitivity is in part due to disrupting existing focal adhesions where several mechanosensors are anchored. As a result, it is unclear how the assembly of actin stress fibers during loading would impact osteocytes mechanosensitivity given the lack of focal adhesions. In osteoblasts, targeting the Rho/Rho kinase (ROCK)1/LIM kinase 2 pathway responsible for ASFF has shown to maintain osteoblasts mechanosensitivity as loading continues and may serve the same purpose in osteocytes [75, 81].

Compared to the actin filaments, re-organization of the intermediate filaments in response to loading is less understood. However, osteocytes' increased expression of vinculin in response to loading is likely to increase cell adhesion and thereby reduce any further deformation of the cell membrane [82–84]. In addition, knockout mice of intermediate filaments have significant bone phenotypes that warrant further investigation regarding their role in regulating osteocytes mechanosensitivity [85].

Osteocytes' response to both cyclic loading and oscillatory fluid flow is characterized by a greater density of microtubules with a more buckled structure [25, 86]. Increasing microtubule density pharmaceutically by way of treating with Taxol increases osteocytes modulus of elasticity while at the same time reducing osteocytes' sensitivity to fluid flow [6]. The increase in cell stiffness is attributed to an increased fraction of detyrosinated tubulin, which binds with other cytoskeletal elements and enables microtubules to bend and buckle under loading [87]. Detyrosinated tubulin is also known to increase microtubule density and structure that may cause a loss in osteocytes' mechanosensitivity under prolonged loading.

Overall, re-organization of the cytoskeleton serves as a potential negative feedback loop by increasing osteocytes' modulus of elasticity and requiring greater strains to elicit a response. Targeting the underlying mechanisms that regulate cytoskeletal dynamics has demonstrated promise to enhancing osteocytes induction of bone formation [75, 81].

Future Directions

Since the seminal work by Robling and Layon in 1984, we have begun to identify potential mechanisms that contribute to "diminishing returns" in the anabolic response to loading

(Fig. 1). However, there is still a lot that remains unknown. In particular, what are the temporal dynamics of the ion channels and ligand receptors involved in mechanotransduction? Answering this question requires further investigation regarding the active and in-active state of each ion channel or receptor beyond the initial 10 min that is commonly reported. Conformational changes as loading persists are extremely lacking for many of the ion channels that contribute to osteocytes' initial response. Also, the endocytosis of receptors and removal from the cell membrane needs to be further examined. Real-time imaging techniques would enable future studies to better understand the dynamics and presence of specific receptors or channels change along the cell membrane as loading persists.

Proteomic and genomic studies are also needed to establish a better understanding of the temporal response to loading by defining how long signaling pathways remain active before becoming saturated or even de-activated. To date, genomic and proteomic studies have only exposed osteocytes to a single bout of loading and examined changes in protein expression thereafter at different time points during what may be a recovery phase [88–90]. As a result, there remains the need to examine variations in gene and protein expression that occurs as loading persists. For example, proteomic analysis comparing variations in protein expression following 0.5, 1, 2, or 3 h of loading would identify key pathways that are quickly shut off or become saturated as loading persists. A similar approach involving RNA-sequencing would also enable further studies to identify which genes are shut off as loading persist.

Defining the temporal changes would not only provide a better understanding of how osteocyte sensitivity to loading changes over time, but also identify potential negative feedback loops that limit osteocytes' anabolic function. In doing so, we can begin to develop novel strategies that prolong osteocytes' anabolic response to loading and enable us to capitalize on the anabolic nature of daily activities.

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