HHS Public Access

Author manuscript

Curr Osteoporos Rep. Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

Curr Osteoporos Rep. 2017 August ; 15(4): 326–334. doi:10.1007/s11914-017-0374-z.

Connexins and Pannexins in Bone and Skeletal Muscle

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Abstract

Purpose of review—To discuss the current knowledge on the role of connexins and pannexins in the musculoskeletal system.

Recent findings—Connexins and pannexins are crucial for the development and maintenance of both bone and skeletal muscle. In bone, the existence of connexin and more recently pannexin channels in osteoblasts, osteoclasts, and osteocytes has been described, and shown to be essential for normal skeletal development and bone adaptation. In skeletal muscles, connexins and pannexins play important roles during development and regeneration through coordinated regulation of metabolic functions via cell-to-cell communication. Further, under pathological conditions, altered expression of these proteins can promote muscle atrophy and degeneration by stimulating inflammasome activity.

Summary—In the current review, we highlight the important roles of connexins and pannexins in the development, maintenance, and regeneration of musculoskeletal tissues, with emphasis on the mechanisms by which these molecules mediate chemical (e.g., ATP and PGE_2) and physical (e.g. mechanical stimulation) stimuli that target the musculoskeletal system and their involvement in the pathophysiological changes in both genetic and acquired diseases.

Keywords

gap junctions; hemichannels; connexon; inflammation

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Conflict of Interest

Juan Sáez, Hannah Davis, Lillian Plotkin, and Bruno Cisterna declare no conflict of interest.

Compliance with Ethical Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

Connexins and pannexins are channel forming proteins that share similar topology, although they do not exhibit sequence homology [1]. Both connexins and pannexins comprise 4 transmembrane domains, two extracellular and one intracellular loop with the amino- and carboxi-terminal regions facing the cytoplasm. Connexins form hexamers or connexons in the cell membrane that mediate the exchange of small molecules between the cells and the extracellular compartment, named hemichannels [2]. Hemichannels present in adjacent cells can align to form gap junction channels, allowing the exchange of molecules between neighboring cells [3]. Pannexins also form hexamers in the cell membrane, but most investigators agree they are not able to form functional gap junction channels [4,5]. The existence of connexin channels in osteoblasts, osteoclasts, and osteocytes has long been recognized, and most recently, the presence of pannexin channels has been described in these bone cells [3]. In muscle, connexins are only expressed in undifferentiated precursors and upon injury in regenerating muscles, whereas pannexins have been reported in both precursors and mature muscle cells [6]. In this review, we discuss the current knowledge on the role of connexins and pannexins in the musculoskeletal system.

CONNEXINS AND PANNEXINS IN BONE

Connexins and pannexins in bone development, maintenance, and regeneration

The development and maintenance of bone tissue depends on the coordinated actions of bone forming osteoblasts and bone resorbing osteoclasts. These actions are controlled by osteocytes, differentiated osteoblasts embedded in the bone matrix [7]. Cell-to-cell communication via gap junctions among these cells was first appreciated in morphological studies using electron microscopy [8]. Connexin 43 (Cx43) is the most abundant member of the connexin family of proteins in bone, and it is expressed in osteoclasts, osteoblasts, and osteocytes [9–11]. Deletion of Cx43 in global Cx43 knockout mice leads to delayed mineralization and deficient osteoblast differentiation in the embryos [12–14]. However, mice with global Cx43 deletion die at birth, and therefore the skeletal phenotype of adult mice cannot be investigated [15]. Deletion of Cx43 in osteochondroprogenitors or in early osteoblast progenitors in mice also results in impaired osteoblast differentiation, suggesting a defect inherent to osteoblastic cells [16,17]. These mice exhibit reduced bone mass and osteoblast numbers [18]. On the other hand, mice with deletion of Cx43 in more mature cells of the osteoblastic lineage (such as cells expressing the col1a1 or DMP1 promoter) exhibit minimal, if any, bone defects [19–21]. Interestingly, Cx43$^{fl/fl}$;Col1a1–2.3kb-Cre mice, in which Cx43 is deleted from osteoblast progenitors, mature osteoblasts, and osteocytes exhibit defective muscle development, with reduced weight, grip strength, and tetanic forces [22]. The decrease in muscle mass results in lower whole body weight, a phenotypic characteristic that is absent in mice with deletion of Cx43 in mature osteoblasts and osteocytes, or in osteocytes only [19,21], suggesting that Cx43 expression in osteoblast precursors is required for optimal muscle development.

Osteoblasts and osteocytes also express Cx45 and Cx46, although the function of these connexins in bone cells is not known [23–25]. More recently, the expression of Cx37 was demonstrated in osteoclasts, osteoblasts, and osteocytes [26,27], and its ubiquitous deletion leads to decreased bone mass due to defective osteoclast differentiation, and altered bone matrix composition [27,28].

Less is known regarding the role of connexins in bone regeneration. A study investigated the consequences of Cx43 deletion from osteoblastic cells expressing the human osteocalcin promoter driving the Cre recombinase (OCN-Cre) in a model of closed femur fracture induced by 3-point bending [29]. Cx43 deficient mice exhibited reduced bone formation and resorption at the fracture site, lower mineralization, and abnormal biomechanical properties, indicating that Cx43 expression in osteoblastic cells is required for proper fracture healing. This phenotype contrasts with the one observed in long bones, in which both bone formation and resorption are increased in mice with deletion of Cx43 in OCN-expressing cells [20,21].

Recent studies describe the skeletal consequences of expression of a truncated Cx43 mutant lacking the carboxi-terminus domain $Cx43^{\Delta CT}$ [30]. The first publication reported that female mice expressing one allele of the truncated Cx43 and one floxed Cx43 allele ($Cx43^{\Delta CT/fl}$ mice) exhibit reduced cancellous bone mass due to defective bone formation, without any apparent cortical bone phenotype [31]. Furthermore, the $Cx43^{\Delta CT}$ mutant reversed the cortical bone phenotype and the increased osteocyte apoptosis resulting from deletion of osteocytic Cx43. On the other hand, a study using male mice showed that $cx43^{\Delta ct/-}$mice expressing only the truncated Cx43 exhibit a phenotype similar to that of mice lacking Cx43 in osteoblasts and osteocytes, with increased periosteal diameter, marrow cavity area, and moment of inertia, and no change in cancellous bone volume/tissue volume [32]. These results indicate a sexually dimorphic role of Cx43 in bone. Whether these differences are due to the presence of different sex steroids in males versus females or to other factors remains to be determined.

The role of pannexins in bone has started to be uncovered in recent years [3]. All three members of this family, pannexin (Panx) 1, Panx2 and Panx3 are expressed in osteoblasts, osteocytes and osteoclasts [1]. Panx1 is the most widely distributed pannexin, found in osteoblastic cells among other cells and tissues. Panx3 exhibits a more restricted pattern of expression in the body, but it is also expressed in osteoblastic cells, and its expression increases as osteoblastic cells differentiate [33]. Panx2 was thought to be expressed only in the central nervous system, but was recently found also in osteoblastic cells and is present in extracellular vesicles released by mineralizing osteoblasts [34]. The role of pannexin channels in bone has not been investigated in great detail. Panx1 knockout mice showed no changes in diaphyseal structure when compared to wild type mice, but, unlike wild type mice, did not exhibit increased intracortical resorption when subjected to fatigue loading [35]. However, the bone mass or the cancellous bone architecture of these mice has not been reported. Panx3 knockout mice exhibit shorter long bones with a higher moment of inertia compared to wild type mice, without changes in bone mineral density [36]. Again, the phenotype of cancellous bone was not investigated in these mice.

Connexins and the aging skeleton

In addition to its role in bone development, reduced expression and function of Cx43 has been shown with aging. The increase in gap junction communication induced by parathyroid hormone is reduced in osteoblastic cells isolated from calvaria bones from old rats (12-, and

24–28-month-old) compared to young, 4-month-old animals [37]. This study did not find changes in Cx43 mRNA or protein in these osteoblastic cells or in basal gap junction communication, suggesting an intrinsic defect on the function of Cx43 gap junctions in response to parathyroid hormone. However, Cx43 expression in osteocyte-enriched whole bone measured by qPCR and by western blotting [38] and in osteocytes assessed by immunohistochemistry [39] is decreased in old mice. The discrepancy among these studies suggests that the reduction in Cx43 expression with aging likely occurs in osteocytes, and not in osteoblasts. Consistent with this notion, Cx43 deletion from osteocytes renders a phenotype that resembles that of old animals [40] with increased osteocyte apoptosis and enhanced endocortical resorption and periosteal apposition [19,21]. Furthermore, deletion of Cx43 from osteocytic MLO-Y4, but not from osteoblastic Ob-6, cells leads to spontaneous cell death in culture [38]. The later study showed that Cx43 restrains osteocyte death by maintaining the levels of the microRNA miR21, with the consequent reduction of the expression of the phosphatase and tensin homolog PTEN, resulting in the preservation of the Akt-survival pathway. Osteocyte apoptosis results in the release of pro-osteoclastogenic molecules RANKL and high mobility group box 1 (HMGB1), leading to increased osteoclastic bone resorption in the vicinity of apoptotic osteocytes. Future studies will determine whether by maintaining Cx43 levels high, some of the deleterious effects of aging on the skeleton can be avoided.

Connexin and pannexin gene mutations and bone disease

Mutations of the Cx43 gene (*GJA1*) were found in individuals with oculodentodigital dysplasia (ODDD), a condition associated with craniofacial and limb abnormalities [41]. These mutations do not have homogeneous consequences, and render proteins unable to form channels or with altered channel permeability. The original study described 17 different mutations, and in the subsequent years at least 76 *GJA1* mutations have been linked to ODDD [41–67]. In the early 2000s, a mouse model of ODDD expressing the mutant Cx43G60S allele was generated using N-ethyl-N-nitrosourea mutagenesis [68]. These mice exhibit craniofacial, limb, and dental abnormalities similar to those of humans with ODDD, including thin cortical bone, enlarged marrow cavity, and reduced bone mineral density, cancellous bone volume and mechanical strength. Another mouse model of ODDD was generated by expressing Cx43G138R in osteochondroprogenitors [17]. These mice exhibit smaller skulls, reduced bone mineral density and cortical thinning.

While all the patients with ODDD were found to exhibit mutations in the Cx43 gene, not all Cx43 mutations lead to ODDD. Thus, the Cx43R239Q mutant results in craniometaphyseal dysplasia [69], and Cx43E42K and Cx43S272P have been associated with sudden infant death [70]. In addition, patients homozygous for the recessive mutation Cx43R76H exhibit Hallermann-Streiff syndrome characterized by small stature, beaked nose, skeletal anomalies, and teeth defects, in addition to characteristics of ODDD [61].

More recently, the first patient with a mutation in the Panx1 gene was described [71]. A female expressing the Panx1R217H mutant exhibited kyphosis, among other abnormalities. *In vitro* studies showed that this mutated gene renders a protein with normal subcellular localization and glycosylation, but with defective channel permeability and reduced ability to release ATP.

In summary, both connexins and pannexins expressed in bone cells are essential for the development and adaptation of bone. Studies summarized elsewhere [72–75] demonstrated the role of Cx43 in the response to several bone-targeting stimuli and the intracellular signaling pathways mediated by Cx43 as part of intercellular gap junction channels, hemichannels, or channel-independent functions. Whether pannexins also can mediate the effect of stimuli that alter bone cell number or function remains to be determined.

CONNEXINS AND PANNEXINS IN SKELETAL MUSCLE

Gap junctions and hemichannels in normal skeletal muscles

Undifferentiated myoblasts express Cx43 and Cx45, proteins that form gap junction channels involved in coordinating the differentiation response induced by extracellular ATP [76]. During the late stage of differentiation myotubes transiently express Cx39 that appears to reduce the rate of differentiation [77], but its functional role remains largely unknown. Also, to the best of our knowledge, the possible role of connexin hemichannels in myoblast proliferation and/or differentiation has not been reported. Upon innervation the expression of the three connexins is down-regulated and are not expressed in differentiated myofibers. In adult muscles, satellite cells express Cx43 and Cx45 and upon muscle damage, satellite cells proliferate and form new myofibers in a connexin-dependent manner, as evidenced by the findings that in the absence of these proteins regeneration response is drastically reduced [78,79].

In undifferentiated myoblasts, Panx3 is required for cell proliferation and is down-regulated in differentiating myoblasts [80]. Moreover, undifferentiated myoblasts express a low amount of Panx1, which increases significantly during differentiation reaching maximal levels in fully differentiated cells [80]. Despite the low levels of Panx1 in undifferentiated myoblasts, the pannexin plays a critical role in acquisition of muscle cell commitment [81] and differentiation [80,81]. Further, electrical stimulation of myotubes promotes opening of Panx1 channels causing ATP release and affecting gene expression [82], suggesting that Panx1 might play a relevant role in muscle plasticity.

Fully differentiated myofibers express Panx1 that is localized in T-tubules and form channels through which ATP is released to the extracellular medium [83,84] where it activates P2 receptors, inducing potentiation of the muscle contraction [83]. This response is not detectable in muscles from Panx1$^{-/-}$ mice and it is blocked by Panx1 channel inhibitors [83].

In resting muscles, Panx1 is phosphorylated and repetitive electrical stimulation of myofibers enhances the phosphorylation state of Panx1 in serine and threonine residues [83]. However, the protein kinase that mediates this effect has not yet been identified.

Connexin and pannexin1-based channels in diseases of skeletal muscles

Although the etiology of skeletal muscle diseases can vary significantly (e.g., denervation or mutations in proteins such as dystrophin or dysferlin) they share several features including progressive muscular fatigue, muscle wasting, inflammation, atrophy and muscle dysfunction. With regard to the inflammatory response, recent studies have unveiled the presence of two processes: 1) infiltration of cells of the innate immune system and 2) activation of the inflammasome expressed by myofibers; however, the relative relevance of each process on muscle degeneration and tissue dysfunction has been poorly studied.

Notably, mutated dystrophin in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) or mutated dsyferlin in limb-girdle muscular dystrophy (LGMD) type 2B are frequently absent or its amount is greatly reduced. The latter appears to be the consequence of protein degradation activated by the inflammatory response. For instance, treatments that reduce the progression of the inflammatory response induce reappearance of the mutated protein [85], suggesting that progression of the inflammatory response promotes degradation of the mutated proteins. A common intracellular signal known to activate protein degradation and inflammation is an elevated intracellular free Ca^{2+} concentration and for that reason in the following section of this review we focus in mechanisms that explain the rise in intracellular Ca^{2+} concentration.

Role of connexin *de novo* expression in muscle atrophy induced by denervation or glucocorticoids

It has been long known that denervated muscles undergo atrophy and this response was preceded by an increase in sarcolemma permeability. Recently, it was demonstrated that adult denervated muscles express *de novo* several poorly selective membrane channels, including connexin hemichannels, $P2X_7$ receptors, and TRPV2 channels, and up-regulation of Panx1 channels [86]. Connexin hemichannels were shown to permeabilize the sarcolemma to small molecules including Evans blue [86]. In addition, all these newly expressed channels are permeable to Ca^{2+} as well as to monovalent ions and therefore can drastically affect the electrochemical gradient across the sarcolemma. Interestingly, the absence of just Cx43 and Cx45 expression was sufficient to avoid the ionic imbalance (e.g., increase in intracellular Ca^{2+} and Na^+ signal) induced by denervation [87], suggesting that despite the persistent expression of Cx39, TRPV2 channels, $P2X_7$ receptors, and Panx1 channels, myofibers can handle the ionic imbalance caused by these channels and/or these channels are not fully functional. Furthermore, Cx39 hemichannels are not permeable to Ca^{2+} [88] and therefore they might be less toxic to denervated myofibers than Cx43 and Cx45 hemichannels.

The permeability of Cx43 and Cx45 hemichannels to Ca^{2+} [89,90] explains the activation of the inflammasome, protein degradation via ubiquitin proteasome pathway and atrophy of denervated fast myofibers [86,87]. The latter is strongly supported by the fact that denervated Cx43/Cx45 KO myofibers show a drastic reduction in protein degradation as well as in protein synthesis (reduced negative protein balance) and atrophy is strongly reduced (by ~75%). In contrast, Panx1 KO myofibers show similar atrophy to that of wild type myofibers upon denervation, indicating that upregulation of this channel does not play a critical role in the denervation-induced muscle degeneration.

An unexpected and recent finding was that *de novo* expression of connexin hemichannels explain the glucocorticoid-induced skeletal muscle atrophy [91], a condition frequently observed in patients under chronic treatment with glucocorticoids due to inflammatory conditions. Myofibers deficient in Cx43 and Cx45 expression do not undergo atrophy after chronic treatment with dexamethasone, a synthetic glucocorticoid widely used in long term clinical treatments. Again, the *de novo* expression of connexin hemichannels promotes activation of the inflammasome in myofibers, indicating that glucocorticoids broadly known as anti-inflammatory agents, indeed act as anti-inflammatory compounds on the immune system but are inflammatory on skeletal muscles, which constitute approximately 50% of the body mass. Hence, inflamed muscles release pro-inflammatory cytokines that might affect other organs including bones.

Connexins in muscular dystrophies

Denervated skeletal muscles undergo a connexin-driven inflammation without the involvement of immune cells [86], but muscular dystrophies present both infiltration of immune system cells and activation of the myofibers inflammasome. For instance, mutations in dystrophin in DMD or BMD muscular dystrophy and mutations of dysfernil in LGMDs lead to severe and still incurable symptoms and lead to progressive myofiber apoptosis and/or necrosis ending in muscle dysfunction. All these diseases present inflammatory responses. In fact, the *mdx* mouse, model of DMD and BMD, shows ~30% of differential expression of genes related to inflammation [92,93], underlying the relevance of inflammation in these condition. In agreement with this statement, depletion or reduction of CD4+ or CD8+ T cells [94] or neutrophils [95] in *mdx* mice reduces the severity of the dystropathology. In addition, *mdx* myofibers were recently shown to express *de novo* three connexins (39, 43 and 45) [96]. Accordingly, myofibers of DMD or BMD patients were found to express connexins 45. 43 and 40.1 (ortholog of mouse Cx39) [85], suggesting that these proteins could play a relevant role in these pathological conditions. Interestingly, streptomycin has been found to reduce stretch-induced membrane permeability in *mdx* muscles [97] and is also known to block connexin hemichannels [98].

In vivo, the newly expressed Cx39, Cx43 and Cx45 in *mdx* myofibers form functional hemichannels in the sarcolemma [85]. Since all these membrane channels found in the sarcolemma are poorly selective, the electrochemical gradient is drastically reduced, which can explain the increase in cytoplasmic Ca^{2+} [99] and Na^{+} [100] concentrations. In addition, it is highly possible that the permeability to Ca^{2+} of the aforementioned channels induce Ca^{2+} overload in myofibers of *mdx* mice as well as DMD and BMD patients and promotes cell death. In agreement, *mdx* myofibers deficient in Cx43 and Cx45 do not exhibit high basal cytoplasmic Ca^{2+} signal or cell death by apoptosis and muscle dysfunction is greatly reduced [85].

Notably, the newly expressed poorly selective channels in *mdx* myofibers are accompanied by an increase in the levels of pro-inflammatory cytokines (e.g., IL-1β and TNF-α), inducible nitric oxide synthase (iNOS) and activated NFκB. All these responses are not detectable in myofibers of *mdx* mice deficient in Cx43/Cx45 expression [85], suggesting that early in the pathogenesis of DMD or BMD, the activation of inflammasome occurs and is induced by the action of functional connexin hemichannels.

Mutations in dysferlin, a protein proposed to participate in membrane repair after damage [101], explain the LGMDs. In adult muscles, dysferlin is clearly expressed in myofibers and is mainly localized in the sarcolemma forming part of the transversal tubule membrane system [102]. With regard to inflammation, animal models show up-regulation of the inflammatory proteins Spp1 and S100a9 [103], suggesting that inflammation play a critical role in muscle degeneration. Also, the absence of dysferlin induced the activation of inflammasome in skeletal muscles [104]. In agreement with a local inflammatory response and the role of connexin hemichannels, it was recently demonstrated that connexin hemichannels also participate in LGMDs. In immortalized myotubes derived from patients harboring dysferlin mutations, it was found that connexin hemichannels are still expressed in mature myotubes and are responsible of an increase (~10%) of basal cytoplasmic Ca^{2+} levels, suggesting that these hemichannels could mediate the posterior muscle atrophy and adult myofibers death [96].

A final product of infiltrated inflammatory cells and activation of the inflammasome of myofibers is the generation and release of pro-inflammatory cytokines, which has been shown to promote the expression of connexin hemichannels in freshly isolated myofibers [105]. Therefore, the expression of connexins in normal differentiated muscles is repressed and several extracellular ligands can de-repress their expression. One of these mechanisms seems to be the lack of a neuron-derived factor in denervated myofibers [106]. A second mechanism could be the direct induction in connexin expression, as in the case of glucocorticoids known to induce the expression of Cx43 [91], and a third mechanism could involve the role of pro-inflammatory mediators, as described above. And of course, under certain conditions two or all three mechanisms could act in an orchestrated fashion with a more negative outcome for skeletal muscle functions.

In summary, the mechanism that induces the expression of connexin hemichannels in denervated muscle, under chronic treatment with glucocorticoids and in muscle dystrophies most likely differ. These three conditions share a common denominator, the expression of connexin hemichannels. Moreover, all of them present an increase in sarcolemma permeability to ions and small molecules leading to activation of the inflammasome. These findings also indicate that a great deal of the muscle dysfunction of all the above mentioned conditions is the result of inflammation rather than the cause of the disease. Therefore, connexin hemichannels could be regarded as new molecular targets to reduce the negative outcome of inflammation and might be beneficial to treat diverse muscle pathological conditions.

Conclusions

Studies of the last decade have revealed that connexins and pannexins are fundamental for the development, maintenance and regeneration of both bone and muscle. Moreover, these molecules, either as part of intercellular gap junction channels, as hemichannels or as channel independent signaling molecules, mediate the effect of stimuli that target the musculoskeletal system and are involved in the pathophysiological changes in both genetic and acquired diseases. The continuous advancements in this field will allow for the development of new strategies that might target the musculoskeletal system to improve bone and skeletal muscle health.

Acknowledgments

This work was supported by the National Institutes of Health R01-AR067210 and R01-AR053643 (to LIP) and T32-AR065971 (to HMD), U.S.A., and by the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) grant 1150291 (to JCS) and ICM-Economía P09-022-F Centro Interdisciplinario de Neurociencias de Valparaíso (to JCS), Chile.

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