

Role of Connexin43 in Osteoblast Response to Physical Load

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ABSTRACT: Gap junctions are hexameric transmembrane channels formed by connexins, and are responsible for direct cell-to-cell communication. The most abundant gap junction protein in bone is connexin43 (Cx43), although connexin45 (Cx45) is also expressed. In the present study, we tested the hypothesis that bone cell responses to mechanical stimulation are dependent on the type of gap junction communication provided by Cx43 *in vitro* and in an *in vivo* model of physical load. Application of cyclic stretch to calvaria osteoblasts results in a modest but detectable increase in PGE₂ levels, and the amount of PGE₂ produced was lower in cells isolated from Cx43 null mice. Mice with an osteoblast-specific deletion of the *Cx43* gene were subjected to an *in vivo* four-point bending protocol on the tibia. This resulted in fast and exuberant formation of woven bone at the region directly below the loading fulcrum in both osteoblast *Cx43*-deleted and wild-type mice. However, indirect measurement of endosteal bone apposition suggested a less pronounced effect of physical load in *Cx43*-deficient than in wild-type mice. Taken together, these results indicate that deficiency of *Cx43* in osteoblasts attenuates but does not abolish anabolic responses to mechanical strain.

KEYWORDS: gap junctions; connexin43; mechanical strain; bone formation

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INTRODUCTION

The mechanisms underlying bone's response to mechanical stimulation are only partially understood. It is generally believed that the osteocyte is the mechanosensory cell and that it transmits signals to bone-forming cells in the surface via gap junctions, to initiate an anabolic response. Gap junctions consist of intercellular channels that serve to connect adjacent cells and allow for the diffusion of small molecules and ions from cell to cell.^{1,2} Each cell contributes half of the intercellular channel (hemichannel), which is composed of six subunits, connexins, arranged in a hexagonal array to form the hemichannel, also called connexon. More than two dozen connexins exist, but the most abundant in bone is connexin43 (Cx43), although osteoblasts also express connexin45 (Cx45).

A variety of approaches have been used to understand how mechanical signals are received and transduced into biochemical responses at the cell surface, and in particular how mechanically generated signals are elaborated and diffused among bone cells. Both cell stretching by substrate deformation and shear stress induced by fluid flow have been applied to cell models of osteoblasts and osteocytes.³⁻¹⁰ *In vivo* loading of bone itself has also been used,¹¹⁻¹³ and although none of these models exactly replicate the stress and strain that bone receives *in vivo* during daily living activities, they do result in osteoblastic responses that can be considered to reflect an anabolic response. In particular, release of prostaglandin E₂ (PGE₂) has been frequently reported following application of fluid flow shear stress.¹⁴⁻¹⁶ PGE₂ has many functions and it can stimulate bone mass in mammals, when used at the appropriate doses. It has also been shown to be involved in mediating new bone formation in response to mechanical load *in vivo*.^{17,18}

New bone formation requires the coordinated activity of osteoblasts at a bone formation site. This coordination can be achieved through direct communication among cells provided by gap junctions, which are also believed to mediate direct osteoblast-osteocyte signaling.^{19,20} Because of the unique microstructural features of osteocytes and their intimate relationship with their microenvironment, it is very difficult to model osteoblast-osteocyte relationships *in vitro*. A more powerful approach is to genetically modify animals to delete one connexin gene specifically in bone cells, and then test whether response to skeletal load is altered in these conditionally connexin-deleted animals. We have recently developed such a mouse model of osteoblast-specific ablation of the Cx43 gene using the Cre/loxP approach.²¹ Because in this model, Cre-induced recombination and gene deletion is driven by a fragment of the $\alpha_1(I)$ collagen promoter, gene ablation occurs in differentiated osteoblasts and thus, osteocytes will be deleted as well.

The goal of the present work was to assess the applicability of one methodology to mechanically load the skeleton in the conditionally Cx43 ablated mice, and begin testing whether this stimulus is dependent on the presence of Cx43

in osteoblasts. We also used Cx43-deficient cells to assess their sensitivity to mechanical stretch *in vitro* in terms of PGE₂ production.

METHODS

Animals

A mouse line with a germline null mutation of Cx43 was used for calvaria cell isolation, as described.²² For the *in vivo* studies, we used a tissue-specific Cx43 gene deletion using Cre-mediated replacement of a “floxed” Cx43 allele (*Cx43^{fl}*) with a *LacZ* reporter cassette.²¹ Homozygous *Cx43^{fl/fl}* mice were crossed with mice expressing Cre driven by a 2.3 kb-fragment of the $\alpha_1(I)$ collagen promoter (abbreviated as *ColCre*) in a heterozygous Cx43 null background, yielding *ColCre;Cx43^{-fl}* mice. Effectiveness of *in vivo* recombination event was demonstrated in osteoblasts by X-gal staining of tibia sections and calvaria cell cultures, and by detection of the deleted allele in osteoblastic cell extracts.²¹

Cell Stretching Studies

Cells from newborn mice calvaria were harvested as described,²² and used for the *in vitro* studies. The Flexercell Strain unit (Flexcell Corporation, McKeesport, PA) was used for application of mechanical stretch to osteoblasts, as detailed in a previous study.⁶ Cells were grown on specially designed 6-well tissue culture dishes with collagen-coated flexible silicone bottoms (Flex I, Flexcell Corporation). Through an air pump, a negative pressure of 22 kPa was applied to the flexible bottomed wells at three cycles/min. During each cycle, a 10-s stretch period was followed by a 10-s relaxation. As the flexible bottoms are pulled downward by the negative pressure, the cells attached to their upper surface are stretched by the deformation of the rubber. Control cultures were grown under the same conditions on dishes of the same size containing a collagen-coated silicone disc identical to the one used for stretch, sitting on a rigid plastic bottom (Flex II, Flexcell Corporation). We optimized this system using two cell lines—ROS 17/2.8 (a rat osteosarcoma cell line) and ROS/Cx45, which stably express the gap junction protein Cx45.²³ Experiments with newborn calvaria cells were performed by applying stretch at a rate of three 10-s cycles/min for different lengths of time. An ELISA assay was used to detect PGE₂ levels in the medium (Cayman Chemical Company Ann Arbor, MI).

In Vivo Studies

To apply mechanical stimulation *in vivo*, a method based on a four-point bending loading paradigm established for mouse tibia was used.²⁴ For these

experiments, 18 mice were used, 6 of each of three genotype, *Cx43*^{+/*fl*} (wild-type equivalent), *Cx43*^{-/*fl*} (heterozygous equivalent), and *ColCre*;*Cx43*^{-/*fl*} (conditional knockout). The right tibia of each mouse was subjected to a bending force of 17N (50% of the breaking point of the bones, calculated experimentally for each genotypes in a pilot study) for 200 cycles, with each cycle comprising 1 s of force application followed by a 10-s rest.^{25,26} This regimen was carried out 5 days per week for 2 weeks. Calcein (5-mg calcein/mL solution at a dose of body weight x 4 μ L) was injected on days 7 and 2 before sacrifice, and the right (loaded) and left (unloaded) tibia used for microstructural and histologic analysis.

Micro computed tomography (μ CT 40, Scanco Medical AG, Basserdorf, Switzerland) was used for three-dimensional reconstruction of the tibial diaphysis and to calculate the internal marrow area, with the assumption that the area is inversely proportional to new endosteal bone apposition. Histomorphometry was used to calculate labeled surface and mineral apposition rate, as described in detail elsewhere.²⁷

RESULTS

In Vitro Studies

Release of PGE₂ has been found to occur rapidly after cell exposure to mechanical stimuli.¹⁴⁻¹⁶ In pilot studies, we tested the medium from cultures of rat osteosarcoma cells (ROS 17/2.8) and cells stably transfected with *Cx45* during and after cyclic stretch (10-s cycles of 1–2% deformation applied for 1–4 h). We could not detect significant PGE₂ release by these cells, either in resting conditions or during stretch (data not shown). Further investigation revealed that these cells did not express inducible *COX-2* (the enzyme isoform that produces PGE₂ upon stimulation), and were thus deemed an inappropriate model to test our hypothesis.

Therefore, we moved to calvaria cells isolated from wild-type and *Cx43*-deficient mice for these studies. We first verified that cell isolates from *Cx43*-deficient mice did not express *Cx43*-specific immunostain, whereas those obtained from wild-type animals did express abundant *Cx43*-specific stain at appositional membranes (a location suggestive of functional channels), as well as in perinuclear areas (FIG. 1). More to the point, there was a tendency toward a minimal increase in PGE₂ production over time with mechanical stretch. Nonetheless, lower amounts of PGE₂ were produced in the *Cx43* knockout cells compared to the wild type (FIG. 2).

In Vivo Studies

The most striking result from the μ CT analysis was an exuberant subperiosteal formation of woven bone in the areas directly underneath the

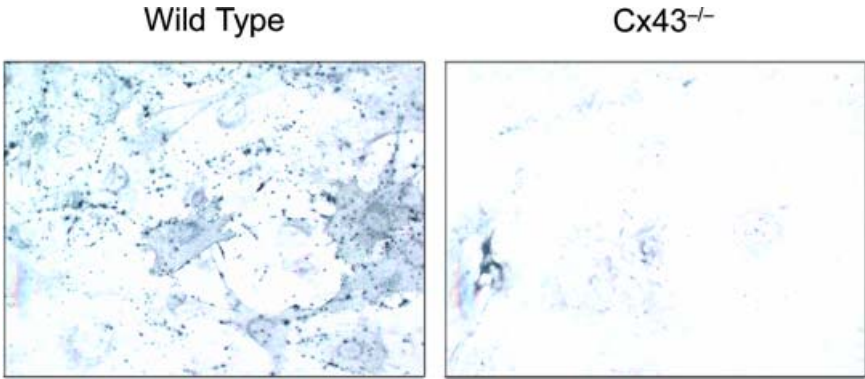


FIGURE 1. Immunohistochemistry of calvaria cells isolated from either wild type or Cx43 null mice. Cells were grown to confluence, fixed with formalin, and immunostained for Cx43. Punctate peroxidase stain was visible only in the wild-type cells, primarily at appositional membranes and in cytoplasmic areas.

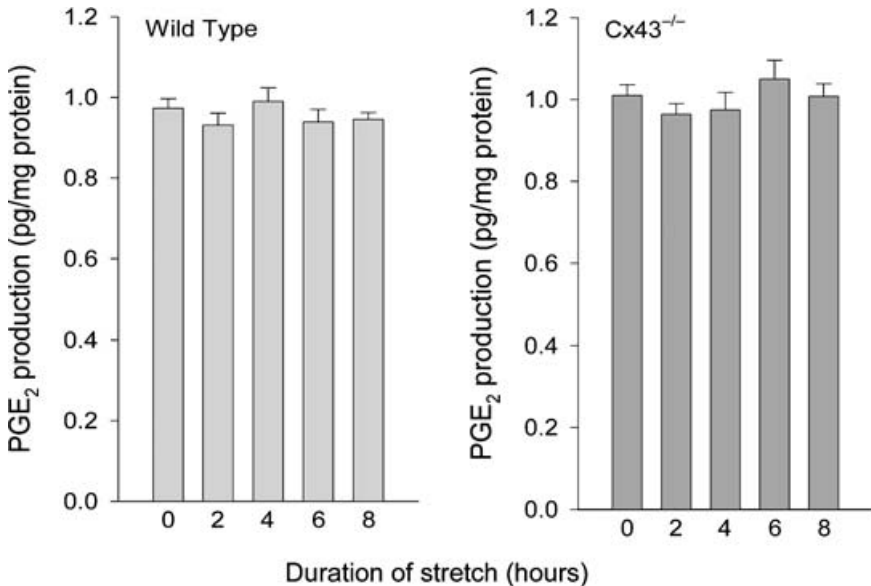


FIGURE 2. Mechanical stretch was applied to primary calvaria cells obtained from wild-type (WT) and Cx43 knockout (KO) mice over a total time course of 8 h with mechanical stretch being applied for durations of 0, 2, 4, 6, and 8 h. Conditioned medium was analyzed for PGE₂ concentration and total protein content, to control for cell density. In this experiment, there was a minimal change of PGE₂ over time.

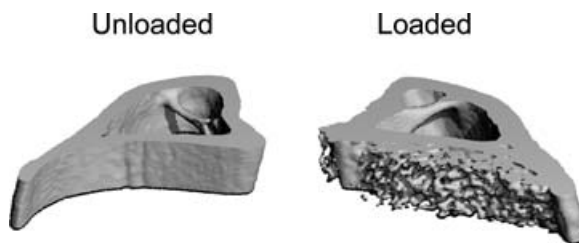


FIGURE 3. Three-dimensional reconstruction of μ CT images of mice tibiae. Tibiae of wild-type mice were subjected to a four-point bending regimen for 16 days under anesthesia, before euthanasia. Note the exuberant subperiosteal bone formation in loaded as compared to unloaded bones.

bending fulcrum in the loaded tibia that was evident in animals of all genotypes (FIG. 3). The degree and extension of this rapid new bone formation may reflect a direct effect of mechanical pressure rather than a reaction to loading, which would be expected to occur primarily on the endosteal surface. Therefore, two types of analyses were performed to determine whether endosteal bone formation was also affected. The first measurement was based on histomorphometry. Qualitative analysis of fluorescent calcein labeling showed higher single and double labeling in the $Cx43^{+/fl}$ relative to the other genotypes (FIG. 4). However, quantitative histomorphometry did not reveal significant differences in labeled surfaces or mineral apposition rates, probably because of the high variability of these results (not shown).

We then measured the area of the medullary space in a zone not directly below the pressure points of the loading device. Assuming that the bone would not expand within the short testing period, this area should be inversely proportional to endosteal new bone apposition. While no differences were detected between loaded and unloaded $Cx43^{-/fl}$ and $ColCre;Cx43^{-/fl}$ tibiae, cross-sectional medullary area was about 5% lower in $Cx43^{+/fl}$ bones relative to the other genotypes, although the difference was not statistically significant (FIG. 5).

DISCUSSION

The underlying hypothesis of this exploratory study was to assess two different approaches to determine whether the anabolic response of the skeleton to mechanical loading is dependent on Cx43. The development of an osteoblast-specific $Cx43$ conditional knockout mouse now allows testing this hypothesis *in vivo*. This is a much more powerful model for assessing the sensitivity of responses to stimulators, such as mechanical load compared to chemical inhibition of gap junctional communication. A recent, very pertinent example of this premise has been provided by the finding of a normal skeletal response

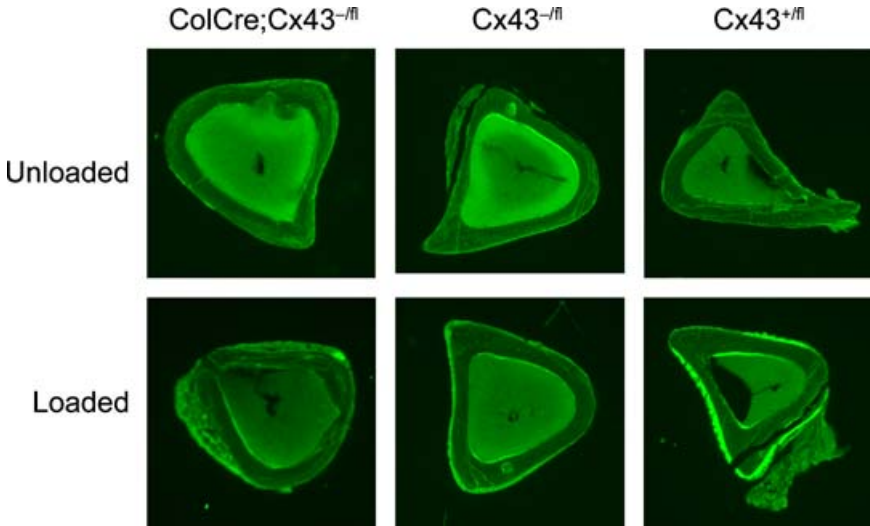


FIGURE 4. Tibiae were subjected to a four-point bending regimen for 16 days under anesthesia. Mice were given double tetracycline labeling injections. Bones were plastic embedded and observed under fluorescent microscopy. Note the exuberant subperiosteal labeling and the more intense fluorescence signal in loaded $Cx43^{+/+}$ bones as compared to the other genotypes and unloaded bones.

to mechanical load in mice genetically deficient of the *COX-2* gene,²⁸ despite previous *in vitro* evidence of the contrary.¹⁴ One caveat here is that animals with germline inactivating mutations may develop compensatory mechanisms if they tolerate the gene defect and survive, whereas short-term inhibition of a gene function may not allow the time for such a compensatory mechanism to develop. We designed this study based on the assumption that long-term responsiveness of bone cells to mechanical stimulation, *in vitro* and *in vivo*, was more biologically relevant than short-term responsiveness.

In the *in vitro* experiments, we used cells harvested from *Cx43* null mice to assess the role of this connexin in biochemical responsiveness of osteoblasts to the application of cyclic stretch. Although *Cx43*-deficient cells did release PGE_2 , the amount released was lower than in wild-type cells, and the increased PGE_2 secretion in response to mechanical stretch was lower than that observed for the wild-type cells. The conclusion of these cell biology experiments is that application of cyclic stretch *in vitro* results in minimal changes in PGE_2 production and this is only marginally affected by the presence of *Cx43* in these cells. The finding of minimal changes in PGE_2 as a result of cell stretching would appear to be in contrast to the findings of previous studies looking at the response of osteoblasts to fluid flow stress, where significant increases in PGE_2 were demonstrated.¹⁴⁻¹⁶ However, cell stretching causes a different type

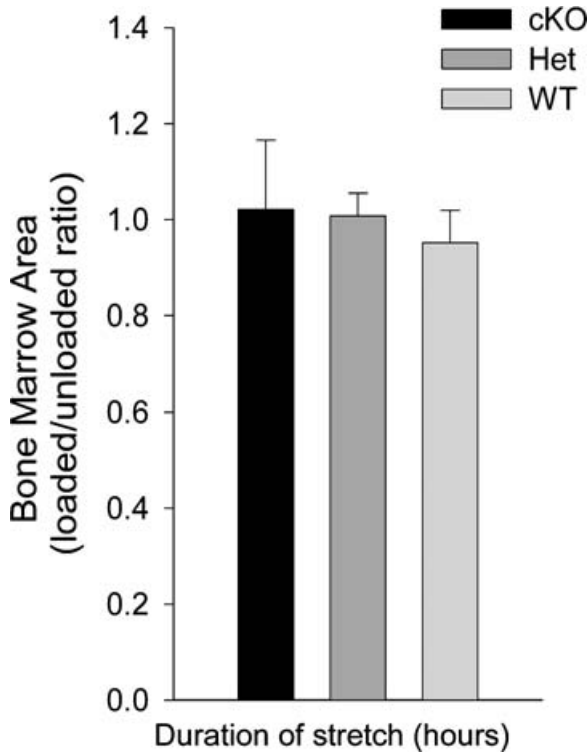


FIGURE 5. Effect of *in vivo* loading on the cross-sectional area of medullary space. μ CT sections of mouse tibiae were obtained after 16 days of *in vivo* loading, and expressed as the ratio of loaded versus unloaded limb in each animal. The bars represent the average \pm 95% confidence interval of three genotypes, *ColCre;Cx43^{-/-}* (cKO); *Cx43^{-/+}* (Het); and *Cx43^{+/+}* (WT). Note the slight decline of the ratio in “wild-type” equivalent mice, indicative of increased new endosteal bone apposition.

of mechanical stimulation than does fluid flow shear stress that may not involve prostaglandin release. For example, cell stretching by substrate deformation induces a reorganization of the cytoskeleton and opening of stretch-activated ion channels.²⁹ Thus, different physical stimuli may differentially affect cellular response.

The most important aspect of our study is the application of a four-point bending model to conditionally *Cx43*-deleted mice. The remarkable and extensive subperiosteal formation of woven bone was certainly not expected to the extent that it was observed. Because this occurred in the area directly below the loading fulcrum of the tibia, it is very likely that this may represent a response to direct pressure from the loading fulcrum on the bone itself. Previous studies using four-point bending methods have used much lower loads than the 17N used in this study.²⁴ Earlier studies in Sprague–Dawley rats used loads

corresponding to 37% of the load necessary to break the tibia.³⁰ In our study, we used a load that was 50% of that required to break the bone, as an initial effort to stimulate a clear response. It is possible that the force applied was in excess of what was required to generate an adaptive response on the periosteal surface, generating instead a reactive response to the pressure of the bending fulcrum. This appears to be a limitation of four-point tibial bending in mice, as others have observed the robust periosteal response.²⁴ In any case, these results emphasize a limitation of the four-point bending model for applying mechanical load to the mouse tibia *in vivo*. Consequently, either lower force or a different approach should be considered.

Although calcein labeling did not reveal significant differences in labeled surfaces or mineralization rates, the finding of slightly larger marrow area in conditionally *Cx43* null mice relative to the other genotypes may reflect a slightly attenuated new endosteal bone apposition after mechanical load in the *Cx43* deficient animals. Obviously, this was a small pilot study and no definitive conclusions can be drawn. Nonetheless, the results are encouraging for a potential involvement of *Cx43* in response to load and a full-scale study is ongoing, using a different model for application of mechanical load.

Taken together these results indicate that lack of osteoblast *Cx43* may attenuate but does not abolish the anabolic skeletal response to mechanical load *in vitro* or *in vivo*.

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