Distinct Pathways Regulate Expression of Cardiac Electrical and Mechanical Junction Proteins in Response to Stretch

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Abstract—To define mechanisms regulating expression of cell–cell junction proteins, we have developed an in vitro system in which neonatal rat ventricular myocytes were subjected to pulsatile stretch. Previously, we showed that expression of the gap junction protein, connexin (Cx) 43, is increased by ~2-fold after 1 hour of stretch, and this response is mediated by stretch-induced secretion of vascular endothelial growth factor (VEGF). Here, we report that the mechanical junction proteins plakoglobin, desmoplakin, and N-cadherin are also upregulated by pulsatile stretch but by a mechanism independent of VEGF or other secreted chemical signals. Stretch-induced upregulation of mechanical junction proteins was blocked by anti–β1 and anti–β3 integrin antibodies. Transfection of cells with adenovirus expressing GFP-FRNK, a dominant-negative inhibitor of focal adhesion kinase (FAK)-dependent signaling, blocked stretch-induced upregulation of Cx43 and mechanical junction proteins but did not block the ability of exogenous VEGF to upregulate Cx43 expression. Conditioned medium removed from uninfected cells after stretch increased Cx43 expression when added to nonstretched cells, and this effect was blocked by anti–VEGF antibodies, but stretch-conditioned medium from GFP-FRNK cells had no effect on Cx43 expression. The src kinase inhibitor 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine blocked stretch-induced upregulation of mechanical junction proteins but not Cx43. Thus, stretch upregulates expression of both electrical and mechanical junction proteins via integrin-dependent activation of FAK. Stretch-induced upregulation of Cx43 expression is mediated by FAK-dependent secretion of VEGF. In contrast, stretch-induced upregulation of adhesion junction proteins involves intracellular mechanotransduction pathways initiated via integrin signaling and acting downstream of src kinase. (Circ Res. 2005;97:346-353.)

Key Words: cell culture ■ pulsatile stretch ■ cell–cell junction proteins ■ mechanotransduction

Cardiomyopathies caused by mutations in plakoglobin or desmoplakin, intercalated disk proteins that link adhesion molecules to the cytoskeleton, are associated with gap-junction remodeling and arrhythmias. These observations are consistent with the hypothesis that normal electrical coupling of cardiac myocytes depends on normal mechanical coupling and that any defect in the overall integrity of cell–cell adhesion junctions or the linkage between intercellular junctions and the cytoskeleton will diminish the stability of gap-junction channel arrays and reduce electrical coupling in cardiac myocytes. To elucidate mechanisms regulating expression of the major ventricular gap-junction protein, connexin (Cx) 43, we developed an in vitro system in which monolayers of neonatal rat ventricular myocytes grown on silicone membranes are subjected to pulsatile stretch. Previously, we reported that after 1 hour of stretch, expression of Cx43 is increased by >2-fold, and this is associated with formation of increased numbers of gap junctions and enhanced impulse propagation velocity. We also showed that stretch-induced upregulation of Cx43 is mediated by an autocrine action of vascular endothelial growth factor (VEGF), which is secreted during stretch.

In the present study, we characterized effects of stretch on expression of mechanical junction proteins including desmoplakin and plakoglobin, linker proteins implicated in the pathogenesis of human cardiomyopathies associated with a high risk of sudden death, and N-cadherin, the major adhesion molecule in adherens junctions of intercalated disks. We asked whether imposition of mechanical load upregulates expression of mechanical junction protein and, if so, whether similar mechanisms were responsible for stretch-induced upregulation of electrical and mechanical junction proteins. We found that brief intervals of pulsatile stretch caused marked upregulation of mechanical junction protein expression associated with formation of more numerous and larger adhesion junctions. Stretch-induced upregulation of both electrical and mechanical junction proteins was initiated by outside-in integrin signaling and activation of focal adhesion kinase (FAK), but, thereafter, divergent downstream signaling pathways were responsible for enhancing Cx43 and...
mechanical junction protein expression. Stretch-induced secretion of VEGF was responsible for upregulating Cx43 expression. In contrast, upregulation of mechanical junction proteins was mediated by intracellular mechanotransduction pathways downstream of src kinase. These results indicate that expression of electrical and mechanical junction proteins in response to load in cardiac myocytes is regulated by distinct signaling pathways.

Materials and Methods

Primary Cultures of Neonatal Rat Ventricular Myocytes

Primary cardiac myocyte cultures were prepared from ventricles of 1-day-old Wistar rat pups (Charles River, Indianapolis, Ind) as described previously. Cells were plated on collagen-coated silicone membranes at a density of 2.4×10^5 cells/cm^2 and grown for 4 days before experimentation. All protocols were approved by the Washington University Animal Studies Committee.

Stretch Protocols and Reagents

Monolayers of ventricular myocytes were subjected to linear pulsatile stretch using a custom-designed apparatus as described in detail in previous studies. Cells were stretched to 110% of resting length at a frequency of 3 Hz for 1 hour in serum-free medium (M-199). In selected experiments, stretch-conditioned medium obtained from selected experiments, the number and size of gap junctions, and the results of the quantitative analysis of intercellular junction protein expression. These anti–integrin antibodies and the anti-VEGF antibody have been shown in previous studies to block integrin or VEGF signaling, respectively. In all experiments, control cultures that were neither stretched nor exposed to exogenous VEGF or other reagents were also incubated in serum-free medium for 1 hour. At the conclusion of each experimental protocol, cells were fixed for confocal immunofluorescence analysis of intercellular junction protein expression.

Quantitative Confocal Immunofluorescence

Analysis of Cell–Cell Junction Protein Expression

Cultures were rinsed in serum-free medium and then fixed in 4% paraformaldehyde. Cells were immunostained with a monoclonal mouse anti-plakoglobin antibody (clone 15F11; Sigma) and polyclonal rabbit anti-desmoplakin antibody (product no. AHP320; SeroTec), anti–N-cadherin (product no. C678; Sigma), and anti-Cx43 (product no. 71-0700; Zymed) antibodies. Specific immunoreactive signal was quantified by laser scanning confocal microscopy as described previously. The amount of each protein signal present in cultures previously stretched for 1 hour in the presence or absence of specific inhibitors was collected and applied to other cultures that had not been stretched. In other experiments, cultures were incubated with 1 or more reagents including recombinant human VEGF (clone 2C9G2; BD Pharmingen) (50 ng/mL), monoclonal blocking antibodies against β1-integrins (clone HA2/5; BP Pharmingen) or β2-integrins (clone 2C9G2; BD Pharmingen) (50 μg/mL for each); or a monoclonal blocking antibody against VEGF (clone 26503; R&D Systems). These anti–β integrin antibodies and the anti-VEGF antibody have been shown in previous studies to block integrin or VEGF signaling, respectively. In all experiments, control cultures that were neither stretched nor exposed to exogenous VEGF or other reagents were also incubated in serum-free medium for 1 hour. At the conclusion of each experimental protocol, cells were fixed for confocal immunofluorescence analysis of intercellular junction protein expression.

Adenoviral Transfection

A replication-defective adenovirus encoding a GFP-FRNK fusion protein has been shown in previous studies to act in a dominant-negative manner to inhibit FAK and block its downstream effects. GFP-FRNK was engineered by cloning chick FRNK cDNA into the EcoRI site of pEGFP-C1 vector. The GFP-FRNK insert was then subcloned into pShuttle-CMV plasmid (Promega) by in vitro blunt ligation. The p Shuttle-CMV expression cassette containing GFP-FRNK was excised by restriction enzyme digestion, subcloned into pAdeno-X viral DNA (Clontech), linearized according to the instructions of the manufacturer, and introduced into HEK293 cells using a liposome-based transient transfection procedure (SuperFect, Qiagen). GFP-FRNK adenovirus was isolated from cell extracts and purified by CsCl gradient centrifugation. The multiplicity of viral infection was determined by viral dilution assays in HEK293 cells grown in 96-well clusters. Cultures of cardiac myocytes were transfected with GFP-FRNK 18 hours after initially being plated. They were rinsed twice in HBSS and infected with the replication-defective adenovirus diluted in M-199 for 60 minutes at 25°C. Thereafter, cells were rinsed and cultured for an additional 72 hours before being subjected to experimental protocols. An adenovirus expressing GFP alone was used to control for nonspecific effects of adenoviral infection.

Electron Microscopy

Stretched and control (nonstretched) neonatal rat ventricular myocytes on silicone membranes were fixed and processed for electron microscopy while remaining on the membranes using methods described previously. Once cells had been infiltrated with plastic resin and the resin had been polymerized, the membranes were peeled away, leaving the cells embedded within the resin. Ultra-thin sections cut in a plane parallel to the plane of the membrane were examined with a JEOL 100SX transmission electron microscope.

Statistical Analysis

All values are expressed as mean±SD. In each confocal microscopy experiment, 3 to 5 randomly selected fields from each individual culture were analyzed to yield a single mean value for each culture. Values for n in each experiment represent the number of individual cultures analyzed. Data were analyzed by 1-way ANOVA with Fischer’s least significance difference test. A probability value of <0.05 was considered significant.

Results

Stretch Increases Expression of Both Electrical and Mechanical Junction Proteins

Neonatal rat ventricular myocytes were subjected to 1 hour of pulsatile stretch and expression of Cx43, and the mechanical junction proteins plakoglobin, desmoplakin, and N-cadherin were analyzed by quantitative confocal microscopy. As shown in Figure 1A and 1B, the amount of immunoreactive signal for each protein was significantly increased at points of cell–cell junctions, as measured by confocal microscopy. Analysis of confocal images showed that stretch increased both the number and size of structures containing plakoglobin and desmoplakin (Table). Thus, stretch not only increased the total amount of mechanical junction protein expressed in the cells but also resulted in more numerous and larger cell–cell adhesion junctions. To further evaluate the effects of stretch on the structure of intercellular adhesion junctions, cells were examined by electron microscopy. Ultrastructural examination of stretched cells revealed marked thickening and enhanced electron density of cell–cell mechanical junctions and an apparent increase in the density of adjacent cytoskeletal filaments (Figure 2). Taken together, these results indicate...
that expression of mechanical junction proteins is dramatically enhanced after only 1 hour of pulsatile stretch, and this is associated with more numerous, larger, and more-mature-appearing adhesion junctions.

**Chemical Signals Secreted in Response to Stretch Uregulate Cx43 Expression but Not Mechanical Junction Protein Expression**

Previously, we showed that VEGF is secreted in response to stretch and acts to upregulate Cx43 expression. To determine whether VEGF plays a role in stretch-induced upregulation of mechanical junction proteins, we incubated cells with exogenous VEGF or stretched them in the presence of anti-VEGF antibodies. As shown in Figure 3, exogenous VEGF upregulated Cx43 expression in nonstretched cells, and stretch-induced upregulation of Cx43 was blocked by anti-VEGF antibodies, as previously reported. In contrast, exogenous VEGF had no effect on expression of mechanical junction proteins, and stretch-induced upregulation of mechanical junction proteins was not blocked by the presence of anti-VEGF antibodies (Figure 3).

To determine whether other chemical signals secreted during stretch might be involved, we performed conditioned-medium experiments. Medium was removed from cells which had been stretched for 1 hour and applied to cultures of nonstretched cells for 1 hour. As demonstrated previously, incubation of cells with stretch-conditioned medium for 1 hour led to a significant increase in Cx43 expression but stretch-conditioned medium had no effect on expression levels of mechanical junction proteins (Figure 4). Taken together, these results indicate that VEGF secreted during stretch is responsible for stretch-induced upregulation of Cx43 expression, but secreted chemicals do not play a role in stretch-induced upregulation of mechanical junction protein expression.
Mechanotransduction Is Initiated by Integrin Signaling and Requires Activation of FAK

Previously, we demonstrated that stretch-induced upregulation of Cx43 expression is blocked by pretreating cells with anti-β1 integrin antibodies. To determine whether outside-in signaling initiated by integrins is responsible for stretch-induced upregulation of mechanical junction proteins, cells were pretreated with anti-β1 and/or anti-β3 integrin blocking antibodies and subjected to pulsatile stretch. As shown in Figure 5, both anti-β1 and anti-β3 integrin antibodies blocked stretch-induced upregulation of mechanical junction proteins. These results indicate that integrins play a critical role as mechanotransducers in stretch-induced upregulation of both electrical and mechanical junction proteins.

Outside-in integrin signaling leads to phosphorylation of FAK, which, in turn, activates multiple intracellular signaling pathways. To investigate the role of FAK in stretch-induced upregulation of cell–cell junction proteins, we infected cultured myocytes with an adenovirus expressing GFP-FRNK, a dominant-negative inhibitor of FAK-dependent signaling, and subjected the myocytes to pulsatile stretch. Examination of cells by fluorescence microscopy to detect expression of GFP indicated that virtually all cells had been infected by virus. As shown in Figure 6A, GFP-FRNK had no effect on basal levels of expression of cell–cell junction proteins. However, GFP-FRNK completely blocked stretch-induced upregulation of Cx43 and the mechanical junction proteins, whereas infection of cells with an adenovirus expressing GFP alone had no effect.

To determine whether inhibition of FAK signaling prevented stretch-induced secretion of VEGF, control cells (neither stretched nor infected with adenovirus) were incubated for 1 hour in conditioned medium obtained from GFP-FRNK–infected cells that had been stretched for 1 hour. The stretch-conditioned medium from GFP-FRNK–infected cells had no effect on Cx43 expression, indicating that secretion of chemical signals during stretch was blocked (Figure 6B). To determine whether GFP-FRNK–infected cells remained sensitive to the effects of VEGF, infected cells were incubated with exogenous VEGF for 1 hour. As shown in Figure 6B, Cx43 expression was increased to roughly the same extent as that in uninfected cells subjected to stretch. Taken together, these results indicate that stretch-induced upregulation of electrical and mechanical junction proteins is mediated by FAK. Inhibition of FAK signaling prevents stretch-induced VEGF secretion but does not block the ability of exogenous VEGF to upregulate Cx43.

Src Kinase Pathways Regulate Expression of Mechanical Junction Proteins

To elucidate pathways downstream from FAK that regulate expression of mechanical junction proteins, we incubated...
cells with the specific src kinase inhibitor PP2. As shown in Figure 7A, PP2 blocked stretch-induced upregulation of mechanical junction protein expression. In contrast, PP2 did not block stretch-induced upregulation Cx43 expression, nor did it prevent upregulation of Cx43 by VEGF or inhibit stretch-induced secretion of chemical signals that upregulated Cx43 when conditioned medium from cells stretched in the presence of PP2 was applied to control cells (Figure 7B).

Discussion
The results of this study show that cardiac myocytes respond to brief intervals of pulsatile stretch by upregulating expression of cell–cell junction proteins and forming more mechanical junctions. Mechanical load leads to enhanced expression of cell–cell junction proteins via outside-in integrin signaling mediated by activation of FAK. However, distinctly different signaling pathways downstream of FAK are responsible for...
upregulating Cx43, the principle gap junction protein of ventricular myocytes and plakoglobin, desmoplakin, and N-cadherin, proteins that form desmosomes and adherens junctions responsible for mechanically linking cardiac myocytes. As previously shown, Cx43 is upregulated by VEGF, which is secreted by cells in response to stretch and acts in an autocrine fashion to increase Cx43 expression and promote formation of more gap junctions. Here, we show that VEGF secretion is dependent on FAK activation, but inhibition of FAK signaling does not affect upregulation of Cx43 expression induced by exogenous VEGF. In contrast, upregulation of mechanical junction protein expression is not mediated by VEGF or any other chemical signaling molecules secreted into the culture medium during stretch. Instead, intracellular-signaling pathways downstream of FAK and involving src kinase are responsible. Previously, we showed that pulsatile stretch for 1 hour increased total Cx43 content as well as Cx43 immunoreactive signal at gap junctions, thus implicating increased synthesis and/or decreased degradation of Cx43. It is not known whether the increased immunoreactive signals for plakoglobin, desmoplakin, and N-cadherin at cell–cell junctions following stretch reflect a similar increase in protein content or, rather, mobilization of intracellular protein and its assembly into new junctions. In either case, however, our results show that cardiac myocytes respond to stretch by assembling more gap junctions and adhesion junctions, but expression of electrical and adhesion junctions proteins and their assembly into junctional organelles is regulated by disparate pathways.

Previous studies have focused on the effects of mechanical stretch on cell–cell junction protein expression. For example, Cowan et al showed that both stretch and fluid shear stress increased expression of Cx43 in cultured vascular smooth muscle cells. Wang et al applied cyclical mechanical stress (20%) to cultured neonatal rat cardiac myocytes and observed increased expression of Cx43 protein and mRNA. And recently, Matsuda et al showed that neonatal cardiac myocytes became aligned in the direction of stretch when stretch was applied early after cell seeding but not at later time points. However, transfection of cells with a dominant-negative N-cadherin restored the ability of cells to align in the direction of stretch at later times after seeding, thus implicating N-cadherin as a determinant of cellular plasticity in response to stretch.

Figure 6. A, Effects of stretch on expression of Cx43 and mechanical junction proteins in cells infected with virus expressing GFP-FRNK (FRNK) or GFP alone (Adv-GFP). n=6 for Cx43 and N-cadherin; n=2 or 3 for plakoglobin and desmoplakin. *P<0.001, †P<0.05 compared with control in each group. B, Effects of stretch-conditioned medium (CM) and exogenous VEGF in cells previously infected with adenovirus expressing GFP-FRNK (FRNK). n=4 for each condition. *P<0.001.
Our results suggest that a major mechanism underlying stretch-induced upregulation of cell–cell junction protein expression involves mechanotransduction by integrin activation via FAK and src kinase pathways. Recently, signaling involving Rac1, which is activated by src kinase pathways, has been implicated in regulating assembly of a cytoskeleton and cell–cell adhesion junctions. For example, small GTPases of the Rho family including RhoA, Rac1, and Cdc42 have been shown to regulate cadherin-based cell adhesion in skeletal muscle development and establish cell polarity, which requires asymmetric distribution of signaling molecules and the cytoskeleton. Our results provide further evidence that pathways acting downstream of src kinase play an important role in assembly of new adhesion junctions and, based on the marked upregulation of plakoglobin and desmoplakin, in promoting more extensive linkage between adhesion junctions and the myocyte cytoskeleton.

Although formation of more numerous and more robust adhesion junctions appears to be an appropriate adaptive response to mechanical load, the reason increased load should also promote formation of gap junctions is not as apparent. We have proposed that the extent to which cardiac myocytes are coupled mechanically by cell–cell adhesion junctions is a major determinant of the extent to which they can become coupled electrically by gap junctions. This hypothesis is supported by numerous observations. For example, reformation of cell–cell junctions in disaggregated cardiac myocytes involves initial formation of adhesion junctions that become well developed before gap junctions can form. Gap junctions are invariably located within intercalated disks and are always flanked by adhesion junctions, consistent with the idea that mechanical junctions act to stabilize the sarcolemmas of neighboring cells, thereby creating conditions that favor formation and maintenance of large arrays of gap junction channels. Thus, genetic deficiency in connexin expression has no effect on expression of mechanical junction proteins or formation of adhesion junctions, but genetic deficiency in mechanical junction proteins has a profound effect on gap junctions, resulting in a marked decrease in the amount of Cx43 immunoreactive signal at cell–cell junctions, reflecting the presence of smaller and fewer gap junctions. Based on these multiple lines of evidence, we propose that pulsatile stretch stimulates expression of both electrical and mechanical junction proteins as part of a compensatory
hypertrophic response but that distinct signaling mechanisms are involved and optimal formation of more and larger gap junctions requires assembly of new mechanical junctions.

Whereas a compensatory hypertrophic response is associated with upregulation of expression of multiple cardiac proteins, which generally leads to a gain in function, a decompensatory or pathological hypertrophic response is associated with downregulation of various cardiac proteins and loss of function. In this regard, numerous studies of failing human myocardium have demonstrated downregulation of Cs43 and remodeling of gap junctions. Whether derangements in cell–cell adhesion or linkage of adhesion junctions to the cytoskeleton is a primary pathogenic event and remodeling of gap junctions occurs as a secondary consequence is unknown, but the results of the present study would favor this sequence. If so, then further investigation of signaling pathways downstream of src kinase as a potential therapeutic target to enhance cell–cell adhesion in diseased cardiac myocytes may be warranted. Stimulation of this pathway could not only improve forced transmission in cardiac myocytes by enhancing mechanical linkage but might also lead to assembly of gap junctions, which could have a salutary effect on conduction and limit arrhythmogenesis.

Imposition of stretch on cultured neonatal cardiac myocytes has been a useful experimental paradigm for investigating mechanisms underlying changes in cardiac structure and function in response to mechanical load. Pulsatile stretch of cultured neonatal rat ventricular myocytes induces what appears to be a physiological hypertrophic response. Accordingly, cells respond to mechanical load by forming increased numbers of more-mature-appearing adhesion junctions. A similar phenomenon occurs in vivo, as part of the hypertrophic response to mechanical load. It should be emphasized, however, that mechanisms underlying responses by neonatal cardiac myocytes to mechanical stretch in vitro may be different from those responsible for physiological and/or pathophysiological responses of the heart to increased load in vivo. Cardiac-specific manipulation of expression of key cell–cell junction proteins or relevant regulatory pathways may provide future insights into this important question.

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References

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