Induction of Tenascin-C in Cardiac Myocytes by Mechanical Deformation

ROLE OF REACTIVE OXYGEN SPECIES*

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Mechanical overload may change cardiac structure through angiotensin II-dependent and angiotensin II-independent mechanisms. We investigated the effects of mechanical strain on the gene expression of tenasin-C, a prominent extracellular molecule in actively remodeling tissues, in neonatal rat cardiac myocytes. Mechanical strain induced tenasin-C mRNA (3.9 ± 0.5-fold, p < 0.01, n = 13) and tenasin-C protein in an amplitude-dependent manner but did not induce secreted protein acidic and rich in cysteine nor fibronectin. RNAse protection assay demonstrated that mechanical strain induced all three alternatively spliced isoforms of tenasin-C. An angiotensin II receptor type 1 antagonist inhibited mechanical induction of brain natriuretic peptide but not tenasin-C. Antioxidants such as N-acetyl-L-cysteine, catalase, and 1,2-dihydroxy-benzene-3,5-disulfonate significantly inhibited induction of tenasin-C. Truncated tenasin-C promoter-reporter assays using dominant negative mutants of IκBα and IκB kinase β and electrophoretic mobility shift assays indicated that mechanical strain increases tenasin-C gene transcription by activating nuclear factor-κB through reactive oxygen species. Our findings demonstrate that mechanical strain induces tenasin-C in cardiac myocytes through a nuclear factor-κB-dependent and angiotensin II-independent mechanism. These data also suggest that reactive oxygen species may participate in mechanically induced left ventricular remodeling.

Cardiac hypertrophy is an independent risk factor of cardiac morbidity and mortality (1) and is characterized by an increase in myocyte mass and volume, as well as an increase of extracellular matrix proteins such as collagen (2). Angiotensin II is a potent stimulator of cardiac hypertrophy (3), and angiotensin-convertase enzyme inhibitors prevent left ventricular hypertrophy in hypertensive animals and humans. For example, Kojima et al. (4) reported that treatment with TCV-116, an angiotensin II receptor type 1 (AT₁) antagonist, decreased left ventricular weight, left ventricular wall thickness, and the transverse diameter of cardiac myocytes in spontaneously hypertensive rats.

Recent studies (5, 6) indicate that angiotensin II-independent mechanisms may also mediate cardiac hypertrophy. Harada et al. (5) demonstrated that acute pressure overload could induce hypertrophic responses such as induction of c-fos, c-jun, and brain natriuretic peptide (BNP) gene expression, mitogen-activated protein (MAP) kinase activation, and increased heart weight/body weight, in the hearts of AT₁A knockout mice. Harada et al. (6) also reported that there were no significant differences between wild-type mice and AT₁A knockout mice in expression levels of fetal-type cardiac genes, in left ventricular wall thickness and systolic function, or in histological changes such as myocyte hypertrophy and fibrosis.

Many intracellular signaling pathways are thought to play important roles in mechanotransduction. Recent studies of myocardial hypertrophy have focused on activation of protein kinases including protein kinase C, Raf-1 kinase, S6 peptide kinase, and MAP kinases, which precede an increase in specific gene expression and protein synthesis (7–11). The MAP kinase signaling pathways consist of three major phosphorylation cascades as follows: the extracellular signaling-regulated-protein kinases, the c-Jun NH2-terminal kinases (JNK), and the p38 MAP kinases (12, 13). JNK and p38 pathways are collectively termed stress-activated protein kinases because they are activated by various stress-related stimuli (14).

Intracellular reactive oxygen species (ROS) may participate in cellular responses to various stimuli including hemodynamic forces and act as signal transduction messengers. ROS in the heart may play a role in pathophysiological conditions such as myocardial ischemia, reperfusion, apoptosis, and heart failure (15). Mechanical stimuli can modulate intracellular ROS in endothelial cells (16–18), vascular smooth muscle cells (19), and cardiac muscle (20). In addition, Kheradmand et al. (21) reported that ROS were essential for nuclear factor (NF)-κB-dependent transcriptional regulation of collagenase-1 gene expression induced by cell shape change.

Ultimately, mechanotransduction events lead to increased cell size and cardiac remodeling. Several extracellular matrix
molecules, including tenasin-C and fibronectin, are induced in remodeling tissues. Tenasin-C is a disulﬁde-linked hexameric extracellular matrix protein with subunit molecular masses of 190–300 kDa depending on the species and on alternative splicing within ﬁbroblast type III repeats (22). Tenasin-C is able to interact with cell surface receptors including integrins (23–25) and also binds to extracellular matrix proteins such as ﬁbronectin (26). Tenasin-C inhibits the attachment of ﬁbroblasts and endothelial cells to various adhesive proteins (27). Thus, tenasin-C may be important in regulating cell-extracellular matrix interactions by promoting cell rounding, migration, and/or differentiation.

By using a mechanical deformation device that applies a highly uniform biaxial strain ﬁeld over the culture substrate, we investigated the effects of mechanical strain on tenasin-C gene expression in cultured neonatal rat cardiac myocytes. We found that tenasin-C is mechanically induced in cardiac myocytes through the activation of NF-xB, suggesting that tenasin-C can be an angiotensin II-independent early marker for cardiac remodeling. In addition, our data suggest that ROS may participate in mechanically induced cardiomyocyte responses.

**ExperimenTal Procedures**

**Materials—**Staurosporine, calphostin C, genistein, PD98059, and SB203580 were purchased from Calbiochem. Recombinant human platelet-derived growth factor-BB (PDGF-BB) was purchased from Beckton Dickinson (Bedford, MA). CP191,166 (Ki = 3.1 nM, AT/AT, IC50 = 3.5 nM, 4.0 μM), an AT, antagonist, was from Pfizer, Inc. (Groton, CT). Fibronectin was purchased from Life Technologies, Inc. Cycolxhime, actinomycin D, N-acetyl-l-cysteine, catalase, and 1,2-dihydroxy-benzene-3,5-disulfonate (Tiron), 7-nitroindazole, and hydrogen peroxide (H2O2) were purchased from Sigma. [α-32P]dATP (3000 Ci/mmol), [α-32P]dCTP (3000 Ci/mmol), [α-32P]dUTP (3000 Ci/mmol), and [3H]thymidine (40 Ci/mmol) were purchased from NEN Life Science Products.

**Culture of Neonatal Rat Ventricular Myocytes (NRVM)—**NRVM from 1-day-old Harlan Sprague-Dawley rats were isolated by previously described methods (28). The ventricles were excised, cut into several pieces, and incubated overnight at 4 °C in 1 ml/1300 trypsin (Life Technologies, Inc.) in Hank’s balanced salt solution in all directions. This approach eliminates locations on the substrate that have very high strains (20–30%) in one direction. We have previously measured membrane strains with a high resolution video device (29); the cans used for this study gave strains of 1, 4, 9, and 14%. We have observed that strains larger than 14% lead to cell injury (data not shown), and the design of this device is limited to a maximum strain of 14%.

NRVM were plated on a fibronectin-coated (2 μg/ml) membrane dish at a density of 2,000,000 cells/dish in 13 ml of DMEM containing 7% FCS and incubated 24 h. Approximate cell confluence was 85–90%.

**Western Analysis of Tenasin-C Protein—**Western analysis was performed using 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane in 25 mM Tris base (pH 8.5), 0.2M glycine, and 20% methanol. The membrane was blocked with 5% nonfat dried milk in TBS-0.1% Tween 20 for 2 h. For the detection of tenasin-C, the membrane was coupled to peroxidase (Bio-Rad), the membrane was developed with the peroxidase Chemiluminescence Detection System (Amersham Pharmacia Biotech).
NRVM were plated on 2 μg/ml fibronectin in DMEM containing 7% FCS for 24 h. After serum deprivation for 24 h, myocytes were exposed to 0 or 9% cyclic mechanical strain (1 Hz) for 24 h. Total RNA was isolated and analyzed by Northern blotting with 32P-labeled tenascin-C, SPARC, fibronectin, and GAPDH cDNA probes. Data are representative of six experiments that gave nearly identical results. At least two tenascin-C mRNA species are increased at 3, 6, and 12 h.

Madison, WI. CAT assays were performed as described by the manufacturer (Promega). The relative CAT activity was calculated as the ratio of CAT to β-galactosidase activity and standardized to unstimulated ~220-CAT promoter activity (fold induction).

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described (35), and the protein concentration of each extract was determined by the Bradford method (Bio-Rad). Oligonucleotide probes for the NF-κB consensus sequences (Promega) were end-labeled with [γ-32P]ATP by incubating with T4 polynucleotide kinase at 37 °C for 10 min. The labeled probe was separated from unincorporated nucleotide using a Sephadex G-50 column (Amersham Pharmacia Biotech). Ten μg of nuclear extract was incubated in 10 μl of binding buffer containing 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC), and 20% glycerol for 10 min at room temperature. 32P-Labeled oligonucleotide probe (50,000–200,000 cpm) was then added, and the reaction mixture was incubated for 20 min at room temperature. DNA-protein complexes were resolved with 6% nondenaturing polyacrylamide gel electrophoresis at 12 V/cm in 0.5× TBE. Specificity was determined by the addition of p50 antibody (15 μg IgG/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or excess unlabeled (cold) NF-κB oligonucleotide (1.75 μg) to the nuclear extracts for 10 min before addition of radiolabeled probe.

Statistical Analysis—Data are expressed as the mean ± S.D. A paired two-tailed Student’s t test was used to analyze differences between two groups, and p values of < 0.05 were considered significant.

RESULTS

Effects of Mechanical Strain on Tenascin-C mRNA Accumulation—We first investigated whether mechanical strain modulated gene expression of tenascin-C as well as SPARC, a remodeling related matrix protein, and fibronectin in NRVM. As shown in Fig. 1, 9% cyclic mechanical strain at 1 Hz induced tenascin-C mRNA accumulation with a maximum peak at 6 h (3.9 ± 0.5-fold by densitometry, p < 0.01, n = 13), whereas mechanical strain did not induce SPARC nor fibronectin mRNA. In addition, when cyclic biaxial strains of 1, 4, 9, and 14% were imposed, induction of tenascin-C mRNA expression in NRVM was amplitude-dependent (Fig. 2). In these studies, no morphologic changes in cardiac myocytes were detected following strains of 1–14%.

LaFleur et al. (36) reported that there are alternatively spliced isoforms of rat tenasin-C, and our Northern analysis suggested that at least two of these species are induced by deformation of cardiac myocytes. We then performed an RNase protection assay with a probe designed to distinguish the three rat tenasin-C cDNA species. We observed three bands of 107, 375, and 409 base pairs in size, corresponding to the three alternatively spliced messages (36); 9% mechanical strain and PDGF-BB (1 ng/ml) induced all three alternatively spliced isoforms of tenasin-C (Fig. 3).

Effects of Mechanical Strain on the Stability of Tenascin-C mRNA—To determine whether mechanical strain increased tenascin-C mRNA accumulation by increasing the rate of synthesis or by decreasing the rate of degradation, NRVM were incubated in the presence or absence of 9% strain for 6 h and then incubated further with actinomycin D (5 μg/ml) to inhibit transcriptional activity. The half-life (t1/2 < 3 h) of tenascin-C mRNA was not affected by mechanical strain (Fig. 4). This experiment suggested that mechanical strain increases the rate of synthesis of tenasin C mRNA.

Effects of Mechanical Strain on Tenascin-C Protein Accumulation—We next investigated whether the increase in tenasin-C mRNA by mechanical strain was accompanied by an increase in tenasin-C protein. The expression of tenasin-C protein was analyzed by immunoblotting with anti-tenasin-C antibody. The tenasin-C protein bands at 220 and 250 kDa were increased in an amplitude-dependent manner (Fig. 5).

Effects of Cycloheximide and Angiotensin II Receptor Type 1 Antagonist on the Induction of Tenascin-C mRNA—Mechanical strain may induce proteins including growth factors and vasoconstrictors in cardiac myocytes. We studied whether new protein synthesis was required for the induction of tenasin-C mRNA expression by strain. The addition of cycloheximide (10 μM; 0% inhibition), a protein synthesis inhibitor, did not affect the induction of tenasin-C mRNA by strain in NRVM (Fig. 6A). This indicates that cyclic mechanical strain induces tenasin-C mRNA expression in NRVM without new protein synthesis. In addition, we investigated whether the effect of cyclic mechanical strain on tenasin-C mRNA expression in NRVM is angiotensin II-dependent. NRVM were subjected to 9% cyclic strain at 1 Hz for 6 h in the presence or absence of an AT1 antagonist. As shown in Fig. 6B, CP191,166 (0.1 μM), an AT1 antagonist, inhibited the induction of BNP mRNA (52% inhibition) but not tenasin-C mRNA (0% inhibition) by cyclic mechanical strain. Therefore, in contrast to the previously reported effect of angiotensin II on strain-mediated BNP induction (37), it is unlikely that angiotensin II is involved in the effect of cyclic mechanical strain on tenasin-C mRNA expression in NRVM.

Involvement of Protein Kinase C, Tyrosine Kinase, and MAP Kinase—Mechanical strain activates protein kinase C, tyrosine kinases, and MAP kinases in cardiac myocytes (7–11). We examined the role of three pathways on the induction of tena-
scin-C mRNA expression by mechanical strain. Staurosporine (100 nM; 12% inhibition), a non-selective protein kinase inhibitor, minimally inhibited tenascin-C mRNA induction by strain, but neither calphostin C (1 μM; 5% inhibition), a selective protein kinase C inhibitor, nor genistein (100 μM; 8% inhibition), a tyrosine kinase inhibitor, inhibited the induction by strain (Fig. 7A). In addition, neither PD98059 (50 μM; 0% inhibition), a MAP kinase kinase inhibitor, nor SB203580 (10 μM; 5% inhibition), a p38 MAP kinase inhibitor, inhibited the effect of mechanical strain (Fig. 7B). These findings suggest that the effect of cyclic mechanical strain is protein kinase C-independent and MAP kinase-independent.

Effects of Antioxidants on the Induction of Tenascin-C mRNA Expression and Tenascin-C Promoter Activity—ROS may participate in mechanotransduction; therefore, we investigated the effects of antioxidants on the induction of tenascin-C mRNA by strain. Antioxidants, such as N-acetyl-L-cysteine (10 mM; 90% inhibition), catalase (500 units/ml; 93% inhibition), and Tiron (10 mM; 95% inhibition) inhibited the effect of strain, whereas inhibition of reactive nitrogen species by 7-nitroindazole (100 μM; 3% inhibition) had no effect (Fig. 8). Furthermore, the addition of H₂O₂ (100 μM) induced tenascin-C mRNA in the absence of strain.

To identify possible sites of induction of the tenascin-C promoter by mechanical strain, we performed transfection studies with promoter reporter constructs containing functional cis-activating elements for activated protein (AP)-1, NF-κB, and GCN4 (AP-1-like site) (Fig. 9A). The tenascin-C promoter contains a consensus AP-1 site at position –875, the function of which has
not been demonstrated (38). The −1175-CAT reporter construct, containing the consensus AP-1 site, displayed a low basal activity and was stimulated 2.6-fold by mechanical strain compared with no strain. A shorter promoter construct, −220-CAT, was stimulated 6.6 ± 0.9-fold by mechanical strain, whereas promoter constructs without the NF-κB site were not activated by strain (Fig. 9A). To investigate the role of NF-κB in strain-induced tenascin-C promoter activity, dominant negative mutants of IκBα (pCMV4-IκBαΔN) or IKKβ (pCDNA3-IKKβΔ34) were cotransfected. Both mutants blocked −220-CAT promoter activation by mechanical strain, whereas control vectors pCMV4 and pCDNA3 had no effect (Fig. 9B).

In addition, N-acetyl-L-cysteine (10 mM), catalase (500 units/ml), and Tiron (10 mM) inhibited the effect of strain, whereas inhibition of reactive nitrogen species by 7-nitroindazole (100 μM) had no effect (Fig. 10). The addition of H₂O₂ (100 mM) also increased −220-CAT promoter activity in the absence of strain. These findings suggest that activation of tenascin-C promoter regions by mechanical strain through the NF-κB-binding site involves ROS.

**Effects of Antioxidant on NF-κB Activation by Mechanical Strain**—Since it is likely that the activation of NF-κB is required for induction of tenascin-C expression by mechanical strain, electrophoretic mobility shift assays were performed using radiolabeled NF-κB oligonucleotide. As shown in Fig. 11, 9% mechanical strain increased the amount of shifted complex. N-acetyl-L-cysteine (10 mM) significantly inhibited the activation of NF-κB by mechanical strain. The shifted complexes were specific for NF-κB since they were supershifted in the presence of antibody to NF-κB subunit and disappeared with excess unlabeled oligonucleotide. Furthermore, the addition of H₂O₂ (100 mM) induced the activation of NF-κB in the absence of strain.

**DISCUSSION**

Tenascin-C is prominent in embryonic and adult tissues that are actively remodeling (39). In cardiovascular tissues, tenas-
mechanical strain. Cardiac myocytes were transfected with −220-CAT construct (10 μg) and CMV β-gal plasmid (3 μg). After transfection for 48 h, myocytes were exposed for 24 h to 0 (open column) or 9% (closed column) cyclic mechanical strain (1 Hz) in the presence or absence of N-acetyl-L-cysteine (NAC, 10 mM), catalase (Cat, 500 units/ml), Tiron (10 mM), 7-nitroindazole (7-NI, 100 μM), or hydrogen peroxide (H₂O₂, 100 μM). N-Acetyl-L-cysteine, catalase, Tiron, or 7-nitroindazole was applied to the myocytes 1 h before mechanical strain. Lysates were prepared for CAT assays and β-galactosidase assays. The relative CAT activity was calculated as the ratio of CAT to β-galactosidase activity and standardized to unstimulated 220-CAT construct (100, 100 M). Specificity was determined by addition of p50 antibody (Ab) (supershift) or unlabeled (cold) NF-κB oligonucleotide (1.75 pm) to the nuclear extracts. Two separate experiments yielded similar results.

**Strain Induces Tenascin-C Expression**

Myocytes were exposed for 1 h to 0 or 9% cyclic mechanical strain (1 Hz) or hydrogen peroxide (H₂O₂, 100 μM) in the presence or absence of N-acetyl-L-cysteine (NAC, 10 mM). N-Acetyl-L-cysteine was applied to the myocytes 1 h before mechanical strain. Specificity was determined by addition of p50 antibody (Ab) (supershift) or unlabeled (cold) NF-κB oligonucleotide (1.75 pm) to the nuclear extracts. Two separate experiments yielded similar results.

Tenascin-C expression has been described in the developing heart (40), in normal blood vessels (41), and in carotid arteries after experimental balloon injury (42). Previous studies reported that mechanical strain may induce tenasin-C in fibroblasts (43) and in pulmonary arteries (44). Chiquet-Ehrismann et al. (43) reported that tenasin-C promoter expression was directly or indirectly activated in fibroblasts generating and experiencing mechanical stress within a restricted collagen matrix. Jones and Rabino (44) demonstrated that the induction of tenasin-C accompanied progressive pulmonary vascular changes. In the present study, cyclic mechanical strain induced tenasin-C mRNA and protein accumulations in cardiac myocytes. These findings suggest that tenasin-C may participate in cardiac remodeling induced by mechanical overload, such as hypertensive heart disease.

The local renin-angiotensin system may play an important role in the adaptation of the heart to pressure and volume overload (45, 46). Sadowski et al. (47) reported that [Sar-1, Ile-8]angiotensin II, a specific angiotensin II receptor antagonist, completely inhibited the stretch-induced c-fos expression in neonatal rat cardiac myocytes. Although angiotensin II secreted from cardiac myocytes is an important mediator of the strain-induced hypertrophic response in vitro, angiotensin II does not appear necessary for cardiac hypertrophy induced by mechanical strain in vivo. Thielen et al. (48) demonstrated that the acute growth responses induced by systolic pressure overload in rat did not depend on AT₁ activation, and Dell'Italia et al. (49) reported that volume overload cardiac hypertrophy in dogs with chronic mitral regurgitation was unaffected by angiotensin-converting enzyme inhibitor treatment. Harada et al. (5, 6) proposed that AT₁-mediated angiotensin II signaling is not essential for the development of pressure overload-induced cardiac hypertrophy. In the present study, AT₁ blockade did not affect the induction of tenasin-C mRNA expression in NRVM, whereas AT₂ blockade partially inhibited BNP mRNA induction by strain. This is consistent with the hypothesis that factors other than angiotensin II are involved in cardiac remodeling induced by mechanical strain.

Mechanical strain rapidly increases protein kinase C and MAP kinase activity in cardiac myocytes (8, 11). Stretch-induced c-fos expression is inhibited by both protein kinase C inhibitors and down-regulation of protein kinase C (7, 11). In addition, Schwachtgen et al. (50) reported that treatment of human endothelial cells with PD98059 (MAP kinase kinase inhibitor) inhibited shear stress activation of egr-1. In the present study, staurosporine and calphostin C (protein kinase C inhibitors), PD98059, and SB203580 (a p38-MAP kinase inhibitor) did not significantly inhibit the stimulatory effect of mechanical strain on tenasin-C mRNA expression in cardiac myocytes, suggesting that this effect is not mediated via activation of protein kinase C nor MAP kinase. However, we cannot exclude the possibility that the JNK pathway, one of three major MAP kinase signaling pathways, might be involved in the effect of mechanical strain on tenasin-C mRNA induction. Furthermore, it is important to recognize that in vitro biaxial strain amplitudes cannot be directly extrapolated to in vivo myocardial deformations. In vivo, the cardiac myocyte is in a three-dimensional tissue with complex active and passive loads, as well as extracellular matrix components that contribute to mechanical behavior.

Many studies have reported that superoxide or its derivative radicals can be demonstrated in reperfused isolated hearts (51–53). Nakamura et al. (54) recently reported that cardiac hypertrophy induced by tumor necrosis factor-α and angiotensin II was inhibited by antioxidants in rat cardiac myocytes and concluded that tumor necrosis factor-α and angiotensin II cause hypertrophy in part via the generation of ROS. Peng et al. (55) demonstrated that nuclear proteins induced by H₂O₂ in rat cardiac myocytes are capable of binding to a DNA probe containing the NF-κB elements. Cheng et al. (20) demonstrated that overstretching produced a 2.4- and a 21-fold increase in the generation of ROS and apoptotic myocytes, respectively. Their findings suggest that ROS production by mechanical deformation may lead to the impairment in force development of the myocardium.

In porcine aortic endothelial cells, cyclic strain induces an oxidant stress, and NADH/NADPH oxidase is a potential source of H₂O₂ release in cyclically strained cells (56). An interesting observation is that many activators of NF-κB have...
been reported to also increase oxidative stress. In cardiac myocytes, the mechanisms by which mechanical strain generates ROS and by which generated ROS activates NF-κB remain unknown. Activation of transcriptional factors NF-κB and AP-1 by ROS during gene induction have been reported (57, 58). However, in this study, the function of a consensus AP-1 site at position −875 was not demonstrated, as described previously (31, 38). NF-κB is retained in the cytoplasm by IκB, which comprises a distinct family of proteins that bind to NF-κB and inhibit nuclear translocation and DNA binding. IKK activation is followed by IκB degradation and NF-κB translocation into the nucleus, resulting in activation of NF-κB. IκBα is the principal regulator of NF-κB activity and can remove bound NF-κB from the nucleus, whereas IκBβ cannot (59, 60). Two closely related kinases designated IKKα and IKKβ have been identified as components of the multiprotein IKK complex (500–900 kDa) that directly phosphorylates the critical Ser residues of IκB proteins (61, 62). In the present study, dominant negative mutants of IκBα and IκBβ significantly inhibited tenascin-C promoter activation by mechanical strain, indicating that NF-κB pathway is involved in the induction of tenascin-C by strain. In addition, antioxidants inhibited the induction of tenascin-C mRNA and gene transcription and NF-κB activation by cyclic mechanical strain in cardiac myocytes. These findings support the premise that mechanical strain induces the generation of ROS, which activates NF-κB in rat cardiac myocytes, and that ROS may be a second messenger in cardiac remodeling. 

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