Reactive Oxygen Species Mediate Amplitude-Dependent Hypertrophic and Apoptotic Responses to Mechanical Stretch in Cardiac Myocytes

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Abstract—Oxidative stress stimulates both growth and apoptosis in cardiac myocytes in vitro. We investigated whether oxidative stress mediates hypertrophy and apoptosis in cyclically stretched ventricular myocytes. Neonatal rat ventricular myocytes cultured on laminin-coated silastic membranes were stretched cyclically (1 Hz) at low (nominal 5%) and high (nominal 25%) amplitudes for 24 hours. Stretch caused a graded increase in superoxide anion production as assessed by superoxide dismutase (SOD)-inhibitable cytochrome c reduction or electron paramagnetic resonance spectroscopy. The role of reactive oxygen species (ROS) was assessed using the cell-permeable SOD/catalase mimetics Mn(II/III)tetraakis(1-methyl-4-peridyl) (MnTMPyP) and EUK-8. Stretch-induced increases in protein synthesis (3H-leucine incorporation) and cellular protein content were completely inhibited by MnTMPyP (0.05 mmol/L) at both low and high amplitudes of stretch. In contrast, while MnTMPyP inhibited basal atrial natriuretic factor (ANF) mRNA expression, the stretch-induced increase in ANF mRNA expression was not inhibited by MnTMPyP. In contrast to hypertrophy, only high-amplitude stretch increased myocyte apoptosis, as reflected by increased DNA fragmentation on gel electrophoresis and an ≈3-fold increase in the number of TUNEL-positive myocytes. Similarly, only high-amplitude stretch increased the expression of bax mRNA. Myocyte apoptosis and bax expression stimulated by high-amplitude stretch were inhibited by MnTMPyP. Both low- and high-amplitude stretch caused rapid phosphorylation of ERK1/2, while high-, but not low-, amplitude stretch caused phosphorylation of JNKs. Activation of both ERK1/2 and JNKs was ROS-dependent. Thus, cyclic strain causes an amplitude-related increase in ROS, associated with differential activation of kinases and induction of hypertrophic and apoptotic phenotypes. (Circ Res. 2001;89:453-460.)

Key Words: oxidant stress ■ remodeling ■ apoptosis ■ hypertrophy

Hemodynamic overload is associated with an increase in myocardial wall stress, which has been implicated as a stimulus for myocardial remodeling. In vitro, both tonic and cyclic mechanical stretch have been used to simulate increased wall stress. In cardiac myocytes in vitro, mechanical stretch stimulates growth,1 alterations in gene expression,2 and apoptosis,3,4 cellular events that are observed in the failing heart.

In animal models of hemodynamic overload leading to myocardial remodeling and failure, there is a chronic increase in myocardial oxidative stress,5 which may contribute to myocardial remodeling.6 Interestingly, tonic mechanical stretch of rat papillary muscle increases the production of reactive oxygen species (ROS), which appear to be involved in mediating myocyte apoptosis in that model.3 An increase in oxidative stress caused by direct addition of ROS also induces myocyte apoptosis.7 We found that a small increase in myocyte oxidative stress caused by partial inhibition of CuZn-superoxide dismutase (SOD) resulted in myocyte hypertrophy, whereas a higher level of oxidative stress due to more complete inhibition of SOD caused apoptosis.8 Angiotensin, tumor necrosis factor-α, α1-adrenergic agonists, and ouabain also appear to increase myocardial ROS that mediate myocyte growth.9–11

These observations led us to hypothesize that oxidative stress mediates the effects of mechanical stretch on myocyte growth and survival. Using an in vitro system to subject cardiac myocytes to cyclic mechanical stretch, we tested whether (a) mechanical stretch causes an increase in the formation of ROS, (b) oxidative stress mediates the effects of mechanical stretch on myocyte hypertrophy and survival, and (c) stretch, acting via ROS, causes activation of mitogen-ac-
Materials and Methods

Preparation of Neonatal Rat Ventricular Myocytes (NRVMs)

Ventricular myocytes were isolated from neonatal rats by a modification of Kasten’s technique, as previously described. Unless otherwise stated, the cells were plated at 800 cells/mm² on 6-well BioFlex (Flexcell International) plates precoated with 0.02 mM/L laminin (GibcoBRL). After 24 hours in DMEM (GibcoBRL) containing 7% (vol/vol) inactivated fetal bovine serum (GibcoBRL) and 1% (vol/vol) penicillin-streptomycin (GibcoBRL), the medium was changed to serum-free DMEM for an additional 24 hours before treatment. Approximately 650 to 700 cells/mm² remained plated after this second medium change.

Mechanical Stretch

NRVMs were subjected to cyclic stretch by means of the Flexcell computer-driven vacuum system as has been previously described. NRVMs plated on BioFlex culture plates were placed on a gasketed baseplate in a 37°C, 5% CO₂ incubator. The plates were subjected to a vacuum of ~5 or ~21 kPa, low and high stretch, respectively, at a frequency of 1 Hz applied in a square-wave pattern of strain lasting 0.5 seconds per cycle for up to 24 hours. Using these parameters, this system produces a gradient of deformation across the membrane, with a maximal deformation of 5% and 25% at ~5 and ~21 kPa, respectively. Parallel BioFlex culture plates not subjected to stretch served as controls.

Measurement of Superoxide Release

Superoxide production was measured as the SOD-inhibitable reduction of cytochrome c. NRVMs were stretched or not stretched for 24 hours. The cells were washed with phosphate-buffered saline (PBS; 137 mM/L NaCl, 2.68 mM/L KCl, 1.47 mM/L KH₂PO₄, 8.1 mM/L Na₂HPO₄, 0.9 mM/L CaCl₂, 0.5 mM/L MgCl₂, and 2 mM/L EDTA [pH 7.4]). Cytochrome c (9.9 mM/L, with 1200 U/mL of catalase and 0.1 mM/L EDTA in phenol-free medium) with or without 300 U/mL SOD was added for 1 hour at 37°C without mechanical stretch. Cytochrome c reduction was measured as the absorbance at 550 nm. The difference in ΔA₅₅₀ between wells with and without SOD was calculated.

Electron Paramagnetic Resonance (EPR)

Measurement of ROS Production

EPR measurements were performed as described by Sorescu et al. Myocytes were washed with ice-cold PBS and scraped in a 50 mM/L phosphate buffer (pH 7.4; pretreated with Chelex [5 g/100 mL] for 2 hours) containing aprotonin (10 μg/mL), pepstatin (0.7 μg/mL), and PMSF (0.5 mM/L). The cells were sonicated for 30 seconds on ice followed by centrifugation at 50 000 g for 30 minutes at 4°C. The membrane pellets were resuspended in 25 μL of lysis buffer and aliquots containing 25 μg of protein (Bradford method) were resuspended with 0.1 mM/L diethylthlenetriaminepentaacetic acid, 300 μM/L NADH or NADPH, and 25 mM/L DEPEPO (OXIS International). The assay mixture was incubated at 37°C for 30 minutes and was stopped by snap-freezing. After thawing the sample at room temperature, EPR measurements were performed at room temperature using a Bruker EMX spectrometer with an ER4119HS high-sensitivity resonator cavity. The instrument settings were as follows: modulation amplitude 3 G; time constant 82 ms; modulation frequency 100 kHz; microwave power 20 mW; and microwave frequency 9.40 GHz. Five scans were accumulated, and the double integral of the second peak of the composite spectrum was used for comparison of conditions.

Measurement of ³H-Leucine Incorporation

Incorporation of ³H-leucine was measured over the final 4 hours of a 24-hour period of cyclic stretch, and total cellular protein content was determined by the Bradford method as previously described. After cell lysis, samples were resuspended in a buffer composed of 100 mM/L Tris, 10 mM/L EDTA, 1 mol/L NaCl, and 10 μg of the fluorochrome H33258 (Calbiochem Corp). In all experiments, cellular DNA content was determined by a fluorometric quantification of the H33258 in comparison to a standard curve with herring sperm DNA, and ³H-leucine incorporation is reported as CPM/μg DNA.

Changes in Total Cellular Protein

After precipitation of myocyte lysates with 10% TCA and resuspension in 0.4 mol/L NaOH buffer, aliquots were removed for determination of cellular protein. Total cellular protein was determined by the Bradford method using Coomassie Blue (BioRad Laboratories). Protein content was normalized to DNA content as described above.

Northern Hybridization

After 24 hours of cyclical stretch or nonstretch control, cells were collected and total RNA isolated as previously described. Northern hybridizations with ³²P-labeled rat cDNAs for prepro-atrial natriuretic peptide (ANP) (courtesy of C. Seidman, Brigham and Women’s Hospital, Boston, Mass) and bax (courtesy of J. Tilly, Massachusetts General Hospital, Boston, Mass) were performed and quantified as previously described. mRNA levels were normalized to 18S ribosomal RNA determined by reprobing blots with ³²P-labeled oligonucleotide complementary to 18S rRNA.

DNA Laddering

After 24 hours of cyclical stretch or nonstretch control, total DNA from NRVMs in a single Flexcell plate (6 wells) was isolated and electrophoresed as previously described to visualize the pattern of DNA fragmentation.

In Situ Nick-End Labeling of DNA Strand Breaks (TUNEL)

NRVMs plated on BioFlex plates were stretched or not stretched for 24 hours as indicated. The cells were TUNEL-labeled and nuclei stained with 10 μg/mL Hoechst as previously described. After three washes in PBS, a glass coverslip was attached to the underside of the Flexcell silastic membrane, which was cut to the size of the coverslip. The membranes were then mounted onto glass slides and viewed with an epifluorescent microscope. Slides were viewed in a blinded fashion and at least 250 total nuclei (Hoechst stained) were counted from each coverslip (a total of 4 coverslips per experiment were viewed), and the number of TUNEL-positive cells was determined for each field.

Immunoblots for Activated MAPK and Bax

The activation of ERKs, JNK, and total bax was examined by Western blot analysis using specific antibodies. Total cellular homogenates from rat myocytes were prepared from one Flexcell plate (6 wells), and equal amounts (50 μg) of the denatured proteins were loaded and separated on 10% or 12% SDS-polyacrylamide gels (Mini Protein II, BioRad) and transferred to polyvinylidine difluoride membranes (Hybond-P, Amersham Life Science). The membrane was blocked with 5% nonfat dry milk (BioRad) in PBS for 1 hour. The membranes were incubated with 1:200 rabbit polyclonal antibody to phospho-p44/42 MAPK, phospho-JNK (Cell Signaling), or bax (Santa Cruz Biotechnology) overnight in 4% BSA in TBS at 4°C. The membranes were washed six times with TBS followed by incubation for 1 hour with a horseradish peroxidase-labeled goat anti-rabbit antibody (Santa Cruz) in 4% BSA in TBS. Afterward, the membranes were exposed to a chemiluminescent reagent (Pierce) and autoradiographed for 1 to 2 minutes.
Materials
SOD, catalase, and cytochrome c were from Sigma. Mn(II/III)tetrakis(1-methyl-4-peridyl)porphyrin (MnTMPyP) was from Calbiochem, Inc. EUK-8 was gift from Eukarion (Bedford, Mass). 32 P-CTP and 3 H-leucine were from New England Nuclear, Inc. All other reagents were from Sigma.

Statistical Analysis
All data are reported as mean±SEM. Significance was determined with the InStat program for student’s unpaired t tests or ANOVA where appropriate. A value of $P<0.05$ was considered to be significant.

Results
Mechanical Stretch Increases Myocyte O$_2^-$ Production
Isolated NRVMs plated onto silastic membranes coated with laminin were subjected to cyclic mechanical stretch (1 Hz) at low (5%) and high (25%) amplitudes for 24 hours. SOD-inhibitable cytochrome c reduction in medium was assessed as a measure of myocyte O$_2^-$ production. There was a graded increase in O$_2^-$ production in stretched cells, with a 230% increase in O$_2^-$ production at low-amplitude stretch and a 405% increase at high-amplitude stretch (Figure 1A).

To confirm the increased production of ROS and examine potential sources, we used EPR. Using DEPMPO as a spin trap with membrane fractions of myocytes incubated with NADPH, we observed the characteristic hydroxyl adduct (DEPMPO-OOH). Formation of the DEPMPO-OOH adduct was increased in myocytes subjected to stretch (Figures 1B and 1C). DEPMPO-OOH adduct formation was completely inhibited with the addition of MnSOD. There was greater formation of DEPMPO-OOH in the presence of NADPH than NADH at baseline, and with stretch the absolute increase was greater with NADPH (versus NADH, Figure 1C). Addition either of the endothelin receptor antagonist (BQ123, 1 µmol/L) or the angiotensin receptor antagonist (losartan, 10 µmol/L) had no effect on baseline levels of ROS production but significantly inhibited the formation of DEPMPO-OOH in stretched myocytes by 42% and 41%, respectively (n=3; $P<0.05$ for both).

Stretch-Induced Myocyte Hypertrophy Is Mediated by ROS
3 H-leucine incorporation and total protein were assessed as measures of myocyte hypertrophy. Cyclic stretch for 24 hours caused a graded increase in 3 H-leucine incorporation, with a 13% increase at low amplitude and a 80% increase at high amplitude (Figures 2A and 2B). Likewise, stretch caused an amplitude-dependent increase in total cellular protein (Figures 2C and 2D).
To examine whether stretch-induced O$_2^-$ production mediates myocyte hypertrophy, we measured $^3$H-leucine incorporation in the presence and absence the ROS scavengers EUK-8 and MnTMPyP.$^{19}$ Both ROS scavengers abolished the increases in $^3$H-leucine uptake (Figures 2A and 2B) and total protein (Figures 2C and 2D) in response to low- and high-amplitude stretch. The ROS scavengers alone had no effect on $^3$H-leucine incorporation or total protein content.

Myocyte hypertrophy is often associated with increased expression of fetal genes such as atrial natriuretic factor (ANF). Stretch at low and high amplitude increased ANF mRNA by 62% and 104%, respectively (Figure 3). While MnTMPyP inhibited baseline ANF expression, MnTMPyP did not decrease the stretch-induced increases in ANF expression.

**Stretch-Induced Myocyte Apoptosis Is Mediated by ROS**

DNA isolated from myocytes stretched at high amplitude for 24 hours showed a ladder pattern on agarose gel electrophoresis indicative of apoptosis (Figure 4A). Minimal DNA laddering was evident in static cells (Figure 4A) or cells stretched at low amplitude (data not shown). Pretreatment with MnTMPyP or EUK-8 prevented DNA laddering with high-amplitude stretch. TUNEL staining of adherent myocytes in separate experiments was performed to quantify the percent of apoptotic myocytes. Low-amplitude stretch tended to decrease the number of myocytes staining positive by the TUNEL method, whereas high-amplitude stretch caused an ≈3-fold increase that was inhibited by MnTMPyP (Figure 4B), suggesting that apoptosis with high-amplitude stretch was ROS-dependent.

The expression of bax is increased in response to apoptotic levels of oxidative stress in cardiac myocytes.$^{8,7}$ We therefore examined whether bax was increased by mechanical stretch. Low-amplitude stretch had no effect on bax mRNA levels, whereas high-amplitude stretch increased bax mRNA expression. Stretch-induced bax expression was prevented by MnTMPyP (Figures 5A and 5B). Likewise, bax protein levels assessed by immunoblotting increased with high-amplitude stretch, and the increase was inhibited by MnTMPyP or EUK-8 (Figure 5C).

**Stretch-Induced Signaling Is Mediated by ROS**

ROS can stimulate MAPK signaling in cardiac myocytes.$^{20,21}$ We examined the effects of low- and high-amplitude stretch on activation of ERK1/2 and JNKs. ERK activation after 8 minutes of stretch, as assessed by immunoblotting for phosphorylated ERK1/2, was increased to similar levels at both low and high amplitudes of myocyte stretch compared with control myocytes. MnTMPyP inhibited the activation ERK1/2 in both amplitudes of stretch (Figures 6A and 6B). Likewise, bax protein levels assessed by immunoblotting increased with high-amplitude stretch, and the increase was inhibited by MnTMPyP or EUK-8 (Figure 5C).
minutes of high-, but not low-amplitude stretch, and this was inhibited by MnTMPyP (Figures 6C and 6D).

Discussion
Mechanical stretch of cardiac myocytes, either tonic or cyclic, leads to hypertrophic growth\(^1\,\text{22}\) as well as apoptosis.\(^3\,\text{4}\) Individually, these effects of mechanical stretch have been studied in some detail. To our knowledge, this is the first report to show an amplitude-dependent shift in myocyte phenotype in response to mechanical stretch. We have previously shown that graded increases in the level of myocyte oxidative stress induce a graded phenotype shift in cardiac myocytes, from hypertrophy and fetal gene expression at low levels of oxidative stress, to apoptosis at high levels of oxidative stress.\(^8\) The response of cardiac myocytes to low and high levels of mechanical stretch is similar to the effect of directly increasing myocyte oxidative stress. First, there is a stretch amplitude-dependent increase in ROS production. Second, there is an amplitude-dependent phenotype shift, from hypertrophy at low levels of stretch, to apoptosis at high levels of mechanical stretch. Finally, the phenotype shift at each level of stretch was inhibited by pharmacologic antioxidants.

**Stretch-Induced ROS Production**
Cyclic stretch increased myocyte ROS production in an amplitude-dependent manner. Static stretch of isolated papillary muscles causes a similar amplitude-dependent increase in myocardial ROS production.\(^3\) We found that ROS production in stretched myocytes was at least in part mediated by angiotensin and endothelin receptor activation, consistent with prior work implicating paracrine release of angiotensin and endothelin in stretch-induced hypertrophy\(^23\) and apoptosis.\(^3\,\text{11}\) Inhibition of either receptor caused a similar reduction in superoxide production consistent with prior reports suggesting that angiotensin acts through endothelin in cardiac myocytes.\(^24\)

The cellular source of increased ROS production in stretched myocytes remains to be determined, but it may involve an NAD(P)H oxidase system and/or changes in mitochondrial production of ROS. Mitochondria are a recognized source of ROS in the myocardium.\(^25\) Mechanical stretch increases myocardial oxygen consumption\(^26\,\text{27}\) and may thus
lead to increased ROS formation by mitochondria. Indeed, in failing myocardium, mitochondria appear to be a source of increased hydroxyl radical (OH·) production, possibly due to a decrease in complex I activity. However, given that the substrate NADPH produced much higher EPR signals than NADH in stretched myocytes, an NADPH-dependent oxidase such as the plasmalemmal NAD(P)H oxidase or nitric oxide synthase seems more likely to be the source of increased ROS in mechanically stretched myocytes. Further work is needed to elucidate the sources of ROS that are involved in the effects of mechanical stretch on myocyte growth and survival.

Role of ROS in Stretch-Induced Myocyte Hypertrophy

The magnitude of the growth response we observed with cyclic stretch is similar to that reported by others using tonic stretch. Both ³H-leucine incorporation and cellular protein content showed a graded increase with the amplitude of stretch. Protein synthesis was associated with increased expression of ANF, suggestive of a fetal phenotype that is typical of myocyte hypertrophy. Stretch-induced protein synthesis (but not ANF mRNA expression) was abolished by antioxidants. These results add to a growing literature suggesting that ROS can act as signaling mediators of growth.

In vascular smooth muscle cells, angiotensin increases ROS production resulting in cell growth through the activation of an NAD(P)H oxidase system. Angiotensin, tumor

Figure 5. Effect of the SOD mimetic MnTMPyP on stretch-induced increases in bax expression. A, Representative Northern blot showing bax mRNA and 18S rRNA expression in myocytes. B, Bax expression was normalized to 18S rRNA expression. C, Bax protein increases with high-level stretch and is inhibited with both MnTMPyP and EUK-8. Immunoblot is representative of 3 separate experiments.

Figure 6. Effect of stretch on activation of ERK1/2 and JNKs. Myocytes were stretched at 5% or 25% for 15 minutes, and levels of phosphorylated ERK and JNK were assessed in cell lysates using antibodies to phosphorylated active ERK1/2 or JNK. A, Representative immunoblots for phosphorylated ERK1/2. B, Densitometric analysis of ERK1/2 is expressed as percent change versus control. Stretch at low and high levels increased levels of phospho-ERK1/2 by 158±55.2% (n=4; *P=0.02) and 136.9±34% (n=6; **P=0.03), respectively. MnTMPyP inhibited the increase in ERK activity at both 5% (*P=0.03) and 25% (##P=0.04) stretch. MnTMPyP alone did not significantly alter ERK activation. C, Representative immunoblots for phosphorylated JNKs. D, Densitometric analysis of JNK phosphorylation is expressed as percent change versus control. Low-level stretch did not change the level of JNK phosphorylation whereas high levels increased JNK by 193.1±23.1% (n=3; *P=0.002). MnTMPyP inhibited the increase in JNK expression at high-level stretch (n=3; ##P=0.008) but had no effect on its own.
necrosis factor-α, and α-adrenergic stimulation likewise have been shown to cause myocyte hypertrophy through an ROS-dependent pathway. Endothelin appears to modulate early-response gene expression through a ROS-dependent pathway involving ras, and ouabain causes hypertrophy via ROS-dependent activation of a ras/MAPK pathway. Stretch-induced hypertrophic signaling has also been shown to involve a rac-dependent pathway. Rac is part of the NAD(P)H oxidase complex, again supporting the thesis that an NAD(P)H oxidase system may be an important source of ROS under these conditions.

Role of ROS in Stretch-Induced Apoptosis
The proapoptotic effects of high-amplitude stretch are complementary to the findings of Anversa and colleagues who reported that stretch of a papillary muscle preparation led to parallel increases in ROS formation and myocyte apoptosis. ROS are well known to induce apoptosis in many cell types including cardiac myocytes. Addition of extracellular sources of ROS, such as H₂O₂ or xanthine/xanthine oxidase, caused apoptosis in neonatal rat myocytes and a myocyte-derived cell line. Similarly, we and others have shown that an increase in intracellular ROS formation due to (a) inhibition of SOD, (b) addition of an O₂⁻ generator (eg, antracyclines), or (c) conditions that favor the formation of intracellular peroxynitrite results in increased myocyte apoptosis. Interestingly, we found that both low- and high-amplitude stretch increased ROS production, apoptosis was increased only with high-amplitude stretch. Likewise, we previously found that graded inhibition of SOD resulting in two levels of ROS caused hypertrophy at both ROS levels, but apoptosis only at the higher level. Taken together, these findings suggest that the quantity and/or quality of ROS is an important determinant of the activation of the apoptotic cascade.

ROS-Dependent Activation of MAPK
In cardiac myocytes, H₂O₂ and superoxide anion activate MAPKs that have been implicated in the regulation of cell growth. We found that mechanical stretch caused activation of ERK1/2 at both low and high amplitude, whereas JNK was activated only at high-amplitude stretch. The activation of both ERK1/2 and JNK was ROS-dependent. ERK1/2 has been implicated in mediating myocyte growth, whereas JNK has been implicated in mediating myocyte growth, death, and hypertrophy. At high versus low-amplitude stretch requires additional signaling pathways (eg, JNKs). Thus, differential activation of MAPK signaling pathways may be involved in the amplitude-dependent effects of stretch on myocyte phenotype.

Conclusion
Our data demonstrate that the effects of cyclic mechanical stretch on myocyte growth and death are amplitude-dependent and mediated by ROS. These findings further support the thesis that oxidative stress mediates important aspects of myocardial remodeling in response to hemodynamic overload. This in vitro system should provide the ability to understand the molecular mechanisms that determine the effects of mechanical overload on myocyte phenotype.

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