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Regulation of Cardiomyocyte Mechanotransduction by the Cardiac Cycle

Keiji Yamamoto, MD; Quynh N. Dang, BS; Yoshikazu Maeda, MD; Hayden Huang, SM; Ralph A. Kelly, MD; Richard T. Lee, MD

Background—Overloading the left ventricle in systole (pressure overload) is associated with a distinct morphological response compared with overload in diastole (volume overload).

Methods and Results—We designed a novel computer-controlled experimental system that interfaces biaxially uniform strain with electrical pacing, so that cellular deformation can be imposed during a specified phase of the cardiac cycle. Cardiomyocytes were exposed to strain (4%) during either the first third (systolic phase) or last third (diastolic phase) of the cardiac cycle. Strain imposed during the systolic phase selectively activated p44/42 mitogen-activated protein kinase (MAPK) and MAPK/extracellular signal-regulated protein kinase kinase (MEK1/2, an activator of p44/42 MAPK) compared with strain imposed during the diastolic phase. In contrast, there was no difference in activation of p38 and c-Jun NH₂-terminal kinases induced by strain imposed during the systolic phase (5.8- and 3.3-fold versus control, n=4) compared with the diastolic phase (5.5- and 3.1-fold). Induction of both brain natriuretic peptide (5.8-fold versus control, $P<0.05$, n=3) and tenascin-C (7.0-fold, $P<0.02$) mRNA expression by strain imposed during the systolic phase was greater than during the diastolic phase (3.9- and 3.6-fold, respectively). [³H]leucine incorporation induced by strain imposed during the systolic phase (4.0-fold versus control) was greater than during the diastolic phase (2.7-fold, $P<0.02$, n=4); a selective inhibitor of MEK1/2 inhibited this difference.

Conclusions—Mechanical activation of p44/42 MAPK and MEK1/2, gene expression, and protein synthesis is regulated by the cardiac cycle, suggesting that mechanotransduction at the cellular level may underlie differences between pressure and volume overload of the heart. (*Circulation*. 2001;103:1459-1464.)

Key Words: hypertrophy ■ strain ■ stress

Hemodynamic loading of the heart induces cardiac hypertrophy,¹⁻³ an independent risk factor of cardiac morbidity and mortality.⁴ Pressure-overload conditions, such as aortic stenosis and hypertension, result in concentric hypertrophy, which is characterized by an increase in ventricular wall thickness, little or no chamber dilation, and the parallel addition of sarcomeres.^{5,6} Conversely, volume-overload conditions, such as mitral regurgitation, promote an eccentric (dilated) form of hypertrophy, which is characterized by relatively little increase in wall thickness, a disproportionately large increase in chamber volume, and the serial addition of sarcomeres.^{7,8} The molecular mechanism by which these different hemodynamic loads lead to distinct forms of cardiac hypertrophy is unclear.

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Recent studies of myocardial hypertrophy have identified potential roles of activation of protein kinases, which precedes an increase in specific gene expression and protein synthesis.⁹⁻¹³ The mitogen-activated protein kinase (MAPK) pathways consist of 3 major phosphorylation cascades: the extracellular signal-regulated protein kinases 1 and 2

(ERK1/2, also called p44/42 MAPK), the c-Jun NH₂-terminal kinases (JNK), and the p38 MAPKs.¹⁴⁻¹⁶ When activated, the MAPKs translocate to the nucleus, where many transcription factors, their primary targets, are located.¹⁷ These transcription factors regulate the induction of genes that determine the ultimate biological response of the cells, including cardiac hypertrophy.

Experiments of mechanically overloading cardiomyocytes have generally been performed by stretching cells with no control of the cardiac cycle, an approach that does not allow distinction between mechanical overload in contraction versus relaxation. In the present study, we designed and constructed a unique experimental system that allows precisely controlled mechanical strains as well as electrical pacing in cultured cardiomyocytes to investigate how cardiomyocyte mechanotransduction is regulated by the cardiac cycle.

Methods

Pacing-Strain Device

The approach to mechanical stimulation used an apparatus that has multiple platens that contact the underside of silicone elastomer

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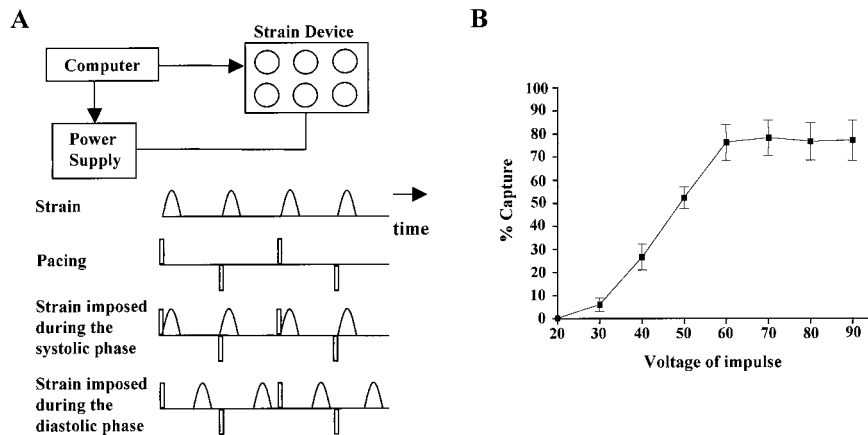


Figure 1. A, Diagram of pacing-strain device. To pace cells, electrical impulses had alternating polarity to minimize electrochemical effects such as pH gradients. Computer controls strain device; in these protocols, we used sinusoidal deformation to a set amplitude ("strain") and a specified "dwell time," during which strain is zero. By controlling both deformation and electrical pacing, device limits mechanical strain to period of contraction or relaxation of cardiomyocyte. Conditions used in this study were strain with no pacing, pacing with no strain, strain in systolic phase, and strain in diastolic phase. B, Capture efficiency in pacing-strain device. Nine locations on each dish were sampled. Capture efficiency was similar at all locations; maximal capture occurred at ≥ 60 V. Points with error bars represent mean \pm SD (n=9).

membranes to apply a spatially isotropic biaxial strain profile to the membrane.¹⁸ Six individual 78-mm membranes can be stretched at once with varying amplitudes of strain by controlling displacement of each platen with a stepper motor. Measured Green strains are accurate to $\pm 0.25\%$ at strains from 1% to 14%.^{19,20} Throughout this study, 4% biaxial strain was used.

To control the timing of mechanical strain relative to the cardiac cycle, the computer paced each dish electrically and controlled the phase between the mechanical strain and the electrical impulse, the electrical impulse duration, and the voltage of the impulse. In addition, the electrical impulses had alternating polarity to minimize electrochemical effects, such as pH gradients, at the electrodes. The 2 outputs were each connected to a single set of electrodes in each dish. The dishes were paced in parallel, with a resistance of $\approx 500 \Omega$ per dish.

The positive and negative voltage sources were provided by 2 power supplies (6545A, Hewlett Packard Co). The control circuit was divided into 2 parts: a high-voltage circuit and a low-voltage or digital-signal circuit. The high-voltage circuit was a gate that switched the output on the basis of the input signal. The low-voltage circuit accepted 2 control signals from the computer and accepted the pulse width from a variable resistor, which controlled both the positive and negative voltage gates. The low-voltage circuit allowed a voltage pulse between 0 and 120 V DC amplitude and 2 and 37 ms duration. Lights provided continuous monitoring of the pulses, and the timing of the circuits and calibration were validated by oscilloscope.

The electrodes for each dish were 2 arc-shaped AgCl₂ wire electrodes at the base of the inner surface of the dish, just above the deformable membrane. The electrodes were premade, ethanol-sterilized, and placed into the dish just before each experiment to minimize potential toxicity from silver. Using this method, we have observed no cellular death or detachment in 24-hour experiments. Each arc is 120°; we performed a 2D finite-element analysis to estimate the uniformity of the potential field with this configuration. These calculations estimate a spatial variation in the potential field of (root mean square)=29%. Thus, this system provides highly uniform biaxial mechanical strain, with a relatively small variation in the voltage field.

Mechanical Stimulation Protocols

We imposed strain only during the first third of the cardiac cycle by electrical stimulation for strain imposed during the "systolic phase," and only during 1 third of the cardiac cycle in the relaxation phase for strain imposed during the "diastolic phase" (Figure 1A). Conditions used in this study were (1) control; (2) strain, no pacing; (3) pacing, no strain; (4) strain imposed during the systolic phase; and (5) strain imposed during the diastolic phase.

Neonatal rat ventricular myocytes (NRVMs) from 1-day-old Sprague-Dawley rats were isolated by previously described meth-

ods.²¹ NRVMs were plated on the coated membrane dish at a density of 2 000 000 cells/dish in DMEM containing 7% FCS and incubated for 24 hours. Approximate cell confluence was 85% to 90%. NRVMs were then made quiescent by washing with 10 mL of HBSS (in mmol/L: NaCl 138, KCl 5.3, NaHCO₃ 4.0, CaCl₂ 1.3, MgCl₂ 0.5, MgSO₄ 0.4, KH₂PO₄ 0.4, Na₂HPO₄ 0.3, and glucose 5.6; Life Technologies, Inc) twice and incubating with 26 mL of DMEM containing 0.2% FCS for 48 to 72 hours.

In these cell culture conditions, cells beat at 40 to 60 bpm. At this rate, we have observed negligible competition when pacing at a rate of 70 bpm. We performed trial capture experiments; 9 locations on each dish were sampled. Capture efficiency was similar at all locations, and maximal capture occurred at ≥ 60 V with 10 ms of pulse width (Figure 1B). Therefore, a voltage of 70 V with 10 ms of impulse duration at a rate of 1.2 Hz (70 bpm) was selected. Under these conditions, we did not observe partial cell detachment.

p44/42 MAPK, MEK1/2, p38 MAPK, and JNK Phosphorylation

Medium was changed to fresh DMEM containing 0.2% FCS 2 hours before experimentation. After exposure to pacing and/or strain, cells were washed 3 times with ice-cold PBS (Life Technologies, Inc) and placed on ice. Cells were lysed with ice-cold buffer (1% Triton X-100; in mmol/L: Tris 20 [pH 7.5], NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β -glycerol phosphate 1, sodium orthovanadate 1, and PMSF 1; and leupeptin 1 μ g/mL). Extracted protein was quantified by the Bradford method (Bio-Rad Laboratories), and equal quantities of total protein were loaded on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane in 25 mmol/L Tris base (pH 8.5), 0.2 mol/L glycine, and 20% methanol. Specific phosphorylated forms of proteins were detected by the Phospho-HRP Western Detection System (New England Biolabs, Inc).

Northern Analysis

Total RNA was isolated by the guanidinium thiocyanate and phenol chloroform method.²² The primer set for the synthesis of the 450-bp tenascin-C cDNA probe contained the 5'-TCTGTCTGG-ACTGCTGATG-3' and 5'-TCTTCAAATCCCTTCATGGC-3' oligonucleotides. Rat brain natriuretic peptide (BNP) cDNA was kindly provided by Dr David Gardner, University of California, San Francisco. These cDNAs were radiolabeled by the random priming method with [α -³²P]dCTP. For Northern blotting, 15 μ g of total RNA was loaded onto a 1.0% formaldehyde gel (2.0 mol/L), transferred to a nylon membrane, and UV cross-linked. The probe was hybridized with QuikHyb solution (Stratagene) at 68°C for 1 hour. Levels of BNP and tenascin-C mRNA were measured by densitometry of the Northern blot autoradiographs using Optimas 5.0 densitometry software.

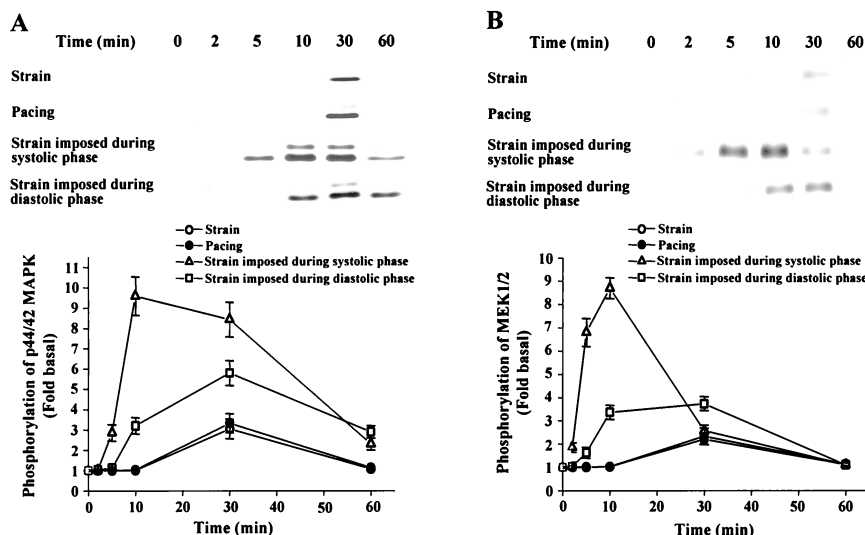


Figure 2. Phosphorylation of p44/42 MAPK and MEK1/2. After exposure to pacing and/or strain for indicated time, cell extracts were subjected to immunoblotting with anti-phospho-p44/42 MAPK (A) and anti-phospho-MEK1/2 (B). A representative immunoblot from 3 independent experiments is shown. Levels of phosphorylation were determined from immunoblots by densitometric analysis (mean ± SD, n=3). Strain imposed during systolic phase led to more rapid and greater phosphorylation of p44/42 MAPK and MEK1/2.

Protein Synthesis

Cells were subjected to pacing and/or mechanical strain for 1 hour and then incubated in 13 mL of fresh DMEM containing 1% insulin, transferrin, selenium media supplement (ITS; Sigma Chemical Co) with 1.0 μCi/mL [³H]leucine for an additional 24 hours. The medium was aspirated, and the cells were washed twice with ice-cold PBS and once with 10% trichloroacetic acid (TCA; Sigma) and fixed for 45 minutes at 4°C with 10% TCA. After 2 washings with cold 95% ethanol, the radioactivity incorporated into the TCA-precipitable material was determined by liquid scintillation counting after solubilization in 0.15N NaOH. In addition, an aliquot was taken for determination of total protein. The total cardiomyocyte protein was not significantly altered by pacing and/or stretch (in μg/dish: control, 171.0 ± 7.4, n=4; strain, 174.8 ± 7.2; pacing, 175.5 ± 7.3; strain imposed during the systolic phase, 179.0 ± 8.5; and strain imposed during the diastolic phase, 176.0 ± 8.3).

Statistical Analysis

Data are expressed as the mean ± SD. Differences were compared by 1-way ANOVA and Dunnett's t test or unpaired 2-tailed Student's t test as appropriate. A value of P < 0.05 was considered significant.

Results

Activation of Kinases

We first investigated the pattern of activation of the various MAPKs in this system. Activation of p44/42 MAPK by strain

imposed during the systolic phase (9.6 ± 1.0-fold versus control) was rapid, with a maximum peak at 10 minutes. In contrast, activation of p44/42 MAPK by strain imposed during the diastolic phase (5.8 ± 0.6-fold, P < 0.01) was later and less intense, with a maximum peak at 30 minutes. Strain only or pacing only marginally activated p44/42 MAPK (Figure 2A). In addition, phosphorylation of MEK1/2, an upstream activator of p44/42 MAPK, by strain imposed during the systolic phase (8.7 ± 0.5-fold) with a maximum peak at 10 minutes was also more rapid and greater than during the diastolic phase (3.7 ± 0.3-fold, P < 0.01), with a maximum peak at 30 minutes, whereas strain only or pacing only marginally activated MEK1/2 (Figure 2B).

In contrast, activation of p38 MAPK by strain imposed during the systolic (5.8 ± 0.5-fold) and diastolic (5.5 ± 0.6-fold) phases with a maximum peak at 10 minutes was not significantly different (Figure 3A). Similarly, strain only or pacing only did not activate JNK, and there was no difference in phosphorylation of JNK, with a maximum peak at 30 minutes induced by strain imposed during the systolic (3.3 ± 0.3-fold) compared with the diastolic (3.1 ± 0.4-fold) phase (Figure 3B). These findings suggest that although

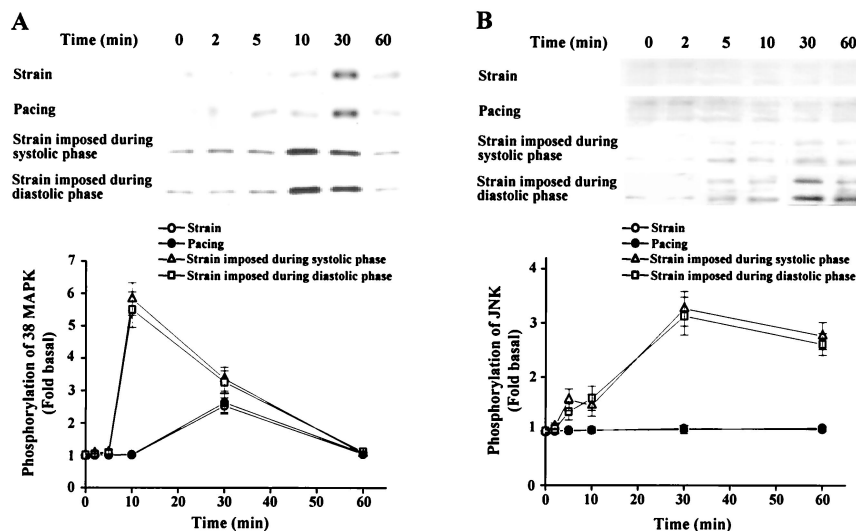


Figure 3. Phosphorylation of p38 MAPK (A) and JNK (B). After exposure to pacing and/or strain for indicated time, cell extracts were subjected to immunoblotting with anti-phospho-p38 MAPK (A) and anti-phospho-JNK (B). Representative immunoblot from 3 independent experiments. Levels of phosphorylation were determined from immunoblots by densitometric analysis (mean ± SD, n=3). In contrast to p44/42 MAPK and MEK1/2, strain imposed during systolic phase did not increase phosphorylation of p38 MAPK and JNK compared with diastolic phase.

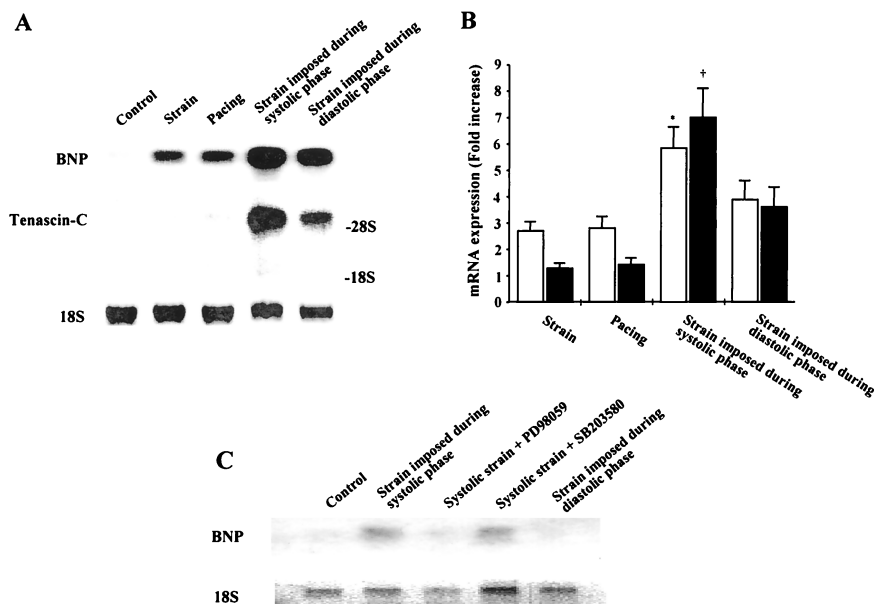


Figure 4. Effects of cardiac cycle on BNP and tenascin-C mRNA expression induced by mechanical strain. **A**, NRVMs were subjected to pacing and/or strain for 1 hour. Total RNA was isolated 6 hours later and analyzed by Northern blotting with ^{32}P -labeled BNP (top) and tenascin-C (middle) cDNA probes. Ethidium bromide-stained 18S ribosomal subunit (bottom) is also shown. Data are representative of 3 experiments that gave nearly identical results. **B**, Results represent fold increase vs control in BNP (open bar) and tenascin-C (solid bar) mRNAs. Bars with error bars represent mean \pm SD ($n=3$). * $P<0.05$ vs strain imposed during diastolic phase; † $P<0.02$ vs strain imposed during diastolic phase. **C**, Effects of MAPK inhibitors on BNP mRNA induction by strain imposed during systolic phase. NRVMs were subjected to pacing and strain for 1 hour in absence or presence of PD98059 (50 $\mu\text{mol/L}$) or SB203580 (10 $\mu\text{mol/L}$). Total RNA was isolated 6 hours later and analyzed by Northern analysis. Ethidium bromide-stained 18S ribosomal subunit is also shown. Data are representative of 2 experiments that gave nearly identical results.

MEK1/2 and p44/42 MAPK pathways may be involved in regulation of the cardiac cycle, activation of the p38 and JNK pathways is not sensitive to the phase of the cardiac cycle.

Gene Expression

We next investigated whether the cardiac cycle affects the expression of BNP mRNA, an angiotensin II-dependent marker for cardiac hypertrophy,^{23,24} and tenascin-C mRNA, an angiotensin II-independent early marker for cardiac remodeling.²⁵ Both BNP and tenascin-C mRNA expressions were induced by strain only or pacing only (Figure 4A and 4B). In addition, induction of both BNP (5.8 ± 0.8 -fold versus control, $P<0.05$) and tenascin-C (7.0 ± 1.1 -fold, $P<0.02$) mRNA expression by strain imposed during the systolic phase at 1.2 Hz was greater than that imposed during the diastolic phase (3.9 ± 0.7 - and 3.6 ± 0.8 -fold, respectively). Next, we investigated the effects of PD98059, a MEK1/2 inhibitor, and SB203580, a p38 MAPK inhibitor, on BNP mRNA induction by strain imposed during the systolic phase. We found that BNP mRNA induction by strain imposed during the systolic phase was inhibited by PD98059 but not by SB203580 (Figure 4C).

Protein Synthesis

Both strain-only (1.8 ± 0.5 -fold versus control, $P<0.05$) and pacing-only (1.9 ± 0.5 -fold, $P<0.02$) conditions increased leucine uptake. Strain imposed during the systolic (4.0 ± 0.6 -fold, $P<0.01$) and diastolic (2.7 ± 0.5 -fold, $P<0.01$) phases induced a significant increase in [^3H]leucine incorporation (Figure 5A). There was a significant difference in [^3H]leucine incorporation between strain imposed during the systolic and

diastolic phases ($P<0.02$, $n=4$). As shown in Figure 5B, PD98059 completely inhibited the activation of p44/42 MAPK by strain imposed during the systolic phase in a dose-dependent manner. PD98059 (50 $\mu\text{mol/L}$) significantly inhibited the increase in [^3H]leucine incorporation induced by strain imposed during the systolic phase (2.3 ± 0.3 -fold, $P<0.01$) but not by strain imposed during the diastolic phase (Figure 5A). In contrast, in the presence of SB203580 (10 $\mu\text{mol/L}$), [^3H]leucine incorporation was significantly different between strain imposed during the systolic phase (3.1 ± 0.4 -fold, $P<0.02$) and strain imposed during the diastolic phase (1.9 ± 0.2 -fold). These findings suggest that the MEK1/2 and p44/42 MAPK pathways play a role in the regulation of mechanically induced protein synthesis by the cardiac cycle.

Discussion

Although Grossman et al²⁶ described morphological differences in the left ventricles of subjects with pressure-overload and volume-overload hypertrophy in 1975, the molecular basis for these differences remains incompletely defined. The present study demonstrated that selective mechanical activation of p44/42 MAPK and MEK1/2, gene expression, and protein synthesis are regulated by the cardiac cycle in vitro.

The MAPK cascades participate in the differentiation and proliferation of many types of cells.²⁷ Although activation of p44/42 MAPK is insufficient to fully promote cardiac hypertrophy,²⁸ p44/42 MAPK is necessary for phenylephrine-induced sarcomerogenesis and increase in cell size.²⁹ Interestingly, Wang et al³⁰ reported that p38 α and p38 β pathways

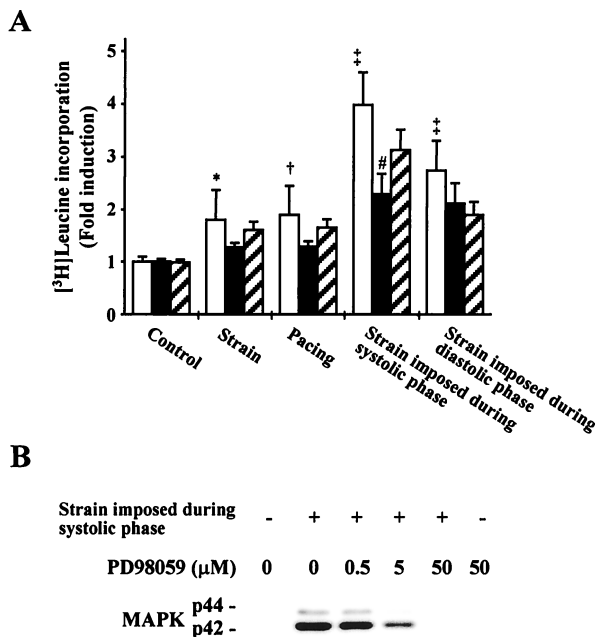


Figure 5. A, Effects of cardiac cycle on protein synthesis. NRVMs were subjected to pacing and/or strain for 1 hour and then incubated with 1.0 $\mu\text{Ci}/\text{mL}$ [^3H]leucine for an additional 24 hours in absence (open bar) or presence of PD98059 (50 $\mu\text{mol}/\text{L}$) or SB203580 (10 $\mu\text{mol}/\text{L}$). PD98059 (solid bar) or SB203580 (hatched bar) was applied to myocytes 30 minutes before experiments. Protein synthesis results are expressed as relative cpm/dish standardized to mean cpm of control cells in each experiment. Bars with error bars represent mean \pm SD ($n=4$). * $P<0.05$ vs control; † $P<0.02$ vs control; ‡ $P<0.01$ vs control; # $P<0.01$ vs nontreated. B, Effects of PD98059 on phosphorylation of p44/42 MAPK by strain imposed during systolic phase. After exposure to strain imposed during systolic phase for 10 minutes, cells were lysed with ice-cold lysis buffer. PD98059 was applied to some myocytes 30 minutes before experiments. Cell extracts were subjected to immunoblotting with anti-phospho-p44/42 MAPK. Representative immunoblot from 2 independent experiments. PD98059 (50 $\mu\text{mol}/\text{L}$) inhibited phosphorylation of p44/42 MAPK and inhibited induction of protein synthesis by strain imposed during systolic phase.

are directly involved in apoptosis and cardiac hypertrophy, respectively, and the role of JNKs is unclear.³¹ We have not observed marked JNK activation with deformation, in contrast to some published reports.^{12,32} In the present study, activation of p44/42 MAPK and MEK1/2 by strain imposed during the systolic phase was more rapid and greater than that imposed during the diastolic phase. Furthermore, a MEK1/2 inhibitor, PD98059, abolished the increase in [^3H]leucine incorporation by strain imposed during the systolic phase compared with the diastolic phase. SB203580 significantly inhibited the increase in the [^3H]leucine incorporation by strain in the systolic as well as the diastolic phase. Even in the presence of SB203580, however, a significant difference remained between strain in the systolic phase and strain in the diastolic phase, in contrast to the results with PD98059. These findings are consistent with the hypothesis that although p38 plays a role in mechanically induced protein synthesis, the MEK1/2-p44/42 MAPK pathway may play a more specific role in the difference between systolic and diastolic phase strain-induced protein synthesis.

Although the effect of the kinase inhibitor PD98059 is informative, it is important to note that this compound may have nonspecific effects.³³ It is also important to note that although a 4% change in tissue strain is a physiologically reasonable value, cellular strains in vivo are poorly described. Therefore, the extrapolation of these results to in vivo loading requires caution. Changes in cytoskeleton and matrix adhesions in cultured cells compared with the intact heart as well as interactions with other cell types and anisotropy of the matrix may be important factors. Finally, we cannot exclude some nonlinear effects on our quantification, because we did not perform internal quantitative controls in every experiment.

It is possible that the increases in MAPK activation, gene expression, and protein synthesis with systolic phase strain are due to sensitivity of the cell to strain energy. Thus, imposing a deformation during systole (when cell stiffness is greater) could deliver more strain energy to the cell and increase the signaling for gene expression and protein synthesis. This is one mechanism by which selective regulation of mechanically induced events by the cardiac cycle may participate in determining differences between pressure overload and volume overload of the left ventricle.

Acknowledgments

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