Activation of Heat-Shock Factor by Stretch-Activated Channels in Rat Hearts

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Background—Previously, we have observed that the isolated, erythrocyte-perfused rabbit heart has increased levels of heat-shock protein (HSP) 72 after a mild mechanical stress. We hypothesized that stretch-activated ion channels (SACs) mediated this increase.

Methods and Results—To test this hypothesis, we subjected isolated, perfused rat hearts to mechanical stretch. Gel mobility shift assay showed that heat-shock factor (HSF) was activated in hearts with mechanical stretch, but not in controls. Supershift experiments demonstrated that HSF1 was the transcription factor. Northern blots revealed the concomitant increase in HSP72 mRNA in stretched rat hearts. In a separate set of experiments, gadolinium, an inhibitor of SACs, was added to the perfusate. Gadolinium inhibited the activation of HSF and decreased HSP72 mRNA level. Because gadolinium can inhibit both SACs and L-type calcium channels, we perfused a group of hearts with diltiazem, a specific L-type calcium channel blocker, to eliminate the involvement of L-type calcium channels. Diltiazem failed to inhibit the activation of HSF.

Conclusions—Stretch in the rat heart results in activation of HSF1 and an increase in HSP72 mRNA through SACs. This represents a novel mechanism of HSF activation and may be an important cardiac signaling pathway for hemodynamic stress. (Circulation. 2001;104:209-214.)

Key Words: stretch n ion channels n calcium n proteins

Cells from all organisms respond to stress by synthesizing heat-shock, or stress, proteins (HSPs). HSPs are a group of highly conserved proteins classified by their molecular weights. Many studies have demonstrated that HSPs play an important role in the protection of stressed organisms.1–4

The regulation of heat-shock response is mediated by cytosolic proteins known as heat-shock factors (HSFs) that interact with a specific regulatory element, the heat-shock element, in the promoter regions of the HSP gene.5 HSF activation is one of the earliest responses observed in many different cells exposed to stresses, such as ischemia, hypoxia, hyperthermia, and mechanical stretch.6–9

Mechanical stretch and pressure overload have been shown to induce HSP72 expression in a variety of different cells and tissues.8,10–12 Several studies reported that stretch resulted in HSF1 activation and HSP72 expression in rat aorta,8,13,14 but few data are available for the heart.9,15 Previously, we observed that even a single myocardial stretch can cause increased expression of HSP72 in the isolated erythrocyte-perfused rabbit heart.15 The mechanism is unknown, but several research groups have postulated that stretch-activated ion channels (SACs) may act as mechanotransducers to mediate stretch-induced gene expression.16,17 As yet, however, there is no direct evidence that stretch-induced HSP72 expression is mediated by SACs.

Therefore, the purpose of this study was 3-fold. First, we used 2 different standard isolated heart perfusion models (Langendorff and working) to demonstrate that stretch alone was sufficient to induce the heat-shock response in rat hearts. These models eliminated any influence of circulating neural and/or humoral factors. Second, we tested the hypothesis that the induction of HSP72 mRNA was via the activation of HSF, specifically HSF1. Third, we used gadolinium, an SAC blocker, to test the hypothesis that the stretch-induced heat-shock response was mediated by SACs.

Methods

Langendorff Perfusion

Male Sprague-Dawley rats (228±9 g, n=43; Harlan Inc, Indianapolis, Ind) were heparinized and anesthetized with sodium pentobarbital. The heart was removed and placed in ice-cold Ringer’s solution (mmol/L: NaCl 120.0, KCl 4.7, NaHCO3 18.0, glucose 11.0, MgSO4 1.5, and CaCl2 2.5, equilibrated with 95% O2/5% CO2). Retrograde perfusion (Langendorff) was quickly established with a perfusion pressure of 95 to 100 cm H2O. The pH and temperature of

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the perfusate were 7.4 and 37°C, respectively. A PE90 tubing was inserted into the left ventricle (LV) and through the apex for thebesian drainage. We placed a fluid-filled latex balloon into the LV via the left atrium and connected it to a transducer to monitor pressures. Initial LV end-diastolic pressure (LVEDP) was adjusted to 10 mm Hg by adjustment of balloon volume. The heart was submerged in a water-jacketed constant-temperature chamber that contained Ringer’s solution equilibrated with 95% O₂/5% CO₂.

The protocol was approved by the Animal Welfare Committee of Texas A&M University.

**Working Heart Perfusion**

The heart was perfused by the Langendorff method first without balloon or ventricular drain. The left atrium was connected to a second cannula and perfused at a preload pressure of 10 to 15 cm H₂O. Afterload was set at 50 to 70 cm H₂O by adjustment of the height of the aortic outflow line above the heart. A compliance chamber was added to the aortic outflow line. The heart was perfused in the working mode (ejecting LV).

**Maximum Ventricular Developed Pressure**

To monitor cardiac contraction, we collected cardiac maximum ventricular developed pressure (P_max) throughout the experiments with a pressure transducer connected to a computerized data-acquisition system (Labview).

**Gel Mobility Shift Assay**

As described previously, a single-strand self-complementary oligonucleotide containing the 5’-nGAAn-3’ repeats was synthesized (5’-CTAGAAGCTTCTAGAAGCTTCTAG-3’), annealed, and end-labeled with [γ-³²P]ATP.¹⁸,¹⁹ Because HSF is normally present in the cell in an inactive form, we were able to use whole-cell lysates for our studies. Samples were processed as previously described. Cold competition and supershift studies were done as previously described.

**RNA Isolation and Northern Blot Analysis**

After each experiment, heart ventricles were rapidly dissected and freeze-clamped in liquid nitrogen. Total RNA isolation was performed with a STAT-60 kit (Tel-test, Inc). Northern blotting was performed as described previously.⁶,¹⁵

**Experimental Protocols**

All hearts were perfused for a 20-minute stabilization period followed by 1 of 7, 60-minute experimental protocols (Figure 1). As a positive control, 2 working hearts were perfused at 43°C for 13 minutes.

**Statistical Analysis**

Data were analyzed by 1-way ANOVA, followed by the Student-Newman-Keuls test (SigmaStat software, SPSS Inc). A value of P<0.05 was considered significant. Data are presented as mean±SEM.

**Results**

**HSF Activation Was Observed in Langendorff Stretched Hearts but Not in Working Hearts**

Previously, we observed that a mechanical stretch induced the expression of HSP72¹⁵ in the rabbit heart. In the present study, we again demonstrated that mechanical stretch, which included stretch from insertion of the apical drain and placement of the ventricular balloon, initiated the heat-shock response in the Langendorff perfusion. The gel mobility shift assays (GMSAs) showed that this stretch activated HSF (Figure 2A). In pilot experiments, the effects of different amounts of stretch on HSF activity were examined. This was done by varying the initial LVEDP in Langendorff perfusions. As shown in Figure 2B, an LVEDP of 10 mm Hg correlated with the strongest HSF activation (lane B in Figure 2B), followed by an LVEDP of 7 to 8 mm Hg (lane C in Figure 2B). Only minimal HSF activation occurred in the
heart with an LVEDP of 5 mm Hg (lane D in Figure 2B). Thus, the increase in HSF activation induced by stretch is correlated with the amount of stretch imposed on the heart. In contrast, working hearts, which are closer to a physiological perfusion, showed little activation of HSF (C lanes in Figure 3A) compared with the Langendorff-perfused hearts (A lanes in Figure 3A). To further test that mechanical stretch can induce the activation of HSF, we perfused a separate set of hearts in the working mode using an aortic cannula with a small diameter (<1 mm in ID) to mimic aortic stenosis. LV afterload was thereby increased, and the ventricular wall stress was enhanced. This resulted in a degree of activation of HSF similar to that observed in Langendorff hearts (B lanes, Figure 3A).

Specific activation of HSF was confirmed by the cold heat-shock element competition assay (B lanes, Figure 3B). The addition of antibodies to HSF1 and HSF2 showed a supershift only with anti-HSF1 (C lanes, Figure 3B), indicating that it is HSF1 that is activated by stretch. HSF2 was not activated (no supershift bands in D lanes, Figure 3B).

Gadolinium but Not Diltiazem Inhibits Mechanical Stretch–Induced Activation of HSF
We perfused hearts in the Langendorff mode with gadolinium, an SAC blocker. As shown in Figure 4 (C and D lanes) and Figure 5 (B and C lanes), gadolinium 10 and 50 μmol/L markedly decreased HSF activation. Densitometric measurement of the GMSA showed a 45% and a 50% decrease in HSF binding activity, respectively, compared with Langendorff perfusion without gadolinium (Figure 5A, P<0.05). Although the high dose of gadolinium tended to suppress HSF activation more than the low dose, this was not statistically significant.

Because gadolinium can block both SACs and L-type calcium channels, it was important to identify whether HSF activation was mediated by L-type calcium channels. To test this, hearts were perfused with diltiazem, a specific L-type calcium channel blocker. Two different concentrations (1 and 3 μmol/L) of diltiazem were used, and both failed to inhibit the activation of HSF (D lanes in Figure 5B, and F and G lanes in Figure 6), indicating that gadolinium attenuates HSF activity via SACs. The high concentration of diltiazem (3 μmol/L), if anything, increased activation of HSF (lane G, Figure 6). Figure 6 summarizes HSF binding activities in hearts from all of the experimental protocols: no perfusion (A), working perfusion (B), Langendorff perfusion (C), 10 μmol/L gadolinium (D), 50 μmol/L gadolinium (E), 1 μmol/L diltiazem (F), and 3 μmol/L diltiazem (G). As a positive control, hearts with heat shock (43°C for 13 minutes) were analyzed and showed similar degrees of activation of HSF (H lanes, Figure 6).

Analysis in HSP72 mRNA Expression
After 60 minutes, we found that the HSP72 mRNA level in Langendorff-perfused rat hearts increased compared with nonperfused control rat hearts (Figure 7). Northern blotting showed that gadolinium attenuated this increase at both doses (Figure 7). No dose-dependent inhibition was observed. This observation is consistent with the GMSA, which showed that HSF activity was decreased by 50% by gadolinium.
Mechanical stresses, such as hemodynamic overload or mechanical stretch, alter the protein expression pattern in cardiac and skeletal muscles. For instance, Peterson and Lesch\(^{20}\) first reported that stretch could accelerate protein synthesis and amino acid transport. A similar phenomenon was observed in rat hearts subjected to high aortic pressures.\(^{21}\) Subsequent studies showed that the HSP72 gene and the “immediate early” (IE) genes, such as c-fos, c-myc, c-jun, JE, and Egr-1, were involved in the early response to mechanical stresses, followed by reexpression of fetal contractile protein genes, such as skeletal α-actin, β-myosin heavy chain (MHC), and atrial natriuretic peptide (ANP) genes.\(^{9,22–25}\) How cells sense mechanical stimuli, transmit, and how this translates into gene expression is not understood. Increased sympathetic nervous activity and increased catecholamine levels have been suggested as potential mediators for induction of cardiac hypertrophy and IE gene expression.\(^{26,27}\) Since Vandenburgh and Kaufman\(^{28}\) reported that mechanical stretch without confounding factors caused increased protein synthesis in cultured skeletal muscle cells, however, many studies have demonstrated that mechanical stimuli alone can directly induce gene expression, including HSPs.\(^9,10,12,15,23,24\)

In our study, we found that mechanical stretch and isovolumic contraction are sufficient to initiate the heat-shock response without neural and humoral factors. These observations extend our previous observations in the erythrocyte-perfused rabbit heart, in which other factors, such as cytokines, could have had an effect, and demonstrate the activation of HSF via SACs in response to stretch.\(^{15}\)

It has been postulated that SACs may function as a mechanotransducer between mechanical load and alterations in protein synthesis in cardiac hypertrophy.\(^{16,29}\) Mechanosensitive ion channels have been found in a broad variety of cell types.\(^{30}\) Activation of stretch-dependent channels results in cation influx, which is associated with gene expression and protein synthesis.\(^{16,31}\) We found that gadolinium decreased HSF activity by half and had a similar effect on HSP72 mRNA levels. Although other studies have shown a dose response,\(^{31}\) the small sample size may account for the lack of dose-dependent inhibition in the present study, or alternatively, a second activating pathway may be involved. Gadolinium 10 μmol/L may have completely inhibited the SACs, even though it did not abolish HSF1 activation. A 5-fold increase in concentration had no further effect; thus, a second pathway may be involved in activation of HSF1 with stretch.

Because it is known that gadolinium also blocks L-type calcium channels,\(^{31,32}\) we used diltiazem, a specific L-type calcium channel blocker, to exclude its effects, and it did not block the activation of HSF. Similar observations, ie, the noninvolvement of L-type calcium channels in the stretch response, were made by Laine et al\(^{33}\) and Komuro et al.\(^{23}\) We did not observe a negative inotropic effect with gadolinium in the concentrations used.

Gadolinium does not affect the stretch-induced expression of IE genes in cultured neonatal cardiac myocytes.\(^{17}\) It has been shown that multiple second messenger factors, such as mitogen-activated protein kinases and protein kinase C, may be involved in the signal transductions for IE gene expression via the activation of serum response factor–P62TCF com-

**Discussion**

In the present study, we demonstrated that stretch initiated the heat-shock response in rat hearts by activating HSF1, and this was followed by an increase in HSP72 mRNA. The intensity of HSF activation correlated with the amount of stretch, suggesting that the magnitude of stretch influences the degree of HSF1 activation. HSF1 activation was blocked by gadolinium, but not by diltiazem, which indicates that the stretch-induced heat-shock response was mediated by SACs. An alternative perfusion system, the working heart, was used to determine whether perfusion alone was sufficient to activate HSF. Perfusion in the working mode, which is closer to physiological perfusion and does not use an apical drain or ventricular balloon, did not activate HSF; however, HSF could be activated by increasing afterload (aortic stenosis), which effectively added an acute stretch.

Maximum Ventricular Developed Pressure

\(P_{\text{max}}\) from all groups was stable during perfusions (Figure 8). There was no significant difference in \(P_{\text{max}}\) between the start and completion of perfusion for any of the groups. Furthermore, there was no significant difference in \(P_{\text{max}}\) among the gadolinium, diltiazem, and working-mode groups compared with Langendorff-perfused control hearts.

**Figure 5.** A, Comparison of HSF activation in (bar A) Langendorff \((n=4)\) and (bar B) Langendorff with 10 μmol/L gadolinium \((n=2)\), (bar C) with 50 μmol/L gadolinium \((n=3)\), and (bar D) with 1 μmol/L diltiazem \((n=3)\) rat hearts. *P<0.05 vs control Langendorff. B, Representative gel shift for gadolinium and diltiazem effects on Langendorff (stretched) rat hearts. Abbreviations as in previous figures.
plex. Induction of these pathways and induction of the IE genes lead to cardiac hypertrophy. 30 Induction of HSPs is not per se associated with hypertrophy, nor does overexpression of the HSPs result in hypertrophy. 34 Thus, the activation of the heat-shock response by stretch may involve pathways distinct from the hypertrophic response. A recent study showed that mitogen-activated protein kinase specific inhibitors did not affect stretch-induced HSF1 activation in vascular smooth muscle cells, and our previous work suggested that protein kinase C does not play an important role in stretch-induced HSP72 expression.12,15

There are several additional differences between our work and the studies of stretch-induced IE genes. Not only are we studying a different set of genes, but we also used the intact, perfused heart as a model. The use of isolated cardiac myocytes, although an excellent model, may eliminate or modulate extracellular signaling. Furthermore, we have focused on the adult heart, and responses in the neonatal heart, in which the induction of IE genes has primarily been studied, may be different. Thus, differences in induction of the heat-shock response and the IE genes may reflect differences in overall regulation and/or differences in models.

Others have observed that volume overload could precondition the heart, and both this stretch-induced preconditioning and classic preconditioning were blocked by gadolinium. 35 They suggested that volume overload, “stretch,” preconditioned the heart through the activation of SACs. Because brief cardiac ischemia/reperfusion results in the upregulation of HSP72 and short ischemia could lead to a transient cardiac dilation (stretching), we suggest that the activation of SACs may be part of the common pathway in the initiation of the cellular stress response in the heart. Certainly, other important factors are present in ischemia, such as denatured proteins, but stretch may be a factor, particularly in the nonischemic region of the ventricle. Previously, we reported that we did not detect a further increase in HSP72 mRNA after ischemia in erythrocyte-perfused hearts compared with control perfusions. 6 We may have already maximally induced the heat-shock response. With the current understanding of cytokines, it may be that in this preparation, which used erythrocytes from cows in the slaughterhouse, cytokines may have had an additive effect; however, perfusion without stretch, but with the erythrocytes, did not induce HSP72. 36 More recently, knowing the effect of stretch, we took measures, such as reduced balloon volume/LVEDP (6 mm Hg), to prevent stretch. This resulted in minimal activation of HSF in control hearts, and we were able to correlate changes in ATP during ischemia/reperfusion with the degree of activation of HSF. 19

In conclusion, we demonstrate that stretch is sufficient to activate HSF1 and increase HSP72 mRNA expression and that SACs are involved in the activation of HSF1 and upregulation of HSP72 mRNA. This represents a novel pathway for the activation of HSF1. Although these results have implications for researchers using the isolated, perfused rat heart preparation, the overall implications are far greater. Stretch is a common phenomenon in both the normal and diseased cardiovascular system. Increased stretch occurs with hypertension, heart failure, and myocardial infarction, for

Figure 6. Comparison of HSF activity in non-perfused (A), working perfusion (B), Langendorff perfusion (C), Langendorff with gadolinium (D, 10 μmol/L; E, 50 μmol/L), Langendorff with diltiazem (F, 1 μmol/L; G, 3 μmol/L), and working perfusion (43°C for 13 minutes) (H). Abbreviations as in previous figures.

Figure 7. Northern blot of HSP72 mRNA present in Langendorff-perfused rat hearts with and without gadolinium.

Figure 8. Pmax at start and end of experiments. There were no significant differences in developed pressure for gadolinium-treated, diltiazem-treated, and 2 working-mode hearts vs Langendorff-perfused control hearts. No difference in Pmax was found between start and end of experiment within each group.
example, and SACs may be an important signaling pathway for the induction of the stress response in these settings. Furthermore, the heart has higher levels of HSP72 than many other tissues (unpublished results), and this may be related to the repetitive stretch that occurs as part of normal cardiac function.

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