Mechanical Stress–Induced Heat Shock Protein 70 Expression in Vascular Smooth Muscle Cells Is Regulated by Rac and Ras Small G Proteins but Not Mitogen-Activated Protein Kinases

Qingbo Xu, Georg Schett, Chaohong Li, Yanhua Hu, Georg Wick

Abstract—Previous studies have documented that acute elevation in blood pressure results in heat shock protein (hsp) 70–mRNA expression followed by hsp70-protein production in rat aortas. In this article, we provide evidence that mechanical forces evoke rapid activation of heat shock transcription factor (HSF) and hsp70 accumulation. In our study, Western blot analysis demonstrated that hsp70-protein induction peaked between 6 and 12 hours after treatment with cyclic stain stress (60 cycles/minute, up to 30% elongation). Elevated protein levels were preceded by hsp70-mRNA transcription, which was associated with HSF1 phosphorylation and activation stimulated by mechanical forces, suggesting that the response was regulated at the transcriptional level. Conditioned medium from cyclic strain–stressed vascular smooth muscle cells (VSMCs) did not result in HSF-DNA–binding activation. Furthermore, mitogen-activated protein kinases (MAPKs), including extracellular signal–regulated kinases, c-Jun NH2-terminal protein kinases or stress-activated protein kinases, and p38 MAPKs, were also highly activated in response to cyclic strain stress. Inhibition of extracellular signal–regulated kinase and p38-MAPK activation by their specific inhibitors (PD 98059 and SB 202190) did not influence HSF1 activation. Interestingly, VSMC lines stably expressing dominant-negative rac (rac N17) abolished hsp-protein production and HSF1 activation induced by cyclic strain stress, whereas a significant reduction of hsp70 expression was seen in ras N17–transfected VSMC lines. Thus, our findings demonstrate that cyclic strain-stress induced hsp70 expression is mediated by HSF1 activation and regulated by rac and ras GTP–binding proteins. Induction of hsp70 could be important in maintaining VSMC homeostasis during vascular remodeling in response to hemodynamic stimulation. (Circ Res. 2000;86:1122-1128.)

Key Words: mechanical stress ▪ smooth muscle cells ▪ heat shock proteins ▪ signaling ▪ G proteins

The passage of blood through the vascular system generates hemodynamic forces. Fluid flow across the cell surface results in shear stress, whereas strain stress, which produces elongational stretch, is caused by circumferential deformations resulting from transmural pressure gradients and vascular smooth muscle tone.1 Vascular smooth muscle cells (VSMCs) are one of the major constituents of blood vessel walls responsible for the maintenance of vascular structure and functions.2 The arterial wall is an integrated, functional component of the circulatory system that is continually remodeling in response to various stressors, such as hemodynamic stress. These stimuli directly or indirectly damage the vessel wall and eventually induce arterial stiffness and obstruction. In fact, VSMCs produce a high level of stress proteins, also known as heat shock proteins (hsp), protecting the host from damage during hemodynamic stress.3

The hsp70s are subdivided into multimember families based on the molecular weights of the proteins encoded, ie, hsp27, hsp60, hsp70, and hsp90. They are highly expressed in cardiovascular tissues in response to stress stimuli.4–6 The hsp production is primarily mediated by heat shock transcription factors (HSFs) that interact with a specific regulatory element, heat shock element (HSE), present in the hsp gene promoters.7 HSF1-null mice exhibit elimination of the classical heat shock response and HSF1 is essential and sufficient for upregulation of hsp70 expression during downregulation of the ubiquitin proteolytic pathway.8,9 Although the activation process seems to involve HSF oligomerization from a monomeric to a trimeric state,7 stress-initiated signal-transduction pathways leading to HSF activation are largely unknown.

Mitogen-activated protein kinases (MAPKs) are thought to play a pivotal role in transmitting transmembrane signals required for gene expression and cell differentiation.10,11 MAPKs comprise a ubiquitous family of tyrosine and threonine kinases and include extracellular signal–regulated ki-
nases (ERKs), stress-activated protein kinases (SAPKs) or c-Jun NH2-terminal protein kinases (JNKs), and p38 MAPKs. They are highly activated in VSMCs in vivo and in vitro in response to cyclic strain stress,2,13 hypertension,14 and angioplasty,15 which are related to altered biomechanical or hemodynamic stress.16

Xu et al17,18 have previously shown that acute hypertension induces a rapid expression of hsp70 mRNA followed by elevated hsp70 proteins in rat aorta. The hsp70 induction is blocked by prevention of elevation in blood pressure, ie, administration of the vasodilator agent sodium nitroprusside. However, it is not known whether hsp70 production is blocked by prevention of elevation in blood pressure, ie, administration of the vasodilator agent sodium nitroprusside.

Materials and Methods

Cell Culture and Cyclic Strain Stress Treatment
VSMCs were isolated from rats by enzymatic digestion of the aorta, as described elsewhere.19 VSMCs were plated on silicone elastomer–bottomed culture plates (Flexcell). Cells were subjected to mechanical stress with the Cyclic Stress Unit, a modification of the unit initially described by Banes et al.20 After treatment at 37°C, cells were harvested for protein preparation or RNA isolation.

Stable Transfection
VSMCs (passages 3 to 5) were transfected with ras N17, rac N17, and neovector plasmids, respectively, by using Superfect Kit (Qiagen) according to the manufacturer’s instructions. Ras N17–transfected, rac N17–transfected, and neotransfected VSMCs were identified by Western blotting analysis with antibodies to H-ras or myc-tagged proteins.

Protein Extractions and Western Blot Analysis
The cells were washed twice with precool (4°C) phosphate-buffered saline and harvested on ice in buffer A, and 50 μg of total VSMC proteins was separated by electrophoresis through a 10% SDS–polyacrylamide gel. The membranes were processed with a monoclonal antibody to hsp70, as described.21 For HSF1 analysis, nuclear proteins (20 μg/lane) and antibodies against mammalian HSF122 were used. Specific antibody-antigen complexes were detected using the ECL Western Blot Detection Kit.

RNA Isolation and Northern Blots
Total RNA was isolated using a standard protocol, as described previously.23 Hybridizations were performed using a fluorescein-labeled cDNA probe for hsp70, as described previously.24 Accuracy of loading and transfer, as well as RNA integrity, was confirmed by quantitative analysis of the 28S and 18S RNAs.

Gel Mobility Shift Assays
For nuclear protein preparation, the procedure used was similar to that described by Schreiber et al,25 with a slight modification.26 The procedure for gel mobility shift assays has been described previously.26 In short, DNA binding was determined after incubation of 5 μg of nuclear protein extracts with an oligonucleotide containing the heat shock element (HSE) sequence from the Drosophila hsp70 promoter (5'-GCCCTCGAATGTTCGCGAAGTTT-3`) labeled with 32P-dCTP. Super-shift assays were performed using antibodies against HSF1, HSF2,22 c-Fos, and ATF2 (Santa Cruz Biotech).

Figure 1. Western blot analysis of hsp70, hsp27, and hsp90 proteins in VSMCs treated with cyclic strain stress. Subconfluent cells cultivated on flexible-bottomed plates were subjected to cyclic strain stress at 37°C. Cells were lysed in the buffer (protein extracts separated on 10% SDS–polyacrylamide gel), transferred to membranes, and probed using a monoclonal antibody to mammalian-inducible hsp70. A, Time course of hsp70 induction in VSMCs treated with cyclic strain stress (60 cycles/minute, 15% elongation). B, Data of hsp70 induction from 3 independent experiments. C, Data of hsp27 and hsp90 expression in VSMCs treated with cyclic strain stress. D, Results from VSMCs treated with various elongations of the original size for 6 hours. Ctl indicates negative control; and Ctl+, positive control treated with 42°C for 30 minutes and 37°C for 5 hours. *Significantly different from untreated controls; P<0.05.

Kinase Assays
For p38 kinase assays, the procedure used was similar to that described previously.27 Briefly, immunocomplexes were incubated with myelin basic protein and γ-P32ATP (5 μCi) for 20 minutes. Proteins in the kinase reaction were resolved by SDS-PAGE (15% gel) and subjected to autoradiography.

Cell Viability Assays
VSMCs were plated in the flexible plates and serum starved. VSMCs were then treated with cyclic strain stress at 37°C for 6 hours or treated with heat shock (42°C) for 30 minutes and recovered at 37°C for 6 hours. H2O2 or nitric oxide donor sodium nitroprusside was added to the culture and incubated at 37°C for 24 hours. Cells were harvested with trypsin-EDTA solution and counted.

Statistical Analysis
A paired Student’s t test was used to assess differences between 2 groups. A value of P<0.05 was considered significant.

Results
hsp70 Protein Induction and mRNA Transcription
Figure 1A shows the time course of hsp70 production in response to mechanical stress. The amount of 70-kDa protein
increased by 1 hour after treatment, reached a plateau in 6 hours, and declined thereafter. Figure 1B summarizes data of hsp70 protein induction as determined by quantification of optical densities from autoradiograms of 3 experiments. Between 6 and 12 hours, 3- to 5-fold increases in hsp70 proteins of VSMCs were observed. Importantly, this effect seemed specific to hsp70, because protein levels for hsp27 and hsp90 were unchanged in response to cyclic strain stress (Figure 1C). These results are concomitant with a report showing that shear stress does not induce hsp27 protein production in endothelial cells.28 Likewise, VSMCs treated with a variety of stress strengths for 6 hours resulted in significant increases in hsp70 proteins (Figure 1D).

hsp70 mRNA levels in stress-treated VSMCs were analyzed by Northern blots. As shown in Figure 2, cyclic strain stress resulted in an increase of hsp70 mRNA transcription in VSMCs. A strength-response analysis indicated that levels of hsp70 mRNA in VSMCs treated with 5% elongation were higher and significantly elevated with 10% or 20% elongation. The lower panel of Figure 2A shows the amount of 18S and 28S RNA from the corresponding blot. Figure 2B summarizes hsp70 mRNA induction as determined by quantification of optical densities from autoradiograms of 2 experiments.

HSF1 Activation
To determine whether mechanical stress induces hsp70 mRNA through HSF activation, nuclear proteins were iso-

Figure 2. Mechanical stress–induced hsp70 mRNA expression. A, Strength course of mRNA expression in VSMCs treated with cyclic strain stress at 37°C for 1 hour. Integrity and quantity of RNA were verified by analysis of 18S and 28S RNAs (bottom). Northern blots were hybridized with hsp70 cDNA probes. B, Statistical data on hsp70-mRNA induction from VSMCs treated with mechanical stress. *Significant difference from control (Ctl); P<0.05.

Figure 3. HSF-binding activity in protein extracts of VSMCs. VSMCs were treated with cyclic strain stress at 37°C. Nuclear proteins were prepared as described in Materials and Methods. Gel mobility shift assay was performed in 4% gel. A, Time course of HSF-DNA–binding activation from VSMCs stressed with 60 cycles/minute and 15% elongation. B, Strength-dependent HSF activation (1 hour). Arrows indicate specific HSE-binding complexes; lines, nonspecific binding.
To verify whether the autocrine and paracrine cytokines are involved in HSF activation, conditioned medium from stressed VSMCs were collected, concentrated, and used to treat cells. The data in Figure 5B show that supernatant or conditioned medium from VSMCs stretch stressed for 1 hour did not result in HSF-DNA–binding activation.

Recent studies have demonstrated that ERK1 phosphorylates HSF1 on serine residues and represses transcriptional activation by HSF1, and that p38 MAPKs can induce hsp27 phosphorylation, which is necessary for hsp27 function. It would be interesting to clarify whether mechanical stress–induced HSF1 activation is regulated by MAPK-signal pathways. As shown in Figure 6A, cyclic strain–stress treatment

MAPK-Independent HSF Activation in VSMCs

Recent studies have demonstrated that ERK1 phosphorylates HSF1 on serine residues and represses transcriptional activation by HSF1, and that p38 MAPKs can induce hsp27 phosphorylation, which is necessary for hsp27 function. It would be interesting to clarify whether mechanical stress–induced HSF1 activation is regulated by MAPK-signal pathways. As shown in Figure 6A, cyclic strain–stress treatment

Figure 4. Specificity of HSE-binding activation in VSMCs in response to mechanical stress. A, Nuclear proteins were incubated with 32P-labeled HSE oligonucleotide in the presence or absence of unlabeled oligonucleotide containing HSE-binding or NF-kB–binding element. B, Results of no addition (−) or addition (+) of various antibodies specific to HSF1, HSF2, c-Fos, or ATF2. Arrows indicate specific protein-DNA complexes; lines, supershifted DNA-binding complexes of HSF1 protein-containing complexes.

Figure 5. A, Western blot analysis of HSF1 proteins. Nuclear proteins prepared from VSMCs treated with heat shock (42°C for 30 minutes) or stretch stress for 1 hour were analyzed by Western immunoblotting for HSF1 proteins. Brackets indicate the position of HSF1 proteins. B, Gel mobility shift assay identifying stress-induced HSF-DNA binding activation. Supernatant 1 indicates nonconcentrated medium; supernatant 10, 10-time–concentrated conditioned medium.

Figure 6. Time course of 3-MAPK phosphorylation in VSMCs exposed to mechanical stress. Serum-starved VSMCs were treated with cyclic strain stress (60 cycles/minute, 15% elongation). Western blot analysis was performed using antibodies to phosphorylated ERK1 and ERK2 (A), phosphorylated JNK1 and JNK2 (B), and phosphorylated p38 MAPK (C). VSMCs were pre-treated with PD 98059 (50 μmol/L), SB 202190 (5 μmol/L), or PD 98059+SB 202190 (PD+SB) for 1 hour. Cells were stressed for 10 minutes (D and E) and 1 hour (F), respectively, and harvested for protein extracts or nuclear protein isolation. p38 MAPK activities were measured based on phosphorylation of myelin basic protein (MBP) (E). F, Results of gel mobility shift assay.
(60 cycles/minute, 15% elongation) resulted in significant increases in ERK phosphorylation. Kinetic analysis indicates that this response occurred as early as 5 minutes, with maximum induction achieved 10 minutes after treatment and declining thereafter (Figure 6A). Similarly, both JNKs/SAPKs and p38 MAPKs were activated in a time-dependent manner (Figures 6B and 6C). PD 98059, a specific inhibitor of ERK kinases, significantly inhibited ERK1 and ERK2 activation in VSMCs in response to mechanical stress but did not inhibit p38 MAPKs (Figure 6D). Likewise, the p38-specific inhibitor SB 202190 abrogated p38 activity stimulated by strain stress, and no effect on ERK phosphorylation was seen (Figure 6E). Surprisingly, both PD 98059 and SB 202190 did not influence HSF-DNA–binding activation induced by mechanical stress in VSMCs (Figure 6F), indicating an MAPK-independent process of HSF1 activation.

**Rac- and Ras-Regulated hsp70 Expression**

To investigate the role of the small GTPase–binding proteins ras and rac in hsp70 expression in stress-stimulated VSMCs, we established VSMC lines stably expressing ras or rac encoding a dominant-negative form (ras N17 or rac1 N17). Rac1 N17–transfected VSMCs expressed a high level of this gene product (Figure 7A). Interestingly, overexpression of rac1 N17 completely inhibited hsp70 protein production stimulated by strain stress and partially blocked heat shock–induced hsp70 expression (Figure 7B). We then assessed the effects of rac1 N17 overexpression on HSF activation in the cell lines treated with mechanical stress. As seen in Figure 7C, strain stress–induced HSF-DNA–binding activities in rac1 N17 cell lines were not detectable. Similarly, HSF1 translocation and phosphorylation stimulated by cyclic strain stress were blocked by overexpression of dominant-negative rac (Figure 7D).

Likewise, ras protein was at a lower level in vector-transfected controls and at a higher level in ras-transfected cells (Figure 8A). Ras-N17 expression largely blocked hsp70-protein induction in the cell lines stimulated by mechanical stress (Figure 8B) and partially inhibited HSF-DNA–binding activation in stressed VSMCs (Figure 8C). Therefore, ras and rac play a role in regulation of hsp70 expression in VSMCs.

**Effect of Cyclic Strain Stress on VSMC Survival After Free Radical Exposure**

To investigate the potential physiological role of cyclic strain stress–induced hsp70 in mediating the protective response to free radical stimulation, we undertook a comparative analysis of cell survival after H2 O2 or sodium nitroprusside exposure in VSMCs with or without cyclic strain stress treatment or preincubation at 42°C for 30 minutes. Data shown in Figure 1 online (available at http://www.circresaha.org) provide evidence that cyclic strain stress or heat shock significantly increased VSMC survival from H2 O2 or sodium nitroprusside–induced cell death.

**Discussion**

In the present study, we provide the first evidence that mechanical stress induces hsp70 expression in VSMCs and demonstrate that this effect relies on HSF1 activation. The nature of the primary signal that activates HSF1 in response to heat shock or stress is not fully elucidated. Current studies support a model for regulating heat shock response in which the hsp7s themselves negatively regulate heat shock gene expression via an autoregulatory loop.7 During mechanical stretch stimulation, multiple and complex pathophysiological changes occur in VSMCs, including PDGF-receptor activation,12 cytoskeletal rearrangement,32 and apoptosis.33 Thus, mechanical stress–induced and heat shock–induced hsp70 productions share many similarities in activation of HSF1 and regulation of hsp70 gene expression.

Much evidence suggests a role for phosphorylation in the conversion of HSF1 from this intermediate state into a transcriptionally active form.7 Recent studies have demonstrated that ERK1 phosphorylates HSF1 on serine residues and represses transcriptional activation by HSF130 and that p38 MAPKs can induce hsp27 phosphorylation, which is necessary for hsp27 function.31 Our findings that mechanical stress rapidly activates 3 members of MAPKs do not support the role of MAPKs in HSF activation or phosphorylation in VSMCs. Thus, mechanical stress–induced hsp70 expression is independent of MAPKs in VSMCs.
activate membrane-bound G proteins by facilitating exchange of GDP to GTP, subsequently leading to HSF1 activation and hsp70 expression.

With regard to the involvement of growth factors and cytokines released by stressed VSMCs in the mechanical stress–induced activation of HSF1, our data do not support ligand-binding activation. The conditioned medium from stressed cells did not result in HSF1 activation. However, the effects of the factors or cytokines on hsp70 production stimulated by mechanical stress cannot be absolutely excluded because of the possible presence of unstable or rapidly inactivated factors in the conditioned medium. In addition, a single myocardial stretch was shown capable of inducing hsp70 expression in isolated perfused rabbit heart,36 and volume overload produced experimentally by banding of the aorta was sufficient to elicit hsp70 induction in the heart.37 However, an in vitro study showed a lack of stretch-induced expression of hsp70 gene in cultured cardiac myocytes.38 In the present study, we demonstrated that VSMCs do express hsp70 in response to stretch stress. These results suggest the significance of potential cell-type specificity in hsp70 induction in the process of cell stretching because of heterogeneous cell compositions of the heart.

Recent studies have demonstrated the presence of VSMC apoptosis in the arterial wall.23,39 Proliferating VSMCs show more apoptotic cell death than nonproliferating VSMCs.40 There is evidence that free radicals, including H2O2 and nitric oxide, involved in the development of vascular diseases can lead to VSMC apoptosis or death.41 Transgenic mice overexpressing hsp70 show enhanced resistance to ischemic injury,42,43 and increased production of hsp70 in atherosclerotic lesions may be beneficial for arterial smooth muscle cell survival.44 HSF1-deficient mice exhibit increased mortality after endotoxin challenge.8 Our studies demonstrate the role of mechanical stress–induced or heat shock–induced hsp expression in protecting VSMCs against free radical–induced death. Thus, hsp7 might influence the process of vascular remodeling or hypertrophy via their effects on VSMC apoptosis and proliferation in response to hemodynamic stress and may exert a role in maintaining cellular homeostasis of the vessel.

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Materials and Methods

Cell culture and cyclic strain stress treatment. VSMCs were isolated by enzymatic digestion of the aorta from rats as described elsewhere (1), and cultured in DMEM medium (Gibco, Grant Island, NY) supplemented with 20% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were passaged by treatment with 0.05% trypsin/0.02% EDTA solution.

VSMCs were plated on silicone elastomer-bottomed culture plates (Flexcell, Meckeesport, PA). Cells achieving 90% confluence were subjected to mechanical stress with the Cyclic Stress Unit, a modification of the unit initially described by Banes et al (2), consisting of a controlled vacuum unit and a base plate to hold the culture plates (FX3000 AFC-CTL, Flexcell). Vacuum (15 to 20 kPa) was repeatedly applied to the elastomer-bottomed plates via the base plate, which was placed in a humidified incubator with 5% CO₂ at 37°C. Cyclic deformation (60 cycles/min) and up to 30% elongation of elastomer-bottomed plates were used (3, 4). After treatment for various periods of time at 37°C, cells were harvested for protein preparation or RNA isolation.

Stable transfection. VSMCs (passages 3-5) were transfected with ras N17, rac N17 and neo-vector plasmids, respectively, by using Superfect Kit (Qiagen) according to the manufacture's instructions. After transfection, the cells were cultured for 24 h, divided one to four, and placed in culture medium supplemented with 20% FCS and 150 µg·ml G418 to select those carrying a neomycin-resistant plasmid. When 80% cell death in a parallel group of normal VSMCs was observed, the medium containing 150 µg·ml G418 was changed into medium containing 50 µg·ml G418 to maintain selection. After 4-8 weeks, living cells were transferred for cell expansion and maintained in culture medium supplemented with 20% FCS and 50 µg·ml G418. Ras N17-, and rac N17- and neo-transfected VSMCs were identified.
by Western blotting analysis with antibodies to H-ras or myc-tagged proteins. The experiments with the cell lines were conducted before passages 20.

**Protein extractions and Western blot analysis.** For heat shock, cells were heated at 42°C for 30 min and then incubated at 37°C for up to 8 h. The cells were washed twice with pre-cold (4°C) PBS (pH7.4), and harvested on ice in buffer A containing 20 mM Hepes (pH 7.5), 0.4 M NaCl, 10% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton, 1 mM DTT, 1 mM Pefablock SC (Boehringer Mannheim, Mannheim, Germany) and 1 μg/ml leupeptin. The suspension was incubated on ice for 20 min and vortexed every 5 min. Cellular debris was then pelleted by centrifugation for 30 minutes at 13,000 rpm (Eppendorf centrifuge) at 4°C. Supernatants were collected and the protein concentration was measured by the Bio-Rad Assay (Bio-Rad, Hercules, CA). Aliquots were frozen in liquid nitrogen and stored at -80°C.

Fifty μg of total VSMC proteins was separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). The membranes were processed with a monoclonal antibody to hsp70 (Catalog no. SPA-810, StressGen Biotechnologies Corp., Victoria, Canada). For HSF1 analysis, nuclear proteins (20 μg/lane) (for preparation see below) were used. Antibodies against mammalian HSF1 (gifts from Dr. R. I. Morimoto, Northwestern University, Evanston, IL; ref. 22) were used in a dilution of 1 to 5,000. Specific antibody-antigen complexes were detected using the ECL Western Blot Detection Kit (Amersham Co., Buckinghamshire, UK).

**RNA isolation and Northern blots.** Total RNA was isolated following a standard protocol, as described previously (5). RNA (10μg/lane) was denatured with formaldehyde (Merck, Darmstadt, Germany), electrophoresed and transferred onto a nylon membrane (Zeta Probe, Biorad, Richmond, CA). RNA was UV-crosslinked in an UV Stratalinker (Stratagene, La Jolla, CA). Hybridizations were performed using a fluorescein-labeled (Amersham Co.) cDNA probe for hsp70, as previously described (6). The membranes were then washed, detected with antifluorescein
alkaline phosphatase conjugate (1:5,000) (Amersham Co.) and exposed to ECL films. Graphs of blots were obtained in the linear range of detection. Accuracy of loading and transfer, as well as RNA integrity, was confirmed by quantitative analysis of the 28s and 18s RNA.

**Gel Mobility Shift Assays.** For nuclear protein preparation, the procedure used was similar to that described by Schreiber (7) with a slight modification (8). Subconfluent (80-90%) VSMCs were treated with cyclic strain stress at 37°C for various periods of times. For heat shock, cells were heated at 42°C for 30 min. The cells were washed and harvested with cold TBS (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH7.4). The cell pellet (2-3x10⁶) was incubated with 400 μl of cold buffer A (10 mM Hapes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, pH7.9) on ice for 15 min, 25 μl (10%) Nonidet P-40 was added, and the pellet was vortexed and centrifuged for 30 sec at 13,000 rpm. The nuclear pellet was incubated with 50 μl of cold buffer B (20 mM Hapes, 0.4 M NaCl, 20% glycerol, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1μg/ml leupeptin, 1μg/ml aprotinin, pH7.9) at 4°C for 15 min on a shaking platform with vigorous rocking. The suspension was centrifuged for 5 min at 4°C at 13,000 rpm. The supernatant was collected as nuclear proteins and protein concentration was determined with Bio-Rad assay.

The procedure for gel mobility shift assays has been described previously (26). In short, DNA binding was determined following incubation of 5 μg of nuclear protein extracts with 10 fmole of an oligonucleotide containing the heat shock element (HSE) sequence from the *Drosophila* hsp70 promoter (5'-GCCTCGAATGTTCGCGAAGT-3') labeled with ³²P-dCTP. The reaction buffer contained 10 mM Hapes, (pH 7.9) 1 mM DTT, 1 mM EDTA, 80 mM KCl, 4% Ficoll and 1 μg poly(dIdC) (Pharmacia Biotech, Uppsala, Sweden) as a non-specific competitor. Samples were electrophoresed through a 4% polyacrylamide gel in 0.5 X TBE (1xTBE: 89 mM Tris, 89 mM Boric acid, 20 mM EDTA, pH 8.3), after which the gel was dried onto DE81 paper and exposed to autoradiographic film. Super-shift
assays were performed using antibodies generated against, and specific to, mammalian HSF1, HSF2 (22), c-Fos and ATF2 (Santa Cruz Biotech. Inc., Santa Cruz, CA). The antibodies were added to samples after the initial binding reactions between protein extracts and oligonucleotides were allowed to take place.

**Kinase Assays.** For p38 kinase assays, 0.5 ml of supernatant containing 0.5 mg proteins were incubated with 10 µl of antibody against mammalian p38 MAPKs (Santa Cruz Biotech. Inc.) for 2 h at 4°C with rotation. Subsequently, 40 µl of protein G-agarose suspension (Santa Cruz Biotech. Inc.) was added and rotation continued for 1 h at 4°C. Immunocomplexes were precipitated by centrifuge and washed twice with buffer A, B (500 mM LiCl, 100 mM Tris, 1 mM DTT, 0.1% Triton X-100; pH 7.6), and C (20 mM Mops, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100; pH 7.2), respectively. p38 activities in the immunocomplexes were measured as described previously (9). Briefly, immunocomplexes were incubated with myelin basic protein (MBP; 6 µg; Upstate Biotech. Inc., Lake placid, NY), and γ-P³²ATP (5 µCi) for 20 min. To stop the reaction, 15 µl of 4x Laemmli buffer was added and the mixture was boiled for 5 min. Proteins in the kinase reaction were resolved by SDS-PAGE (15% gel) and subjected to autoradiography.

**Cell viability assays.** VSMCs were plated at a density of 1 x 10⁶ cells/plate in the medium containing 10% fetal calf serum and incubated at 37°C for 24 h. The culture medium was replaced with the medium containing 0.1% fetal calf serum, and incubated for 48 h. VSMCs were then treated with cyclic strain stress at 37°C for 6 h or treated with heat shock (42°C) for 30 min and recovered at 37°C for 6 h. H₂O₂ (50 nM) or nitric oxide donor sodium nitroprusside (0.5 mM) was added to the culture and incubated at 37°C for 24 h. The cells were washed 3 times with PBS, harvested with trypsin-EDTA solution and counted.

**Statistical analysis.** A paired Student’s t test was used to assess differences between two groups. A p value less than 0.05 was considered significant.
References:


Figure Legend:

Fig. 1 Online. Comparative effect of heat shock and cyclic strain stress on VSMC viability. VSMCs were preincubated with the medium containing 0.1% bovine calf serum for 48 h, and treated with heat shock (42°C) for 30 min and incubated at 37°C for 6 h or cyclic strain stress (60 cycles/min, 15% elongation) at 37°C for 6 h. VSMCs with or without treatments were incubated with H₂O₂ (50 nM), or nitric oxide donor sodium nitroprusside (SNP; 0.5 mM) at 37°C for 24 h, washed 3 times with PBS and released with trypsin-EDTA solution. The survival cells were counted. The graph is % means±SD of 3 experiments. *Significant difference from VSMCs treated with H₂O₂ or SNP alone.