Rapid Induction and Translocation of Egr-1 in Response to Mechanical Strain in Vascular Smooth Muscle Cells

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Abstract—The effect of mechanical strain on transcription and expression of the immediate-early genes, early growth response gene-1 (Egr-1), c-jun, and c-fos, was investigated in neonatal rat aortic vascular smooth muscle (VSM) cells. Cells grown on silicone elastomer plates were subjected to cyclic mechanical strain (1 Hz) at various durations and magnitudes. Egr-1 mRNA increased rapidly in response to cyclic strain, reached a maximum of 10-fold after 30 minutes, and returned to baseline after 4 hours. c-jun exhibited a similar pattern, whereas c-fos mRNA expression was unaffected by strain. Cycloheximide prolonged the increase in Egr-1 and c-jun mRNA and caused superinduction of both. The threshold level of continuous cyclic strain needed to induce expression was 5% for Egr-1 and c-jun. Even a single cycle of mechanical strain that lasted 1 second was sufficient to induce Egr-1 and c-jun mRNA. Strain also increased expression of a transiently transfected Egr-1 promoter-reporter construct. The effect of varying extracellular matrices on strain-induced Egr-1 and c-jun mRNA was examined. In contrast to collagen type 1- and pronectin-coated plates, strain did not significantly alter expression of Egr-1 and c-jun was less induced on laminin-coated plates. On collagen type 1, strain increased Egr-1 protein levels by 2.1-fold at 60 minutes. Immunofluorescence microscopy revealed translocation of Egr-1 to the nucleus in response to strain. These observations indicate that Egr-1 expression and translocation are sensitive to mechanical perturbation of the cell. c-jun is also induced by strain, but c-fos is not. The signal for this induction may involve specific cell-matrix interactions. (Circ Res. 1999;84:678-687.)

Key Words: mechanical stimulation • cyclic strain • genes • muscle, smooth, vascular • transcription

Vascular smooth muscle (VSM) cells in large arteries are exposed to substantial mechanical forces during the cardiac cycle. These forces may participate in developmental processes and in the vascular hypertrophy associated with disease states.1–3 Recently, the application of mechanical strain to VSM cells in vitro has yielded a variety of descriptive data on alterations in the growth and phenotype of VSM cells subjected to strain.3

Previous work from this laboratory has shown that cyclic mechanical strain, applied by a vacuum to cells cultured on silicone elastomer membranes, induces DNA synthesis and expression of gene transcripts, such as PDGF-A, PDGF-B, and the PDGF-β receptor.4–7 Under certain conditions, smooth muscle myosin SM-1 and SM-2 are induced.8 These responses vary depending on the extracellular matrix. On laminin (LN), DNA synthesis and expression of PDGF are minimal8 but induction of smooth muscle myosins SM-1 and SM-2 become more apparent.8

To date, only minimal information exists on the transcription factors that transduce mechanical signals into gene activity. One example is the shear stress–responsive element, which is a modified nuclear factor (NF)-κB site found in the promoter region of several genes induced by shear stress in endothelial cells.9,10 Recently, the TPA-responsive element and the binding site of early growth response gene-1 (Egr-1) were found to be involved in shear stress–induced gene expression in endothelial cells.11,12 In cardiac myocytes, Egr-1, c-jun, and c-fos expression are increased by mechanical forces.13–15

On the basis of VSM cells transfected with various PDGF-A–chain promoter truncations and subjected to strain, data from this laboratory identified a 92-bp region of the promoter that is responsive to strain6 This region contains a binding site for the Egr-1 protein.

Egr-1 is thought to be involved in the regulation of mitosis and differentiation.16,17 Egr-1, like c-jun and c-fos, belong to the class of transcription factors called immediate-early genes. The expression of these transcription factors is induced by a variety of stimuli, including growth factors, serum, or vasoconstrictors.17,18 After activation, Egr-1 protein is translocated from the perinuclear space to the nucleus, at which it can bind to specific promoters and regulate gene expression.19,20

Received November 20, 1998; accepted January 7, 1999.

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In VSM cells, the effect of mechanical strain on Egr-1 and c-fos is unknown. The cellular induction of c-fos by mechanical strain is controversial.\(^{21,22}\) We therefore examined the induction of Egr-1, c-fos, and c-jun expression in VSM cells subjected to varying mechanical forces. We find that Egr-1 and c-jun but not c-fos mRNA expression is dramatically upregulated by exposure to even brief mechanical stimuli. Extracellular matrix composition may determine both the magnitude and direction of the effect of strain on Egr-1 and c-jun transcription.

### Materials and Methods

**Materials**

All materials were purchased from Sigma Chemical Co unless otherwise specified. BSA was fraction V, fatty acid poor (Miles Inc). Flex plates were from FlexCell Inc. Transfection reagent (DOTAP) was purchased from Boehringer Mannheim, and Bicinchoninic Acid solution protein assay reagent was purchased from Pierce Corp. The Egr-1 antibody used was a rabbit affinity-purified polyclonal antibody raised against a peptide of 14 amino acids mapping at the carboxy terminus of mouse Egr-1 p82 (Santa Cruz Biotechnology, Inc). The Egr-1 antibody was shown to react with the rat Egr-1 p82 by Western blot analysis. β-Galactosidase assay kit was purchased from Promega.

**Cell Culture**

Primary cultures of VSM cells from newborn rat aorta were established by Dr Peter Jones (University of Southern California, Los Angeles, Calif). From these primary cultures, the cell line R22D was established\(^{23}\) and at passage 15 generously supplied by Dr Jones. The cells were maintained in medium with 10% serum (MEM with 10% FBS, 2% tryptose phosphate broth, 50 U/mL penicillin, and 50 U/mL streptomycin) in a humidified atmosphere of 5% CO\(_2\) at 37°C. Culture medium was changed every other day until cells were confluent. Confluent cells were subcultured with trypsin-versenese. Cells were used from passages 17 to 25 for these studies. In most experiments, cells were plated on 6 well (5 cm\(^2\) per well) collagen-type I (Col I) silicone elastomer plates (Flex I, FlexCell Inc) in medium with 10% serum and cultured until confluent. Three days before experimentation, the medium was replaced with serum-free medium (MEM with 0.5 g/L BSA, 0.5 mg/L apo-transferrin, 2% tryptose phosphate broth, 50 U/mL penicillin, and 50 U/mL streptomycin). Medium was subsequently replaced with fresh serum-free medium every 24 hours and again 3 hours before the strain experiment. In experiments on different extracellular matrices, confluent cells in conventional plastic flasks were detached with trypsin-versenes and trypsin was then inactivated with serum-containing medium. The cells were then centrifuged for 5 minutes at 1000 rpm, washed once with serum-free medium, and the cells of one 9-cm plastic dish were plated in serum-free medium on one 6-well silicone elastomer plate coated with Col I, pronectin (FN, a fibronectin-like poly RGD matrix), or LN. Serum-free medium was replaced 24 hours later (3 hours before application of mechanical strain).

**Application of Cyclic Strain to Cultured Cells**

Confluent VSM cells on the silicone elastomer culture plates were subjected to mechanical deformation with the Flexercell Stress Unit (FlexCell Inc). The stress unit is a modification of the unit initially described by Banes and coworkers\(^{24,25}\) and consists of a computer-controlled vacuum unit and a base plate to hold the culture dishes. Vacuum is repetitively applied to the rubber-bottomed dishes through the base plate, which is placed in a humidified incubator with 5% CO\(_2\) at 37°C. The computer system controls the frequency of deformation and the negative pressure applied to the culture plates. A negative pressure of 20 kPa results in a maximal 25% elongation of cells at the periphery of the dishes. After 24 hours of continuous cyclic mechanical strain at the maximum attainable level, no increase in lactate dehydrogenase activity (In vitro toxicity assay kit, Sigma) was measurable in the supernatant (H.M., H.E.I., unpublished data, 1998).

**Immunofluorescence Microscopy**

VSM cells were grown on Col I–coated silicone elastomer plates and subjected to 30 minutes of cyclic mechanical strain (1 Hz, 25% strain) followed by 30 minutes in the resting state. In other experiments, cells were subjected to 60 minutes of continuous cyclic strain. After being rinsed briefly with PBS, cells were incubated with 4% paraformaldehyde/PBS at room temperature for 10 minutes followed by incubation with methanol at −20°C for 6 minutes. All subsequent steps were performed in a humidified chamber at room temperature. The fixed cells were incubated with 10% serum/PBS for 20 minutes, washed with 1% BSA/PBS, and then incubated with rabbit polyclonal anti-Egr-1 antibody (2 μg/mL, Santa Cruz Biotechnology, Inc) for 1 hour. After the primary antibody was removed, the cells were gently washed with 1% BSA/PBS, incubated with a fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (1:200 dilution, Sigma) in the dark for 45 minutes and washed with PBS. The silicone bottoms of the culture plates were removed and trimmed to allow the material to be flattened for microscopic observation. The silicone disks were then mounted in gel/mount (Biomeda) on a glass slide. The stained cells were visualized with a ×20 Fluor objective (Nikon) with epifluorescence illumination. Photographs were taken with the use of Kodak Tmax 100 ASA film.

**cDNA Clones**

The Egr-1 cDNA clone contains a 1.6 kb BglII mouse Egr-1 cDNA fragment cloned into the BamHI sites of pUC19 (Stratagene).\(^{16}\) The Egr-1 plasmid DNA was isolated with the QIAGEN Plasmid Maxi Kit (QIAGEN Inc), digested with HindIII and EcoRI, and the 1.6-kb Egr-1 fragment was eluted after agarose gel electrophoresis with the QIAquick Gel Extraction Kit (QIAGEN Inc). The c-jun cDNA clone is the mouse 2.6-kb JAC.1 in the pGEM-2 vector (ATCC). The c-fos cDNA clone includes the pTRE–c-fos/exon 2 from mouse (Ambion Inc). In addition, a human c-fos cDNA fragment was cloned from human umbilical vein endothelial cells by reverse-transcriptase PCR and its identity confirmed by DNA sequencing (H.M., H.E.I., unpublished data, 1998). The rat GAPDH clone is a 250-bp EcoRV fragment cloned into the pT7Blue vector (Novagen Inc).\(^{26}\)

The Egr-1 promoter deletion chloramphenicol acetyltransferase (CAT) construct pEgr-1 P1.2 contains the “full-length” Egr-1 promoter (from position −957 to +248 bp, relative to the Egr-1 promoter transcriptional start site) fused with the reporter gene CAT.\(^{27}\) The Egr-1 promoter–deletion CAT construct pE50 contains the minimal Egr-1 promoter (from position −50 to +65 bp, relative to the Egr-1 promoter transcriptional start site) fused with the reporter gene CAT.\(^{28}\) The pRSV.CAT plasmid\(^{29}\) was used as a positive control for CAT protein after transfection, and the pRSV-β-Gal plasmid\(^{30}\) was used as a control for transfection efficiency.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from VSM cells with the RNA STAT-60 reagent (Tel-Test “B,” Inc). RNA samples (10 μg per lane) were separated by electrophoresis through 1.2% agarose gels after denaturation of the RNA with glyoxal and dimethylsulfoxide.\(^{31}\) RNA was transferred with 20× SSC buffer to Hybond-N nylon membranes (Amersham Life Science Inc). The isolated cDNA fragments were labeled with an oligolabeling kit (Pharmacia Biotech Inc) with [α-\(^{32}\)P]dCTP (Amersham Life Science Inc), purified with MicroSpin Columns (Pharmacia Biotech Inc), and hybridized with the RNA membranes in hybridization solution (1 mol/L NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA) at 65°C for 16 hours. The membranes were washed twice for 15 minutes with 2× SSC, 0.1% SDS at 60°C and once for 15 minutes with 0.2× SSC, 0.1% SDS at 55°C and exposed to Hyperfilm MP (Amersham Life Science Inc) at −80°C. Blots were quantified by
scanning of autoradiographs with a laser densitometer (Molecular Dynamics).

**Protein Isolation and Western Blot Analysis**

After mechanical strain, cells were washed and harvested in PBS and centrifuged for 5 minutes at 3000 rpm at 4°C. The cell pellet was lysed in lysis buffer (0.5% SDS in PBS) with a syringe, boiled for 10 minutes, and centrifuged for 10 minutes at 13 000 rpm and 4°C. The protein concentration was determined with the BCA protein assay reagent. Proteins (10 μg per lane) were separated in SDS-PAGE (7.5%) and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Science Inc). The membranes were incubated with a primary Egr-1 antibody (Santa Cruz Biotechnology, Inc), secondary horseradish peroxidase–linked rabbit Ig, and detected with ECL Western blotting detection reagent (Amersham Life Science Inc).

**Transfections and CAT Assays**

Transfections were performed with the transfection reagent DOTAP. VSM cells were grown on Col I-coated silicone elastomer plates until 60% to 80% confluent. They were then transfected for 10 hours with DOTAP; 5 μg RSV—β-galactosidase–DNA; and either (1) 10 μg Egr-1 promoter CAT-DNA, (2) 10 μg pRSV-CAT, or (3) without CAT-DNA. Cells were then incubated in Opti-Mem I culture medium (Gibco-BRL Life Sciences) for 48 hours and subjected to cyclic mechanical strain (1 Hz, 25% strain) for 8 hours or 30 minutes strain after incubation in the relaxed position for 7.5 hours. The cells were lysed, and the protein concentration was determined with the Coomasie plus protein assay reagent (Pierce Corp). The β-galactosidase activity of the equal protein amounts of the cell lysate was measured with the β-galactosidase enzyme assay system (Promega), and the CAT protein was determined with CAT ELISA (Boehringer Mannheim).

**Statistics**

Dimensionless quantities (ie, band densities) from multiple similar experiments were combined by calculation of the fold increase (or decrease) versus control under each experimental condition. Data are given as mean±SEM (n=3 in all cases). Statistical analysis was performed with Student’s t test. A value of P<.05 was considered statistically significant.

**Results**

**Egr-1 and c-jun mRNA Induction by Continuous Cyclic Mechanical Strain**

Exposure of cultured neonatal aortic VSM cells to varying time periods of cyclic mechanical strain (1 Hz, 25%) resulted in a dramatic upregulation of Egr-1 and c-jun mRNA expression (Figure 1). Increased Egr-1 and c-jun mRNA were first detectable at 15 minutes (Egr-1: 6.1±1.3-fold increase in band density versus control, P<.001, n=6; c-jun: 1.4±0.09-fold increase versus control, P<.001, n=4), reached its maximum for both genes after 30 minutes (Egr-1: 9.7±1.1-fold increase versus control, P<.001, n=6; c-jun: 5.1±0.6-fold increase versus control, P<.001, n=4), and returned to baseline after 4 hours (Egr-1) or 2 hours (c-jun) continuous exposure to cyclic strain. In contrast, c-fos mRNA was not significantly affected by mechanical strain in VSM cells. This result was confirmed with a independently cloned human c-fos cDNA probe (data not shown).

To assess the role of de novo protein synthesis in the downregulation of Egr-1 and c-jun mRNA during continuous exposure to strain, the strain protocol was repeated in the presence of cycloheximide (Figure 2). Cycloheximide (35 μmol/L) prevented the downregulation of Egr-1 and c-jun mRNA expression and caused superinduction of both genes.
at times >1 hour and for at least 4 hours. In the absence of strain, cycloheximide caused only a minimal induction of Egr-1 and c-jun mRNA at 4 hours (Figure 2). In contrast, even in the presence of cycloheximide, the expression of c-fos in response to strain was not affected (data not shown). Thus, downregulation of Egr-1 and c-jun mRNA expression during continuous exposure to strain requires de novo protein synthesis.

The threshold magnitude of strain necessary to induce Egr-1 and c-jun mRNA was determined by exposing cells to 5% to 25% cyclic strain for 30 minutes (Figure 3). Induction of Egr-1 mRNA was first detectable with 5% cyclic strain (2.4 ± 0.3-fold increase in band density versus unstrained control, P < 0.05, n = 3), increased rapidly up to 10% strain (6.9 ± 0.4-fold increase versus control, P < 0.001, n = 3), and increased slightly further (9.4 ± 0.6-fold versus control, P < 0.001, n = 3) with strain ≥ 25%. The threshold level of c-jun induction by cyclic mechanical strain was similar (5% strain: 1.4 ± 0.2-fold induction versus control, P < 0.001, n = 4), which at 10% strain reached a level of induction (4.2 ± 0.3-fold induction versus control, P < 0.001, n = 4) that did not increase significantly ≥ 25% strain (4.5 ± 0.6-fold induction). Therefore, Egr-1 mRNA is ≈ 2-fold higher induced than c-jun (each versus control) in response to mechanical strain in VSM cells.

**Induction of Egr-1 and c-jun mRNA by a Single Cycle of Strain/Relaxation**

We next determined the minimum number of strain/relaxation cycles necessary to induce Egr-1 and c-jun mRNA expression. Because the maximal expression of Egr-1 mRNA was previously found to occur after 30 minutes of continuous cyclic strain (Figure 1), we exposed cells to strain (25%) for a varied number of strain/relaxation cycles and then incubated cells in the relaxed position to achieve a total incubation time of 30 minutes (Figure 4). Surprisingly, even a single cycle of strain/relaxation that lasted only 1 second was sufficient to induce Egr-1 (4.6 ± 1.2-fold versus control, P < 0.05, n = 4) and c-jun (2.4 ± 0.3-fold versus control, P < 0.001, n = 6) mRNA. Egr-1 (5.7 ± 0.4-fold) and c-jun (3.7 ± 0.4-fold) mRNA was further increased after application of 5 minutes of cyclic strain and reached its maximum after 30 minutes of continuous cyclic strain (Egr-1: 9.6 ± 0.1-fold versus control, P < 0.001, n = 4; c-jun: 5.6 ± 0.6-fold induction, compared with control without strain, P < 0.001, n = 6).

The threshold percent strain needed from single mechanical stimulus to induce Egr-1 mRNA was 10% (1.5 ± 0.07-fold induction versus control, P < 0.05, n = 3) and to induce c-jun mRNA was 20% (1.7 ± 0.2-fold induction versus control, P < 0.01, n = 3; Figure 5). Expression was dose-dependently increased and reached its maximum after a single deformation of 25% (Egr-1: 4.9 ± 0.3-fold induction versus control, P < 0.01, n = 3; c-jun: 3.4 ± 0.8-fold induction versus control, P < 0.05, n = 3). Higher degrees of mechanical deformation were not tested because they were not relevant to the in vivo situation. The c-fos mRNA was not affected by a single mechanical stimulus of increasing magnitude (data not shown).

We next examined the induction of Egr-1 by a single cycle of strain/relaxation in more detail. The time course of Egr-1 mRNA induction after a single cycle of mechanical strain (1-second duration, 25% strain; Figure 6) was similar to the time course during continuous cyclic strain (Figure 1). Increased Egr-1 mRNA was first detectable after 15 minutes (2.6 ± 0.5-fold induction versus control, P < 0.05, n = 6), reached its maximum level at 30 minutes (4.0 ± 1.0-fold induction versus control, P < 0.05, n = 6), and returned to baseline 2 hours after the single mechanical stimulus.

**Induction of Egr-1 Promoter CAT Construct by Mechanical Strain**

In later studies, we examined the effect of mechanical strain on Egr-1 in more detail. To determine whether increased Egr-1 mRNA expression in response to strain is due to increased transcription, we examined the effect of strain on expression of transiently transfected Egr-1 promoter CAT constructs (Figure 7). The full-length Egr-1 promoter construct (pEgr-1P1.2) was induced by mechanical strain (5.9 ± 0.7-fold versus control without strain, P < 0.05, n = 4). A similar induction of pEgr-1P1.2 was detectable after 30 minutes of cyclic strain after incubation in the relaxed position to reach a total time of 8 hours (4.5 ± 0.7-fold versus control without strain; P < 0.05, n = 4, data not shown). Neither a truncated Egr-1 promoter construct (pE50) nor pRSV-CAT was induced by strain. Thus, Egr-1 transcription is induced by mechanical strain at least in part through an element located between −957 to −50 bp of the promoter.

**Effect of Extracellular Matrix Protein on Induction of Egr-1 and c-jun mRNA by Strain**

Previous studies from our laboratory showed that mechanical strain of VSM cells is sensed by interactions with specific
extracellular matrix proteins. Therefore, we examined the effect of extracellular matrix protein composition on the induction of Egr-1 and c-jun mRNA by mechanical strain (Figure 8). To minimize the potential impact of extracellular matrix protein synthesis by the cells after plating, experiments were performed 24 hours after plating, compared with 72 hours after reaching confluence for the experiment reported above. This led to lower levels of Egr-1 and c-jun induction after strain than was observed above. Egr-1 and c-jun mRNA were induced on Col I– (Egr-1; 2.8±0.2-fold induction, \(P<0.01, n=3\); c-jun; 2.4±0.1-fold induction versus control on the same matrix without strain, \(P<0.001, n=4\)
and FN- (Egr-1; 2.6 ± 0.4-fold induction; c-jun; 2.1 ± 0.2-fold induction versus control; \( P < 0.001, n = 4 \)) coated silicone elastomer plates after 30 minutes of cyclic mechanical strain (1 Hz, 25% strain). The time course of Egr-1 mRNA induction by cyclic strain was also similar on Col I- and FN-coated plates (unpublished data, 1998). In contrast, Egr-1 mRNA was not significantly induced by strain in cells cultured on LN (0.9 ± 0.1-fold expression versus control on the same matrix without strain, \( n = 3 \)). c-jun mRNA was induced by cyclic strain on LN-coated plates (1.6 ± 0.1-fold induction versus control on LN without strain, \( P < 0.001, n = 4 \)) to a significantly reduced extent compared with Col I–coated plates (2.4 ± 0.1-fold induction; \( P < 0.01, n = 3 \)) and FN-coated plates (2.1 ± 0.2-fold induction, \( P < 0.05, n = 4 \)). This was due in part to increased basal expression of Egr-1 and c-jun mRNA on LN-coated plates (Egr-1; 1.7 ± 0.3-fold; c-jun; 1.4 ± 0.3-fold expression versus expression without strain on Col I– or FN-coated plates, \( P < 0.05, n = 3 \)). Despite this fact, expression of Egr-1 after strain on LN-coated plates was lower than on Col I– or FN-coated plates (LN: 1.6 ± 0.4-fold; Col I: 2.8 ± 0.1-fold; and FN: 2.6 ± 0.2-fold induction versus expression without strain on Col I; LN versus collagen

Figure 5. Threshold of Egr-1 and c-jun induction by a single cycle of mechanical strain. VSM cells were grown on collagen-coated silicone elastomer plates and subjected to a single cycle (duration, 1 second) of mechanical strain of the indicated magnitude followed by 30 minutes incubation in the relaxed position. A, Total RNA was isolated and analyzed as in Figure 1A. B, Densitometrical analysis of 3 similar experiments as described in panel A. Data are given as mean ± SEM. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \) vs basal.

Figure 6. Time course of Egr-1 mRNA induction after exposure to a single cycle of mechanical strain. VSM cells on collagen-coated silicone elastomer plates were exposed to a single cycle (1 second) of mechanical strain (25%) and then incubated in the relaxed position for the indicated times. A, Total RNA was harvested and hybridized with Egr-1 and GAPDH as in Figure 1A. B, Densitometric analysis of experiments as described in panel A. Bars represent the mean ± SEM for 3 similar experiments. * \( P < 0.05 \) vs basal.
or FN, *P < 0.05, n = 3). In contradiction, the expression level of c-jun after 30 minutes of mechanical strain was similar on all 3 different extracellular matrices (Col I: 2.5 ± 0.2-fold; FN: 2.3 ± 0.2-fold; and LN: 2.1 ± 0.2-fold induction versus expression without strain on Col I, n = 4, respectively).

Figure 7. Induction of an Egr-1 promoter CAT construct by mechanical strain. VSM cells on Col I–coated silicone elastomer plates were transiently transfected with RSVβ-Gal and either RSV-CAT (RSV) or 1 of the Egr-1 promoter constructs pE50 or p1.2, whose structures are indicated. 48 hours after transfection, cells were subjected to cyclic mechanical strain for 8 hours. CAT expression was measured as described. At this time point, Egr-1 promoter-induced CAT protein expression was maximal* without reaching saturation (H.M., H.E.I., unpublished data, 1998). A similar induction of Egr-1 promoter–induced CAT protein expression was found after 30 minutes of mechanical strain following incubation in the relaxed position to reach a total incubation time of 8 hours. Bars represent the mean ± SEM for 4 similar experiments. *P < 0.05 vs basal.

Increase of Egr-1 Protein Expression After Mechanical Strain
We next examined Egr-1 protein expression after exposure of cells to various periods of continuous cyclic mechanical strain on Col I–coated plates. Western blots (Figure 9) demonstrated first increases in Egr-1 protein after 30 minutes of cyclic strain (1.3 ± 0.01-fold versus control without strain; *P < 0.01, n = 5) and a maximal 2.1 ± 0.2-fold increase at 60 minutes (**P < 0.001, n = 5). Protein expression subsequently declined toward basal levels. This pattern of expression was temporally similar to that observed for the mRNA in Figure 1.

Translocation of Egr-1 Protein to the Nucleus in Response to Mechanical Strain
Immunofluorescence imaging of Egr-1 protein was performed after 30 or 60 minutes of cyclic mechanical strain (Figure 10). In resting controls, immunofluorescence demonstrated a granular pattern of Egr-1 protein in the perinuclear space. After 30 minutes of mechanical strain, there was no change in Egr-1 distribution at the center of the strain dishes, at which strain on the cells is nearly zero. However, at the periphery of the dishes, at which strain is maximal, Egr-1 protein became uniformly distributed in the nucleus. Similar translocation was observed after 1 hour of continuous mechanical strain (data not shown). Thus, both Egr-1 expression and translocation are highly sensitive to mechanical forces in VSM cells.

Figure 8. Effect of extracellular matrix protein on induction of Egr-1 and c-jun mRNA by strain. VSM cells were plated in serum-free medium on Col I–, FN–, or LN–coated silicone elastomer plates and were subjected to 30 minutes of continuous cyclic mechanical strain (+) or no strain (−). Total RNA was isolated and analyzed as in Figure 1A. Densitometrical analysis of experiments was performed on Northern blots. Bars represent the mean ± SEM for 3 similar experiments. *P < 0.05, **P < 0.01 vs basal.

Figure 9. Expression of Egr-1 protein in response to mechanical strain. VSM cells on collagen-coated silicone elastomer plates were subjected to cyclic strain for the indicated times. A, Protein was harvested from total cell lysates and Western blots were performed with an Ab to Egr-1. B, Bars represent the mean ± SEM for 3 similar experiments. *P < 0.01, **P < 0.001 vs basal.
This study shows that mechanical strain of cultured neonatal rat VSM cells induces immediate-early gene transcription, protein synthesis, and protein translocation from the perinuclear space to the nucleus, respectively.

The time and the level of maximum induction of the immediate-early gene Egr-1 mRNA in VSM cells exposed to cyclic strain is similar to the induction found in stretched cardiac myocytes and in cyclically stretched mesangial cells. While performing a more detailed time course in VSM cells, we found that Egr-1 and c-jun transcription after mechanical strain starts between 5 and 15 minutes, reaches its maximum at 30 minutes, and returns to baseline after 4 hours or after 2 hours of continuous cyclic mechanical strain. The rapid decline in Egr-1 and c-jun mRNA during continuous cyclic strain might be due to the subsequent binding of transcription factors that downregulate the transcription of both genes and raises the possibility of an enzyme that degrades these mRNA molecules. This hypothesis is supported by the prolongation and superinduction of Egr-1 and c-jun mRNA after exposure to strain in the presence of the inhibitor of de novo protein biosynthesis, cycloheximide. This effect might be due to the inhibition of de novo synthesis of proteins that downregulate the transcription of both immediate-early genes. In addition, cycloheximide prevents synthesis of a protein, which degrades the poly (A) tail of certain mRNAs and thus superinduces many immediate-early response genes.

Evidence that the induction of Egr-1 mRNA by strain is due to increased transcription was obtained with a 957-bp full-length promoter CAT construct. Known responsive elements in this promoter region include 2 AP-1 sites, 4 SP-1 sites, 2 cAMP response elements, 6 CARG-boxes or serum response elements, and 4 Egr-1 binding sites. Any of these, or as yet undefined sequences, could be "strain responsive elements." Because the AP-1 transcription factor is composed of c-jun homodimers or c-jun/c-fos heterodimers, the induction of c-jun but not c-fos by mechanical strain suggests the activation of c-jun homodimers in response to strain in VSM cells. The involvement of c-jun in the cellular response to mechanical strain is supported by recent data from this laboratory, which show the activation of c-jun amino terminal kinase by cyclic strain in VSM cells. In additional preliminary studies, we could show that the SP-1 protein is induced by strain, possibly also implicating SP-1 sites in the strain response. Recently, a functional interplay between Egr-1 and SP-1 in the PDGF-A chain promoter has been elegantly demonstrated in bovine aortic endothelial cells in response to shear stress. In these studies, the binding of Egr-1 to the PDGF-A chain promoter was induced by shear stress, which displaced SP-1 from their overlapping recognition elements. Ongoing work from our laboratory suggests a similar binding of the Egr-1 protein to its binding site in response to mechanical strain in VSM cells (E.W., H.E.I., unpublished data, 1998).

In this work, we attempted to determine the minimal mechanical stimulus needed to activate Egr-1 gene activity. A single, transient, mechanical stimulus that lasted 5 minutes was shown to be sufficient to induce DNA synthesis in adult human VSM cells. In cardiac myocytes, c-fos mRNA was induced by as little as 1 minute of continuous mechanical strain. Surprisingly, in our system, even a single cycle of strain/relaxation that lasted only 1 second was sufficient to induce Egr-1 and c-jun mRNA. To our knowledge, this is the shortest duration of any mechanical perturbation reported to induce gene activity. The minimal magnitude of strain necessary to induce Egr-1 or c-jun mRNA by single cycle of strain/relaxation was between 5% and 10% (or 15% and 20% at the dish periphery). Because the strain profile on the dishes we used is not homogeneous and many of the cells are actually exposed to lesser degrees of strain, this value of 10% sets the upper limit of strain necessary to elicit a cellular response. After application of a single stretch that lasted 1 second to VSM cells grown on uniformly distensible silicone membranes and after incubation of these cells for 30 minutes in the relaxed position, we found a similar threshold level of Egr-1 mRNA induction in response to strain (H.M., D.D., H.E.I., unpublished data, 1998). The threshold strain level needed to induce Egr-1 mRNA by continuous cyclic strain was 5% lower than for a single perturbation. Although the system we studied cannot be directly compared with VSM cells in vivo, the threshold strain levels we determined are comparable to strains of 6% to 22% observed in certain intact blood vessels. It is also possible that the in vivo threshold for Egr-1 induction is reached only during injury to the vessel wall or in severe hypertension, when strain of the vessel wall is abnormally increased.

Additional studies were aimed at determining the mechanism by which strain is sensed in VSM cells. Previous work from this laboratory indicates that strain is sensed by specific cell–extracellular matrix interactions. Induction of DNA synthesis and activation of MAP kinase was found in neonatal VSM cells stretched on collagen or FN but not in cells on LN. This is not due to a failure of the cells to detect strain on LN, because strain increased expression of smooth muscle myosin isofoms and activated c-jun amino terminal kinase.
in cells on LN. Thus, cells respond differently to strain when they are plated on different matrix proteins.

In the current work, we demonstrate that the induction of Egr-1 follows the pattern previously observed for the proliferative response to strain. Egr-1 mRNA was significantly increased in cells on Col I and the fibronectin-like protein FN but was not increased on LN. The maximal magnitude of Egr-1 induction on collagen I and FN was somewhat lower in this group of experiments than in the earlier experiments on Col I. This was probably due to the shorter period (24 hours versus 72 hours) allowed for the cells to achieve quiescence. The shorter time period was chosen to minimize the production of extracellular matrix proteins, which might have interfered with the impact of the specific matrix proteins used to coat the silicone dishes. We do not yet know the significance of the higher basal (without strain) expression of Egr-1 in cells plated on LN, but it is consistent with higher basal rates of thymidine incorporation in cells plated on LN than on Col I or fibronectin. The basal expression of c-jun was increased on LN in a similar way. In contrast, c-jun mRNA expression after application of mechanical strain is similar on all 3 extracellular matrices tested. Thus, the effect of strain on Egr-1 expression is closely correlated with its effect on DNA synthesis in cells on various extracellular matrix proteins. This observation supports the hypothesis that Egr-1 may contribute to the induction of PDGF-A transcription and ultimately secretion of PDGF in response to strain. On the other hand, c-jun mRNA transcription and c-jun amino terminal kinase are activated on collagen, FN, and LN. This pattern of activation might reflect a matrix-independent, more general response to extracellular matrix stress in VSM cells. VSM cells in the media of large arteries in vivo (eg, in the aorta, in which mechanical stimuli of 6% to 22% strain are relevant) and in the periphery of the Flexcell plates at which strain was maximal (25%) and not at the center of the plates, at which strain was less than 3%. This finding suggests an initial direct effect of strain on Egr-1 translocation rather than an indirect effect of secreted factors, such as PDGF. PDGF has previously been shown to be secreted into the medium after several hours exposure of VSM cells to strain. In addition, these data suggest that translocation of Egr-1 requires strain, which, as noted above for mRNA induction, is in the range observed in intact blood vessels.

In summary, our results indicate that Egr-1 and c-jun expression and translocation are exquisitely sensitive to mechanical strain in VSM cells. The signal transduction pathway for this induction involves specific cell/matrix interactions. In response to strain, Egr-1 and c-jun may then participate in the regulation of other genes as part of developmental processes or in the adaptive response to injury or hypertension. The inducible expression of Egr-1–dependent genes has been proposed as a paradigm of transcriptional activation in vascular endothelium and VSM cells. In particular, we propose that early induction of Egr-1 in response to strain participates in the induction of PDGF-A expression, which subsequently leads to secretion of PDGF and proliferation of cells exposed to cyclic mechanical strain.

Acknowledgments

This work was supported by National Institutes of Health grants HL48474 (H.E.I.) and DK07219 (Y.-H.M.) and the German Academic Exchange Service and the Deutsche Forschungsgemeinschaft (H.M.).

References


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Circ Res. 1999;84:678-687
doi: 10.1161/01.RES.84.6.678

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