Mechano-Sensitive Transcriptional Factor Egr-1 Regulates Insulin-Like Growth Factor-1 Receptor Expression and Contributes to Neointima Formation in Vein Grafts

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Objective—Vein grafts in a coronary bypass or a hemodialysis access often develop obliterative growth of the neointima. We previously reported that the mechanical stretch-activated insulin-like growth factor-1 receptor (IGF-1/IGF-1R) pathway plays an important role in this remodeling. However, the transcriptional mechanism(s) regulating IGF-1R expression and neointima formation have not been identified.

Methods and Results—Deletion and site-specific mutagenesis analysis of IGF-1R promoter identified that the minimal mecano-responsive promoter element (−270−−130) contains 2 consensus sequences for binding of early growth response-1 (Egr-1) transcriptional factor. Mechanical stretch stimulated both Egr-1 mRNA (4.6-fold) and protein (5.2-fold) in vascular smooth muscle cells. Interposition of a vein into an artery increased Egr-1 mRNA (7.8±2.6-fold vs sham). In vascular smooth muscle cells isolated from Egr-1 knockout mice, mechanical stretch could not increase IGF-1R, and vascular smooth muscle cells proliferation was decreased by 47% compared to wild-type cells. Importantly, the neointima area was reduced by at least 50%, and the lumen-to-media ratio increased by 55% in vein grafts of Egr-1 knockout mice compared with results of wild-type mice.

Conclusion—Egr-1 is a mechano-sensitive transcriptional factor that stimulates IGF-1R transcription, resulting in vascular remodeling of vein grafts. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: early growth response ■ insulin-like growth factor-1 receptor ■ mechanical stretch ■ transcription ■ vein grafts

Vein segments in a coronary bypass graft or in venous anastomoses for hemodialysis access often develop obliterative stenosis because of proliferative thickening of the intima. Unfortunately, there is no effective strategy that prevents this clinical problem. One prominent factor in these venous grafts is the mechanical stress imposed by arterial blood pressure.1–4 Elevated mechanical stress is a main etiologic factor that induces neointima proliferation in vein grafts.1–4 Earlier, we demonstrated that insulin-like growth factor-1 (IGF-1) is essential for vascular smooth muscle cell (VSMC) proliferation.5 Subsequently, we found that mechanical stretch activates IGF-1 insulin-like growth factor-1 receptor (IGF-1R) signaling, causing VSMC proliferation. In fact, when IGF-1R is knocked-down in mouse, there was significant suppression of neointima formation in vein grafts.8 It is not known, however, what transcriptional signal/mechanism is responsible for mechanical stretch-induced activation of IGF-1/IGF-1R and VSMC proliferation in vein grafts.

Multiple transcription factors can regulate IGF-1R transcription in response to different stimuli. Previously, we and others7 reported that angiotensin II induces IGF-1R transcription in VSMC by a mechanism involving activation of NF-κB, leading to IGF-1R expression by Ras/MAP kinase 1-dependent and inhibitory-κB kinase-dependent pathways. Thrombin also transcriptionally regulates the IGF-1R via a redox-sensitive protein tyrosine kinase-dependent pathway.8 In addition, transcription factors STAT1, STAT3, and c-Jun are required for basic fibroblast growth factor-induced transcription of IGF-1R.9 The transcriptional factors Wilms tumor 1 (a member of the early growth response gene family) and p53 can also regulate IGF-1R gene expression.10–12

Egr-1 is a member of the early growth response gene family and can function as a key transcriptional factor mediating gene expression after vascular injury.13 Early growth response-1 (Egr-1) is not expressed in the normal artery wall but is expressed in response to acute injury.13,14 or angiotensin II.15 Mechanical stretch can increase Egr-1 transcription in endothelial cells or smooth muscle cells.16,17 The impact of Egr-1 on mechanical stretch-induced IGF-1R expression and neointima formation is not known.

We investigated how mechanical stretch stimulates IGF-1R transcription and VSMC proliferation in vein grafts; we found that Egr-1 is a transcriptional mediator that regulates IGF-1R expression. Knockout of Egr-1 significantly suppressed VSMC proliferation and neointima formation in vein grafts.

Materials and Methods

Animals

Egr-1 knockout and control C57/BL6 mice were purchased from Taconic Farm (Germantown, NY). Briefly, Egr-1 knockout mouse...
was targeted by creating the Egr-1 gene in D3 embryonic stem cells derived from 129S2/SvPas mice. The targeting vector introduced several in-frame stop codons into the Egr-1 coding sequence upstream of the zinc finger DNA binding domain. Resultant chimeras were backcrossed to C57BL/6 for 12 generations. The experiments were approved by the Institutional Animal Care and Use Committee.

Vein grafts were created as described. In brief, the vena cava from a donor mouse was grafted between 2 ends of a carotid artery by “sleeving” the ends of the vein over the artery cuff and secured with 8.0 silk sutures. After 4 weeks, the vein grafts were harvested and the intima plus media were measured as the region between the lumen and the adventitia. Vessel wall thickness was measured as the cross-sectional area of the vessel minus that of the lumen using NIH Image Program Image J and AxioVision software. Five cross-sections were examined as described by Cheng et al.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated by Tri-reagent (Sigma-Aldrich) and real-time polymerase chain reaction was performed as described.

**Western Blot Analysis**

Cells were lysed in RIPA buffer (25 mmol/L Tris.HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and equal amounts of protein were loaded onto 10% SDS-polyacrylamide gel, separated by electrophoresis and transferred onto PVDF membranes. Membranes were incubated with specific antibodies as described.

**Statistics**

Results are presented as mean ± SEM. Comparison between groups was made using 1-way ANOVA; P < 0.05 was considered statistically significant.

**Other Methods and Materials**

Detailed information can be found in the online supplemental Methods section.

**Results**

**Mechanical Stretch Increases Egr-1 Expression in Both the Grafted Vein and Cultured VSMC**

To identify mechanical stretch-regulated genes in VSMC, we exposed cultured vein VSMC to mechanical stretch for 2 hours and examined mRNA levels by Affymetrix gene array. Mechanical stretch significantly increased the expression of the transcriptional factor Egr-1 (unpublished data, 2009). To extend this to an in vivo model, we created vein grafts in mice as shown in Figure 1B, as early as 5 minutes after being applied, mechanical stretch stimulated Egr-1 mRNA; the peak was present at 30 minutes (4.6-fold vs control; n = 4; P < 0.05). The increase in Egr-1 mRNA was followed up with a peak level of protein at 1 hour (2.2-fold vs control; n = 5; P < 0.01; Figure 1C).

To determine how mechanical stretch regulates Egr-1 expression, we examined the time course of Egr-1 expression in VSMC subjected to mechanical stretch. As shown in Figure 1B, Egr-1 mRNA was increased in the grafted vein compared to control normal vein (7.8 ± 2.6-fold; n = 4; P < 0.01; Figure 1A).

**Knockout of Egr-1 Suppresses Mechanical Stretch-Induced VSMC Proliferation**

To explore the functional consequence of mechanical stretch activation of Egr-1, we isolated VSMC from veins of wild-type and Egr-1 knockout mice. After mechanical stretch treatment, BrdUrd-positive cells in wild-type VSMC increased 8.3-fold (Figure 2). This response was significantly suppressed (52.6% ± 8.6% of wild-type stretch value; n = 4; P < 0.01) in VSMC isolated from Egr-1 knockout mice. This result links activated Egr-1 to mechanical stretch-induced VSMC proliferation.

**Egr-1 Is Involved in Mechanical Stretch-Stimulated IGF-1R Transcription**

Since we previously reported that mechanical stretch stimulates transcription of IGF-1R, which plays an important role in neointima formation in vein grafts, we investigated the role of transcriptional factor Egr-1 in regulating IGF-1R transcription. Venous VSMC isolated from wild-type and Egr-1 knockout mice were subjected to mechanical stretch for 0 to 2 hours, and the changes of IGF-1R mRNA were determined by real-time quantitative polymerase chain reaction. The mechanical stretch-induced increase in IGF-1R mRNA was significantly suppressed in VSMC isolated from Egr-1 knockout mice (64% ± 6.6% of wild-type stretch value; n = 4; P < 0.01; Figure 3A). We also found that suppression of mechanical stretch-stimulated IGF-1R...
mRNA was mirrored by changes in IGF-1R protein (46.61 ± 7% of wild-type value; n=5; P<0.01; Figure 3B) at 24 hours. Note that knockout of Egr-1 did not completely inhibit mechanical stretch-induced IGF-1R transcription; therefore, we measured other transcriptional factor activity in response to mechanical stretch. As shown in Figure I, mechanical stretch also significantly increased NF-κB transcriptional activity (4.0±0.6-fold vs control; without mechanical stretch; n=4), consistent with other reports.19,20 The effect of mechanical stretch on activation of both Egr-1 and NF-κB is specific because mechanical stretch did not increase other transcriptional factor activity. Next, we examined if Egr-1 regulates IGF-1R expression in vein grafts when mechanical stretch is high. As shown in Figure 3C, interposing a vein graft into the carotid artery significantly increased IGF-1R mRNA (3.8±0.6-fold vs sham surgery; n=4; P<0.01). In contrast, increase of IGF-1R mRNA in vein grafts created in Egr-1 knockout mice was significantly reduced (53.0±6.8% of the control; wild-type vein graft value; n=4; P<0.05). These results indicate that Egr-1 plays an important role in stretch-activated IGF-1R transcription in vein grafts.

**Mechanical Stretch-Activated Egr-1 Regulates IGF-1R Promoter Activity**

To identify the mechanical stretch-responsive transcriptional region(s) in the IGF-1R promoter, we performed 5′-end deletion analysis of IGF-1R promoter (−460/+21). Chinese hamster ovary (CHO) cells were transiently transfected with these promoter–luciferase reporter constructs and with pRL-TK (as an internal transfection control). As shown in Figure 4A, mechanical stretch significantly increased IGF-1R promoter (−460/+21) activity in CHO cells. The mechanical stretch-responsive element was shown to be located between nucleotides −270 and −130 of the 5′ flanking region of IGF-1R promoter.

To further determine the mechanical stretch-responsive element(s) in the IGF-1R promoter, we replaced consecutive 20-bp stretches of IGF-1R sequence with the same length of linker between −270/+21 of the IGF-1R promoter and subcloned these mutations into the pGL2 basic luciferase vector. Mechanical stretch did not stimulate promoter activity in mutations at −170/−150 (1.2±0.4-fold increase; l; not significant; n=5) or at −210/−190 (1.7±0.3-fold increase; not significant; n=5) compared with control without stretch basal promoter activity (Figure 4B). These results suggest that mutations of the Egr-1 site of −170/−150 or −210/−190 in IGF-1R promoter significantly suppress mechanical stretch-stimulated transcriptional activity. As shown in Figure 4C, there is an exact Egr-1 binding site (GGCGGGCGCG, −196/−188) located within −210/−190 and an Egr-1 like site (GGCGGGCGCG, −162/−154) located within −170/−150 of IGF-1R promoter.
Mechanical Stretch Increases Egr-1 Binding to the Egr-1 Site in the IGF-1R Promoter

To determine if mechanical stretch also stimulates Egr-1 promoter binding activity, we performed EMSA using a p32-labeled Egr-1 classic probe. As shown in Figure 4E, mechanical stretch increased Egr-1 promoter binding activity in a time-dependent manner. Egr-1 binding was eliminated when an excess (100-fold) of Egr-1 cold probe was present (left lane, Figure 4E). Notably, the mechanical stretch stimulated Egr-1 binding to the Egr-1 site derived from the IGF-1R promoter; however, when this site is mutated there is no binding at all (left lane, Figure 4F).

To further demonstrate that Egr-1 binds to the IGF-1R promoter region, we performed chromosome immunoprecipitation assay in VSMC subjected to mechanical stretch. Chromatin was immunoprecipitated with an anti-Egr-1 antibody, and followed-up by polymerase chain reaction amplification of the 106-bp promoter region of IGF-1R. We found that Egr-1 binds to the IGF-1R promoter as early as 15 minutes and lasts for 30 minutes of VSMC treatment with mechanical stretch (Figure 4D,E). Finally, to detect mechanical stretch activation of Egr-1 transcriptional activity in vein grafts, we measured the nuclear accumulation of Egr-1 in vein grafts at 3 hours after placement of vein into artery. As shown in Figure 4G, there was a rapid nuclei translocation of Egr-1 in VSMC of grated veins assessed.
by immunofluorescence staining. These results indicate that Egr-1 induced by mechanical stretch binds to the transcription site in the IGF-1R promoter and, hence, could be involved in mechanical stretch-stimulated IGF-1R transcription in grafted veins.

Loss of Egr-1 Reduces Neointima Formation in Vein Grafts

To assess the pathophysiological importance of mechanical stretch-activated Egr-1, we placed vein grafts from donor Egr-1 knockout mice into a littermate Egr-1 knockout mice. After 4 weeks, the grafted veins were collected and analyzed. As shown in Figure 5, there was a significant reduction in the IGF-1R protein level in these vein grafts compared to results from vein grafts of wild-type control mouse; the neointima area of vein grafts in Egr-1 knockout mice was reduced by at least 50% vs grafts in wild-type mice (Figure 5B). Likewise, the lumen-to-media ratio was increased by 55% in vein grafts in Egr-1 knockout mice (Figure 5C). Our results indicate that Egr-1 plays an important role in regulating IGF-1R expression and neointima formation.

Because Egr-1 is also known to regulate several vascular remodeling genes, we immunochemically stained vein grafts and found platelet-derived growth factor-A level in the neointima in Egr-1 knockout mice was lower than that resulting in wild-type mice (Figure II).

Discussion

Previously, we demonstrated that mechanical stretch stimulates IGF-1/IGF-1R expression and activates its downstream signaling, a process that stimulates venous VSMC proliferation and contributes to neointima formation in vein grafts. However, the transcriptional mechanisms by which venous VSMC respond to elevated mechanical stretch have not been characterized. Our present study identified Egr-1 as a mechano-sensitive transcription factor that stimulates IGF-1R expression in venous VSMC. We also demonstrated that mechanical stretch activation of Egr-1 is critical for neointima formation in vein grafts.

Activation of IGF-1 receptor plays an essential role in growth factor-induced VSMC proliferation. Our present results provide insights into how mechanical stretch stimulates IGF-1R transcription. Using a deletion and mutagenesis analysis, we showed that $-170/-150$ or $-210/-190$ of the IGF-1R promoter is responsible for the mechanical stretch-induced transcriptional response of VSMC (Figure 4A,B). Within these regions, there are 2 Egr-1 sites: $-196/-188$ (GCCGGGGCGC) and $-162/-154$ (GCCGGCGCGC) located within IGF-1R promoter (Figure 4C). An essential role of Egr-1 in regulating IGF-1R transcription was supported by our findings. First, mechanical stretch stimulated binding of Egr-1 to the IGF-1R promoter (Figure 4). Second, mechanical stretch could not induce IGF-1R transcription either in venous smooth muscle cells isolated from Egr-1 knockout mouse or in the vein grafts of Egr-1 knockout mice (Figure 3). However, knockout of Egr-1 did not completely abolish mechanical stretch-induced increases in IGF-1R transcription, suggesting other transcriptional factors are also involved in this process. We found that mechanical stretch significantly increased NF-$\kappa$B activity (Figure I). In lieu of our earlier report that NF-$\kappa$B regulates IGF-1R transcription, it is no surprise that knockout of Egr-1 does not completely inhibit mechanical stretch-induced IGF-1R transcription. Taken together, our results demonstrate that Egr-1 mediates mechanical stretch-stimulated IGF-1R transcription. This conclusion adds to the established roles that is played by Egr-1, which include the established roles that is played by Egr-1, which include regulation of inflammatory genes (eg, CD44, TNF-$\alpha$) and growth factors (eg, TGF-$\beta$, platelet-derived growth factor-A, and platelet-derived growth factor-B).

The effect of knockout of Egr-1 on neointima formation in vein graft is similar to the effect of that knockout of IGF-1R, suggesting a critical link of Egr-1 in mechanical stretch-induced IGF-1R expression and neointima formation in vein graft. However, it should be pointed out that it is unlikely that the Egr-1 effect is mediated by solely regulating IGF-1R expression, because Egr-1 can also regulate other vascular remodeling genes.

We found that the level of platelet-derived growth factor-A in the neointima of vein grafts in Egr-1 knockout mice was also lower than that of wild-type mouse (Figure II). There are also studies demonstrating that Egr-1 can be activated by mechanical stretch in cultured vascular cells. In the present study, we extended these findings to in vivo studies. We found that Egr-1 expression was rapidly increased in vein grafts 3 hours after surgery (Figure 1), and this event involves transcriptional activation of IGF-1R. These in vivo results indicate that mechanical stretch stimulates Egr-1 transcription and its activity not only in cultured VSMC but also in the grafted veins. How could mechanical stretch increase Egr-1 transcription? Mechanical stretch can directly activate the AT1 receptor without the involvement of angiotensin II. Additionally, mechanical stretch increase Egr-1 transcription by protein kinase C-dependent, calcium-dependent, and p44/42 MAPK-dependent mechanisms. Therefore, it will be interesting to test if AT-1 is the mechano-sensor for mechanical stretch-stimulated Egr-1 transcription in the future.

Conclusion

Our study also elucidates the consequence of mechanical stretch activation of Egr-1 in neointima formation in vein graft. It is known that neointimal cells in vein grafts derived are not only from the grafted vein but also from cells outside of grafted vein (eg, bone marrow-derived cells). Our goal is to understand the mechanism by which mechanical stretch activates Egr-1 and its role in neointima formation in vein graft.
is to study the role of Egr-1 in neointima formation (accumulation of neointimal cells of both sources); therefore, we design our experiments by placing vein graft from Egr-1 knockout mice into littermate Egr-1 knockout mice. As shown in Figure 5, both neointima formation and IGF-1R expression in vein graft are significantly suppressed in Egr-1 knockout mice compared with wild-type mice.

In summary, we identified transcriptional factor Egr-1, by which mechanical stretch activates IGF-1R transcription in the vein graft. This pathway contributes to neointima formation, causing the failure of hemodialysis accesses or coronary bypass grafts.

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Disclosure
None.

References
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Supplemental methods

*Immunohistochemistry* After removing paraffin and rehydrating, sections were incubated for 30 minutes in 3% H$_2$O$_2$ at room temperature (RT), washed with doubly distilled water and PBS and heated in 1% citric acid to 199°F for 25 minutes. Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes and then incubated with rabbit anti-IGF-1R (Santa cruz), mouse anti-SMA(Cell signaling) or goat anti-PDGF(Santa cruz) overnight. After washing, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) at RT for 40 minutes. After washes, sections were incubated with the ABCElite reagent (Vector Laboratories) for 10 minutes. The immunostaining was visualized using a peroxidase substrate kit (Vector Laboratories) according to the manufacturer's protocol. The sections were also counterstained with hematoxylin, dehydrated in alcohol, cleared with Histo-clear, and examined by light microscopy. For a negative control, the same protocol was used with antigen dilution reagent instead of the primary antibodies. The staining density was analyzed with SigmaScan Pro. 5.0.

*Vein Smooth Muscle Cells and Mechanical Stretch* VSMCs isolated from the vena cava of wild-type or Egr-1 knockout mice were kept in Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and humidified 95% air/5% CO$_2$. To apply mechanical stretch, 90% confluent VSMCs on silicone elastomer-bottomed and collagen-coated plates (Flexcell,
McKeesport, PA) were subjected to mechanical stretch using a computer-controlled Cyclic Stress Unit (Flexcell 4000) with a defined cyclic deformation (60 cycles/min) and 15% elongation.

Primers for Quantitative Real-time PCR Primers for mouse Egr-1 were: forward, 5'-AAC AAC CCT ATG AGC ACC TGA CCA-3', and reverse, 5'-AGT CGT TTG GCT GGG ATA ACT CGT-3'. For mouse IGF-1R amplification primers used were: forward, 5'-TGC CAG CAG AAG GAG CAG ATG ATA-3' and reverse, 5'-ATC CTC GAC TTG CGA CCC GTA TTT-3'. For mouse GAPDH amplification primers used were: forward, 5'-CAT GGC CTT CCG TGT TCC TA-3', and reverse, 5'-GCG GCA CGT CAG ATC CA-3'.

BrdU Incorporation BrdU immunostaining was performed according to the manufacturer's protocol (Roche, Indianapolis, IN). We counted 4~5 different views under 100X magnification and we repeated the experiment for four times. The minimum 7000 cells in each group were counted.

IGF-1R promoter construct: The IGF-1R promoter reporter gene (-476/+640-Luc) was used to generate series 5'-end deletion fragments. These fragments were subcloned into the firefly luciferase pGL2 basic vector as described\(^1,2\). The mutants for IGF-1R promoter in mut -250/-230, -230/-210, -210/-190, -190/-170 or -170/-150 construct were created by a standard PCR-based linker-scan mutagenesis. We replaced the these sequences with a same-length linker: 5' - GAG ATC TTG ATG AGA TAT CA-3'. Briefly, the linker sequence was incorporated so that it flanked the PCR primer of the
IGF-IR promoter and the mutated fragments were subcloned into pGL2 luciferase vector.

**Transient Transfection and Luciferase Assay** The minimal IGF-1R promoter has low promoter activity and VSMC is difficult to transfect. CHO cell is an alternative cell line for studying the transcriptional regulation of IGF-1R\(^1\)\(^3\). Adherent CHO cells (10\(^7\)) were transfected with 1 µg IGF-IR-Luc and 2.5 ng Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were then incubated in serum-free media for 24 hours before being treated with or without mechanical stretch for 16 hours. by Promega (Madison, WI) dual luciferase system. pRL-TK was co-transfected and the level of firefly luciferase activity was normalized to Renilla luciferase activity measured.

**Electrophoretic Mobility Shift Assay (EMSA)** Extracts from nuclei of VSMCs were prepared as described\(^2\). A double-stranded oligonucleotide containing the Egr-1-like sequences (underlined), 5'-TCC CCA CGC CCG CGC TCC G-3', and 5'- CGG AGC GCG GGC G TG GGG AG -3', was used to measure the DNA-binding activity of Egr-1 by EMSA. To test for Egr-1-specific binding to the IGF-1R promoter, we replaced the above oligonucleotide with a double-stranded oligonucleotide containing a linker sequence: 5'-TCC CCA CGT TAC GAC TCC G-3', and 5'-CGG AGT CGT AAC GTG GGG AG-3' in the binding mixture.

**Chromatin Immunoprecipitation Assay (ChIP)** ChIP analysis was performed according the manufacture's protocol (Upstate Biotechnology, Lake Placid, NY).
with a slight modification as described\textsuperscript{2}. Precipitated DNA were amplified by PCR (38 cycles) using the following promoter-specific primers: forward (\textsuperscript{-236 to -220}), 5'-GCG TGG CTC AGT GTG CG-3', and reverse (\textsuperscript{-146 to -130}), 5'-GGG ATC GCA GGG ACG TG-3' to amplify a 106-bp region of the mouse IGF-IR promoter.

**Reference**


Supplemental figures

Figure I  The effect of mechanical stretch on promoter activity of different transcriptional factors. pNFκB-luc, pE2F-luc, pRb-luc, pmyc-luc and pP53-luc were transfected into CHO cells, and promoter activities were measured after treatment with/without mechanical stretch for 24 hours. pRL-TK was co-transfected and the level of firefly luciferase activity was normalized to Renilla luciferase activity. The fold changes were calculated by comparing them with the value that was obtained in CHO cells without mechanical stretch treatment. Data are expressed as mean ± SE (*, *P*<0.05 vs. control, n=5).

Figure II  Loss of Egr-1 suppresses PDGF expression in vein graft. The grafted vein from wild type mice and Egr-1 knockout mice were immunostained with anti-PDGF antibody. Positive signaling was shown in Brown (magnification, 400×).
Wu et al. supplemental Figure 1
Wu et al. supplemental Figure 2