Regulation of Discoidin Domain Receptor 2 by Cyclic Mechanical Stretch in Cultured Rat Vascular Smooth Muscle Cells

Kou-Gi Shyu, Ya-Meng Chao, Bao-Wei Wang, Peiliang Kuan

Abstract—Discoidin domain receptor 2 (DDR2) plays potential roles in the regulation of collagen turnover mediated by smooth muscle cells in atherosclerosis. How mechanical stretch affects the regulation of DDR2 in smooth muscle cells is not fully understood. We sought to investigate the cellular and molecular mechanisms of regulation of DDR2 by cyclic stretch in smooth muscle cells. Rat vascular smooth muscle cells grown on a flexible membrane base were stretched by vacuum to 20% of maximum elongation, at 60 cycles/min. Cyclic stretch significantly increased DDR2 protein and mRNA expression after stretch. Cyclic stretch also significantly increased DNA–protein binding activity of Myc-Max. Addition of SB203580, transforming growth factor-β1 (TGF-β1) monoclonal antibody, p38 small interfering RNA (siRNA), and c-myc siRNA 30 minutes before stretch inhibited the induction of DDR2 protein and abolished the DNA–protein binding activity induced by cyclic stretch. Cyclic stretch increased, whereas SB203580 abolished the phosphorylated p38 protein. Conditioned medium from stretched smooth muscle cells and exogenous administration of angiotensin II and TGF-β1 recombinant proteins to the nonstretched cells increased DDR2 protein expression similar to that seen after stretch. In conclusion, cyclic mechanical stretch enhances DDR2 expression in cultured rat smooth muscle cells. The stretch-induced DDR2 is mediated by angiotensin II and TGF-β1, at least in part, through p38 mitogen-activated protein kinase and Myc pathway. (Hypertension. 2005;46:614-621.)

Key Words: muscle, smooth, vascular • protein kinases • angiotensin II • transforming growth factors

Discoidin domain receptor 1 (DDR1) and DDR2 are unusual receptor tyrosine kinases in that their ligands are fibrillar collagen rather than growth factor–like peptides. DDR1 is expressed mainly in epithelial cells, whereas DDR2 is found in mesenchymal cells. Evidences from the generation of DDR1 and DDR2-null mice and in vitro studies suggest that DDR can regulate cell proliferation and extracellular matrix remodeling mediated by matrix metalloproteinase (MMP) activities during normal development or pathological conditions. Prolonged stimulation of DDR2 has been associated with the upregulation of MMP-1 expression. DDR2 also plays an important role in mediating hepatic stellate cells and fibroblast migration and proliferation by MMP-2–dependent mechanisms. Recently, Ferri et al showed that DDR 1 and DDR2 play potential roles in the regulation of collagen turnover mediated by vascular smooth muscle cells (VSMCs) in obstructive diseases of blood vessel. However, the clear picture of DDR-signaling pathways is not known.

Transforming growth factor-β (TGF-β) plays an important role in maintaining normal vessel wall structure, and that loss of this protective effect contributes to the development of atherosclerosis. TGF-β inhibits smooth muscle proliferation, inhibits VSMC migration, and promotes the expression of an array of proteins that make up the contractile apparatus of the cell. Reduced TGF-β activity is a common consequence of a range of environmental and genetic factors associated with development of atherosclerosis. It is not known whether there is a link between DDR and TGF-β. The Myc oncoprotein is a transcription factor that can activate and repress genes. TGF-β has been shown to regulate Myc oncoprotein. The link between DDR2 and Myc has not been reported previously.

VSMCs are the major cellular components of the blood vessel wall and are subjected to a dynamic mechanical environment modulated by pulsatile pressure and oscillatory shear forces. The accompanying stress may regulate normal vascular tone and contribute to atherogenesis, the vascular hypertrophy associated with hypertension, and the acute rupture of atherosclerotic lesions. How cyclic mechanical stretch affects the regulation of DR2 in smooth muscle cells (SMCs) has not been characterized previously. The present study was designed to investigate the cellular and molecular mechanisms of regulation of DDRs by mechanical cyclic stretch.
stretch in VSMCs and to seek the possible signal pathways mediating the expression of DRRs by cyclic mechanical stretch.

Methods

VSMC Culture

Primary cultures of VSMCs were grown by the explant technique from the thoracic aorta of 200- to 250-g male Sprague-Dawley rats, as described previously. Cultures were maintained in medium 199 containing 20% FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C under 5% CO2/95% air in a humidified incubator. When confluent, VSMC monolayers were passaged every 6 to 7 days after trypsinization and were used for experiment from the third to sixth passages. These third to sixth passage cells were then cultured in Flexcell I flexible membrane dish in medium 199 containing 0.5% FCS, and the cells were incubated for an additional 2 days to render them quiescent before the initiation of each experiment.

In Vitro Cyclic Strain on Cultured SMCs

The strain unit Flexcell FX-2000 (Flexcell International Co.) consists of a vacuum unit linked to a valve controlled by a computer program. VSMCs cultured on the flexible membrane base were subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure as described previously. VSMCs cultured on the flexible membrane base but not subjected to stretch were used as control. The cells were placed in a humidified incubator with an atmosphere of 5% CO2 at 37°C. Pretreatment of cells with different inhibitor sources and specificity of action are available in the online data verification and then eluted and served as probe to detect respective targets.

Immunoprecipitation and Western Blot

VSMCs exposed to cyclic stretch at 20% elongation were harvested by scraping and then centrifuged (300g) for 10 minutes at 4°C. The pellet was resuspended and homogenized in a reporter lysis buffer (Promega Corp.), centrifuging at 10 600 g for 20 minutes. Protein content of the supernatant was determined by the Bio-Rad protein electrophoresis. Western blot was performed as described previously. Rabbit polyclonal anti-DDR2 antibody (Chemicon), polyclonal anti-p38 mitogen-activated protein (MAP) kinase and monoclonal anti-phospho p38 MAP kinase antibodies (Cell Signaling) were used.

Reverse Transcription, Polymerase Chain Reaction, and Northern Blot

Total RNA was isolated from VSMCs using the single-step acid guanidium thiocyanate/phenol/chloroform extraction method. Reverse transcription (RT) was performed as described previously. The cDNA produced by RT was used to generate DDR2 cDNA probe by polymerase chain reaction (PCR). PCR primer sequences were chosen as the following: DDR2, forward, 5'-GGCGAGAACAAATTGCTT-3'; reverse, 5'-ACCCTGACAAAACCGGG-3'. In brief, 10 µL of the RT reaction solution was used in the PCR. PCR was performed in a final volume of 50 µL containing 200 mM/L of each dATP, dCTP, dGTP, and dTTP, 5 pmol of each primer, 1.25 U of Taq polymerase, 20 mM/L Tris-Cl, pH 8.4, 1.5 mM/L MgCl2, and 50 mM/L KCl. The mixture was incubated in a thermal cycler for 35 cycles using the following profile: 94°C for 7 minutes, then repeat cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 7 minutes and cooled to 40°C. PCR products (291 bp) were ran on 2% agarose gel for DNA fragment size verification and then eluted and served as probe to detect respective mRNA in Northern blot analysis. Northern blot was performed as described previously.

RNA Interference

Rat VSMCs were transfected with 800 ng p38-annaled small interfering RNA (siRNA), c-myc siRNA oligonucleotide, or DDR2 siRNA (Santa Cruz Biotechnology). p38 or c-myc siRNA is a target-specific 20- to 25 nt siRNA designed to knock down gene expression. DDR2 sense and antisense of siRNA sequences were GGAUAGAAUACACUGCGAUU and 5'-PUCCGAGUGUUG-CUAUCAUU, respectively. As a negative control, a nontargeting siRNA (control siRNA) purchased from Dharmacon Inc. was used. After incubation at 37°C for 24 hours, cells were stretched for 18 hours and subjected to analysis.

Cytotoxicity Studies

VSMCs were adjusted to 1×10^3 cells/mL in DMEM medium. Aliquots of 20 mL of cell suspension were plated in 40-mm Petri dishes. After incubation for 24 hours, the medium was replaced with fresh medium containing SB203580 and TGF-β1 monoclonal antibody at a concentration of 20 µmol/L and 5 ng/mL, respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described previously. For detection of cell injury possibly induced by stretch, cell viability after application of cyclic stretch was monitored constantly by trypan blue staining and measurement of release of lactate dehydrogenase (LDH) into culture medium and total VSMC LDH.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed to detect the formation of Myc-Max–DNA complexes. Nuclear protein concentrations from VSMCs were determined by Bio-Rad protein assay. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labeled by polynucleotides kinase incorporation of [γ³²P]-dATP. Oligonucleotides sequences for Myc-Max consensus were 5'-GGAACGCAGACGTGCTGCTC-3'. The Myc-Max mutant oligonucleotide sequences were 5'-GGAACGAG-ACCAGGAGTCTGCTC-3'. EMSA was performed as described previously. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to labeled sequences.

Migration Assay

The migration activity of VSMCs was determined using the growth factor–reduced Matrigel invasion system (Becton Dickinson) following the protocol provided by the manufacturer. Migration assay was performed as described previously.

Statistical Analysis

The data are expressed as mean±SEM. Statistical significance was performed with Student’s t test or ANOVA (GraphPad Software Inc.) where appropriate. Dunnett’s test was used to compare multiple groups to a single control group. Tukey–Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. A value of P<0.05 was considered to denote statistical significance.

Results

Cyclic Stretch Enhances DDR2 Protein and mRNA Expression in Cultured VSMCs

Immunoprecipitation/Western blot for anti-DDR2 demonstrated that cyclic stretch increased DDR2 protein expression. Western blot with antiphosphotyrosine demonstrated that DDR2 is phosphorylated after cyclic stretch. The levels of DDR2 protein began to increase as early as 6 hours after stretch at 20% elongation, was applied, and reached a maximum of 5.7-fold over the control by 24 hours (Figure 1).
Stretch-induced DDR2 protein expression was load dependent. When VSMCs were stretched at 10% elongation, the levels of DDR2 protein did not increase significantly compared with control cells without stretch (Figure 1).

The Northern blots showed that DDR2 messages increased significantly after 6 hours and 24 hours of stretch at 20% elongation (Figure 1C). The GAPDH mRNA levels were relatively constant when VSMCs were subjected to cyclic stretch. No increase in release of LDH was observed after cyclic stretch at 20% elongation for 24 hours, and trypan blue staining also did not show any significant cell damage under these conditions. These data demonstrated that cyclic stretch at 20% elongation did not induce serious injury on VSMCs.

**Stretch-Induced DDR2 Protein Expression in VSMCs Is Mediated by TGF-β1 and p38 MAP Kinase**

To investigate the possible signal pathway that mediates the stretch-induced DDR2 in VSMCs, VSMCs were stretched 20% for 24 hours in the presence or absence of inhibitors or antibody. As shown in Figure 2A, the stretch-induced increase of DDR2 protein was significantly reduced after the addition of TGF-β1 antibody (5 μg/mL) or SB203580 30 minutes before stretch. However, the DDR2 protein induced by stretch was not affected by the addition of SP600125 or PD98059. The phosphorylated c-Jun N-terminal kinase (JNK) was blocked after addition of SP600125, and the phospho-p42/p44 MAP kinase was diminished after addition of PD98059. These findings confirmed the biological activity and correct dose of SP600125 and PD98059. To test the specific effect of p38 MAP kinase pathway mediating the expression of DDR2, p38 siRNA was transfected to VSMCs before cyclic stretch. As shown in Figure 2B, p38 siRNA also completely blocked the DDR2 expression induced by cyclic stretch (P<0.01). The control siRNA did not affect the DDR2 expression induced by cyclic stretch. These findings implicated that p38 MAP kinase pathway, but not JNK and p42/p44 MAP kinases, mediated the induction of DDR2 protein by cyclic stretch in VSMCs. Addition of TGF-β1 monoclonal antibody but not rabbit IgG antibody 30 minutes before stretch significantly blocked the induction of DDR2 expression by cyclic stretch. The conditioned medium from stretched VSMCs could induce the same increase in DDR2 protein expression in nonstretched VSMCs. The upregulation of DDR2 in static cells after addition of conditioned media was also blocked by TGF-β1 antibody. These findings suggested that cyclic stretch regulated DDR2 protein in VSMCs possibly via autocrine or paracrine mechanisms.

As shown in Figure 2C, phosphorylated p38 protein was induced by cyclic stretch for 20% elongation. The phosphorylated p38 protein induced by stretch was abolished by SB203580. Total p38 protein was not affected by cyclic stretch. MTT assay showed that the absorbency at 570 nm demonstrated no difference among control cells and cells treated with SB203580 and TGF-β1 antibody at different concentrations for up to 24 hours. These data demonstrated no cytotoxicity of SB203580 and TGF-β1 antibody on VSMCs.

**Cyclic Stretch Increases Myc-Max–Binding Activity**

Cyclic stretch of VSMCs for 6 to 24 hours significantly increased the DNA–protein binding activity of Myc-Max (Figure 3). An excess of unlabeled Myc-Max oligonucleotide competed with the probe for binding Myc-Max protein,
whereas an oligonucleotide containing a 2-bp substitution in the Myc-Max binding site did not compete for binding. Addition of SB203580, TGF-β1 monoclonal antibody, and losartan (100 nmol/L), an angiotensin II receptor blocker, 30 minutes before stretch abolished the DNA–protein binding activity induced by cyclic stretch. p38 siRNA, similar to SB203580, also abolished the DNA–protein binding activity induced by cyclic stretch. To test the significance of activation of Myc-Max transcriptional complexes for the elevation in DDR2 expression, myc siRNA was transfected to VSMCs before cyclic stretch. Myc siRNA significantly abolished the DDR2 protein expression induced by cyclic stretch (decreased from 5.1 ± 0.3-fold to 1.3 ± 0.1-fold compared with control; P < 0.001; n = 3). This finding implicates that Myc-Max is directly inducing DDR2 expression.

Cyclic Stretch Increases Angiotensin II and TGF-β1 Protein Secretion
Angiotensin II released from stretched VSMCs at 20% elongation for 18 hours increased significantly compared with control cells without stretch (75 ± 5 ng/mL versus 46 ± 4 ng/mL; P < 0.01; n = 4). However, stretch at 10% did not significantly increase angiotensin II secretion (57 ± 5 ng/mL) compared with nonstretched cells. This finding demonstrated that 20% stretch was required for DDR2 expression because it required higher concentrations of angiotensin II. TGF-β1 released from cultured medium after cyclic stretch at 20% elongation for 18 hours was significantly higher than that without stretch (2.12 ± 0.07 ng/mL versus 1.10 ± 0.07 ng/mL; P < 0.001; n = 3) as measured by ELISA (R & D Systems). Addition of losartan (100 nmol/L) 30 minutes before stretch abolished the DDR2 protein expression induced by cyclic stretch (Figure 4). The effect of TGF-β1 on DDR2 protein expression was dose dependent (data not shown). Exogenous addition of angiotensin II at 10 nmol/L to the VSMCs without stretch also increased DDR2 protein expression. Addition of losartan (100 nmol/L) 30 minutes before stretch abolished the DDR2 protein expression induced by cyclic stretch.
Addition of p38 siRNA or c-myc siRNA blocked the direct stimulation of DDR2 by angiotensin II and TGF-β1 recombinant proteins. Addition of TGF-β1 monoclonal antibody 30 minutes before angiotensin II treatment also abolished the DDR2 protein expression induced by angiotensin II. These data indicate that angiotensin II enhances DDR2 expression through angiotensin II receptor and via TGF-β1 in VSMCs. The effects of p38 and c-myc are on DDR2 regulation itself.

**DDR2 Increases Proliferation and Migration of VSMCs**

When the same numbers of VSMCs were cultured, cyclic stretch increased the cell number of VSMCs measured by a cell counter. After stretch for 24 hours, the cell number of VSMCs increased from $5.9 \pm 0.1 \times 10^5$ to $7.1 \pm 0.1 \times 10^5$ ($P<0.01$). Addition of SB203580 or losartan 30 minutes before stretch completely attenuated the increased cell num-

![Figure 3. Cyclic stretch increases Myc-Max– binding activity. Representative EMSA showing protein binding to the Myc-Max oligonucleotide in nuclear extracts of VSMCs after cyclic stretch in the presence or absence of inhibitors. Similar results were observed in another 2 independent experiments. Cold oligo means unlabeled Myc-Mac oligonucleotides.](image)

![Figure 4. Exogenous administration of angiotensin II (Ang2) and TGF-β1 increases DDR2 protein expression. Top, Representative Western blots for DDR2 in VSMCs after exogenous administration of Ang2 and TGF-β1. Bottom, Quantitative analysis of DDR2 protein levels after addition of Ang2 and TGF-β1. *$P<0.01$ vs control. 24 hour indicates cyclic stretch at 20% elongation for 24 hours (n=4).](image)
number of VSMCs induced by stretch (5.7 ± 0.1 × 10^5 and 5.8 ± 0.1 × 10^5, respectively). Rat VSMCs cultured in the conditioned medium generated from stretched cells migrated significantly through the filter membrane compared with those cultured in usual medium. Inhibition of DDR2 activity by siRNA and inhibition of TGF-β1 activity by TGF-β1 monoclonal antibody decreased the SMC migration activity (Figure 5). These findings implicate that DDR2 mediates the proliferation and migration of VSMCs induced by cyclic stretch.

**Discussion**

In this study, we demonstrated several significant findings. First, cyclic stretch upregulates DDR2 expression in rat VSMCs; second, cyclic stretch induces angiotensin II and TGF-β1 expression; third, angiotensin II and TGF-β1 act as autocrine factors to mediate the increased DDR2 expression induced by cyclic stretch; fourth, p38 MAP kinase and Myc-Max transcription factor are involved in the signaling pathway of DDR2 induction; and fifth, cyclic stretch increases VSMC proliferation and migration via DDR2. DDR2 was upregulated in a time- and load-dependent manner by cyclic stretch. Cyclic stretch of VSMCs increased DDR2 protein and mRNA expression. Because rhythmic distension of the vessel wall is a component of pulsatile flow, our study indicates that cyclic mechanical stretch is an important factor regulating DDR2 in vascular wall cells.

Cyclic stretch has been shown to induce collagen synthesis in VSMCs via angiotensin II and TGF-β. TGF-β has been shown to be modulated by cyclic stretch in VSMCs, and cyclic stretch has been shown to modulate the signaling and growth responses of SMCs to angiotensin II. However, the signaling pathway mediated by angiotensin II and TGF-β was not fully understood. DDR2, a family of receptor tyrosine kinase, is a collagen receptor. Our study established that stretch-induced DDR2 was also mediated via an autocrine-paracrine mechanism of angiotensin II and TGF-β1 and further explored the downstream signaling pathway mediating the DDR2 expression. Angiotensin II stimulates proliferation of VSMCs. TGF-β, a pleiotropic cytokine, plays a controversial role in VSMCs. Some studies reported that TGF-β promotes proliferation of VSMCs, whereas the other studies demonstrated that TGF-β inhibits proliferation and migration of VSMCs. In our study, cyclic stretch...
increased TGF-β1 protein synthesis and induced proliferation of VSMCs. Therefore, TGF-β1 seems to play a role in proliferation but not inhibition of VSMCs. Inhibition of p38 MAP kinase, angiotensin II receptor antagonist, and DDR2 siRNA inhibited the proliferation and migration of VSMCs induced by stretch. These data indicated that DDR2 may play an important role in the remodeling of vascular disease. Li et al reported that 10% cyclic stretch at 30 cycles/min increased angiotensin II secretion in the VSMCs. 27 However, 20% cyclic stretch but not 10% cyclic stretch at 60 cycles/min increased angiotensin II secretion in our study. Li et al used rabbit aortic SMCs between passages 4 and 11. Our study used rat aortic SMCs between passages 3 and 6. Different species, cell age, and stretched frequency may explain the discrepancy.

Myc proteins are basic helix-loop-helix-leucine zipper (bHLH-ZIP) transcription factors, the known biological activities of which require that they heterodimerize with the bHLH-ZIP protein Max. 33 The Myc transcription factor can activate and repress genes. 17 In this study, we demonstrated that cyclic stretch stimulation of Myc-Max–DNA binding activity required at least phosphorylation of the p38 because p38 inhibitor and p38 siRNA abolished the Myc-Max binding activity. SB203580, a potent and specific inhibitor of p38 MAP kinase, inhibited the DDR2 expression induced by stretch, whereas both inhibitors of JNK and p42/p44 MAP kinases did not have the inhibitory effect. TGF-β normally inhibits myc transcription and the subsequent formation of Myc-Max heterodimers. 17 However, in this study, we demonstrated that the TGF-β1–neutralizing antibody attenuated the Myc-Max transcriptional complexes induced by cyclic stretch. Differences in culture methods and cell types may explain this discrepancy.

In this study, we also demonstrated the complete inhibition of DDR2 by p38 siRNA. Double-stranded RNA interference can regulate gene expression at a translational level through interactions with its target messenger RNA. 34 These data implicate that the p38 MAP kinase pathway, but not the JNK and p42/p44 MAP kinases, mediates the increased transcriptional activity of Myc-Max. However, in this study, we did not demonstrate the transcriptional activity by Myc-Max–dependent reporter gene assay. Therefore, binding activity of Myc-Max was not equaled to its transcriptional activity. However, inhibition of myc messenger RNA by siRNA abolished the DDR2 protein expression induced by cyclic stretch. This finding confirmed the significance of activation of Myc-Max transcriptional complexes in inducing DDR2 expression.

In summary, our study reports for the first time that cyclic mechanical stretch enhances DDR2 expression in cultured rat VSMCs. The stretch-induced DDR2 is mediated by angiotensin II and TGF-β1, at least in part, through p38 MAP kinase and Myc-Max pathway.

**Perspectives**

DDR2 expression is regulated by cyclic stretch of VSMCs mediated by increases in TGF-β1 and angiotensin II production. These results clearly indicate that hemodynamic forces can play a significant role in the modulation of DDR2 expression of VSMCs. When blood pressure is fluctuating, high blood pressure will increase the vessel wall tension and stretch the vascular wall. Therefore, the transient increase in DDR2 gene expression after cyclic stretch may be important in patients with fluctuating high blood pressure. DDR2 is expressed in VSMCs in lesions of atherosclerosis. Based on this viewpoint, our study implicates that control of hypertension to prevent fluctuation of blood pressure and morning surge in hypertensive patients is very important.

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