Cyclic Strain Increases Protease-Activated Receptor-1 Expression in Vascular Smooth Muscle Cells

Kytai T. Nguyen, Stacie R. Frye, Suzanne G. Eskin, Cam Patterson, Marschall S. Runge, Larry V. McIntire

Abstract—Cyclic strain regulates many vascular smooth muscle cell (VSMC) functions through changing gene expression. This study investigated the effects of cyclic strain on protease-activated receptor-1 (PAR-1) expression in VSMCs and the possible signaling pathways involved, on the basis of the hypothesis that cyclic strain would enhance PAR-1 expression, reflecting increased thrombin activity. Uniaxial cyclic strain (1 Hz, 20%) of cells cultured on elastic membranes induced a 2-fold increase in both PAR-1 mRNA and protein levels. Functional activity of PAR-1, as assessed by cell proliferation in response to thrombin, was also increased by cyclic strain. In addition, treatment of cells with antioxidants or an NADPH oxidase inhibitor blocked strain-induced PAR-1 expression. Preincubation of cells with protein kinase inhibitors (staurosporine or Ro 31-8220) enhanced strain-increased PAR-1 expression, whereas inhibitors of NO synthase, tyrosine kinase, and mitogen-activated protein kinases had no effect. Cyclic strain in the presence of basic fibroblast growth factor induced PAR-1 mRNA levels beyond the effect of cyclic strain alone, whereas no additive effect was observed between cyclic strain and platelet-derived growth factor-AB. Our findings that cyclic strain upregulates PAR-1 mRNA expression but that shear stress downregulates this gene in VSMCs provide an opportunity to elucidate signaling differences by which VSMCs respond to different mechanical forces. (Hypertension. 2001;38:1038-1043.)

Key Words: muscle, smooth, vascular ▪ stress mechanical ▪ gene expression ▪ thrombin ▪ protein kinases ▪ oxidative stress

Vascular smooth muscle cells (VSMCs) in the major arteries are constantly exposed to cyclic strain (2% to 18%) that is caused by pulsatile blood flow. Recent studies indicate that cyclic strain plays an important role in growth and gene expression in VSMCs under both normal and pathological conditions.1–3 In hypertension, cyclic strain increases by as much as 30%, resulting in marked alterations in signal transduction and gene expression that contribute to VSMC hypertrophy and hyperplasia.3–5 In vein grafts, intimal and medial thickening occur in the regions of increased circumferential deformation.6 Moreover, VSMCs exposed to mechanical strain in vitro exhibit alterations in cell morphology, proliferation, production of vasoactive substances,2,3 and gene expression.7–9

Many of the effects of thrombin, including inflammation and cell proliferation, are mediated by protease-activated receptor-1 (PAR-1). Furthermore, PAR-1 expression is increased in atherosclerotic and balloon-injured arteries and in arteries of hypertensive animals10–12 indicating its contribution to the development of arterial diseases. We postulated that the PAR-1 gene in VSMCs would be regulated by cyclic strain, on the basis of the important role that PAR-1 plays in controlling VSMC functions and proliferation in vivo. Cyclic strain (20%) induced PAR-1 mRNA expression, leading to an increase in surface PAR-1 protein and an increase in cell proliferation in response to thrombin. In addition, strain-enhanced PAR-1 expression may be mediated by reactive oxygen species (ROS) through the NADPH oxidase pathway and is negatively regulated through protein kinase C (PKC). We found no evidence that NO synthase, tyrosine kinase, or mitogen-activated protein kinase (MAPK) pathways regulated strain-induced PAR-1 expression. Basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF-AB) have been shown to induce PAR-1 expression.12,13 bFGF and cyclic strain applied together increased PAR-1 expression beyond the increase induced by either stimulus alone; whereas PDGF-AB, when applied to cells concomitantly with cyclic strain, had no additional effect on PAR-1 expression.

Methods

Cell Culture and Cyclic Strain

Human aortic smooth muscle cells (HASMCs, Cascade) were cultured as described previously.14 For experiments, HASMCs (P5 to P10) were seeded (3×10^4 cells/cm²) on silicone membranes by guest on July 9, 2017 http://hyper.ahajournals.org/ Downloaded from
(0.005-in thickness, Specialty Manufacturing) coated with 5 μg/cm² human plasma fibronectin (Collaborative). At confluence, cell-cultured membranes were maintained under static conditions; cyclically strained at 5%, 10%, or 20% of the resting length at 1 Hz (60 cycles/min); or exposed to fluid agitation (motion control) in humidified 95% air/5% CO₂ at 37°C.

Northern Analysis and Flow Cytometry
Total RNA was isolated by using the fast-RNA isolation kit (BIO101) and analyzed as described previously. To correct for differences in RNA loading, the signal intensity of PAR-1 for each sample was normalized to GAPDH. For flow cytometry, HASMCs were preincubated with thrombin (4 U/mL, 15 minutes), exposed to 20% cyclic strain for 24 hours, and detached and labeled as previously described. The geometric mean fluorescence of each sample (10,000 cells) was calculated as a percentage of matched static controls.

Cell Proliferation in Response to Thrombin
HASMCs were exposed to 20% strain in serum-containing medium for 24 or 48 hours, washed, and incubated in serum-free medium (30 minutes); then thrombin (5 U/mL in serum-free DMEM) was added or not, and cells were further incubated for 24 hours under static conditions and counted (Coulter).

O₂⁻ Production
HASMCs were preincubated in serum-containing medium without phenol red (30 minutes, 37°C), and cytochrome c (final concentration, 1 mg/mL with or without superoxide dismutase (final concentration, 500 U/mL) was added. Cells were then exposed to cyclic strain; medium was collected at 0, 20, 40, and 60 minutes; and absorbance was read (550 nm). Equivalent superoxide (O₂⁻) production was estimated by converting the optical density difference between samples with or without superoxide dismutase by using the molar extinction coefficient for cytochrome c: 21 × 10⁴ (mol/L)⁻¹ · cm⁻¹.

ROS, NO, and Protein Kinase Inhibitors
HASMCs were pretreated (1 hour) with N-acetyl-L-cysteine (NAC, 1 to 20 μmol/L; antioxidant), pyrrolidine dithiocarbamate (PDTC, 50 μmol/L, antioxidant), diphenyleneiodonium chloride (DPI, 1 to 20 μmol/L; NADPH oxidase inhibitor), indomethacin (10 μmol/L, cyclooxygenase inhibitor), oxyipurinol (10 μmol/L, xanthine oxidase inhibitor), N⁶-methyl-L-arginine (L-NMMA, 1 mmol/L; NO synthase inhibitor), staurosporine (1 to 20 nmol/L, nonspecific protein kinase inhibitor), Ro 31-8220 (0.1 μmol/L, PKC inhibitor), herbimycin A (2.0 μmol/L, tyrosine kinase inhibitor), or PD 098,059 (50 μmol/L, MAPK kinase inhibitor). Cells were subjected to 20% strain in the presence of the same inhibitor for 12 hours. In separate experiments, HASMCs were treated with the PKC activator phorbol 12-myristate 13-acetate (PMA, 0.3 μmol/L) for 12 hours.

Effects of Cyclic Strain and Growth Factors
HASMCs were incubated in serum-free medium for 24 hours and then subjected to cyclic strain alone, 15 ng/mL bFGF alone, 20 ng/mL PDGF-AB alone, or a combination of cyclic strain with either growth factor for 12 hours.

Statistical Analysis
Results are mean±SEM. Statistical analysis between 2 groups was determined by a factorial ANOVA followed by the Fischer protected least significant difference test. Values of P<0.05 were considered significantly different.

Results
Cyclic Strain Induced PAR-1 mRNA and Protein Levels in HASMCs
Northern blot analysis showed that high levels of cyclic strain (20% strain) increased the expression of PAR-1 mRNA 2-fold after 6 hours (Figure 1A, P<0.05), whereas low and moderate strain (5% and 10% strain, respectively) did not induce PAR-1 mRNA significantly compared with expression in static controls (Figure 1B). Fluid motion controls corresponding to 5%, 10%, and 20% strain did not change PAR-1 mRNA significantly compared with static controls (data not shown). To investigate whether the cyclic strain–induced increase in PAR-1 mRNA was followed by an increase in PAR-1 protein, we measured cell surface PAR-1 expression. Cyclic strain (20%) increased cell surface PAR-1 protein >250% in HASMCs after 24 hours (Figure 2, 20.3±2.6 fluorescence units for 20% strain versus 7.1±0.3 fluorescence units for static controls, n=4, P<0.05).

HASMC Proliferation in Response to Thrombin
The proliferation of static and cyclically strained HASMCs in response to thrombin was measured to determine the functional consequences of cyclic strain–induced PAR-1 expression. Compared with cell proliferation in static control cells, thrombin (5 U/mL) increased cell proliferation in HASMCs after exposure to 20% strain for 48 hours (P<0.05) but not in cells strained for 24 hours (Figure 3). Without thrombin stimulation, the cell number was not significantly different between cyclically strained and static cells at either time.

Role of ROS and NO Synthase in Strain-Induced PAR-1 Expression
Cyclic strain (20%) enhanced O₂⁻ production in a time-dependent manner, reaching the maximum after 60 minutes...
of exposure (Figure 4). Two antioxidants, NAC and PDTC, significantly inhibited strain-enhanced PAR-1 mRNA (Figure 5C), whereas neither NAC (Figure 5A) nor PDTC (data not shown) inhibited PAR-1 expression in static HASMCs. Furthermore, DPI, an NADPH oxidase inhibitor, significantly blocked strain-increased PAR-1 mRNA (Figure 5B), whereas inhibitors of xanthine oxidase (oxypurinol) and cyclooxygenase (indomethacin) had no significant effects (Figure 5C). Inhibiting NO synthases with L-NMMA showed no significant effect on the increase of PAR-1 mRNA by cyclic strain (Figure 5C).

Role of Protein Kinases in Strain-Induced PAR-1 Expression

Strain-increased PAR-1 mRNA was enhanced by the nonspecific protein kinase inhibitor staurosporine at all concentrations (Figure 6A and 6B) and by the specific PKC inhibitor Ro 31-8220 (Figure 6B, P<0.05). Neither herbimycin A (tyrosine kinase inhibitor) nor PD 098,095 (MAPK kinase inhibitor) had significant effects on strain-induced PAR-1 expression (Figure 6B). None of the inhibitors had significant effects on PAR-1 mRNA in static cells nor did the PKC activator PMA (data not shown).

Effects of Cyclic Strain and Growth Factors on PAR-1 Expression

Treatment of HASMCs with 20% strain, bFGF, or PDGF-AB alone caused a marked increase in PAR-1 mRNA compared with the control condition (Figure 7, P<0.05). Exposure of cells to 20% strain together with bFGF induced a 4-fold increase in PAR-1 mRNA compared with the effect of 20% strain or bFGF alone (Figure 7, P<0.05), indicating an additive effect of cyclic strain and bFGF on PAR-1 expression. Unlike treatment with bFGF, treatment of cells with 20% strain plus PDGF-AB did not significantly increase PAR-1 mRNA compared with treatment of cells with 20% strain or PDGF-AB alone.

Discussion

Mechanical strain on the artery wall is increased up to 30% in hypertension and is postulated to play a role in vascular injury.2-5 Thrombin is concentrated in vivo at sites of vascular injury, and its effects are mediated chiefly through PAR-1.21 PAR-1 is expressed at very low levels in normal arteries but increases after vascular injury 12,18,22 and is increased in the arteries of hypertensive rats.11 Thus, we hypothesized that cyclic strain administered to VSMCs in vitro would increase PAR-1 expression. Cyclic strain increased PAR-1 mRNA and protein levels (Figures 1 and 2), and after 48 hours of cyclic strain, thrombin also produced a 50% increase in cell number (Figure 3). The significance of these findings is based on the opposite responses of PAR-1 expression to cyclic strain and shear stress. PAR-1 expression increases 2-fold under 20% cyclic strain.
strain for 24 hours, whereas it decreases by 4-fold under shear stress (25 dyne/cm²) for 24 hours. Thus, mechanical forces regulate the PAR-1 gene differently, depending on the balance of mechanical forces to which the cells are subjected over the cardiac cycle. These differences must be signaled through different pathways, or a critical on/off switch along a common pathway must play a role in producing opposing responses. Another possible explanation for differing directions of PAR-1 response is that the threshold of injury to cyclic strain might be lower than that to shear stress. Cyclic strain produces greater membrane perturbations than does shear stress. Cheng et al found that membrane disruption of SMCs occurred under 14% cyclic strain, 1 Hz, for 10 minutes, whereas Rhoads et al found less membrane disruption under 25 dyne/cm² shear stress for 15 minutes by use of the same techniques (fluorescent dextran markers).

The observations that PAR-1 promoter regions contain antioxidant response element–like consensus sequences and that cyclic strain rapidly increases superoxide production suggest an important role of oxidant-mediated mechanisms in regulating PAR-1 expression in VSMCs. In the present study, cyclic strain stimulated superoxide production in VSMCs after a short exposure (Figure 4), in agreement with previous work, and antioxidants significantly inhibited strain-induced PAR-1 expression (Figure 5). Furthermore, strain-enhanced PAR-1 expression decreased after treatment of cells with the NADPH oxidase inhibitor DPI (Figure 5). Treatment of cells with the NO synthase inhibitor L-NMMA or the xanthine oxidase (oxypurinol) or cyclooxygenase (indomethacin) inhibitors showed no effect on strain-induced PAR-1 expression.

Figure 5. Effects of ROS and NO inhibition on cyclic strain–induced PAR-1 expression. Cells were pretreated with inhibitors for 1 hour and then exposed to 20% strain or maintained in stationary conditions for 12 hours in the presence of the same inhibitor. A and B, Dose responses of PAR-1 expression to NAC (A) and DPI (B) in the presence or absence of cyclic strain. Ratio of PAR-1 to GAPDH densitometry provides normalized values. C, Effects of inhibitors on cyclic strain–increased PAR-1 expression: NAC (20 mmol/L), PDTC (50 μmol/L), DPI (10 μmol/L), indomethacin (INDO, 10 μmol/L), oxyrinol (OXY, 10 μmol/L), and L-NMMA (1 mmol/L). Results of densitometry of PAR-1 mRNA normalized to corresponding GAPDH mRNA are shown (n=3 to 6). *Significant differences from static controls (P<0.05). \[Significant differences from strained samples (P<0.05).\]
expression. This provides evidence that the PAR-1 response to cyclic strain is mediated by ROS in VSMCs through the NADPH oxidase pathway.

Besides ROS, protein kinases may act as second messengers for cyclic strain signaling. Our results with staurosporine and Ro 31-8220 were unexpected (Figure 6), with both inhibitors increasing expression at least 3-fold over stationary control levels rather than inhibiting cyclic strain–induced PAR-1 expression. Staurosporine is a nonselective PKC inhibitor (inhibiting protein kinase A, myosin light chain kinase, and phosphorylase kinase, among other enzymes), whereas Ro 31-8220 is a potent and selective inhibitor of PKC. The corroboration between the results obtained with Ro 31-8220 and staurosporine provides evidence that cyclic strain signaling is occurring through PKC.

Stimulatory responses of VSMC to staurosporine and Ro 31-8220 are not without precedent. Hecker et al found that staurosporine “paradoxically” potentiated rather than inhibited interleukin-1β–induced NO₃⁻ formation in VSMCs, whereas Ro 31-8220 downregulated NO₂⁻ production. NO₂⁻ formation is a reflection of inducible NO synthase mRNA synthesis. They suggest that an unidentified PKC plays a role in the negative control of the inducible NO synthase gene expression or that there is a protein kinase that prevents inducible NO synthase gene expression. Fisslthaler et al found that angiotensin II increases PAR-1 mRNA 3-fold. Administering staurosporine or Ro 31-8220 (at the same concentrations used in the present study) enhanced angiotensin II–stimulated PAR-1 expression, suggesting that PKC negatively regulates the signaling of the angiotensin II–stimulated increase in PAR-1 expression. Mills et al found no effect of up to 20 ng/mL staurosporine on cyclic strain–increased proliferation of bovine aortic smooth muscle cells. Hishikawa et al found that treatment with the PKC inhibitor chelerythrine significantly inhibited cyclic strain O₂⁻ production in VSMCs.

It is tempting to speculate that the synergistic effect of PKC inhibitors on cyclic strain–induced PAR-1 gene expression is mediated by ROS in VSMCs through the NADPH oxidase pathway. Because PAR-1 expression is induced by growth factors in addition to cyclic strain, it is possible that cyclic strain acts together with growth factors released from platelets and vascular cells to alter gene expression maintaining VSMCs in a proliferative state. Aggregating platelets have been shown to induce thrombin receptor expression in cultured VSMCs through the release of transforming growth factor-β1 and PDGF-AB. In vascular cells, the growth-promoting effects of hemodynamic forces increase the release of growth factors, such as PDGF and bFGF, which also enhances the expression of PAR-1. This additive effect of cyclic strain and bFGF on PAR-1 expression (Figure 7) suggests that cyclic strain and bFGF may cooperatively promote mitogenesis of VSMCs in response to thrombin.

Although one must appreciate the fact that results obtained with cultured cells are questionably relevant to in vivo events, in vitro findings, such as those in the present study, provide investigative tools that will lead to more relevant in vivo models for cardiovascular diseases.

In summary, we have demonstrated that cyclic strain induces the expression of PAR-1, leading to the increase of VSMC proliferation in response to thrombin. The cyclic
strain–increased PAR-1 expression may be mediated via ROS and PKC signaling pathways. Because PAR-1 expression in VSMCs mediates many effects of thrombin on the blood vessel wall, an understanding of the PAR-1 gene regulation by the mechanical forces of cyclic strain and shear stress may offer insight into vascular proliferative diseases.

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