INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Effects of Shear Stress and Cyclic Strain on the Expression of Thrombin Receptor Gene in Human Vascular Endothelial and Smooth Muscle Cells

by

Kyta Truong Nguyen

Doctor of Philosophy

Houston, Texas
May, 2000
RICE UNIVERSITY

Effects of Shear Stress and Cyclic Strain on the Expression of Thrombin Receptor Gene in Human Vascular Endothelial and Smooth Muscle Cells

by

Kytaі Truong Nyguen

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED. THESIS COMMITTEE:

Larry V. McIntire. E.D. Butcher Professor
Chair. Department of Bioengineering
Chair. Institute for Biosciences and Bioengineering

Suzanne G. Eskin. Adjunct Professor
Department of Bioengineering
President. Vascular Solutions Consulting Group

Antonios Mikos. John W. Cox Professor
Director. Cox Laboratory for Biomedical Engineering

Jennifer L. West. T. N. Law Assistant Professor
Department of Bioengineering

Houston. Texas
May. 2000
ABSTRACT

Effects of Shear Stress and Cyclic Strain on the Expression of Thrombin Receptor Gene in Human Vascular Endothelial and Smooth Muscle Cells

by

Kytai Truong Nguyen

Mechanical forces such as shear stress and cyclic strain have been shown to regulate expression of many genes that can alter vascular functions such as cell proliferation, leading to the development of vascular diseases including atherosclerosis. Thrombin receptor gene, protease-activated receptor-1 (PAR-1), mediates many important vascular functions such as thrombin-stimulated thrombosis, inflammation, and proliferation of vascular cells; however, the regulation of PAR-1 by mechanical forces has not previously been studied. This thesis investigates effects of shear stress and cyclic strain on gene regulation of PAR-1 in human vascular cells such as endothelial (ECs) and smooth muscle cells (VSMCs) and the molecular mechanisms involved in this regulation.

This work finds that shear stress and cyclic strain differentially regulated PAR-1 expression in vascular cells, leading to alterations of cell functions in response to thrombin, and that these processes were mediated through various signaling pathways. Cultured cells were exposed to different levels of shear stress or cyclic strain using the
parallel flow plate chamber or uni-axial cyclic strain system. After exposure, PAR-1 mRNA and protein were quantified by Northern blot and flow cytometry, respectively. In addition, inhibitors of various signal pathways such as protein kinases were used to investigate the molecular mechanisms. Arterial shear stresses decreased PAR-1 mRNA and protein both time- and dose-dependently in both macro- and microvascular ECs, leading to attenuation of thrombin-stimulated nitric oxide and endothelin-1 releases. Furthermore, protein kinase C partly mediated shear-reduced PAR-1 expression in both cell types. As in ECs, shear-downregulated PAR-1 expression in VSMCs caused decreases in thrombin-stimulated calcium mobilization and cell proliferation. The transcription mechanism, but not mRNA stability, regulated shear-reduced PAR-1 expression in VSMCs. In contrast to shear stress, high levels of cyclic strain increased PAR-1 expression in VSMCs time-dependently, leading to induction of cell proliferation in response to thrombin, and this process was mediated by reactive oxygen species, possibly through the NADPH pathway. These findings indicate important roles of mechanical forces in regulating vascular functions and thus provide a better understanding of how mechanical factors act to promote vascular diseases.
In remembrance of my father and
the people who could not reach the land of freedom.

To my beloved mother, all of my brothers and sisters, and my husband Sang.

"Dad and Mom, words cannot say what I feel: this work is my love for you!"
ACKNOWLEDGMENTS

I would like to take this opportunity to express my gratitude to many individuals who have contributed to many aspects in my studies, work, and life:

Dr. Larry V. McIntire and Dr. Suzanne G. Eskin for providing me the guidance and focus, giving me the opportunity to explore different directions in this project, as well as for being patient and encouraging when I made mistakes or some experiments did not work out.

Dr. Marshall S. Runge and Dr. Cam Patterson for their excellent collaboration and discussion, especially Dr. Patterson, who always gave his help very quickly.

Dr. Antonios Mikos and Dr. Jennifer L. West for spending their valuable time to serve on my thesis committee.

Dr. Robert T. Tranquillo of the University of Minnesota and Dr. Richard Robb of the Mayo Clinic, for giving me the opportunities to work in their groups and “planting” my curiosity in biomedical fields, leading to my graduate study later.

Dr. Carol Ballinger for technical advice in Northern blot, Dr. Thomas Chow in the flow cytometer, Dr. Sue McCormick in the RNA isolation and flow-loop setup, Sarah Baptist-Nguyen in cell culture techniques, Dr. Yang-Ting Shiu in the endothelial cell isolation, Dr. Geogios Stamatas in the Fura-2 imaging system, and Lydia Sturgus in the cytofluorometer. I would especially like to thank Dick Chronister for constructing the uni-axial cyclic strain system; Nancy A. Turner and Marcella E. Estrella for technical assistance: Arnez Washington, Maria Modelska, Julie Kudelka, and Sherry Nassar, for administrative assistance.

My friends at Rice, the Cox Laboratory, and biochemistry department for their advice and support, Dr. Becky Jo Fredrickson, Dr. Maria Papadaki, Richard Payne, and David Rhoads for being nice, especially to my good friends: Shih-Hsin Kao for his helpful discussion and encouragement in this project, Author Bergman for his support and honest
critiques that help me to improve my communication. Dickson Tanzil and Shih-Yi Yang for being nice and supportive.

Staff members in the Cain project at Rice, especially Dr. Jan Hewitt and Tracy Volz for helping me individually and teaching me the grammar, thesis writing, and presentation skills.

My beloved father for his love and belief that not only his sons but also his daughters could have the courage to escape the country for their freedom and higher education. My beloved mother for giving me life and sacrificing her youth to raise us since my father passed away. My brothers Tuan, Thanh, and Phi; my sisters Hien, Nhan, The, Thuy, and Thai; and the rest of my family for their love and support. My family’s love and encouragement have helped me to live through the difficult times in refugee camps, as well as to study hard and live more meaningfully here in the U.S.

My beloved Aunt Chin and Uncle Sa in Vietnam for giving me the gold to pay for my second and third escapes until I succeeded in the third time. I could not be here today without their risky support (their saving and fortune to risk for my freedom). My Uncles Tu and Ha, and my Aunt Phan in the U.S. for their advices on keeping my culture values, especially Uncle Ha in taking my father’s place (advising and giving me away on my wedding day as required by Vietnamese tradition).

My American parents, Carl and Miriam Manfield and Allan and Donna Anderson, for their love and support since I came here. They not only taught me American culture and English but also made me feel as home and helped me gain back my belief in humanity and love with their care and kindness.

Last but not least, my husband, Sang, for his love, understanding, and patience throughout my studies and the rest of his family for their love and support.

This work was supported through NIH grants HL18672 and NS23327, NASA grant NAG5-4072, and Welch Foundation grant C-0938.
# TABLE OF CONTENTS

Abstract.......................................................................................................................... ii
Acknowledgements......................................................................................................... v
Table of contents........................................................................................................... vii
List of Figures and Tables............................................................................................... ix
Abbreviations.................................................................................................................. xi
Preface............................................................................................................................ xiii

1. Introduction 1
   1.1. The vascular cells in health and cardiovascular diseases........ 1
   1.2. Hemodynamic forces in the blood vessels......................... 5
   1.3. In vitro devices to study effects of shear stress and
        cyclic strain on vascular cells........................................... 8
   1.4. In vitro studies of shear stress on endothelial cells.......... 11
   1.5. In vitro studies of cyclic strain on endothelial cells........... 32
   1.6. In vitro studies of shear stress on vascular smooth
        muscle cells (VSMCs)................................................... 35
   1.7. In vitro studies of cyclic strain on VSMCs......................... 37
   1.8. Thrombin and protease activated receptor-1 (PAR-1).......... 44
   1.9. General objectives............................................................ 47

2. Effects of shear stress on PAR-1 expression in endothelial cells 48
   2.1. Introduction................................................................................. 48
   2.2. Procedures.................................................................................. 49
   2.3. Results....................................................................................... 56
2.4. Discussion.................................................................64

3. Effects of shear stress on PAR-1 expression in VSMCs 71
   3.1. Introduction...........................................................71
   3.2. Procedures............................................................71
   3.3. Results.................................................................75
   3.4. Discussion............................................................80

4. Effects of cyclic strain on PAR-1 expression in VSMCs 82
   4.1. Introduction...........................................................82
   4.2. Procedures............................................................83
   4.3. Results.................................................................88
   4.4. Discussion............................................................97

5. Future work 105
   5.1. Effects of complex shear stress on vascular cells.............105
   5.2. Effects of shear stress and cyclic strain on vascular cells....108
   5.3. Studies of stress- and strain-responsive elements.............109
   5.4. Mechanotransduction and intracellular signal mechanisms.....110
   5.5. Effects of mechanical and humoral factors on vascular cells...111

Closing remarks 113

References 114

Appendix 137
LIST OF FIGURES AND TABLES

Figures

Fig. 1.1. Endothelial cell functions in health and in cardiovascular diseases. 3
Fig. 1.2. Hemodynamic forces and the resulting mechanical stresses in the blood vessel wall. 7
Fig. 1.3. The parallel plate flow chamber to generate shear stress in vitro. 10
Fig. 1.4. Proposed mechanotransduction pathways in vascular cells. 12
Fig. 1.5. G-protein linked receptors and their downstream signal pathways. 15
Fig. 1.6. Integrin activation pathways in vascular cells in response to mechanical factors. 17
Fig. 1.7. Various MAPKs pathways regulated by fluid shear stress in ECs. 22
Fig. 2.1. Schematic of a parallel plate flow chamber system. 51
Fig. 2.2. PAR-1 mRNA levels in HUVECs subjected to shear stress. 57
Fig. 2.3. PAR-1 mRNA levels in HMECs subjected to shear stress. 58
Fig. 2.4. Flow cytometry of PAR-1 protein on the cell surface in static and sheared HUVECs and HMECs. 59
Fig. 2.5. Cell surface PAR-1 expression in HUVECs and HMECs is reduced by shear stress. 60
Fig. 2.6. Effects of thrombin on nitrite formation from shear-stressed HUVECs and HMECs. 62
Fig. 2.7. Effects of thrombin on endothelin-1 production from sheared HUVECs and HMECs. 63
Fig. 2.8. Effects of protein kinases on shear stress-reduced PAR-1 expression. 65
Fig. 3.1. Schematic of the fluorescence ratio imaging system. 74
Fig. 3.2. Effects of shear stress on PAR-1 mRNA stability.

Fig. 3.3. Shear stress attenuated Ca\(^{2+}\) mobilization in response to thrombin in HASMCs.

Fig. 3.4. Shear stress inhibited HASMC proliferation in response to thrombin.

Fig. 4.1. Schematic of the uni-axial cyclic strain system.

Fig. 4.2. PAR-1 mRNA levels in HASMCs subjected to cyclic strain.

Fig. 4.3. Flow cytometry of PAR-1 protein on the cell surface in static and strained (20%) HASMCs.

Fig. 4.4. Thrombin-stimulated cell proliferation in static and strained HASMCs.

Fig. 4.5. Cyclic strain induced superoxide production in HASMCs.

Fig. 4.6. Effects of ROS and NO inhibitors on cyclic strain-induced PAR-1 expression.

Fig. 4.7. Effects of various protein kinase inhibitors on cyclic strain-increased PAR-1 expression.

Fig. 4.8. Synergistic stimulation of PAR-1 mRNA expression in HASMCs by cyclic strain and bFGF.

Tables

Table 1.1. Effects of shear stress on growth, morphology, and gene expression in endothelial cells.

Table 1.2. Effects of cyclic strain on growth, morphology, and gene expression in endothelial cells.

Table 1.3. Effects of cyclic strain on growth, morphology, and gene expression in vascular smooth muscle cells.
ABREVIATIONS

AA  arachidonic acid
AC  adenylate cyclase
Ang II angiotensin II
AP-1 activator protein 1 (c-fos/c-jun complex)
ATP adenosine triphosphate
bFGF basic fibroblast growth factor
BMK-1 big mitogen-activated protein kinase-1
cAMP cyclic adenosine 3',5'-monophosphate
Cas p130 Crk-associated substrate, a putative c-Src substrate
cdk cyclin-dependent kinase
cGMP cyclic guanosine monophosphate
CNP C-type natriuretic peptide
CO2 carbon dioxide
CRE cAMP response elements
DAG diacylglycerol
ECM extracellular matrix
ECs endothelial cells
EGFR epidermal growth factor receptor
Egr-1 early growth response-1
ELAM-1 endothelial leukocyte adhesion molecule-1
ERK1/2 extracellular signal-regulated kinase 1/2
ET-1 endothelin-1
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GC guanosine cyclase
HASMCs human aortic smooth muscle cells
HB-EGF heparin binding endothelial growth factor
HMECs human microvascular endothelial cells
HO-1 heme oxygenase-1
HUVECs human umbilical vein endothelial cells
ICAM-1 intercellular adhesion molecule-1
IGF-1 insulin-like growth factor-1
IL interleukin
IP3 inositol 1,4,5 trisphosphate
JNK 3-Jun NH(2)-terminal kinase
LDL low-density lipoprotein
LOX-1 lectin-like oxidized LDL receptor-1
MAPK mitogen-activated protein kinases
MCAF monocyte chemotactic and activating factor
MCP-1 monocyte chemoattractant protein-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKP-1</td>
<td>MAPK phosphatase-1</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light-chain kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor-kB</td>
</tr>
<tr>
<td>NO/NOS</td>
<td>nitric oxide/nitric oxide synthase</td>
</tr>
<tr>
<td>NSM-A/NSM-B</td>
<td>non-skeletal muscle myosin heavy chains A and B</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor type-1</td>
</tr>
<tr>
<td>PAR-1</td>
<td>protease-activated receptor-1</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PG12/PGL2S</td>
<td>prostacyclin/prostacyclin synthase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PKA/PKC/PKG</td>
<td>protein kinase A, C, G</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PTHrP</td>
<td>parathyroid hormone-related peptide</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>Rac1</td>
<td>a Ras-related small GTP-binding protein</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SA channels</td>
<td>stress-activated cation channels</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SM-1/SM-2</td>
<td>smooth muscle myosin heavy chain isoforms 1 and 2</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SSRE</td>
<td>shear stress responsive elements</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>TGF-b1</td>
<td>transforming growth factor-b1</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>TM</td>
<td>thrombomodulin</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TRE</td>
<td>tumor promoting agent response elements</td>
</tr>
<tr>
<td>TXA2</td>
<td>thromboxan A2</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cellular adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
</tr>
</tbody>
</table>
Preface

Mechanical forces such as shear stress and cyclic strain regulate expression of many vasoactive genes that can alter vascular functions, leading to the prevention or development of cardiovascular diseases. The thrombin receptor gene, protease-activated receptor-1 (PAR-1), is involved in many vascular processes including thrombosis; however, the regulation of PAR-1 by these forces has not previously been studied. This thesis investigates the effects of shear stress and cyclic strain on the gene regulation of PAR-1 on vascular cells. PAR-1 is selected due to the dramatic increases of its expression in injured and hypertensive arteries and to its well-known role in mediating many thrombin-stimulated events such as proliferation, thrombosis, and inflammation in vascular cells that would lead to the development of vascular diseases.

Cardiovascular diseases including atherosclerosis and thrombosis are the leading causes of morbidity and mortality in the Western societies, causing not only high numbers of deaths but also incurring extremely expensive associated costs. In fact, these diseases remain responsible for about 40% of all US deaths (more than 600,000 fatalities each year).¹ In 1998 alone, associated costs in the U.S. were estimated to be more than $200 billion, according to the American Heart Association. Understanding the risk factors involved in cardiovascular diseases and how these factors act on the vascular cells and change their functions becomes an important issue in order to better design therapeutic strategies, and thereby to reduce the number of fatalities and associated costs.
Ultimately, much evidence demonstrates that mechanical factors such as fluid-induced shear stress and pressure-enhanced cyclic strain may play an important role both in the early development of vascular diseases and in the plaque rupture leading to possible fatalities. Numerous in vivo studies show that atherosclerotic lesions are not developed at random areas but often occur at arterial branch ostia, bifurcations, and bends, regions of low or complex shear stresses. In addition, atheromatous plaques do not usually form in veins that are exposed to low pressure. However, when veins are exposed to arterial pressure as in venous grafts, atherosclerotic plaques readily develop.\textsuperscript{2} Furthermore, computer modeling of the distribution of the mechanical stress in arteries with atherosclerotic lesions shows that cyclic strain is highest near the plaque cap edge, which is the typical site of plaque rupture.\textsuperscript{3,4} These observations suggest that shear stress and cyclic strain are major factors in maintaining vascular functions and in developing underlying atherosclerotic formation.

Over the last decade, much research has been focused on investigating the effects of these two major mechanical factors. In confirmation of those seen in vivo, results from in vitro studies show that mechanical stresses regulate both normal cellular functions and pathophysiological changes leading to vascular diseases. In particular, shear stress and cyclic strain induce morphologic changes, affect the synthesis and secretion of macromolecules, and alter expression of vasoactive genes in vascular cells such as endothelial and smooth muscle cells. However, many questions related to mechanical stimuli and their relations with vascular diseases remain unanswered. For example, some genes such as endothelin, the tissue plasminogen activator, and the platelet-derived
growth factor regulated by mechanical forces have been discovered, yet many important genes remain to be studied. This project focuses on one of those genes, PAR-1.

In this thesis, chapter 1 introduces the background of the biology and physiology of vascular cells such as endothelial cells and smooth muscle cells. It also discusses how vascular cells sense mechanical factors such as shear stress and cyclic strain, leading to changes in cell functions and gene expression. Moreover, this chapter describes the biological properties of PAR-1 and its roles in vascular functions. The effects of shear stress on PAR-1 expression in both macro- and micro-vascular endothelial cells are identified in chapter 2. In addition, this chapter presents the consequences of shear stress on the PAR-1 functional activities such as thrombin-stimulated releases of nitric oxide and endothelin-1. It then evaluates the mechanisms by which the cells sense shear stress and alter gene expression. Chapter 3 elucidates the consequences of shear stress in thrombin-induced responses, including calcium mobilization and cell proliferation, and the mechanisms of shear stress-reduced PAR-1 expression in vascular smooth muscle cells. Chapter 4 demonstrates the effects of cyclic strain on PAR-1 expression in human vascular smooth muscle cells and the mechanisms involved in this process. Finally, chapter 5 suggests some future research directions.
CHAPTER 1

Introduction

Understanding the focus of this thesis on regulation of thrombin receptor gene by mechanical forces on vascular cells requires some background of vascular cells in the cardiovascular system. Hemodynamic forces exerted on the vessel wall, and devices used to generate shear stress and cyclic strain in vitro. It is also important to review previous in vitro studies of shear stress and cyclic strain on vascular cells and the roles of PAR-1 in the blood vessels.

1.1 The vascular cells in health and cardiovascular diseases:

The normal arterial wall consists of three layers: the inner intima, media, and outer adventitia. The inner intima is lined on its luminal surface by the endothelium. The medial layer is mainly composed of smooth muscle cells, resident macrophages, and the secreted extracellular matrix. The adventitia, a connective tissue layer, contains the vasa vasorum, the vascular supply to the arterial wall. Atherosclerosis normally occurs in the intimal and medial layers of the vessel wall; therefore, the biological properties of endothelial (ECs) and smooth muscle cells (VSMCs) will be reviewed briefly.

Endothelial cells

In contrast to their previous static barrier function, it is now well known that ECs are actively involved in inflammation, hemostasis and thrombosis, as well as in control of
vascular tone and growth. In order to perform its multiple functions, the endothelium is capable of producing many endothelium-derived factors that affect both the blood cells and underlying VSMCs in response to stimuli (Fig. 1.1). For example, ECs offer antithrombotic surfaces by producing many substances such as prostacyclin (PGI₂) and nitric oxide (NO) that have anticoagulant effects. In addition, the endothelium modulates VSMC tone by regulating endothelium-derived relaxing factors including endothelium-derived hyperpolarization factors (EDHF), PGI₂, and NO, as well as releasing endothelium-derived constricting factors such as thromboxane A₂ (TXA₂), endothelin-1 (ET-1), angiotensin II (Ang II), and reactive oxygen species (ROS) that include peroxide (O₂⁻) and hydrogen peroxide (H₂O₂). ECs can also regulate the proliferation and migration of underlying VSMC by releases of growth regulators such as transforming growth factors-β1 (TGF-β1), NO, basic fibroblast growth factor (bFGF), and platelet-derived growth factors (PDGF). These multiple functions of the endothelium suggest its key role in health and in cardiovascular diseases.

Dysfunction of the endothelium, considered as a basis for the development of many vascular diseases, is the result of an imbalance production of endothelial-derived factors. These include the imbalance between the relaxing and contracting factors and between anti- and pro-coagulant mediators. These imbalances lead to the activation and migration of circulating monocytes into the intima where they take up the oxidized extracellular low-density lipoprotein (LDL) and develop into foam cells. Concurrently, growth factors and extracellular matrix (ECM) proteins produced by platelets. ECs, and
Figure 1.1. *Endothelial cell functions in health and in vascular diseases*. The endothelial cells, in contact with both blood cells and the underlying vascular smooth muscle cells, are capable of producing many factors such as growth factors, vasoactive factors, and chemotactic and adhesive molecules upon activation.
macrophages promote migration and proliferation of VSMCs, leading to the occurrence of vascular diseases.

**Vascular smooth muscle cells**

VSMCs play an important role in maintaining vascular structural integrity and tone by converting from one to the other of contractile and synthetic phenotypes *in vivo.*\(^9,10\) In normal arterial media, VSMCs are usually in the contractile phenotype. Under this state, VSMCs are normally quiescent and rarely exhibit mitogenesis. They can also regulate peripheral resistance and venous capacitance, thereby modulating cardiac output, maintaining systemic blood pressure, and regulating organ blood flow. Upon activation, VSMCs can modulate from the contractile phenotype to form cells with the synthetic phenotype, shown by an increase in the amount of synthetic organelles and a decrease in contractile filaments, and thus resulting in a loss of the ability to contract. The expression of a synthetic phenotype has great importance to the development of intimal thickening because of the enhanced synthetic capabilities of VSMCs, leading to cell proliferation and migration.

Dysfunction of VSMCs, included VSMC proliferation and migration that are the key events in the development of atherosclerosis and restenosis, may be related to the synthetic phenotype.\(^11-13\) VSMCs are capable of proliferation at very high and rapid rates in the synthetic phenotype. In addition, they have the potential to migrate from the media into the intimal of the vessel wall. Like macrophages, VSMCs can also uptake
LDL and become cholesterol-laden foam cells. Moreover, they can synthesize a large amount of ECM proteins, which enhance even more cell migration and proliferation into the injury sites.

1.2 **Hemodynamic forces in the blood vessels:**

Two key hemodynamic forces exerted on vascular cells *in vivo* are fluid-enhanced shear stress and pressure-induced cyclic strain (Fig. 1.2). Shear stress mainly acts on ECs, whereas cyclic strain (or circumferential stress) exerts on both ECs and VSMCs. Typical venous and arterial shear stresses range between 1-5 and 6-40 dyn/cm², respectively. Whereas physiological arterial levels of cyclic strain are between 2-18% at 1 Hz. Wall shear stress (τₜ) experienced by ECs in the blood vessels can be estimated using the Poiseuille-Hågen’s law in a uniform rigid cylinder, assuming the simple fully developed laminar flow of a Newtonian fluid

\[
\tau_w = \frac{4\mu Q}{\pi R^3}
\]

where μ is the viscosity of the blood flow, Q is the volumetric blood flow rate, and R is the vessel radius. Shear stress can be increased by increased velocity of blood flow or increased hematocrit. Wall shear stress is proportional to blood flow Q and inversely proportional to the third power of the internal vessel radius R, implying that small changes in the internal radius can offset quite large changes in volume blood flow to maintain shear stress τₜ at a constant level.
In addition, wall stress ($\sigma$) due to transmural pressure differences can be approximated as described by Laplace’s law, assuming isotropic properties of the vessel wall and a thin tube wall ($W << R$):

$$\sigma = \frac{\pi RP}{W}$$

where $R$ is the internal vessel radius, $P$ is the transmural pressure, and $W$ is the vessel wall thickness. Vessel wall tension can be increased by either increased pressure or internal radius (Fig. 1.2). Local wall stress is proportional to transmural pressure $P$ and inversely proportional to the vessel wall thickness $W$, implying that an increase in the vessel wall thickness in response to an increase in transmural pressure can maintain wall stress $\sigma$ at a constant level. This wall stress generates a material strain, the magnitude of which depends on the wall elastic modulus or inversely with wall compliance. Since the arterial pressure varies cyclically, the resulting wall strain is also cyclic, with magnitude dependently on pulse pressure and effective wall elasticity. Both of these formulas are only approximation for blood flow in vessels. Blood is not strictly Newtonian (though nearly so at high shear rate) and the vessel wall is certainly not isotropic and often of significant thickness relative to the vessel radius.

For an ideal elastic tube, the circumferential strain ($\varepsilon$) is related to the wall stress $\sigma$ by the Hook’s law:

$$\varepsilon = \frac{(1 - \nu^2)RP}{EW}$$

where $\nu$ is the Poisson ratio and $E$ is the Yong’s modulus.
Figure 1.2. Hemodynamic forces and the resulting mechanical stresses in the blood vessel wall. Two major hemodynamic forces exerted on the vessel wall are the blood flow ($Q$) and transmural pressure ($P$). Fluid shear stress ($\tau_w$) can be estimated using Poiseuille’s law, assuming fully-developed laminar flow; shear stress is proportional to the volume flow $Q$ and inversely proportional to the third power of the internal vessel radius ($R$). Assuming isotropic properties of the vessel wall, the pressure-induced wall stress ($\sigma$) can be approximated using the Laplace’s law. Wall stress is proportional to transmural pressure $P$ and the internal radius $R$ and inversely proportional to the vessel wall thickness ($W$).
Shear stress and cyclic strain have been shown to actively influence vessel wall remodeling and pathobiology *in vivo*. For example, chronic increases in blood flow and consequently in shear stress as seen in the radial artery of dialysis patients proximal to their arteriovenous fistula lead to expansion of the luminal radius so that mean shear stress is returned to its baseline levels. Decreased shear stress resulting from lower flow or blood viscosity also induces a decrease in internal vessel radius.\(^{16}\) In addition, plaques often occur at the carotid bifurcation, branches, vessel narrowing, and curvatures where flow is slow, disturbed, or separated.\(^{17}\) Furthermore, a series of vein graft studies demonstrates that intimal hyperplasia is strongly associated with low shear stress, whereas medial thickening is associated with elevated cyclic strain.\(^{3}\) These observations suggest that shear stress and cyclic strain have direct effects on the vascular cells such as ECs and VSMCs, leading to alterations in cell functions and structure.

### 1.3 *In vitro* devices to study effects of shear stress and cyclic strain on vascular cells:

To individually elucidate the effects of shear stress and cyclic strain on vascular cells, several devices have been designed to simulate shear stress or cyclic strain for *in vitro* studies. The parallel-plate flow chamber and modified cone-plate devices are widely used experimental systems for studies of shear stress in cultured cell systems. Wall shear stress is created by gravity in the parallel-plate flow chamber system, whereas it is generated by adjusting cone angle, medium viscosity, and cone rotation speed in the modified cone-plate viscometer.
The parallel plate flow chamber system is particularly used in this thesis due to its advantages: being easy to operate, producing constant shear stress on vascular cells over a long time period, and providing continuous medium samples for measuring of secreted molecules.\textsuperscript{18} A schematic of the parallel flow chamber is shown in Figure 1.3. The wall shear stress, $\tau_w$, experienced by cells in this system is estimated using the momentum balance and assuming a Newtonian fluid\textsuperscript{18}:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

where $\mu$ is the viscosity of the culture medium, Q is volumetric flow rate, $w$ is the channel width, and $h$ is the channel height.

For in vitro cyclic strain studies, the Flexercell stress and uni-axial cyclic strain systems have been used. The Flexercell stress unit permits stretching the flexible membrane seeded with vascular cells by applying a vacuum pressure underneath the membrane. In this system, cyclic strain increases in the direction from the center to the periphery of the well with the maximum strain at the edge of the well (radial strain).\textsuperscript{19} Unlike the Flexercell stress unit, the uni-axial strain system, used in this thesis, provides a uniform uni-axial cyclic strain to vascular cells seeded on the membrane (except at the edge of the membrane).\textsuperscript{20} This system stretches the elastic membrane by converting the rotation movement of rotating cylinders to the cyclic strain motion with the use of a camshaft. Cyclic strain is usually measured as a change in length relative to an initial reference length for a given elastic material.
Figure 1.3. The parallel-plate flow chamber to generate shear stress in vitro. This flow chamber consists of the glass slide, the silicon gasket, and the polycarbonate plate. They are held together by a vacuum, which is at the periphery of the chamber plate. The velocity profile is parabolic in this system, w is the channel width, h is the channel height, and l is the channel length.
1.4 *In vitro* studies of shear stress on endothelial cells:

To date, cyclic strain and shear stress have been shown to regulate gene expression and functions of ECs. Some cellular mechanisms linking physical forces and altered gene expression have been studied *in vitro*, although the precise signal mechanism is not clearly understood. Generally, vascular cells sense mechanical stimuli at the cell membrane through mechanoreceptors such as ion channels. G-protein linked receptors, integrins, and cytoskeletal mechanoreceptors. Signals from the cell surface are transduced through a cascade of signaling molecules, including second messengers and protein kinases. Activation of this cascade ultimately leads to the transcription of early response genes and transcription factors, as well as to the interactions of these factors with their corresponding binding elements. These, in turn, induce transcription of genes that are important in vascular injury, including vasoactive factors, growth regulators, mediators of thrombosis and fibrinolysis, inflammatory mediators, and adhesion molecules (Fig. 1.4).

**How do vascular cells sense shear stress at the membranes?**

In recent years, there has been developed a great interest in understanding the mechanisms that transmit mechanical stimuli on the cell membrane into intracellular signals, which ultimately regulate gene expression in vascular cells. The known mechanoreceptors are ion channels, G-protein linked receptors, integrins, and mechanoreceptors that are tensionally integrated with the cytoskeleton network. Loss of the cell abilities to sense stimuli (or impaired mechanoreceptors) may lead to the development of vascular diseases.
Figure 1.4. **Proposed mechanotransduction pathways in vascular cells.** Vascular cells sense mechanical stresses such as shear stress and cyclic strain at the cell membrane by means of mechanoreceptors. Signals from the cell surface are transduced by a cascade of second messengers and protein kinases. This cascade leads to the activation of many nuclear transcription factors and the interactions of these factors with their corresponding binding elements. These, in turn, cause the activation of many vasoactive genes, leading to altered cell responses, as well as to feedback and adaptation.
**Ion channels**: Ion channels, responsible for the transfer of a specific ion across the cell membrane, are common mechanisms used by ECs to sense mechanical stimuli. Several ion channels are mechanical sensitive including potassium (K⁺), calcium (Ca²⁺), and sodium (Na⁺) channels. The most rapid responses of ECs to shear stress involve the activation of the apical K⁺ channels.\(^{21}\) Blockage of these channels attenuates shear-induced NO production,\(^ {22}\) shear-activated transforming growth factors β1 (TGF-β1) expression,\(^ {23}\) shear-elevated cyclic guanosine monophosphate (cGMP),\(^ {24}\) and shear-downregulated ET-1 expression.\(^ {25}\) Recent work shows that shear stress also activates both K⁺ and chloride channels and the net membrane potential is determined by the balance of these responses.\(^ {26}\)

In addition to K⁺ channels, Ca²⁺ channels are another potential mechanotransduction. Although voltage-gated Ca²⁺ channels are not found in cultured ECs, evidence shows that hyperpolarization resulting from activation of K⁺ channels, which can lead to the increased Ca²⁺ influx via a Ca²⁺-permeable channels that are voltage independent.\(^ {5,27}\) Shear stress rapidly induces Ca²⁺ influx through these channels,\(^ {28}\) and blockage of these permeable channels inhibits shear stress dependent morphology change and migration in ECs.\(^ {29}\) Other putative mechanisms of shear transduction include Na⁺ channels, which mediate the shear activation of extracellular signal-regulated kinases (ERKs) in ECs,\(^ {30}\) and other ion exchangers including HCO₃⁻/Cl⁻ transporters, which lead to changes in cytosolic pH that may serve as second messengers.\(^ {14,31}\)
G protein-linked receptors: Belonging to families of transmembrane receptors. G protein-linked receptors respond to extracellular stimuli by activation of G proteins. G proteins then mediate many downstream signals in response to stimuli. For example, Gi proteins inhibit adenylyl cyclase (AC) and activate K+ channels, whereas Gs proteins stimulate AC and activate Ca^{2+}-permeable channels. In addition, both Gi and Gs act on AC or guanosine cyclase (GC) to bring about the synthesis of cyclic adenosine 3'-5' monophosphate (cAMP) or cGMP, which later activates protein kinase A (PKA) or G (PKG), respectively. Furthermore, Gq proteins stimulate phospholipase C (PLC) to release 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG further mediates activation of the protein kinase C (PKC) family, while IP_3 stimulates the release of [Ca^{2+}]_i from the intracellular calcium-sequestering compartments (Fig. 1.5).

Results from a number of studies on shear-mediated responses in ECs support a role for G protein-linked receptors as mechanosensors. Shear stress rapidly enhances levels of G-proteins in a dose-dependent manner, a process that is dependent on the phospholipid bilayer physical properties and independent of the intact cytoskeleton. Furthermore, G-protein signals are required for shear induction of endothelial nitric oxide synthase (eNOS) and the burst NO production, as well as for the activation of ERKs and 3-Jun NH(2)-terminal kinase (JNK).
Figure 1.5. **G-protein linked receptors and their downstream signal pathways.**

Activation of G-protein linked receptors (GR) activates PLC to produce IP₃ and DAG. IP₃, in turn, stimulates Ca²⁺ release from calcium-sequestering compartments. whereas DAG can activate PKC and produce PGI₂. Activation of GRs also can regulate PKA and PKG. and these protein kinases (PKA, PKC, and PKG) may lead to additional protein kinase activation.
**Integrins:** Integrins, ubiquitous α/β heterodimeric transmembrane glycoproteins, act as adhesion receptors in the interactions between vascular cells and the extracellular matrix (ECM), including fibronectin (a ligand for α5β1) and collagen (a ligand for α2β1). Integrin-ECM activation leads to phosphorylation of many focal adhesion proteins (e.g. α-actin, vinculin, talin, tensin, and paxillin) and additional kinases, including focal adhesion kinase (FAK). Src-like protein kinases Src, fyn, and csk. Possible downstream signal pathways involved in integrins are shown in Figure 1.6. Integrins convert the mechanical stimuli into biochemical signals by association with FAK, Src, Shc, or rho, leading to activation of Ras and later ERK and JNK pathways. Downstream ERK and JNK pathways include the activation of nuclear transcription factors and the interactions of these factors with their corresponding DNA binding elements.

Integrins as important mechanoreceptors are supported by many following studies. Shear stress has been shown to enhance an aggregation of vitronectin receptors, increases the tyrosine phosphorylation of FAK, and stimulates the tyrosine kinase activities of Src and Fyn. In addition, shear stress activates ERK only when ECs adhere to fibronectin, but not when ECs adhere to poly-L-lysine, nor when they are in suspension. Furthermore, flow-induced vascular tone could be inhibited with RGD (Arg-Gly-Asp) peptides, which compete with the ECM for integrin interactions, or with antibodies against the β3 integrins. Integrins are also involved in shear activation of IκB kinase, ERK, and JNK.
**Figure 1.6.** Integrin activation pathways in vascular cells in response to mechanical factors. Integrins transduce the mechanical stimulation into biochemical signals by association with FAK, Src, or Shc. The activated signals induce the association with SH2-containing docking proteins such as Grb2 and Sos to activate Ras and subsequently ERK and JNK pathways. These, in turn, lead to the activation of the nuclear transcription factors such as AP-1 and to the interactions of these factors with their corresponding binding elements such as TRE to activate gene expression. Other signal pathways through FAK/Src and rho will also cause cytoskeletal changes and activation of other second messengers such as PKC.
**Cytoskeletal mechanoreceptors:** Another possible candidate for transduction of shear stress into biochemical signals is the mechanoreceptors that are tensionally integrated with the cytoskeleton.\(^5\) Mechanical stimuli transmission can occur through any cytoskeleton contact sites such as focal adhesion sites, cell-cell junctions, and nuclear membranes. Of these three, focal adhesion sites are most intensively studied due to their location. Formed at the linkage sites of the cytoplasmic sides of integrins with cytoskeleton proteins, focal adhesion sites provide a connection between the cytoskeleton and the ECM structure in ECs upon shear stress activation.\(^46,47\) Recent work indicates that cell-cell junctions also appear as other major mechanotransduction sites in ECs.\(^48\) Shear stress causes the cytoskeletal reorganization to align in the direction of flow that includes the redistribution of F-actin stress fibers or microfilaments, microtubules, and intermediate filaments.\(^49\) The precise role of these cytoskeleton structures in mechanotransduction are poorly understood, although changes in cell shapes and cytoskeleton reorganization are one of the first reported endothelial responses to steady laminar shear stress. Yet the cytoskeletal mechanoreceptors are involved in many shear responses in ECs such as shear-activated calcium influx,\(^50\) shear-reduced ET-1 expression,\(^25\) and shear-altered EC shape.\(^51\).

**How do signals from the cell membrane get into the cell cytoplasm?**

As shown above, ECs can sense mechanical stimuli through many mechanoreceptors. One of the next important questions concerning the precise molecular mechanisms in the cytosol leading to the gene regulation by shear stress remains to be elucidated. We are
just now beginning to understand that cultured vascular ECs respond to mechanical stimuli by increasing the activities of the signaling cascade within the cell cytosol including second messengers, protein kinases (PK), tyrosine kinases (TK), and mitogen-activated protein kinases (MAPK).

**Second messengers:** Second messengers generated upon activation of mechanoreceptors include IP$_3$, DAG, intracellular Ca$^{2+}$, ROS, NO, cGMP, and cAMP. Releases of IP$_3$, DAG, and PKC by shear stress are rapid in the order of seconds to minutes. However, many studies do not observe any significant increases of the cytosolic Ca$^{2+}$ in ECs exposed to shear stress in the absence of calcium mobilizing agonists such as adenosine triphosphate (ATP).$^{5,14}$ Nevertheless, intracellular Ca$^{2+}$ has been found to mediate shear stress-reduced ET-1$^{25}$ and shear-induced eNOS expression.$^{33}$ Recently, ROS and NO can also serve as second messengers. Shear stress rapidly induces the production of both NO$^{52}$ and O$_2^-$.$^{53}$ NO is necessary for flow-reduced EC adhesiveness$^{54}$ and shear-induced expression of early growth response-1 (Egr-1),$^{55}$ whereas ROS are critical for shear activation of many genes including c-fos,$^{56}$ MAPKs,$^{53,57}$ and intercellular adhesion molecule-1 (ICAM-1).$^{58}$

**Protein kinases A/C/G:** Other signal molecules involved in shear stress are protein kinases such as PKA, PKC, and PKG. Of these three, the family of PKC enzymes appears important in the EC response to fluid shear stress. PKC enzymes can be classified into different isoforms: Ca$^{2+}$ dependent and phorbol ester responsive (PKC-$\alpha$-,
\(\beta_1, \beta_III, \gamma\), Ca\(^{2+}\) independent and phorbol ester responsive (PKC-\(\delta, \epsilon, \theta, \eta\)), and Ca\(^{2+}\) independent and phorbol ester unresponsive (PKC-\(\zeta, \lambda, \iota\)). ECs express primarily three PKC isoforms: PKC-\(\alpha, \epsilon, \zeta\).\(^{59}\) The importance of PKC in signal pathways of shear stress is shown from the findings that PKC activity (PKC-\(\epsilon\) and/or PKC-\(\zeta\)), but not intracellular Ca\(^{2+}\), is critical for shear activation of ERK.\(^{60}\) In addition, PKC is involved in the releases of endothelin-1,\(^{61}\) as well as in shear activation of c-fos\(^{62}\) and PDGF gene expression.\(^{63}\)

**Tyrosine kinases:** Besides PKC, TKs are other signal mechanisms for shear stress in the cytosol. TKs can be separated into two major categories: receptor tyrosine kinases and nonreceptor tyrosine kinases (e.g., FAK and c-Src). Shear stress increases the tyrosine phosphorylation of FAK and Src in ECs.\(^{38,45}\) Inhibition of TKs attenuates shear-activated ERKs and JNK expression.\(^{35}\) shear-induced NO release,\(^{64}\) shear-reduced ET-1 expression.\(^{25}\) and shear-mediated EC shape and fiber organization.\(^{51}\) These findings indicate the important role of TKs in shear stress-mediated signal pathways. In addition, mutation of TKs (e.g., FAK and Src) inhibits shear stress activation of MAPKs (e.g., ERK and JNK), indicating a critical link between TKs and MAPKs.\(^{45}\) This link may be an integration point for several physiological stimuli such as mechanical factors and growth factors.

**Mitogen-activated protein kinases:** In addition to TKs, MAPKs can also serve as other signal pathways upon shear stimuli. Belonging to a group of small Serine/small
Threonine kinases. MAPKs are activated by extracellular stimuli through dual-phosphorylation at specific threonine and tyrosine residues. MAPKs are activated by MAPK kinases (MEKs), which are in turn activated by MAPK kinase kinase (MEKKs). The known MAPKs regulated by shear stress include ERKs (ERK1/2), JNK, p38 MAPK, and big MAP kinase 1 (BMK1 or ERK5). The upstream regulatory pathways of MAPKs include G-proteins, Ras, Sos, TKs, and PKC. On the downstream side of MAPKs, ERK activation leads to c-fos gene expression, whereas JNK activation induces c-jun that further increases AP-1/TRE (activator protein-1/12-O-tetradecanoylphorbol 13-acetate-responsive elements) transcription activity (Fig. 1.7).

Shear stress has been shown to regulate MAPK pathways, leading to gene alterations. Shear stress activates ERK1/2, JNK, and BMK1 in a rapid and transient fashion. The shear dual activation of ERK and JNK in ECs appear to have significant implications in the vessel wall because ERK are involved in the activated cell growth, whereas JNK appear to be related to programmed cell death. MAPK pathways are involved in shear-induced gene expression of c-jun and Egr-1. In addition, MAPKs and TKs are activated by shear stress in the absence of Ca\(^{2+}\) mobilization, suggesting that they represent a novel, calcium-independent pathway for stimulation of EC responses by flow, which may be a convergence point for several physiological stimuli.
Figure 1.7. Various MAPK pathways regulated by fluid shear stress in ECs. Activation of mechanoreceptors leads to activation of a cascade of protein kinases MAPKs. MAPKs are activated by MAPK kinases (MEK), which are in turn activated by MAPK kinase kinase (MEKK). The cascade of protein kinases ultimately activates signal downstream events such as activation of transcription factors, leading to alterations in gene expression. Major MAPK pathways regulated by shear stress in ECs include ERK1/2, JNK, and p38 MAPK.
How do signals get into the nucleus of vascular cells?

Downstream of the signaling cascades in the cytosol, shear stress stimulates the activation of multiple immediate early genes and transcription factors. It also induces the interactions of these factors with their target binding elements of the gene promoter region in the nucleus. These in turn result in the induction of genes encoding for vasoactive factors, growth regulators, mediators of thrombosis and fibrinolysis, chemokines, and adhesion molecules.

Immediate early genes: Immediate early (IE) genes rapidly activated by stimuli are involved in the propagation of the response by acting as modulators of transcriptional activity. Shear stress transiently upregulates the IE genes such as c-myc, c-fos, c-jun, and Egr-1.62,72 c-fos and c-jun can form nuclear transcription factors such as nuclear activator protein 1 (AP-1), while c-myc protein can associate with other proteins to form a "putative" transcription factor.

Nuclear transcription factors: Many transcription factors involved in shear stress-regulated gene expression have been identified. These include activator protein-1 (AP-1) and the Rel-related nuclear factor kappa B (NF-κB).73 AP-1, which binds to the TRE elements, is formed from homodimers (c-jun/c-jun) or heterodimers (c-fos/c-jun). Evidence shows that the c-jun/c-jun homodimers rather than the heterodimers are the activators of the down-stream AP-1/TRE in ECs exposed to shear stress.66 Although many shear stress responsive genes contain AP-1 in ECs, only MCP-1 and VCAM-1
respond to shear stress through AP-1/DNA binding in ECs.\textsuperscript{74,75} In addition to AP-1 sites, NF-κB also involves in shear signal mechanisms.\textsuperscript{73} NF-κB is a heterodimer of a p50 and a p65 protein, bound to an inhibitory cytoplasmic IκB protein. Upon activation, the p50/p65 protein complex rapidly dissociates from IκB, allowing transport of free NF-κB to the nucleus where it can bind to specific NF-κB consensus sites at certain genes, including VCAM-1, TF, ILs, and c-myc. The importance of NF-κB can be seen from the findings that NF-κB is critical for low sheared-induced VCAM-1\textsuperscript{76} and shear-activated PDGF-B expression.\textsuperscript{77}

Besides regulating transcription factors, mechanical stimuli can modulate gene expression through mRNA stability. In the 3' untranslated region of many genes there are dispersed AUUUA repeated sequences, which are known to induce mRNA instability.\textsuperscript{5} Shear stress enhances the production of the granulocyte-macrophage colony-stimulating factor in ECs by increasing its mRNA stabilization.\textsuperscript{78} In contrast, shear stress reduces ET-1 expression through the decrease in the transcription, but not the mRNA stability.\textsuperscript{79} The mRNA instability may be important to genes that are downregulated by shear stress. Whether mRNA stability is involved in the suppression of other genes exposed to shear stress in ECs remains to be elucidated.

**Shear stress-inducible binding elements:** Regulation of gene expression by shear stress results from the binding of transcription factors to their corresponding cis-elements in the promoter region of the gene. The ability to respond to shear stresses can be due to the
presence of specific, cis-acting sequence elements termed as the shear stress response elements (SSREs). Studies of reporter gene constructs found that the SSRE, a defined 6 bp core binding sequence (GAGACC), is necessary for shear stress responsiveness of the human PDGF-B promoter. In addition, NF-κB binds to the SSRE to activate the expression of PDGF-B by shear stress. Besides PDGF-B, several endothelial genes that are responsive to shear stress also contain the SSRE. Those include ICAM-1, tPA, NOS, c-fos, c-jun, TGF-β1, and MCP-1. Of interest, too, is the finding that although the MCP-1 gene contains the SSRE, the activation of MCP-1 is mediated via the TRE elements, independently of SSRE elements. Furthermore, many genes have been reported to be responsive to shear stress, but did not have this SSRE sequence. This led to the discovery of other shear response elements such as GC-rich sequences bound with Egr-1 (Egr-1 binding sites) and Sp-1 sites, which are responsible for the shear stress mediated induction of the PDGF-A and TF genes, respectively.

How do endothelial cells respond to shear stress?

*In vitro* studies show that shear stress affects both endothelial biological functions and structure. The biological responses of ECs in response to shear stress include gene regulation and synthesis of vasoactive factors, growth regulators, mediators of thrombosis and fibrinolysis, and mediators of inflammation and adhesion. In addition, shear stress modulates changes in morphology and reorganization of the stress fibers, as well as regulates cell apoptosis and proliferation. A summary of EC responses to shear stress can be found in Table 1.1.
Table 1.1. Effects of shear stress on growth, morphology, and gene expression in endothelial cells.

<table>
<thead>
<tr>
<th>Process</th>
<th>Responses</th>
<th>Signal pathways involved</th>
<th>Pathways not involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>Decrease</td>
<td>Cu/Zn SOD, NOS, Akt kinase, cdk inhibitor p21</td>
<td>-</td>
<td>81, 82, 83</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Decrease</td>
<td>CKI/p21</td>
<td>-</td>
<td>83</td>
</tr>
<tr>
<td>Morphology</td>
<td>Elongation</td>
<td>TKs, [Ca2+]i, intact microtubules</td>
<td>PKC, SA channels, intermediate filaments</td>
<td>51</td>
</tr>
<tr>
<td>Cytoskeleton organization (with flow)</td>
<td>Increase</td>
<td>Rho-p160ROCK, TKs, [Ca2+]i</td>
<td>PKC, SA channels, intermediate filaments</td>
<td>84, 51</td>
</tr>
<tr>
<td>Migration (with flow)</td>
<td>Increase</td>
<td>[Ca2+]i</td>
<td>-</td>
<td>29</td>
</tr>
</tbody>
</table>

Gene expression

Vasoactive factors

<table>
<thead>
<tr>
<th>Vasoactive factor</th>
<th>Response</th>
<th>Pathways not involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenomedullin</td>
<td>Decrease/Increase*</td>
<td>-</td>
<td>85, 86</td>
</tr>
<tr>
<td>CNP</td>
<td>Increase</td>
<td>[Ca2+]i</td>
<td>NO, TK</td>
</tr>
<tr>
<td>NO/NO synthase</td>
<td>Increase</td>
<td>[Ca2+]i/G-protein, PI3Kinase, cell-matrix interactions</td>
<td>Calmodulin, microtubules, TK</td>
</tr>
<tr>
<td>PGI2/PGI2 synthase</td>
<td>Increase</td>
<td>NO, NOS</td>
<td>-</td>
</tr>
<tr>
<td>ACE</td>
<td>Decrease</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ET-1/ECE</td>
<td>Decrease</td>
<td>[Ca2+]i, TK, intact microtubules, K+ channels</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 1.1. Effects of shear stress on growth, morphology, and gene expression in endothelial cells (continued).

<table>
<thead>
<tr>
<th>Process</th>
<th>Responses</th>
<th>Signal pathways involved</th>
<th>Pathways not involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth regulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF</td>
<td>T. Increase</td>
<td>-</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>T. Increase</td>
<td>-</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>NC/T. Increase*</td>
<td>Egr-1</td>
<td>-</td>
<td>72, 95, 96</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Increase/ Decrease*</td>
<td>SSRE, NF-κB, PKC</td>
<td>-</td>
<td>93, 96, 77, 63</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Increase</td>
<td>K+ channels</td>
<td>-</td>
<td>23, 97</td>
</tr>
<tr>
<td>Thrombosis/Fibrinolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>98</td>
</tr>
<tr>
<td>tPA</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>98</td>
</tr>
<tr>
<td>TF</td>
<td>Increase</td>
<td>Erg-1</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td>TM</td>
<td>Decrease</td>
<td>[Ca2+]i</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Inflammation/Adhesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Increase</td>
<td>ROS</td>
<td>-</td>
<td>58, 101</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>NC/Decrease*</td>
<td>NO</td>
<td>-</td>
<td>101, 102, 103, 104</td>
</tr>
<tr>
<td>MCP-1</td>
<td>NC/T. Increase*</td>
<td>AP-1/TRE</td>
<td>-</td>
<td>75, 95, 105</td>
</tr>
<tr>
<td>LOX-1</td>
<td>Increase</td>
<td>[Ca2+]i</td>
<td>-</td>
<td>106</td>
</tr>
<tr>
<td>ILs (IL-1, IL-6)</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>107</td>
</tr>
</tbody>
</table>

T. Increase = transient increase and NC = no change. * two different responses have been reported.
**Vasoactive factors:** Shear stress regulates not only vasodilators but also vasoconstrictors, suggesting a balanced interplay regulated by mechanical stimuli on these vasoactive substance production. Shear stress induces the release of many vasodilators such as PGI₂\textsuperscript{108} and NO.\textsuperscript{52} as well as the inducing gene expression of their enzymes, prostacyclin synthase (PGI₂S)\textsuperscript{109} and eNOS.\textsuperscript{22} In addition, shear stress-induced PGI₂ production appears to depend on NO signaling.\textsuperscript{90} suggesting that shear stress tightly controls the vascular tone by coupled regulating of these vasodilators. Shear stress also enhances the expression of C-type natriuretic peptide (CNP) that has vasodilator activity.\textsuperscript{87} On the other hand, arterial shear stress reduces the expression of vasoconstrictors such as ET-1\textsuperscript{179,92} and angiotensin-converting enzyme (ACE).\textsuperscript{91} Arterial shear stress may play an antiatherogenic action on ECs by inducing releases of vasodilators while reducing production of vasoconstrictors to maintain the vessels in a dilation state.

**Growth factors:** Many researchers have investigated effects of shear stress on growth factors in addition to vasoactive factors due to the important role of heparin-binding growth factors in long-term control of vascular structure. Shear stress enhances the expression of PDGF-A and PDGF-B, paracrine growth factors that induce VSMC growth.\textsuperscript{77,110} In addition, it induces a transient activation of heparin-binding epidermal-like growth factor (HB-EGF)\textsuperscript{94} and bFGF.\textsuperscript{93} Moreover, TGF-β1 is induced by shear stress in a sustained manner, and these shear-induced releases of TGF-β1 have significant inhibitory effects on subcultured VSMCs.\textsuperscript{97} Shear stress also increases the production of
many growth inhibitors including NO. These results suggest that a sudden increase of shear stress results in a transient induction of mitogenic responses of vascular cells by inducing growth factors, whereas chronic exposure of ECs to shear stress, which is normally the physiological condition in vivo, may be antiproliferative by enhancing growth inhibitors and thereby atheroprotective.

Mediators of thrombosis and fibrinolysis: Besides changes in vasoactive factors and growth factors, flow-regulated changes in mediators of thrombosis and fibrinolysis have been studied due to the importance of the anti-thrombogenic properties of the endothelium. Diamond et al. initially showed that arterial shear stress but not venous shear stress increased the production of tissue plasminogen activator (tPA), an antithrombotic glycoprotein, with an unchanged production of plasminogen activator inhibitor 1 (PAI-1). In addition, another thrombomodulator, thrombomodulin (TM) that serves to counteract thrombin-induced clot formation, is downregulated by shear in a time- and magnitude-dependent fashion in bovine aortic ECs. However, another study reports a sustained increase in TM expression in human vein ECs. These different responses of TM may be due to species disparity, and the reason for these differences is unclear. Shear stress also causes a transient induction of TF, an initiator of the coagulation cascade. These findings indicate that shear stress can modulate the thrombosis and fibrinolysis of ECs by regulating an intricate balance of these factors.
**Inflammation and adhesion molecules:** Other interested responses of ECs to shear stress are changes in inflammatory mediators and adhesion molecules. The endothelium recruits the adhesion and migration of blood-borne inflammatory cells such as monocytes, lymphocytes, and leukocytes into the blood vessel wall by secretion of chemotactic factors and expression of cell surface adhesion molecules. Shear stress induces a transient activation of the chemoattractant MCP-1 in HUVECs\(^1\) and increases the cell surface expression of ICAM-1.\(^{101,104}\) In contrast, several investigators demonstrate that shear stress reduces VCAM-1 expression in ECs.\(^{74,102,103,113}\) Recent work demonstrates that shear stress also inhibits the complement activation of the inflammatory responses in ECs by inducing the complement-inhibitory protein clusterin.\(^{114}\) These findings suggest that shear stress generated by blood flow acts as a regulator of cell inflammation and adhesion expression in the vessel wall.

**Morphological changes:** Besides causing alterations in the gene expression, shear stress also modulates changes in the morphology of ECs shown in both *in vivo* and *in vitro* studies. ECs at high shear regions *in vivo* studies are elongated in the direction of blood flow, whereas ECs at branch points, where shear stresses are low, possess a polygonal shape with no discernable orientation.\(^{16,115}\) Numerous *in vitro* studies also confirm these results. In response to a sufficient magnitude and duration of laminar shear stress, ECs demonstrate an ellipsoidal morphology with the long axis aligned with the direction of flow, whereas cells grown in static conditions or exposed to low shear conditions have
a polygonal morphology with random orientation. Concurrently, actin filaments and focal adhesion contacts in sheared cells are concentrated in the proximal end of the cells (with respect to the flow direction), whereas they are randomly distributed throughout the cytosol of static cells. Adherence junction proteins (e.g., cadherin, α-catenin, and β-catenin) that mediate cell-cell adhesion are also intense and localized with the end of shear fibers. The reorientation of ECs into the streamlines and rearrangement of the stress fibers and adherence junction proteins in the flow direction may decrease the effective resistance, thus causing lower shear stress experienced by ECs.

**Cell apoptosis and proliferation**: Finally, shear stress regulates the endothelial cell survival by reducing both cell growth and apoptosis. Several studies have demonstrated that laminar shear stress not only inhibits the cell proliferation but also reduces the cell apoptosis in cultured ECs. In addition, shear stress is critical for optimal regeneration of an injured endothelium. ECs at the wound edge region fail to maintain contact with neighboring cells, randomly orient, as well as slowly spread and migrate into wound sites under low shear stress conditions. Shear stress also stimulates the cell migration in the direction of flow. These observations suggest that shear stress regulates the EC survival rather than either cell growth or apoptosis.

A growing number of important vasoactive genes modulated by fluid shear stress in ECs have been identified. Alterations of these genes by extracellular stimuli will regulate
processes such as thrombosis and fibrinolysis, vascular tone, and growth, as well as inflammation and adhesion. These findings suggest fluid shear stress profoundly influences ability of the endothelium to carry out many of its vital functions in health, and alterations in these abilities are likely important in vascular diseases.

1.5 *In vitro* studies of cyclic strain on endothelial cells:

The mechanotransduction induced by cyclic strain, including mechanoreceptors at the cell membrane and signal molecules in the cytoplasm and nucleus, are similar to those seen in shear stress. Major mechanoreceptors involved in cyclic strain include the ion channels such as stretch-activated Ca\(^{2+}\)-permeable channels\(^{123,124}\) and integrins including \(\alpha_5\beta_1\) and \(\alpha_2\beta_1\).\(^{125}\) In addition, cyclic strain activates many second messengers such as intracellular Ca\(^{2+}\). ROS, as well as stimulates PKs, TKs and MAPKs pathways.\(^{14,126-128}\) Of these signal molecules, ROS appears important as shown in the findings that ROS is critical for strain-induced expression of many genes such as MCP-1,\(^{129}\) ICAM-1,\(^{130}\) PAI-1,\(^{131}\) and heme oxygenase-1 (HO-1).\(^{132}\) These findings suggest that cyclic strain imposes an oxidative stress on ECs, possibly through NADH/NADPH oxidase, leading to alterations of many vasoactive genes. Similar to shear stress, cyclic strain also causes the induction of many immediate early genes including Egr-1\(^{133}\) and transcription factors such as AP-1 and NF-\(\kappa\)B.\(^{129,134,135}\) However, the SSRE binding elements, which are critical for the induced transcription by shear stress of many genes including PDGF-B and tPA, are not necessary for the inducible expression of these genes by cyclic strain.\(^{135,136}\) These findings suggest that
shear stress and cyclic strain may differently regulate some signal pathways and thus resulting in various cell responses in ECs.

In addition to causing alterations in signal pathways, exposure of ECs to cyclic strain in vitro also results in changes in cell functions and structure (Table 1.2). Cyclic strain increases the production of vasoactive factors, including NO, \(^{137}\) PGI\(_2\), and ET-1, \(^{20}\) as well as enhances the synthesis of thrombosis and fibrinolysis mediators such as PAI-1 \(^{131}\) and TF. \(^{138}\) In addition, cyclic strain induces the expression of growth factors including PDGF-B \(^{136}\) and many regulators of inflammation and adhesion such as IL-8 \(^{139}\), MCAF \(^{139}\), MCP-1 \(^{140}\) and ICAM-1 \(^{141}\). Cyclic strain also alters EC morphology from a polygonal shape to an elongated spindle-like shape whose long axis is aligned perpendicular to the stretch axis. \(^{142}\) Furthermore, ECs respond to cyclic strain in a biphasic manner such that decreases in DNA synthesis and cell growth correlate with physiologic levels of cyclic strain (less than 18% strain). whereas increases in DNA synthesis and cell growth correspond to high levels of cyclic strain (above 20% strain). \(^{143}\) These findings suggest that cyclic strain can specifically modulate gene expression and cell functions.

Although shear stress and cyclic strain share many similar mechanoreceptors, signaling pathways, and transcription factors, some of the cyclic strain effects on ECs are different from those seen in shear stress. For example, both cyclic strain and shear stress increase
Table 1.2. Effects of cyclic strain on growth, morphology, and gene expression in endothelial cells.

<table>
<thead>
<tr>
<th>Process</th>
<th>Responses</th>
<th>Signal pathways involved</th>
<th>Signal pathways not involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>Increase</td>
<td>protein phosphatase 2A</td>
<td>ERK1/2</td>
<td>127. 144</td>
</tr>
<tr>
<td>Morphology</td>
<td>Elongation</td>
<td>[Ca2+]/pp125FAK, rho p21/pp125FAK</td>
<td>-</td>
<td>145, 146</td>
</tr>
<tr>
<td>Alignment (perpendicular to strain direction)</td>
<td>Increase</td>
<td>[Ca2+], SA channels, actin filaments, AC</td>
<td>ERK1/2</td>
<td>127. 147, 142. 148</td>
</tr>
<tr>
<td>Gene expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>20. 149</td>
</tr>
<tr>
<td>ROS</td>
<td>Increase</td>
<td>NADH/NADPH oxidase, NOS III</td>
<td>-</td>
<td>150. 151</td>
</tr>
<tr>
<td>NO/NOS</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>152</td>
</tr>
<tr>
<td>PGI2/PGI2S</td>
<td>NC/weakly induce*</td>
<td>-</td>
<td>-</td>
<td>19, 153</td>
</tr>
<tr>
<td>PAI-1</td>
<td>NC/Increase*</td>
<td>ROS</td>
<td>-</td>
<td>154. 131</td>
</tr>
<tr>
<td>tPA</td>
<td>NC/Increase*</td>
<td>AP-2, CRE</td>
<td>SSRE</td>
<td>20. 135</td>
</tr>
<tr>
<td>TF</td>
<td>Increase</td>
<td>-</td>
<td>PKC, cAMP</td>
<td>138</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>Increase</td>
<td>-</td>
<td>-</td>
<td>155</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Increase</td>
<td>ROS</td>
<td>-</td>
<td>130. 141</td>
</tr>
<tr>
<td>IL-8</td>
<td>Increase</td>
<td>actin cytoskeletons, PLC, PKC, TK</td>
<td>microtubules</td>
<td>139</td>
</tr>
<tr>
<td>MCAF/MCP-1</td>
<td>Increase</td>
<td>PLC, PKA/C/G. TK, ROS/AP-1/TRE, [Ca2+]/i</td>
<td>microtubules</td>
<td>139. 129. 140</td>
</tr>
<tr>
<td>bFGF</td>
<td>NC</td>
<td>-</td>
<td>-</td>
<td>93</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>NC/Increase</td>
<td>-</td>
<td>SSRE</td>
<td>136. 93</td>
</tr>
</tbody>
</table>

NC: no change. * two different responses have been reported
activities of TKs and PKC. activation of transcription factors: however, shear stress reduces ET-1 expression.\textsuperscript{25} but cyclic strain causes an increase of ET-1 in ECs.\textsuperscript{20} In addition, shear stress transiently increases the expression of MCP-1 gene.\textsuperscript{105} whereas cyclic strain enhances MCP-1 expression in a sustained manner.\textsuperscript{140} Unlike shear stress, cyclic strain is a weak inducer of prostacyclin synthase\textsuperscript{153} and cyclooxygenase-2.\textsuperscript{156} Furthermore, shear stress results in an increase in tPA with an unchanged PAI-1 production.\textsuperscript{98} whereas cyclic strain causes an increase in PAI-1 with an unchanged tPA release.\textsuperscript{20,131} These different cell responses to shear stress and cyclic strain imply either that these mechanical factors may regulate different signal pathways for specific genes or that they may differently perturbate and injure the cell membrane, thereby modulating various responses.

1.6 \textit{In vitro} studies of shear stress on VSMCs:

Although effects of shear stress on endothelial function and gene expression have been investigated extensively, little is known of the effects of shear stress on VSMC function and gene regulation. This may be due to the location of VSMCs in the vessel wall. Covered by the endothelium, VSMCs are rarely exposed to blood flow-exposed sites and thus shear stress. However, under certain conditions such as endothelial denudation after angioplasty, VSMCs can be directly exposed to blood flow, thereby affected by shear stress. There is also evidence that VSMCs can be exposed to shear stress even in the
presence of intact ECs. Modeling systems of arterial blood flow indicates that VSMCs could also experience shear stress due to the pressure gradients of the transmural interstitial fluid flow.\textsuperscript{157} This transmural flow is normally low; however, the small interstitial spaces and the flow resistances created by the surrounding extracellular matrix can lead to thin boundary layers and shear stresses of high magnitudes that could affect VSMC function and gene regulation.

The reported responses of VSMCs to shear stress are similar to those seen in ECs, i.e., changes in cell proliferation and functions. \textit{In vitro} flow experiments on VSMCs demonstrate an increased cell proliferation under low shear conditions and a decreased proliferation under high shear, possibly by the autocrine action of TGF-\textbeta 1.\textsuperscript{158-160} Similarly, low shear stresses transiently increase PAR-1, whereas high shear conditions reduce PAR-1 expression.\textsuperscript{161} In contrast, an inverse pattern of regulation by shear stress is observed (e.g., tPA expression is increased by high shear stress, but decreased at low shear).\textsuperscript{161} Other changes in VSMCs in response to shear stress include increases of PDGF, bFGF, NO, and PGI\textsubscript{2} releases,\textsuperscript{162-165} as well as activation of HO-1 and PDGF receptor alpha expression.\textsuperscript{166,167} These findings suggest that shear stress can modulate gene expression as well as cell functions in VSMCs, thus indicating its important role in vascular physiology and pathophysiology. Whether the intracellular signal mechanisms regulated by shear stress in VSMCs are similar to those in ECs remains to be elucidated.
1.7 *In vitro* studies of cyclic strain on VSMCs:

It is now recognized that elevated cyclic strain can elicit VSMC responses that ultimately contribute to maintaining vascular structure and progressive intimal thickening. As the range and complexity of VSMC biological responses to mechanical strain has emerged, it is important to investigate the mechanisms whereby VSMCs sense changes in cyclic strain and translate this mechanical stimulus into intracellular biochemical signals and ultimately alter biological responses. The original work on cyclic strain effects in VSMCs, however, focuses only on end-point responses such as cell proliferation, morphology, cytoskeleton arrangement, differentiation, and ECM protein synthesis. More recently, studies have been developed to investigate mechanical strain-induced signal transduction pathways that lead to alterations in cell proliferation and gene expression. Similar to ECs, VSMCs may demonstrate a hierarchy of cell signaling responses to mechanical strain including enhanced-activation of various mechano-receptors at the membrane such as ion channels and integrins, elevated-activities of numerous cell-signaling molecules in the cytoplasm, and increased levels of specific transcription factors in the nucleus (Fig. 1.2).

**How do VSMCs sense cyclic strain at the membrane?**

Ion channels and integrins are the key molecules in the initiation of mechanotransduction and ultimately biological responses of VSMCs in response to cyclic strain. Mechanical strain can directly influence Ca$^{2+}$ and K+ conductance (ion channels) in VSMCs and thus modulate vascular tone.168,169 In addition, stress-activated (SA) ion channels are
involved in strain-induced ERK1/2 activation\textsuperscript{170} and strain-activated PLC activity.\textsuperscript{171} Besides ion channels, integrins are also shown as major candidates for mechanoreception. Mechanical strain increases DNA synthesis in VSMCs cultured on collagen, fibronectin, or vitronectin, but not in cells cultured on elastin or laminin.\textsuperscript{172} Moreover, strain-induced expression of PDGF-A can be abrogated by integrin-binding RGD peptides.\textsuperscript{172} Interestingly, the phenotypic modulation of VSMCs (in a more differentiated state) and activation of MAPKs. Egr-1. and c-jun by mechanical strain are strongly influenced by ECM composition.\textsuperscript{173-175} These findings suggest that specific integrin-ECM interactions serve as mechanotransducers. The basis mechanism of the specific integrin-dependence in VSMCs is presently unknown, but it is likely that integrin-ECM interactions will activate specific intracellular signaling pathways leading to alterations in cell responses. In support of this concept, mechanical strain has been shown to activate the MAPK pathway via integrin-dependent pathways.\textsuperscript{174}

\textbf{How do signals from the cell membrane get into the cytoplasm and nucleus?}

Multiple signal pathways in the cytosol activated by mechanical strain in VSMCs include second messengers such as ROS, PKs, and MAPKs. Cyclic strain results in the increases of ROS production in VSMCs that mediate cell proliferation\textsuperscript{176} and LDL oxidation,\textsuperscript{177} suggesting that the increased oxidant stress by cyclic strain is the potential mechanism whereby hypertension facilitates atherosclerosis. In addition to ROS, PKs are other signal pathways regulated by cyclic strain. Cyclic strain increases IP\textsubscript{3}, DAG, and PKC levels.\textsuperscript{178,179} Mechanical strain induces a decrease in the catalytic activity of AC that
generates cAMP. Since cAMP is generally associated with VSMC relaxation, the strain-induced decrease in AC activity would promote contraction. Although stretch activates both PKA and PKC pathways in VSMCs, inhibition of PKA or PKC alone fails to prevent strain-induced alignment and proliferation. Yet PKC is shown to mediate the synergistic stimulation of parathyroid hormone-related peptide gene (PTHrP) expression by cyclic strain and Ang II as well as the strain activation of extracellular Ca²⁺-dependent vascular tone. Cyclic strain has been shown to induce TK signal pathways in ECs; however, these events have not been specifically studied in VSMCs. Nevertheless, TKs are necessary for strain-stimulated MCP-1 in VSMCs.

Of all signal pathways regulated by cyclic strain in VSMCs, MAPK pathways are best studied. Cyclic strain induces the rapid and transient activation of all three members of MAPKs: ERKs. JNK that is dependent on mechanisms involving autocrine stimulation of purinoceptors by ATP and p38 MAPKs in VSMCs. Cyclic strain can also activate induction of MAPK phosphatase-1 (MKP-1), which serves as a negative regulator of MAPK signaling pathways to cause the dephosphorylation and inactivation of ERK, JNK, and p38 MAPK, as well as inhibition of DNA synthesis, suggesting a strain-induced feedback loop to limit MAPK activation. MAPK pathways are critical for the strain-induced proliferation and migration of VSMCs. The MAPK cascade is also activated by numerous growth factors and Ang II thus providing a potential point of convergence and synergism between growth factors and mechanical strain in the regulation of VSMC growth. The upstream and downstream mechanisms whereby cyclic
strain actives MAPK pathways are presently ill-defined. These signal mechanisms may 
be similar to those seen in ECs. For example, cyclic strain stimulates integrin:ECM 
interactions or G protein-linked couple receptors. subsequently activates the protein 
kinease cascades, leading to alterations in gene expression. However, this hypothesis 
remains to be elucidated. Recent work demonstrates that cyclic strain rapidly activates 
p38 MAPKs via activation of PKC/ras/rac signal pathways\textsuperscript{186} and MKP-1 via Ras/Rac-
MAPK pathways.\textsuperscript{184} 

Besides inducing rapid increases in signal molecules in the cytoplasm, cyclic strain also 
results in the rapid increases of many immediate early genes and transcription factors 
such as Egr-1 and c-jun.\textsuperscript{175} Furthermore, Wilson et al.\textsuperscript{188} has shown that Egr-1 and 
Sp-1 in VSMCs are upregulated by mechanical strain. and Egr-1 and possibly Sp-1 bind 
to the putative mechanical strain response element within the PDGF-A promoter to 
gradually induce PDGF-A expression.

\textbf{VSMC orientation, growth, and differentiation in response to cyclic strain}

Exposure of cultured VSMCs to mechanical strain alters not only cell growth but also cell 
orientation and differentiation. Cyclic strain has been shown to induce VSMC growth, 
which is dependent on the autocrine action of PDGF.\textsuperscript{189} Furthermore, others have 
reported that strain-induced VSMC proliferation is dependent on both Ang II production 
and PDGF expression.\textsuperscript{190} insulin-like growth factor 1 (IGF-1).\textsuperscript{191} and epidermal growth
factor receptor (EGFR) in an autocrine manner. Cyclic strain also increases oxidative stress as shown in increases of ROS production, which in turn stimulates DNA synthesis via NF-κB in cultured VSMCs. In contrast, physiological levels of cyclic strain (10% strain) have been shown to inhibit VSMC growth by an increase in cyclin-dependent kinase inhibitor p21. These suggest that physiological levels of cyclic strain contribute to vascular homeostasis by inhibiting the cell cycle progression, whereas overstretch can contribute to intimal thickening by promoting VSMC growth via production of many mitogens that act as an autocrine manner. In addition, VSMC growth in response to mechanical strain in vitro is synergistically enhanced by VSMC mitogens such as Ang II and thrombin, indicating the potential for interaction between mechanical and non-hemodynamic factors in the regulation of vascular structure in vivo.

In addition to regulating cell proliferation, cyclic strain also alters cell morphology and orientation, as well as increases the expression of highly specific VSMC differentiation in vitro. VSMCs exposed to high cyclic strain are elongated spindle shape and aligned perpendicular to the strain gradient, whereas VSMCs at the low strain regions remain aligned randomly. In addition, mechanical strain increases the expression of smooth muscle myosin heavy chain (both the SM-1 and SM-2 isoforms) and decreases the expression of the non-muscle forms of myosin (NM-A and NM-B), suggesting that cyclic strain can alter myosin isoform expression toward a more differentiated state. These findings are consistent with the observation that the development of a well-differentiated contractile phenotype is found in VSMCs exposed to mechanical strain.
Cyclic strain thus appears as the most important factor regulating VSMC differentiation and the maintenance of the contractile phenotype within the vessel wall. As suggested, "no stretch" (or static culture) results in a loss of contractile phenotype and a gain of the proliferative and dedifferentiated VSMCs; "optimal physiological stretch" maintains VSMCs in the healthy, quiescent, and contractile phenotype; and "overstretch" (i.e., hypertension) causes the induction of VSMC hypertrophy and increase of matrix synthesis.194

**Genes regulated by cyclic strain in VSMCs**

Mechanical strain not only induces synthesis of ECM proteins but also regulates many vasoactive genes in VSMCs *in vitro* such as growth factors (Table 1.3). Cyclic strain has been shown to increase synthesis of ECM proteins such as collagen and fibronectin in VSMCs.194,197 Mechanical strain can increase ECM accumulation in VSMCs by releasing a soluble factor from VSMCs that is capable of inducing VSMC matrix synthesis. This is supported through the findings that Ang II and TGF-β1 released from VSMCs mediate strain-induced collagen synthesis.198 These findings suggest that cyclic strain not only can induce the VSMC contractile phenotype but also be able to stimulate matrix deposition and thus VSMC proliferation.
Table 1.3. Effects of cyclic strain on growth, morphology, and gene expression in vascular smooth muscle cells.

<table>
<thead>
<tr>
<th>Process</th>
<th>Responses</th>
<th>Signal pathways involved</th>
<th>Pathways not involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA synthesis</td>
<td>Increase</td>
<td>ERK, ROS/NF-κB</td>
<td>-</td>
<td>199, 176</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Increase</td>
<td>PDGF, IGF-1, EGFR, AngII, cell-matrix interaction, SA channels</td>
<td>PKA, PKC</td>
<td>191, 170, 190, 179, 172, 189, 170</td>
</tr>
<tr>
<td>Morphology</td>
<td>Elongation</td>
<td>-</td>
<td>-</td>
<td>179, 195</td>
</tr>
<tr>
<td>Orientation (perpendicular to strain)</td>
<td>Increase</td>
<td>-</td>
<td>PKA, PKC</td>
<td>179</td>
</tr>
</tbody>
</table>

Gene expression
- CO/HO-1: Increase - 166
- PTHrP: Increase - PKC - 200
- COX-1: Increase - 201
- ROS (O2-): Increase NADPH oxidase, NF-κB - 176
- PAI-1: Increase - 201
- VEGF: Increase - 201
- PDGF-A: Increase Egr-1 sites - 188
- PDGF-α receptor: Increase MAPK, AP-1 - 167
- PDGF-B/β receptor: Increase Ang II - 190, 202
- IGF-1: Increase - 191
- MCP-1: Increase TK - 183
- LDL oxidation: Increase ROS - 177
- Collagen synthesis: Increase Ang II, TGF-β1 - 198
- SM-1/SM-2: Increase cell-matrix interactions - 173
- NSMA/NSMB: Decrease - 173
In addition to its effects on matrix deposition, cyclic strain can also influence many other aspects of gene expression. Cyclic strain induces the expression of growth factors and their receptors such as PDGF-B, the PDGF-β receptor,\textsuperscript{202} PDGF-A,\textsuperscript{188} the PDGF-α receptor,\textsuperscript{167} and bFGF.\textsuperscript{203} Cyclic strain also increases the expression of vascular endothelial growth factor (VEGF), a potent EC specific mitogen that induces marked increases in vascular endothelial permeability.\textsuperscript{201} This induction may act in a paracrine loop to regulate EC growth and permeability. Besides inducing growth factors, mechanical strain also stimulates expression of chemotaxis and adhesion molecules including MCP-1.\textsuperscript{183} These findings indicate that increased cyclic strain in VSMCs can elicit cell responses such as alterations in gene expression and cell proliferation that ultimately contribute to progressive hyperplasia. The diversity of VSMC responses to cyclic strain is remarkable, and the signal pathways that regulate these responses are clearly complex. One of the challenges in this research in the future will be the investigation of the intracellular signal mechanisms linking cyclic strain and altered gene expression or the elucidation of how cyclic strain selectively activates the signal molecules within the cells and thereby elicits differential biological responses.

\section{1.8 Thrombin and PAR-1}

As shown above, shear stress and cyclic strain can regulate many vasoactive genes in vascular cells, leading to alterations in vascular functions. PAR-1, a family of thrombin receptors, can modulate many vascular functions; however, the effects of shear stress and cyclic strain on its expression have not previously been examined. Thrombin, a trypsin-
like serine protease generated at the site of vascular injury, plays an important role in the development of atherosclerosis, restenosis, and thrombosis. Besides its well-known role in blood coagulation, thrombin elicits a variety of cellular events. It induces platelet aggregation and increases monocyte and neutrophil chemotaxis. Thrombin also promotes mitogenesis for lymphocytes and mesenchymal cells including VSMCs. In addition, it stimulates endothelial cells to produce prothrombotic factors, antithrombotic factors such as NO, and growth factors including PDGF. Most of these effects are mediated by members of the thrombin receptor family, protease-activated receptors (PARs). PARs belong to the G protein-coupled receptors that contain seven-transmembrane domains, a cytosolic C-terminal domain, and an extracellular N-terminal segment. Up to now, four protease-activated families of G protein-coupled receptors, PAR-1, PAR-2, PAR-3, and PAR-4, have been cloned. PAR-1, PAR-3, and PAR-4 are activated by thrombin, whereas PAR-2 is activated by trypsin and tryptase. Of these four, PAR-1 expression is highest in human ECs, VSMCs, and platelets. Besides these cell types, PAR-1 is also expressed in fibroblasts, monocytes, T-cell lines, osteoblast-like cells, neurons and glial cells in the brain and periphery, and certain tumour cell lines.

PAR-1, one of four known PARs, plays an important role in the development of vascular diseases and vascular functions. In normal arteries, PAR-1 is usually present in the adventitia, less often found in the endothelium, but does not exit in the media. However, there is a marked increase in PAR-1 expression in medial SMCs after a balloon catheter injury. In situ hybridization studies of human arteries also show high levels of PAR-1 expression in human atheromas, especially in areas of proliferating VSMCs and in areas rich in macrophages, suggesting the important role of PAR-1 in the development of atherosclerosis and restenosis. In addition, PAR-1 mediates many of the biological effects of thrombin including inflammation, proliferation, and thrombosis.
agonists cause platelet secretion and aggregation and induce the proliferation of many cell types including VSMCs. PAR-1 regulates most of thrombin's cellular effects in ECs and VSMCs such as the release of NO, PGI₂, and ET-1, endothelial permeability, and Ca²⁺ mobilization.

How did PAR-1 mediate these effects of thrombin? Thrombin, cleaving the N-terminus of PAR-1 at specific sites (Arg41-Ser42, PR-SFLLRN), exposes the newly exposed N-terminal sequences that interact with the cleaved receptors to initiate transmembrane down-stream signaling events. PAR-1 activated by thrombin interacts with at least 2 G-proteins: a Gi subtype coupled to AC and a Gq subtype coupled to PLC. PLC activation induces at least 2 messengers: IP₃, which causes a rise in cytosolic free Ca²⁺, and DAG that leads to the activation of PKC. This kinase cascade causes a consequent activation of TKs and MAPKs and results in an activation of NF-κB, leading to the expression of many genes such as inflammatory cytokines, growth factors, and cell adhesion molecules. After proteolytic activation by thrombin, PAR-1 is quickly desensitized by several mechanisms including receptor phosphorylation, internalization and degradation. The resulting rapid inactivation suggests that the receptor signaling is dependent on the rate of receptor cleavage by thrombin and not the cumulative number of cleavage receptors. About 90% of PAR-1 is internalized within 10 minutes of thrombin or PAR-1 peptide treatment. These internalized receptors are shuttled to lysosomes for degradation, whereas few of thrombin-cleaved receptors that did not degrade recycle to the cell surface in an inactive state. Unlike most other G protein-coupled receptors, the PARs are activated by proteolysis that occurs only once: i.e., the availability of newly synthesized cell surface PAR-1 is required for the recovery of cellular responses to thrombin. In vascular cells, two general mechanisms account for
this resensitization of PAR-1: the synthesis of new receptors and the mobilization of intracellular pools of receptors in the Golgi vesicles. 213.216

1.9 General objectives

Due to the importance of PAR-1 in the maintenance of vascular integrity, this project investigated the effects of shear stress and cyclic strain on PAR-1 gene regulation in human vascular ECs and VSMCs. First, effects of shear stress on PAR-1 expression in both human macro- and microvascular ECs were investigated using an in vitro flow system, the parallel plate flow chamber system. In addition, this study evaluated changes in thrombin-induced events after cell exposure to these mechanical factors, including releases of NO and ET-1, as well as investigated the roles of signal pathways involved in this process including autocrine factors, protein kinases, TKs, and MAPKs. Second, this project further investigated the mechanism involved in shear-reduced PAR-1 expression in human aortic smooth muscle cells (HASMCs) as shown by previous work in our group and evaluated thrombin-stimulated events including Ca²⁺ mobilization and cell proliferation. Finally, this work also investigated the effects of cyclic strain on PAR-1 expression in HASMCs and the signal mechanisms by which these cells sensed the force and altered PAR-1 gene expression, leading to changes in cell proliferation in response to thrombin.
CHAPTER 2

Effects of shear stress on PAR-1 expression in endothelial cells

2.1. Introduction

This study investigates the effects of shear stress on PAR-1 expression in both human macro- and microvascular ECs and the signal intracellular pathways that may be involved in this process. Less intimal hyperplasia was observed in areas of high shear stress after balloon catheter injury and in vascular grafts.\textsuperscript{217} even though PAR-1 expression and thrombin levels increase significantly in injured arteries.\textsuperscript{204} Therefore, we hypothesized that high shear stress decreases the expression of PAR-1 in ECs. If true, this could reduce the secretion of vasoactive substances from ECs in response to thrombin, including ET-1 that is mitogenic for VSMCs. Most studies of human ECs \textit{in vitro} have been conducted on cells obtained from macrovascular ECs such as human umbilical vein ECs (HUVECs). However, the microvascular bed has large surface areas, and thus plays an important part in producing and delivering endothelium-derived products into the circulation and in regulating hemostasis.\textsuperscript{218} Thus it is essential to investigate effects of shear stress on PAR-1 expression not only on macrovascular ECs but also on microvascular ECs.

In this work, arterial shear stresses decreased PAR-1 expression in both HUVECs and human microvascular cells (HMECs), but with different thresholds. In addition, down-
regulation of PAR-1 expression by shear stress altered subsequent endothelial cell functions, as shown by decreases in thrombin-stimulated nitric oxide (NO) and endothelin-1 (ET-1) secretion. The transduction pathways whereby ECs sense shear stress and reduce gene expression of PAR-1 were also studied. PKC but neither tyrosine kinases nor autocrine factors was found to mediate shear stress-reduced PAR-1 expression in either macro- or microvascular ECs.

2.2. Procedures

To investigate the effects of shear stress on PAR-1 expression in HUVECs and HMECs, cells were isolated (HUVECs) or purchased (HMECs), subcultured following cell culture technique, and exposed to different levels of shear stress as described in shear stress experiments. After the experiments, PAR-1 mRNA was quantified using Northern analysis, whereas surface PAR-1 protein was detected using flow cytometry. In addition, two functional studies, nitrite assays and ET-1 radioimmunoassays, were applied to study the subsequent abilities of sheared cells to release NO and ET-1 in response to thrombin. Finally, preliminary work was conducted to investigate the role of autocrine factors in shear stress-reduced PAR-1 expression using conditioned media and the role of protein kinases using various protein kinase inhibitors.

Materials

Herbimycin A, PD 98059, staurosporine, KT 5720, Ro 31-8220, and KT 5823 were purchased from Calbiochem. Media and antibiotics were from Gibco. Other chemicals (if not specified) were obtained from Sigma Chemical Co.
Cell culture

After isolation from umbilical veins by collagenase treatment, primary HUVECs were grown in medium 199 (M199) supplemented with 20% fetal bovine serum (HyClone), 200 μg/ml L-glutamine, 200 U/ml of penicillin, and 100 μg/ml streptomycin (HUVEC complete medium). HUVECs were subcultured using 0.05% trypsin-EDTA and were used from P2 to P5 for experiments. Human dermal microvascular endothelial cells (Cascade Biotechnology) were grown in microvascular endothelial cell basal medium (Cascade) with the above serum and antibiotic concentrations (HMEC complete medium). Passages between 5 and 9 were used for experiments.

Shear stress experiments

Cells were seeded at 5 x 10⁴ cells/cm² onto glass slides (75x38 mm. Fisher) coated with 1% (w/w) gelatin crosslinked with 0.5% glutaraldehyde in PBS. After reaching confluence, cells were either maintained under static conditions or exposed to shear stress in complete media using the parallel plate chamber system described previously (Fig. 2.1). Three levels of shear stress were used in these experiments: 5, 15, and 25 dyn/cm². Experiments were performed in a 37°C humidified room for 3, 6, or 24 hours, and the pH was maintained at physiologic levels by gassing the medium with a mixture of 95% air and 5% CO₂.
Figure 2.1. **Schematic of a parallel plate flow chamber system.** The flow loop apparatus consists of two reservoirs connected with each other by an insert and the parallel plate flow chamber. The parallel plate flow chamber contains a polycarbonate template, a gasket, and the glass-slide seeded with cells. These were held together by a vacuum. Flow is driven through the chamber by the pressure head created by the vertical distance between the upper and lower reservoirs. Media were recirculated through the flow loop system by a peristaltic pump.
Northern Analysis

Total RNA was isolated immediately after the experiments by using the FastRNA prep kit (Bio101). RNA samples were fractionated on a 1.3% formaldehyde agarose gel. transferred to nylon membranes (MSI) using a vacuum transfer system (Stratagene), and immobilized by UV irradiation (Stratagene). The cDNA PAR-1 and GAPDH probes\textsuperscript{207} were labeled with $\left[\alpha-^{32}\text{P}\right]$dCTP (DuPont NEN) by random priming. Blots were hybridized with the labeled probes for 1 hour in QuikHyb solutions (Stratagene) at 68°C. For quantitative analysis of expression, the blots were scanned by a densitometer and analyzed using the ImageQuant software (Molecular Dynamics). To correct for differences in RNA loading and transfer, the signal intensity for each RNA sample was normalized to GAPDH mRNA, which is not affected by fluid shear stress in human ECs.\textsuperscript{220}

Flow cytometry

For flow cytometry experiments, cells were pre-incubated with thrombin (4 U/ml) in a complete medium for 15 minutes at 37°C to deplete intracellular receptor pools and cell surface PAR-1. Cells were then either maintained in complete media under static conditions or exposed to 25 dyn/cm\textsuperscript{2} for 24 hours. After detachment from slides by 50 mM EDTA, cells were centrifuged at 3000 rpm for 10 minutes and resuspended in PBS containing 2% bovine serum albumin (BSA). Phycoerythrin(PE)-conjugated monoclonal human thrombin receptor antibody, SPAN12 (Coulter), a "cleavage-sensitive" antibody, which reacted only with the uncleaved-thrombin receptor, was added to the cell
suspension at a final concentration of 20 μg/ml per 5x10^5 cells and incubated at 4°C for 30 minutes. Purified PE-conjugated anti-mouse IgG1 antibody (Coulter) was added to cells as a negative control. After labeling, cells were washed once in PBS, fixed in PBS/1% formaldehyde, and analyzed on a fluorescence analyzer (Becton Dickinson). The geometric mean fluorescence of each sample, correlated to cell surface PAR-1 density, was calculated as a percent of static controls to determine the effect of shear stress on cell surface-associated PAR-1 protein.

Nitrite assays

Cells were exposed to 25 dyn/cm² for 24 hours or maintained under static conditions in complete media. The cells were then washed with serum-free M199 without L-arginine and phenol-red and equilibrated for 30 minutes at 37°C/5% CO₂/95% humidified air in the above medium. Thrombin (2U/ml) was added and medium was collected at 0, 1, 3, 5, or 10 minutes after adding thrombin. NO production in response to thrombin was estimated by measuring nitrite, an oxidation product of NO. Nitrite levels were quantified using a fluorometric assay, which uses 2,3-diaminonaphthalene (DAN purchased from Aldrich) to convert nitrite into a fluorescent compound 1-((H))-naphthotriazole. In brief, 20μl of freshly prepared DAN at 0.05 mg/ml in 0.62 M HCl was added to 100μl of diluted sample. After incubation in the dark at room temperature for 10 minutes, the reaction was terminated by adding 10 μl 2.8 N NaOH. Fluorescence was measured on a 96-well plate reader (Cytofluor 2350, Millipore) with excitation at 360nm and emission at 460nm. Sodium nitrite standards were made fresh in phenol red-
free M199 and kept on ice until use. Nitrite levels in collected media were calculated using a standard curve. Changes in nitrite formation at each time point after adding thrombin were normalized to the cell number for each sample.

**ET-1 Radioimmunoassay (RIA)**

After exposure to 25 dyn/cm² for 24 hours, cells were washed and equilibrated at 37°C for 30 minutes in complete media. Cells were then treated with thrombin (2U/ml), and medium was collected at 0, 1, 3, 5, and 24 hours. To extract endothelin, the collected medium was mixed with an equal volume of 20% acetic acid solution and applied to 200mg C-18 extraction columns (Varian), which were pre-washed successively with methanol, water, and 10% acetic acid solution. Extraction columns were then washed with 10% acetic acid and ethyl acetate. After the washing step, samples were eluted with methanol-0.05 M ammonium bicarbonate solution (80:20), vacuum dried, and resuspended in an assay buffer provided in the ET-1 RIA kit (NEN Life Science). ET-1 levels from sample extracts were determined by competitive RIA, following the manufacturer’s instructions. The radioactivity was read using a gamma counter (Nuclear Medical Laboratory). Changes in ET-1 protein levels were then determined against ET-1 standard curves and were normalized to the cell number for each sample.

**Conditioned medium experiments**

Cells were exposed to 25 dyn/cm² for 24 hours or maintained under static conditions in the complete medium. The conditioned medium from static or sheared cells was added to fresh confluent cells of the matching cell type in stationary culture for 24 hours. Cells
fed with fresh medium were used as controls. After 24 hours of exposure to fresh or conditioned medium, total RNA was isolated and PAR-1 mRNA was measured.

**Role of protein kinases on shear stress-reduced PAR-1 expression**

To investigate the roles of protein kinases in shear stress-decreased gene expression of PAR-1, ECs were pretreated with protein kinase inhibitors for one hour each and then exposed to 25 dyn/cm² for 24 hours in the presence of the same inhibitor. Cells were treated with either the non-specific protein kinase inhibitor staurosporine (10 nmol/L), MAPK kinase (MEK) inhibitor PD 98059 (50 μmol/L) that inhibits the activation of MAPK, or tyrosine kinase inhibitor Herbinycin A (2.0 μmol/L). The role of the AGC kinase group was further investigated using PKA inhibitor KT 5720 (0.5 μmol/L); PKG inhibitor KT 5823 (1.0 μmol/L); and PKC inhibitor Ro 31-8220 (0.1 μmol/L). Pilot experiments determined the concentrations of each inhibitor used, which was not toxic to cells and was well above twice of the manufacturer’s recommended inhibition curve (IC₅₀).

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical analysis between multiple groups was determined by a factorial ANOVA followed by Fischer's protected least significant difference test. Values of P<0.05 were considered significantly different.
3.3 Results

Shear stress downregulated PAR-1 mRNA levels in HUVECs and HMECs

Arterial shear stresses downregulated PAR-1 mRNA levels in a time-dependent manner in both HUVECs and HMECs (Figs. 2.2 and 2.3, respectively). At 24 hours, PAR-1 mRNA expression was decreased 2.5 fold under high shear stress (25 dyn/cm²) compared to that in HUVECs under static conditions, low, or moderate shear (5 or 15 dyn/cm²; Fig. 2.2 B) High shear stress also reduced PAR-1 mRNA a 3-fold decrease in HMECs after 6 hours of exposure compared to those of static HMECs (Fig. 2.3 B). Unlike HUVECs, PAR-1 mRNA levels in HMECs were significantly downregulated by 15 dyn/cm². Low shear stress did not change PAR-1 mRNA levels relative to those of static controls in either cell type.

Shear stress reduced cell surface PAR-1 expression in HUVECs and HMECs

PAR-1 surface expression decreased by 45% in HUVECs after exposure to 25 dyn/cm² for 24 hours (20.3±2.7 fluorescence units for control versus 13.1±3.3 for shear-stressed cells. n=4) and by 60% in HMECs (35.7±1.6 for static and 14.5±1.4 for shear-stressed cells) (Figs. 2.4 and 2.5). More total PAR-1 was expressed on the surface of static HMECs compared to HUVECs (35.7±1.6 fluorescent units for HMECs versus 20.3±2.7 for HUVECs, P<0.05).

Post-shear stress NO production in response to thrombin

After exposure to 25 dyn/cm² for 24 hours, cells were stimulated with thrombin to release NO. Thrombin stimulated half as much NO release from shear stressed HUVECs
Figure 2.2. PAR-1 mRNA levels in HUVECs subjected to shear stress. mRNA expression of PAR-1 and GAPDH was determined by Northern blot analysis. A. An autoradiogram representative of 3-5 experiments, in which the top band represents PAR-1 mRNA and the bottom band displays the corresponding GAPDH of static control (C) and 5, 15, and 25 dyn/cm² for 24 hours. B. Time course of mean PAR-1 mRNA band densities normalized for loading variation with GAPDH. Results are expressed relative to static controls as mean ± SEM. Significantly different from: * static control (P<0.05) and +5 dyn/cm² at 24 hours (P<0.05).
Figure 2.3. PAR-1 mRNA expression in HMECs subjected to shear stress. **A.** Representative experiment from 3-5 individual Northern blot experiments after exposure to shear stress for 24 hours. **B.** Time course of mean PAR-1 mRNA band densities normalized for loading variation with GAPDH. Results are expressed relative to static controls as mean ± SEM. Significantly different from: * static control (P<0.05) and † 5 dyn/cm² at 24 hours (P<0.05).
Figure 2.4. Flow cytometry of PAR-1 protein on the cell surface in static and sheared HUVECs (A) and HMECs (B). The PAR-1 levels were detected with PE-labeled PAR-1 antibody and the results analyzed by FACS. On the same plot, curves for nonspecific antibody (PE-labeled mouse IgG antibody) are shown as negative controls. Each curve is based on 10,000 cells.
Figure 2.5. Cell surface PAR-1 expression in HUVECs and HMECs is reduced by shear stress. HUVECs or HMECs were exposed to shear stress (25 dyn/cm²) for 24 hours or maintained under static conditions. The percentage of control geometric mean fluorescence of sheared cells was calculated by normalizing with matched stationary controls. Results are shown as mean ± SEM (n=4). Significant differences in PAR-1 between static cells and cells exposed to shear stress: *P<0.05 and **P<0.005.
compared to static controls at 1 minute (43.7±4.2 nmol/10^6 cells for sheared cells versus 76.9±4.7 for static controls). Oxidation of nitrite to nitrate probably accounts for the decrease after this time point, indicating a thrombin-induced burst, but little sustained NO production induced by thrombin (Fig. 2.6 A). HMECs released very little NO compared to HUVECs (6.3±1.3 nmol/10^6 cells for HMECs versus 76.9±4.7 for HUVECs at one minute after adding thrombin), and there was no difference between NO levels released by shear-stressed and static HMECs (Fig. 2.6 B).

**Post shear stress ET-1 production in response to thrombin**

To give further evidence that shear stress reduces functional PAR-1 expression, we measured ET-1 production in response to thrombin in static and shear-stressed HUVECs and HMECs at 1, 3, and 5 hours. Thrombin caused a time-dependent release of ET-1 in both HUVECs and HMECs (Fig. 2.7). ET-1 production in response to thrombin was reduced significantly in sheared HUVECs and HMECs compared to static controls (P<0.05).

**Effects of conditioned medium on PAR-1 expression**

Hemodynamic forces regulate gene expression and cell growth via an autocrine action of many factors. To study whether the decrease in PAR-1 expression by shear stress is mediated via such autocoids, conditioned medium from cells exposed to shear stress was used. If autocrine pathways were involved, we would expect to see decreases in PAR-1 mRNA in static cells exposed to shear conditioned medium. However, PAR-1 mRNA
Figure 2.6. Effects of thrombin on nitrite formation from shear-stressed HUVECs (A) and HMECs (B). Thrombin (2U/ml) was added to sheared (25 dyn/cm², 24 hours) and static cells. Medium was collected for nitrite assays at the times indicated. Nitrite formation was normalized to the cell number for each sample. Results are shown as mean ± SEM (n=3-4). Filled symbols (circles for HUVECs and triangles for HMECs) represent static cells whereas open symbols indicate sheared cells. The asterisk indicates significant differences between static cells and cells exposed to shear stress (P<0.05).
Figure 2.7. Effects of thrombin on ET-1 production from shear-stressed HUVECs (A) and HMECs (B). Thrombin (2U/ml) was added to sheared (25 dyn/cm², 24 hours) and static cells. Medium was collected for ET-1 RIA at the times indicated. ET-1 production was normalized to the cell number for each sample. Results are shown as mean ± SEM (n=3-4). Filled symbols (circles for HUVECs and triangles for HMECs) represent static cells whereas open symbols indicate sheared cells (25 dyn/cm², 24 hours). The asterisk indicates significant differences between static cells and cells exposed to shear stress (P<0.05). Note that at 1-hour time point, ET-1 release was 2.2±0.1 pg for static HMECs and 1.4±0.2 pg for sheared HMECs (p = 0.03).
levels were not decreased in HUVECs or HMECs: in fact, there was an increase in
PAR-1 mRNA expression in HMECs (1.6 ± 0.2 fold induction versus fresh medium).
These findings indicate that autocrine factors are not responsible for the shear stress-
reduced PAR-1 expression.

**Role of protein kinases in shear stress-reduced PAR-1 expression**

Using inhibitors of various protein kinases, we investigated in a preliminary way the
intracellular signaling pathways involved in shear stress-reduced PAR-1 mRNA
expression. Shear stress-downregulation of PAR-1 mRNA expression in HUVECs was
eliminated by the non-specific protein kinase inhibitor staurosporine, the PKA inhibitor
KT5720, or the PKC inhibitor Ro31-8220 (Fig. 2.8 A, P<0.05). The decrease in PAR-1
mRNA expression by shear stress in HMECs was inhibited by the non-specific protein
kinase inhibitor staurosporine, the MEK inhibitor PD98059, the PKC inhibitor Ro31-
8220, or the PKG inhibitor KT5823 (Fig. 2.8 B, P<0.05). The tyrosine kinase inhibitor
Herbimycin A did not alter the shear stress-reduced PAR-1 expression in either HUVECs
or HMECs (0.6±0.1-fold of static controls when sheared cells pretreated with HA vs.
0.5±0.1-fold of static controls in sheared HUVECs). None of the inhibitors used had
significant effects on PAR-1 mRNA levels in static cells (results not shown).

**3.4. Discussion**

This study demonstrates that laminar shear stress decreases PAR-1 expression in
endothelial cells, leading to altered secretion of NO and ET-1 in response to thrombin, in
part through a PKC mediated mechanism. Previous *in vivo* and *in vitro* studies showed
Figure 2.8. **Effects of protein kinases on shear stress-reduced PAR-1 expression.** Cells were pre-treated with PD98059 (PD), Staurosporine (Sta), KT5720 (K57), Ro 31-8220 (Ro), or KT5823 (K58) for one hour, followed by exposure to 25 dyn/cm² for 24 hours in the presence of the inhibitor (see procedures). Results of densitometry of PAR-1 mRNA normalized to corresponding GAPDH mRNA are shown (A) for HUVECs and (B) for HMECs (n=3-4). The asterisk indicates significant differences from static controls. C. and † indicates significant differences from sheared samples. S.
that PAR-1 mediates many of the known thrombin-induced effects, including the inflammatory and proliferative responses. The increase of PAR-1 expression in atherosclerosis lesions indicates an important role for PAR-1 in restenosis and atherogenesis. Knowing the mechanisms that regulate PAR-1 expression will give a better understanding of vascular diseases and potentially provide therapeutic tools that will selectively inhibit the effects of thrombin on vascular cells.

Shear stress has been shown to regulate expression of many vasoactive genes in ECs. leading to alterations in vascular functions. In the current study, shear stress-reduced PAR-1 expression in both HUVECs and HMECs is time- and dose-dependent. Arterial shear stress downregulated the expression of PAR-1 at both the mRNA (Figs. 2.2 and 2.3) and protein levels (Figs. 2.4 and 2.5) in both HUVECs and HMECs. The decrease of PAR-1 expression by shear stress was more pronounced in HMECs than in HUVECs (Fig. 2.5). Our results support the hypothesis that high shear stress reduces the expression of atherogenesis-related genes and thus may inhibit neointimal hyperplasia in injured arteries. Laminar shear stress also downregulates other atherogenesis-related genes including ET-1,92 and VCAM-1.103 Previously, high shear stress had been found to decrease PAR-1 expression in human aortic smooth muscle cells (HASMCs).161 The downregulation of PAR-1 expression by high shear stress in HUVECs, HMECs, and HASMCs suggests that these cell types may share common signal transduction mechanisms in PAR-1 gene regulation.

PAR-1 mediates thrombin-stimulated processes in ECs including releases of NO and
ET-1. Thrombin exposure increased ET-1 production in a time-dependent manner and also increased transient NO release in ECs. In this study, exposure of cells to high shear stress for 24 hours significantly decreased both thrombin-induced release of ET-1 in HUVECs and HMECs (Fig. 2.7) and of NO in HUVECs (Fig. 2.6A). The decrease in thrombin-induced responses from shear stressed HUVECs or HMECs indicates that shear stress also reduces the expression of functional PAR-1 in HUVECs and HMECs. This may lead to the decrease in thrombogenic effects of thrombin on vascular cells and to the decrease in vascular permeability changes associated with thrombin exposure.

Although high shear stress had similar effects on the downregulation of PAR-1 expression in both HUVECs and HMECs, marked differences in NO formation in response to thrombin were observed in these cell types. Thrombin released a high amount of NO in HUVECs (76.9±4.7 nmol/10⁶ cells), but it stimulated very little NO release in HMECs (6.3±1.3 nmol/10⁶ cells), even though HUVECs expressed less surface PAR-1 than HMECs (Fig. 2.4). This small release of NO in thrombin-stimulated HMECs cannot be attributed to the inability of HMECs to synthesize NO. Human dermal microvascular endothelial cells do express nitric oxide synthases and release NO in response to other stimuli, including vasodilator neuropeptides. Apparently, the biochemical mechanisms in HMECs for the formation of NO are not responding to thrombin. HMECs may alternatively produce other factors than NO in response to thrombin. Endothelium-derived hyperpolarizing factor (EDHF), not NO, has been found
to control microvascular tone, and the importance of EDHF increases as the vessel size decreases.226

Besides the difference in the thrombin-induced NO production between these cell types, the different expression of PAR-1 in response to a moderate shear stress was also observed. Moderate shear stress (15 dyn/cm²) resulted in a time-dependent decrease of PAR-1 mRNA in HMECs, but not in HUVECs. The physiological significance of this difference may be related to the different amount of PAR-1 expressed on these cell types. Microvascular ECs express more thrombin receptors than macrovascular ECs, and the mitogenic activity of thrombin has been related to the number of binding sites on the endothelial surface.218,227 Microvascular ECs have been reported to respond to thrombin differently than do endothelial cells from large vessels such as HUVECs. Thrombin induces prostacyclin production and IP₃ formation in HUVECs, but not in microvascular ECs, whereas it stimulates proliferation in microvascular ECs but not in ECs from large veins or arteries.228,229

Although many effects of shear stress on ECs have been reported, the mechanisms by which ECs sense mechanical stimuli and alter gene regulation are not well understood. Mechanical stimuli have been shown to alter gene expression by producing autocrine factors or by regulating a cascade of protein kinases. Shear stress reduction of PAR-1 expression in this study is unlikely to be the result of autocrine action of any endothelium-secreted factors. PAR-1 mRNA was not significantly different between cells fed with either fresh medium or conditioned medium from static or sheared
HUVECs. whereas PAR-1 mRNA expression was increased in HMECs fed with conditioned medium from sheared HMECs. Shear stress apparently stimulates intracellular signaling pathways, leading to a dramatic inhibition of PAR-1 expression, and these pathways do not appear to depend on autocrine factors.

In addition to autocrine factors, protein kinases can also be involved in gene regulation by shear stress in ECs. Shear stress activates protein kinases in ECs including PKC, TK, and MAPK, and protein kinases have been previously involved in shear stress-regulated endothelin-1, monocyte chemotactic protein-1, and PDGF expression. These findings, along with the observation that activators of PKC and AC mediate the loss of PAR-1, suggest the involvement of protein kinases in shear stress-reduced PAR-1 expression. The results from this study demonstrate that shear stress-reduced PAR-1 expression is mediated, at least in part, through the AGC kinase group in both HUVECs and HMECs (Fig. 2.8). Protein kinase C is required for both EC types. However, shear stress-downregulated PAR-1 expression involves MEK in HMECs (Fig. 2.8B), but not in HUVECs (Fig. 2.8A). Despite numerous instances suggesting a role for tyrosine kinases in the intracellular signaling pathways activated by shear stress, shear stress-reduced PAR-1 expression was not significantly altered by the tyrosine kinase inhibitor Herbimycin A (data not shown) and therefore does not appear to involve tyrosine kinase activation.

In summary, the results from this study indicate that arterial shear stress can reduce endothelial cell surface PAR-1 expression and decrease EC responsiveness to thrombin in
both macro- and microvascular endothelial cells. This process is mediated through a mechanism that is dependent on protein kinases, but not on either tyrosine kinases or autocrine factors. In addition, the observation that macro- and microvascular ECs had different stress thresholds and responded differently to thrombin stimulation of NO production after stress exposure further emphasizes the need to study the endothelial cells derived from the vascular bed of interest rather than to extrapolate the results obtained from HUVECs or other macrovessel-derived endothelium.
CHAPTER 3

Effects of shear stress on PAR-1 expression in human vascular smooth muscle cells

3.1 Introduction

Although previous work in our group demonstrated that arterial shear stress reduced PAR-1 expression in VSMCs, the mechanisms of this process had not been studied. This work further investigated whether shear stress-reduced PAR-1 expression was due to either the transcription mechanism or mRNA stability. The subsequent abilities of sheared cells to alter Ca\(^{2+}\) mobilization and proliferation in response to thrombin were also examined. We found that the reduction of PAR-1 expression by shear stress was due to the transcription mechanism. In addition, shear stress-decreased PAR-1 expression resulted in functional consequences of VSMCs in response to thrombin, including reduction in Ca\(^{2+}\) mobilization and cell proliferation.

3.2 Procedures

To study the effects of shear stress on PAR-1 expression in VSMCs, cells were seeded on glass slides and exposed to shear stress using the parallel plate flow chamber system (Fig. 2.1). The mRNA stability experiments were used to study the mechanism of shear stress-reduced PAR-1 mRNA levels, which were quantified using Northern blot analysis. Furthermore, functional studies such as Ca\(^{2+}\) measurements and mitogenic assays were
conducted to investigate thrombin-induced events including Ca\(^{2+}\) mobilization and cell proliferation in sheared cells.

**Cell Culture and Shear Stress Experiments**

Human aortic smooth muscle cells (HASMCs) were obtained from the abdominal aorta of a 9-year-old patient or purchased from Cascade. Cells were cultured in DMEM supplemented with 20% heat-inactivated FBS (Hyclone). 2 mmol/L L-glutamine. 200 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL). HASMCs used at passages P3 to P10 were seeded on glass slides (75x38 mm, Fisher) coated with 1 µg/cm\(^2\) human plasma fibronectin (Collaborative). Upon reaching confluence, cells were either maintained in static conditions or exposed to shear stress (25 dyn/cm\(^2\)) using the parallel plate chamber system. Shear stress experiments were performed at determined time points in a 37\(^{0}\)C humidified room.

**mRNA Stability Experiments**

To estimate the half-life of mRNA, actinomycin D was used to inhibit RNA transcription.\(^{232,233}\) After HASMCs were maintained under static conditions or exposed to 25 dyn/cm\(^2\) for 12 hours, they were exposed to 4 µg/mL actinomycin-D for 0, 2, 4, or 6 hours. Total RNA was isolated, and PAR-1 mRNA levels were quantified using Northern blot analysis as described in chapter 2. The degradation rate of PAR-1 mRNA was then determined over indicated time points.
**Ca\textsuperscript{2+} Measurements**

HASMCs were seeded on glass coverslips (24x50 mm. No. 1 thickness. Sigma) coated with 2 µg/cm\textsuperscript{2} human plasma fibronectin. After reaching confluence, cells were either exposed to 25 dyn/cm\textsuperscript{2} for 24 hours or maintained under stationary conditions. HASMCs were then washed with HBSS (Gibco), loaded with Fura-2 AM (Molecular Probes: 1 µmol/L final concentration in HBSS) and Pluronic (Gibco: 0.1% of final volume), and incubated for 30 minutes at 37\textdegree C. After this incubation period, cells were washed twice with HBSS and incubated for another 30 minutes to allow the hydrolyzation of Fura-2 AM to its nonpermeable fluorescent form, Fura-2. Fura-2 measurements were conducted using the fluorescence ratio imaging system (Fig. 3.1) as described previously.\textsuperscript{234} In brief, two images were acquired for each time point at two different excitation wavelengths (340 and 380 nm) with the time interval of 20 second each and the range from 0 to 400 seconds. The background was subtracted, and the ratio (340nm/380nm) value corresponding to the intracellular change in Ca\textsuperscript{2+} was calculated for each time point. Thrombin (5 U/mL) was added to both control and sheared cells after 100 seconds of the imaging process.

**Mitogenic Assays**

After HASMCs were exposed to 25 dyn/cm\textsuperscript{2} for 24 hours or maintained under static conditions, the cells were washed and incubated in DMEM with or without thrombin (5 U/mL) for 24 hours. After the thrombin treatment period, the cell number for each sample was measured by a Coulter Counter.
Figure 3.1. Schematic of the fluorescence ratio imaging system.
In separate experiments, static or sheared cells were labeled with 2 μCi/mL [³H]thymidine in DMEM in the presence or absence of thrombin (5 U/mL) for 24 hours. Cells were harvested after incubation, and the relative [³H]thymidine incorporation was determined using a liquid scintillation counter (Packard).

**Statistical Analysis**

Results are expressed as mean±SEM. When data from more than two groups were compared, a 2-way ANOVA was used following by the Fisher least significant difference post-hoc test. A 2-tailed Student t test for paired samples was used to find significance between treatment and control groups. Differences were considered significant when P<0.05. All calculations were performed using SuperANOVA (Macintosh).

### 3.3 Results

**Effects of shear stress on PAR-1 mRNA stability**

To determine whether the reduction of PAR-1 mRNA by shear stress was due to the transcriptional mechanism or to mRNA stability, actinomycin D was used to study the degradation rate of PAR-1 mRNA in static and sheared cells. Normalized PAR-1 mRNA levels in both static and sheared cells gradually declined over time after actinomycin-D treatment (Fig. 3.2). The half-life of PAR-1 mRNA was 4.8±0.7 hours for static HASMCs and 4.4±0.8 hours for cells exposed to shear stress, thus indicating that shear stress-reduced PAR-1 was not due to PAR-1 mRNA stability.
Figure 3.2. **Effects of shear stress on PAR-1 mRNA stability.** Static or shear-stressed HASMCs (25 dyn/cm² for 12 hours) were incubated with 4 mg/mL actinomycin-D for 0, 2, 4, and 6 hours. The PAR-1 mRNA levels were quantified by Northern blot analysis and normalized to GAPDH mRNA for differences in gel loading and transfer. The results are presented as the percentage of the PAR-1 presented at the time of actinomycin-D addition (n=4). The PAR-1 mRNA half-life was 4.8±0.7 hours for stationary control HASMCs whereas it was 4.4±0.8 hours for sheared HASMCs.
**Effects of shear stress on Ca\(^{2+}\) mobilization in response to thrombin**

To investigate the functional consequences of shear stress-reduced PAR-1 expression, we investigated Ca\(^{2+}\) mobilization in response to thrombin in both static and sheared cells. The chosen thrombin concentration (5 U/ml) has been shown to induce maximal increases in Ca\(^{2+}\) mobilization and mitogenic responses.\(^{211,235}\) Thrombin rapidly induced increases in Ca\(^{2+}\) in both control and sheared HASMCs, reaching a maximum peak within 50 to 100 seconds after addition and returning to basal levels after 200 seconds (Fig. 3.3). In addition, exposing cells to shear stress (25 dyn/cm\(^2\) for 24 hours) resulted in a significant decrease in Ca\(^{2+}\) mobilization in response to thrombin compared to those of static controls (P<0.02 at 160 and 180-second time points).

**Effects of shear stress on cell proliferation in response to thrombin**

Cell proliferation and \(^{3}H\)thymidine incorporation were used to assess the thrombin-induced proliferation of sheared cells. Stimulation with thrombin significantly increased cell proliferation in both static and sheared HASMCs (Fig. 3.4A). In addition, thrombin-induced proliferation was slightly decreased in cells exposed to shear stress compared to those of static controls. Similarly, thrombin-induced \(^{3}H\)thymidine in sheared cells was lower than those of static controls (Fig 3.4B). Without thrombin treatment, cell numbers were not significantly different between sheared and static cells, indicating shear stress-inhibited growth\(^{158,159}\) as a reversible process.
Figure 3.3. **Shear stress attenuated Ca\(^{2+}\) mobilization in response to thrombin in HASMCs.** Cells were exposed to 25 dyn/cm\(^2\) for 24 hours, and thrombin (5 U/ml) was added. Ca\(^{2+}\) release by thrombin in sheared cells was compared to static control cells. Data are presented as the average ratio of Fura-2 (340 nm/380 nm)\(_{t=0}\) at each time point after adding thrombin to Fura-2 at time zero (340 nm/380 nm)\(_{t=0}\), numbers of cells n>20. The asterisks show the significant differences between static and sheared cultures (P<0.05).
Figure 3.4. Shear stress inhibited HASMC proliferation in response to thrombin. HASMCs were exposed to 25 dyn/cm² for 24 hours and then incubated with or without thrombin (5 U/mL). Cell numbers (A) and [3H]thymidine incorporation (B) were compared to static cells. Results are shown as mean±SEM (n=4). The asterisks show the differences respective to unstimulated controls, whereas † stands for differences respective to thrombin-stimulated sheared cultures (P<0.05).
3.4 Discussion

Shear stress regulates gene expression in VSMCs, leading to alterations in VSMC functions and proliferation. Shear stress inhibits proliferation in VSMCs by autocrine induction of TGF-β1.\textsuperscript{158} It also induces production of vasoactive substances such as NO,\textsuperscript{162} tPA,\textsuperscript{158,161} HO-1,\textsuperscript{166} and prostaglandins.\textsuperscript{163} These vasoactive substances including NO and prostaglandins are known to inhibit VSMC proliferation, and thus the induction of these vasoactive substances would promote the anti-proliferative process of high shear stress on the vessel wall. In addition, high shear stresses downregulate PAR-1 expression, whereas low shear stresses transiently upregulate PAR-1 expression.\textsuperscript{161} This observation is consistent with the fact that lesions often occur at regions of low shear stresses whereas areas of high shear stress are free of deposits.

Shear stress regulates gene expression in vascular cells through various mechanisms including altering mRNA stability. For examples, shear stress modulates the granulocyte-macrophage colony-stimulating factors in ECs through increasing mRNA stability. Although shear stress has been shown to affect gene regulation in VSMCs, the mechanisms of this regulation are unknown. In particular, shear stress downregulates PAR-1 expression in HASMCs, but the mechanism that reduces PAR-1 gene expression is not known. The decrease of PAR-1 expression by shear stress on VSMCs could be due to either the transcription mechanism or mRNA stability. Shear stress did not show any significant effects on the stability of PAR-1 expression (Fig. 3.2), thus indicating that the reduction of PAR-1 by shear stress is due to the transcription mechanism.
Shear stress has been shown to regulate gene expression in vascular cells, leading to alterations in vascular functions. The findings reported here demonstrate that changes in PAR-1 expression by shear stress resulted in the alterations of VSMC functions in response to thrombin. PAR-1 mediates many thrombin-stimulated events in VSMCs including cell proliferation and Ca\(^{2+}\) mobilization.\(^{211,235}\) Therefore, these thrombin-induced processes were used to investigate the physiological consequence of shear stress-reduced PAR-1 expression. Exposure of HASMCs to high shear stress (25 dyn/cm\(^2\)) for 24 hours significantly decreased Ca\(^{2+}\) mobilization and slightly reduced cell proliferation and DNA synthesis in response to thrombin. These findings suggest that arterial shear stresses cause the reduction of PAR-1 expression in HASMCs, leading to the attenuation of thrombin-stimulated responses, including Ca\(^{2+}\) mobilization and cell proliferation.

In conclusion, our results indicate that arterial shear stress reduces PAR-1 gene expression through the transcriptional mechanism and that the reduction of PAR-1 expression by shear stress subsequently results in the decreases in VSMC responsiveness to thrombin including cell proliferation and Ca\(^{2+}\) mobilization. Because high expression of PAR-1 in vascular lesions indicates its involvement in the development of atherosclerosis, understanding the regulation of PAR-1 expression by stimuli such as shear stress in VSMCs may lead to provide future directions for the development of therapeutic approaches to cure these diseases.
CHAPTER 4

Effects of cyclic strain on PAR-1 expression in vascular smooth muscle cells

4.1. Introduction

This project investigates the effects of cyclic strain on PAR-1 expression in HASMCs and the possible signal pathways involved in this process. PAR-1 expression is increased in atherosclerotic and balloon-injured arteries and in arteries of hypertensive animals\textsuperscript{208,236,237}. In addition, PAR-1 antisense sequences inhibit proliferation of many cell types including VSMCs in response to thrombin\textsuperscript{238,239}. These findings indicate the important role of PAR-1 in the development of arterial diseases; therefore, it is important to investigate regulation of PAR-1 by cyclic strain in HASMCs.

In the current study, cyclic strain (20\% strain) was found to induce PAR-1 expression in a time-dependent manner, leading to alterations in thrombin-induced cell proliferation. In addition, cyclic strain-enhanced PAR-1 expression appeared to be mediated by ROS and PKC pathways, but not by NO, TK, or MAPK pathways. Since growth factors such as bFGF and PDGF-AB have been shown to induce PAR-1 expression\textsuperscript{208,240}, the potential additive effects of two major stimulus types, growth factors and cyclic strain, on PAR-1 expression was further investigated. Using combinations of bFGF or PDGF-AB with cyclic strain, bFGF and cyclic strain acted in an additive manner to increase PAR-1
expression. whereas PDGF-AB and cyclic strain. when applied to cells concomitantly.
had no additional effects on PAR-1 expression.

4.2. Procedure

HASMCs were seeded on the membrane of the uni-axial cyclic strain system and
exposed to different levels of cyclic strain as described in the cell culture section. After
the experiments. PAR-1 mRNA was quantified using Northern blot analysis. whereas
PAR-1 protein was detected using the flow cytometry technique (as described in chapter
2). Cell proliferation in response to thrombin was also used to assess the functional
surface PAR-1 expression. To elucidate whether the ROS pathways were involved in the
cyclic strain process. ROS production by cyclic strain was measured. and PAR-1 mRNA
levels in cells after the treatments with antioxidants or various ROS inhibitors were
quantified. In addition. inhibitors of various pathways were used to investigate the
involvement of other pathways including NO. TK. PKC. and MAPKs. Finally. the
additive effects of cyclic strain and growth factors was investigated by measuring
changes in PAR-1 mRNA levels when cyclic strain and growth factors applied to cells
concomitantly.

Uni-axial cyclic strain system

The uni-axial cyclic strain system\textsuperscript{241} was used to produce different levels of cyclic strain
on HASMCs. In brief. the strain chamber unit. which was made of polycarbonate. had
two identical wells: cyclic strain and motion control. For cyclic strain. elastic silicone
membranes (0.005 in. thick. Specialty Manufacturing) were sandwiched between stainless steel plates. which were fastened to the rear of the strain chamber and connected to stretch bars at the front of the unit (Fig. 4.1A). For motion control, the membrane was fixed into the metal frame, so that fluid agitation was produced without cyclic strain (Fig. 1B). The stretch bars were set into the stroke whose displacement was regulated through a fixed pin and a crank pin mounted atop a rotating cylinder. Placement of the adjustable offset pin on the arm of the stroke determined levels of cyclic strain. Displacement frequency was driven by a direct current (DC) motor (Fig. 4.1B).

Cell culture
HASMCs (Cascade), passages P5-P10, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Hyclone). 200 μg/ml L-glutamine. 200 U/ml penicillin and 100 μg/ml streptomycin (cDMEM). HASMCs were subcultured using 0.05% trypsin-EDTA, and seeded at 3.0×10⁴ cells/cm² on silicone membranes that were coated with 5 μg/cm² human plasma fibronectin (Collaborative). Cells were grown to confluence and were then either maintained under static conditions, cyclically strained, or exposed to fluid agitation (motion control) in humidified 95% air/5% CO₂ at 37⁰C. Experiments were conducted at 5, 10, or 20% cyclic strain at 1 Hz for 3, 6, 12, and 24 hours.
Figure 4.1. Schematic of the uni-axial cyclic strain system. A. Side view of the system showing strain chamber unit connected to the drive unit. B. Overhead view showing strain chamber, which has two identical wells: cyclic strain and motion control, and the drive unit, which contains motor gear assembly and stroke. Placement of adjustable offset pin on the stroke determines level of cyclic strain exerted on the elastic membranes. Frequency is regulated by controlling current to direct current (DC) motor.
Cell proliferation in response to thrombin

To test the effect of cyclic strain on the subsequent ability of cells to proliferate in response to thrombin, HASMCs were maintained under static conditions or exposed to 20% strain (1 Hz) in cDMEM for 24 or 48 hours, washed, and equilibrated with serum-free DMEM media for 30 minutes. Cells were then incubated in DMEM with or without thrombin (5 U/ml) for 24 hours under static conditions, and cell number was determined by an electronic counter (Coulter Electronics).

Measurement of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) production

Superoxide production was measured using the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c as previously described. In brief, HASMCs were preincubated in DMEM without phenol red for 30 minutes at 37°C, and then cytochrome c (final concentration, 1 mg/mL) with or without SOD (final concentration, 500 U/mL) was added. Cells were then exposed to 20% strain or maintained under static conditions. Medium was collected at 0, 20, 40, and 60 minutes, and the absorbance was read at 550 nm. The equivalent O$_2^-$ production was estimated by converting the optical density difference between comparable samples with or without SOD using the molar extinction coefficient for cytochrome c [21.0x10$^3$ (mol/L)$^{-1}$ cm$^{-1}$]. In separate experiments, cells were exposed to 20% strain or maintained under static conditions in DMEM without phenol red, and media were collected at 0, 20, 40, and 60 minutes. Hydrogen peroxide production was measured using the H$_2$O$_2$ assay (OXIS), following the manufacturer’s instructions.
Roles of ROS and NO

To study the role of ROS in strain-induced PAR-1 expression, HASMCs were pretreated with one of two antioxidants: N-acetyl-L-cysteine (NAC, 20 mmol/L) or pyrrolidinedithiocarbamate (PDTC, 50 μmol/L). In addition, the possible specific ROS pathways involved in strain-increased PAR-1 expression were investigated using diphenyleneiodoniumchloride (DPI, 10 μmol/L), an NADPH oxidase inhibitor; indomethacin (10 μmol/L), a cyclooxygenase inhibitor; and oxypurinol (10 μmol/L), a xanthine oxidase inhibitor. We further examined the contribution of NO in strain-upregulated PAR-1 using N\textsuperscript{G}-Methyl-L-Arginine (L-NMMA). Since DMEM contains L-arginine, we chose a high concentration of L-NMMA inhibitor (1 mmol/L). Cells were pretreated with each inhibitor for 1 hour before the application of 20% strain in the presence of the same inhibitor for 12 hours. Concentrations of each inhibitor were chosen above the IC\textsubscript{50} from manufacturer’s information and were not toxic to cells.

Role of protein kinases (PK)

To determine the roles of PK, MAPK, and TK in strain-induced PAR-1 expression, we used the non-specific protein kinase inhibitor staurosporine (10 nmol/L). TK inhibitor Herbimycin A (2.0 μmol/L), MAPK kinase (MEK) inhibitor PD 98059 (50 μmol/L), and PKC inhibitor Ro 31-8220 (0.1 μmol/L).\textsuperscript{242} After treatment with each inhibitor for 1 hour, cells were exposed to 20% strain for 12 hours in the presence of the same inhibitor. In separate experiments, HASMCs were treated with PKC activator phorbol myristate acetate (PMA, 0.3 μmol/L) for 12 hours.
Effects of cyclic strain and growth factors on PAR-1 expression

HASMCs were seeded on membranes and incubated in DMEM supplemented with 20% FBS to allow for cell adhesion and spreading. Serum containing medium was replaced with serum-free DMEM 24 hours before the experiments to minimize residual growth factors. Cells were then maintained under static conditions, stimulated with either cyclic strain alone (20% strain at 1 Hz), growth factors (R & D systems) alone: bFGF (15 ng/ml) or PDGF-AB (20 ng/ml), or a combination of cyclic strain with either one of growth factors for 12 hours.

4.3. Results

Cyclic strain increased PAR-1 mRNA and protein in HASMCs

Northern blot analysis showed that high cyclic strain (20% strain) produced about 2-fold increase in PAR-1 mRNA after 6 hours of exposure (Fig. 4.2A. P<0.05), whereas low and moderate strain (5% and 10% strain, respectively) did not induce PAR-1 mRNA significantly compared to static controls (Fig. 4.2B). Fluid motion induced by the strain clamp movement corresponding to 5, 10, and 20% strain did not change PAR-1 mRNA significantly compared to static controls. Cyclic strain (20% strain) also increased cell surface PAR-1 protein as shown by flow cytometry (Fig. 4.3) in addition to inducing PAR-1 mRNA. PAR-1 surface expression in HASMCs after exposure to 20% strain for 24 hours increased 150% above that seen in static cells (20.3±2.6 fluorescence units for 20% strain versus 7.1±0.3 for static controls, n=3-5, P<0.05).
Figure 4.2. PAR-1 mRNA levels in HASMCs subjected to cyclic strain. mRNA expression of PAR-1 and GAPDH was determined by Northern blot analysis. A. An autoradiogram representative of 3-5 experiments, top lane represents PAR-1 mRNA and bottom lane displays the corresponding GAPDH of static control (C) at 24 hours and 20% strain for 3, 6, 12, and 24 hours. B. Time course of mean PAR-1 mRNA band densities normalized for loading variation with GAPDH at different levels of cyclic strain: 5, 10, and 20%. Results are expressed relative to static controls as mean ± SEM. Significantly different from: * static control (P<0.05).
Figure 4.3. Flow cytometry of PAR-1 protein on the cell surface in static and strained (20%) HASMCs. PAR-1 level was detected with PE-labeled PAR-1 antibody and the results analyzed by a flow cytometer. On the same plot, the histogram curve for nonspecific antibodies (PE-labeled mouse IgG antibodies) is shown as a negative control. Each curve is based on 10,000 cells.
HASMC proliferation in response to thrombin

The proliferation of static and cyclic strained HASMCs in response to thrombin was measured to investigate the functional consequences of cyclic strain-induced PAR-1 expression. Thrombin (5U/ml) increased cell proliferation in HASMCs exposed to 20% strain for 48 hours (P<0.05), but not for 24 hours compared to static control cells (Fig. 4.4). Without thrombin stimulation, cell proliferation was not significantly different between cyclic-strained and static cells in either time period. The later response to thrombin may be due to the use of serum-contained media in these cyclic strain experiments.

Role of ROS and NO in strain-induced PAR-1 expression

To study the role of ROS in strain-induced PAR-1 expression, the production of ROS by cyclic strain and the changes in cyclic strain-enhanced PAR-1 expression when treated with ROS inhibitors including antioxidants were quantified. Cyclic strain (20%) enhanced $O_2^-$ production in a time-dependent manner, reaching the maximum after 60 minutes of exposure. In contrast to $O_2^-$, the production of $H_2O_2$ was not changed by 20% strain (Fig. 4.5). These results suggest that cyclic strain-stimulated ROS production may be involved in strain-induced PAR-1 expression. To test this hypothesis, we used two different antioxidants and inhibitors of various ROS enzymatic pathways. Two antioxidants, NAC and PDTC, significantly inhibited strain-enhanced PAR-1 mRNA (Fig. 4.6. P<0.05), whereas neither NAC nor PDTC showed significant effects on static HASMCs (data not shown). Furthermore, DPI, an NADPH oxidase inhibitor, significantly reduced strain-increased PAR-1 mRNA, whereas inhibitors for other
Figure 4.4. Thrombin-stimulated cell proliferation in static and strained HASMCs. Cells were exposed to 20% strain for 24 or 48 hours or maintained under static conditions. The cell number was counted for each sample after the incubation period of cells in DMEM with or without thrombin (5 U/ml) for 24 hours. The cell number for each sample was normalized to those of the corresponding thrombin-untreated static cells. Results are shown as mean ± SEM (n=3-4). * indicates significant differences between cells treated with and without thrombin, whereas † indicates significant differences from thrombin-treated static samples (P<0.05).
Figure 4.5. Cyclic strain induced superoxide production in HASMCs. HASMCs were exposed to 20% strain or maintained under static conditions. Superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) production were measured as described in procedures and normalized to those of static controls. Squares are for O$_2^-$, and diamonds are for H$_2$O$_2$. Results are shown as mean ± SEM (n=3-4). * indicates significant differences between static and strained cells (P<0.05).
Figure 4.6. Effects of ROS and NO inhibitors on cyclic strain-induced PAR-1 expression. Cells were pretreated with antioxidants (NAC or PDTC): NADPH oxidase inhibitor. DPI: cyclooxygenase inhibitor, indomethacin (INDO): xanthine oxidase inhibitor, oxypurinol (OXY): or NOS inhibitor. L-NMMA (LMMA): for one hour, followed by exposure to 20% strain for 12 hours in the presence of the same inhibitor (see procedures). Results of densitometry of PAR-1 mRNA normalized to corresponding GAPDH mRNA are shown (n=3-4). * indicates significant differences from static controls and † indicates significant differences from strained samples.
enzymatic pathways of ROS such as oxypurinol for xanthine oxidase and indomethacin for cyclooxygenase did not have significant effects (Fig. 4.6). NO may also play a role in cyclic strain-mediated gene expression in addition to ROS. To investigate the role of NO in cyclic strain-induced PAR-1 expression, we used L-NMMA as a competitive substrate, which occupies the substrate binding site of nitric oxide synthase (NOS) to inhibit NO production. L-NMMA showed no significant effect on the increase of PAR-1 by cyclic strain, indicating that NO was not involved in strain-induced PAR-1 expression.

Role of protein kinases in strain-induced PAR-1 expression

Inhibitors of various protein kinase pathways were used to investigate the involvement of protein kinase pathways in strain-induced PAR-1 expression in addition to ROS pathways. Strain-increased PAR-1 mRNA was enhanced about 2-fold over those seen in cyclic strain by the non-specific protein kinase inhibitor staurosporine and the PKC inhibitor Ro 31-8220 (Fig. 4.7, P<0.05). However, incubation of HASMCs with the PKC activator PMA did not alter PAR-1 mRNA (data not shown). In contrast to PKC inhibitors, neither tyrosine kinase inhibitor Herbimycin A nor MEK inhibitor PD98095 had significant effects on strain-induced PAR-1 expression (Fig. 4.7). None of the inhibitors used had significant effects on PAR-1 mRNA in static cells (data not shown).

Effects of cyclic strain and growth factors on PAR-1 expression

To investigate whether cyclic strain and growth factors have an additive effect on PAR-1 expression, growth factors, bFGF or PDGF-AB, were added either alone or together with
Figure 4.7. Effects of various protein kinase inhibitors on cyclic strain-increased PAR-1 expression. Cells were pre-treated with a non-specific protein kinase inhibitor, Staurosporine (STAU); tyrosine kinase inhibitor, Herbimycin A (HA); MEK inhibitor, PD98059 (PD); or PKC inhibitor, Ro 31-8220 (RO); for one hour, followed by exposure to 20% strain for 12 hours in the presence of the same inhibitor (see procedures). Results of densitometry of PAR-1 mRNA normalized to corresponding GAPDH mRNA are shown (n=3-4). * indicates significant differences from static controls and § indicates significant differences from strained samples (P>0.05).
cyclic strain in HASMCs. Treatment of HASMCs with 20% strain, bFGF, or PDGF-AB alone caused a marked increase in PAR-1 mRNA compared to those without treatment (Fig. 4.8, P<0.05). Cyclic strain (20%) of cells in the presence of bFGF elicited a 4-fold increase in PAR-1 expression compared to non-bFGF static cells. Exposure of cells to 20% strain and bFGF induced PAR-1 mRNA significantly more than 2-fold compared with those of 20% strain or bFGF alone (Fig. 4.8, P<0.05), thus indicating an additive effect of cyclic strain and bFGF on PAR-1 expression. Unlike bFGF, treatment of cells with 20% strain and PDGF-AB did not significantly increase PAR-1 mRNA compared to those exposed to 20% strain or PDGF-AB alone.

4.4 Discussion

The focus of this work was to investigate the effects of cyclic strain on PAR-1 expression in HASMCs due to the following reasons. Mechanical strain on the vessel wall is increased up to 30% in hypertension and it is postulated that cyclic strain plays a role in vascular injury. In addition, thrombin is concentrated in vivo at sites of vascular injury, and the effects of thrombin are mediated chiefly through PAR-1,204,244 which is rapidly increased after vascular injury, both experimentally and clinically.207,208 Also, there is increased PAR-1 expression in the arteries of hypertensive rats. These findings suggest that cyclic strain administered to VSMCs in vitro would lead to increased PAR-1 expression.
Figure 4.8. **Stimulation of PAR-1 mRNA expression in HASMCs by cyclic strain and bFGF.** Cells were untreated, stimulated with either 20% strain or growth factor alone (bFGF or PDGF-AB), or a combination of 20% strain with one of the growth factors for 12 hours as described in procedures. Results of densitometry of PAR-1 mRNA normalized to corresponding GAPDH mRNA are shown (n=3-4). * indicates significant differences from static controls (C) and † indicates significant differences from static samples treated with the corresponding growth factor or from strained samples.
This study demonstrates four important findings: (1) 20% cyclic strain induces PAR-1 expression, leading to changes in thrombin-induced cell proliferation. (2) ROS but not NO production appears to mediate strain-induced PAR-1 expression, possibly through NADPH oxidase. (3) PKC pathway may provide a negative regulation (feedback) for strain-induced PAR-1 expression, whereas neither TK nor MAPK pathways appear to be involved. and (4) cyclic strain and bFGF elicit synergistic stimulation of PAR-1 gene expression.

Cyclic strain has been implicated as a major regulator of gene expression in VSMCs, leading to altering VSMC functions and proliferation. In this study, cyclic strain not only induced PAR-1 mRNA and protein levels (Figs. 4.2 and 4.3), but also increased the functional activity of PAR-1, as assessed by cell proliferation in response to thrombin (Fig. 4.4). These results support the hypothesis that elevated cyclic strain promotes vascular injury by inducing expression of vasoactive genes including PAR-1 to enhance VSMC proliferation. In addition to the findings herein, other studies have shown these proliferative effects of cyclic strain in vitro. For example, cyclic strain increases VSMC proliferation via the autocrine production of PDGF, both Ang II and PDGF, IGF-1, or EGFR. In addition, it enhances expression of many vasoactive genes including PDGF-A and –B mRNA and protein; platelet-activating factor receptor; insulin-like growth factor 1; MCP-1; VEGF, cyclooxygenase-1, and tenascin-C. The increases of many genes including PAR-1 by cyclic strain in VSMCs may lead to the occurrence of intimal hyperplasia and medial thickening in injured and hypertensive arteries.
Although many effects of cyclic strain on gene regulation in VSMCs have been reported, little is known about the mechanisms by which mechanical deformation leads to an altered gene expression in VSMCs. Cyclic strain has been shown to stimulate MCP-1 in VSMCs through a TK-dependent pathway. In addition, MAPK pathway mediates strain-increased mitogen-activated protein kinase phosphatase-1 expression. Rho kinase is also involved in strain-induced extracellular signal-regulated kinase. Cyclic strain increases activation of PKC and MAPK as well as production of ROS in VSMCs, thus suggesting that these signal elements may be involved in cyclic strain-regulated gene expression in VSMCs.

The hypothesis that ROS mediates cyclic strain-induced PAR-1 expression in VSMCs is supported by many findings. The observations that PAR-1 promoter regions contain many antioxidant-response element-like consensus sequences and that cyclic strain rapidly increases superoxide production in VSMCs suggest an important role of oxidant-mediated mechanisms in regulating PAR-1 expression in VSMCs. In this study, cyclic strain stimulated superoxide production in HASMCs after short exposure (Fig. 4.5), similar to previous work. In addition, antioxidants, NAC or PDTC, significantly inhibited strain-induced PAR-1 expression (Fig. 4.6). Furthermore, the inhibitor DPI (of NADPH oxidase, an enzymatic pathway to produce superoxide) diminished strain-enhanced PAR-1 expression (Fig. 4.6). These findings clearly indicate that cyclic strain-induced PAR-1 expression is mediated by ROS in HASMCs, possibly through the
NADH/NADPH oxidase pathway. Cyclic strain has also been found to induce an oxidant stress in ECs by the potential source. NADH/NADPH oxidase.\textsuperscript{150} Similar to those seen in VSMCs findings herein, involvement of ROS production in cyclic strain-induced expression of some oxidant-sensitive genes such as MCP-1,\textsuperscript{129} PAI-1,\textsuperscript{131} and ICAM-1\textsuperscript{130} has been shown in ECs. In contrast to ROS, NO was not involved in cyclic strain-induced PAR-1 expression in this study since treatment of cells with NOS inhibitor did not show any effect.

Besides ROS, protein kinases and tyrosine kinases may also act as second messengers to alter gene expression by cyclic strain. Previous work has shown that both a PLC inhibitor 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate and a selective PKC inhibitor chelerythrine inhibited strain-stimulated \( \text{O}_2^- \) production in VSMCs.\textsuperscript{176} From this one would expect that blockage of PKC may inhibit cyclic strain-induced PAR-1 expression. In contrast, inhibition of PKC using either Ro 31-8220 or staurosporine enhanced the cyclic strain-mediated increase in PAR-1 mRNA in this study (Fig. 4.7), indicating that strain-induced PAR-1 mRNA in HASMCs may be negatively regulated by the PKC pathway. However, treatment of cells with PKC activator PMA did not have any effect on PAR-1 expression (data not shown). It is possible that PMA-insensitive isoforms of PKC are involved in the cyclic strain-induced PAR-1 expression since some PKC isoforms such as PKC-\( \xi \) do not bind diacylglycerol or phorbol esters.\textsuperscript{59,246} Unlike ROS inhibitors, PKC inhibitors enhanced cyclic strain-induced PAR-1 in this study, suggesting that several mechanisms take place in the regulation of PAR-1 expression by cyclic
strain. In support of our finding, it has been found that PKC negatively regulated angiotensin II-induced thrombin receptor expression in VSMCs.\textsuperscript{242}

In addition to protein kinases, tyrosine kinase and MAPK pathways have also been shown to mediate gene regulation by cyclic strain. However, despite evidence suggesting a role for tyrosine kinases and MAPK in the intracellular signaling pathways activated by cyclic strain,\textsuperscript{187,194} strain-induced PAR-1 expression was insensitive to Herbinycin A and PD98059 in this study (Fig. 4.7); therefore, the response does not appear to involve tyrosine kinase or MAPK pathways. MAPKs are activated by ROS in several cell types including VSMCs. They are also thought to play a pivotal role in transmitting transmembrane signals required for cell growth and differentiation.\textsuperscript{187} Perhaps tyrosine kinases and MAPKs, which may be insensitive to the inhibitors used, are involved in mediating strain-induced PAR-1 expression. In support of this possibility, a previous study shows that the MEK inhibitor PD98059 is not completely specific for the MAPK pathway despite its apparent high selectivity in inhibiting the MAPK pathway.\textsuperscript{247}

Other non-mechanical factors such as Ang II have demonstrated additive effects with mechanical stimuli in gene regulation in vascular cells. Because PAR-1 expression is induced by growth factors in addition to cyclic strain,\textsuperscript{244} 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 throughout intimal thickening. Aggregating platelets have been shown to induce thrombin receptor expression in
cultured VSMCs through the release of TGF-β1 and PDGF-AB.\textsuperscript{240} In vascular cells, the growth-promoting effects of hemodynamic forces increase the release of growth factors such as PDGF\textsuperscript{189} and bFGF.\textsuperscript{203} which also enhances the expression of PAR-1.\textsuperscript{208} In this study, cyclic strain and bFGF have an additive effect on PAR-1 expression (Fig. 4.8). The additive effect of cyclic strain with bFGF, but not PDGF-AB was found on PAR-1 expression suggests that cyclic strain and bFGF may cooperatively promote the extensive mitogenesis of VSMCs in response to thrombin. The mechanisms account for this additive effect remains to be elucidated.

VSMCs have been found to respond differently to various mechanical stimuli such as shear stress and cyclic strain. Shear stress has been shown to differently regulate VSMC functions and gene regulation compared to cyclic strain. For example, high shear stress decreases VSMC proliferation, whereas high cyclic strain increases cell proliferation.\textsuperscript{159} In addition, shear stress down-regulates PAR-1 expression in HASMCs.\textsuperscript{161} whereas cyclic strain up-regulates PAR-1 expression in this study. Similar, other differential responses are also observed in ECs including ET-1.\textsuperscript{20,25} Opposite responses of the same cell types to cyclic strain and shear stress suggest either that these mechanical factors are mediated by different signaling pathways, or that cyclic strain at the magnitudes used produce greater cell injury than does shear stress. Evidence for the second possibility can be found by comparing the work of Cheng, et al.\textsuperscript{203} to that of Rhoads, et al.\textsuperscript{164} Cyclic strain injured VSMC membranes as indicated by FITC-dextran release.\textsuperscript{203} whereas membrane perturbation caused by shear stress was sufficient to release bFGF, but not
FITC-dextran. On the other hand, some responses to shear stress and cyclic strain are the same (e.g. both induce activation of the PDGF receptor alpha in VSMCs and gene expression of hemeoxygenase-1). suggesting cross talk between signaling pathways for these specific genes.

In summary, we have demonstrated that cyclic strain induces expression of PAR-1, leading to the increase of VSMC proliferation in response to thrombin, a process that is mediated by ROS 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 stimuli such as cyclic strain may offer insight into potential therapeutic invention.
CHAPTER 5

Future Work

5.1. Effects of complex shear stresses on vascular cells

Recent studies show that many antithrombotic and prothrombotic responses of the vessel wall depend on the local flow conditions. In general, the endothelium is more actively antithrombotic and more fibrinolytic under physiological shear conditions (e.g. >15 dyn/cm²). Atherosclerotic lesions, however, tend to localize at curvatures, branch points, or regions of irregular geometry in the circulation. ECs in such regions become prothrombotic, leading to localized platelet adhesion and aggregation and smooth muscle cell migration and proliferation.

In these predilection sites of thrombosis and atherosclerosis, the local flow is slow, disturbed, or irregular. Low levels of fluid shear stresses (e.g. ±0-4 dyn/cm²), large gradients in fluid shear stress, oscillatory shear stress, and turbulent shear stress all become important. Studies of these stress responses in vascular cells not only add new insight to the pathogenesis of cardiovascular diseases, but also lead to the discovery of new genes for atherosclerosis and thus to increase the probability of finding new therapeutic targets. As shown in chapter 1 (introduction), many studies have been developed over the last decade to investigate the effects of laminar shear stress (especially the physiological ranges of shear stress from 4 to 25 dyn/cm²) on vascular cell functions and gene expression. The effects of low shear stress (0-4 dyn/cm²), large
gradients in fluid shear stress, and turbulent shear stress have not yet been fully investigated.

Recently, much evidence shows that oscillatory shear stress, but not steady laminar shear stress, has been implicated in the genesis of atherosclerosis. Temporal gradient in shear but not steady shear stress induces PDGF-A and MCP-1 expression in endothelial cells. Moreover, oscillatory shear stress causes a sustained activation of pro-oxidant processes (e.g., NADH oxidase activity and the redox-sensitive gene HO-1) whereas steady laminar shear stress initially activated these processes but also induced compensatory antioxidant defenses (e.g., increase of Cu/Zn superoxide dismutase, an antioxidant defense enzyme). In addition, other genes such as ET-1, gap junction protein connexin43, VCAM-1, ICAM-1, and E-selectin are induced more by disturbed flow than laminar steady shear stress. So are transcription factors such as NF-kB, Erg-1, c-Jun, and c-Fos. These observations indicate that spatial shear stress gradients represent important local modulators of endothelial gene expression at anatomic sites predisposed for atherosclerotic development. Oscillatory shear stress applied to the lining of blood vessels may cause endothelial cell injury, leading to the development of atherosclerosis.

Although a strong correlation exists between the location of developing arterial lesions and regions where flow is slow, disturbed, or separated, studies of effects of low shear stress (0-4 dyn/cm²) or complex patterns of shear stress have not yet attracted much attention due to the complexity of these flow systems. The effects of slow, disturbed, or
separated flow on vascular gene expression could be studied \textit{in vitro} by modifying the parallel flow system. In particular, one could selectively change the gap thickness of the flow system by varying the gasket thickness to create low shear stress. Moreover, the laminar flow system could be modified into the oscillatory flow system by inserting a motor-driven pump that generates longitudinal oscillatory motion. The oscillatory component allows pulsatility to be superimposed on the steady flow via a displacement of the medium back and forth above the cell surface. In addition, the disturbed flow could be studied using a vertical-step flow channel.\textsuperscript{254} Cells would be exposed to disturbed flow fields that model spatial variations in fluid shear stress found at arterial bifurcation. Detailed shear stress distributions and flow structures could be computed by using the finite volume method in general curvilinear coordinate system. It should be possible to isolate small numbers of endothelial cells within the disturbed or separated flow regions. Spatial localization of vascular cell gene expression in the context of hemodynamics is a promising approach.\textsuperscript{255}

To study transcription profiling of endothelial cells as a function of location, one will need to use the combination of computation modeling, molecular biology techniques, and microarray technology. The computation modeling will provide the spatial shear stress distributions and flow structures whereas molecular biology technique and microarray technology will give the expression levels of thousands of genes in vascular cells spatially localized. To obtain sufficient mRNA from a single cell or small groups of cells in spatially relevant locations, the amplified antisense mRNA techniques can be used to provide near-linear amplification of the original mRNA population of the cell.\textsuperscript{256-259}
The microarray technology could then be applied to screen many genes simultaneously to get quantitative profiles of these gene expressions in detailed results.

5.2. Effects of shear stress and cyclic strain on vascular cells

Vascular cells are continuously subjected to a wide combination of ranges of fluid shear stress and cyclical wall strain due to the pulsatile blood flow in vivo, dependent on their anatomic location. In animal studies, intimal thickening occurs at areas of disturbed or slow flow recirculation, whereas marked intimal hyperplasia and medial thickening are often located in regions of increased circumferential deformity.260,261 Recently, a study in vivo has found that reduced wall tension and increased shear stress using an external tube support greatly reduce intimal thickening.262 These observations support the importance of studying the effects of the dynamic mechanical environment, which simulates both shear stress and cyclic strain on vascular cells. The results from these studies will provide a better understanding of the relationship between mechanical factors and cellular physiology and functions, as well as the relevance to atherogenesis.

Most previous in vitro studies over the last decade were devoted to the investigation of the effects of either wall shear stress or cyclic strain on vascular cells. The results from these studies have shown the different cell responses to these mechanical forces. For example, the cells are seen to elongate and align with shear stress, but they are perpendicular to the direction of stretching. In addition, shear stress reduces expression of many genes such as ET-1, VCAM-1, and PAR-1 expression whereas cyclic strain has
been found to induce these gene expressions. Furthermore, cell proliferation is decreased by shear stress whereas it is increased by cyclic strain. *In vivo,* however, vascular cells are simultaneously subjected to both fluid shear stress and cyclic strain with possible regions of low shear stress and elevated cyclic strain. Therefore, a more complete investigation of mechanical influence on vascular cell responses should include both shear stress and cyclic strain.

To date, only two groups have tried to develop a compliant tubular device for subjecting vascular cells to both fluid shear stress and cyclic strain *in vitro.*\(^{263,264}\) Although the cells in these studies remained healthy and attached to the tubular wall throughout the 24-hour experiments, only one preliminary result was published. The alignment of endothelial cells subjected to shear stress was enhanced by the addition of cyclic strain. Many systematic problems have been encountered in these studies. The tubes (Dacron) designed were both either inherently stiff or incompliant when mounted to the holders. In addition, it was difficult to seed and spread cells evenly. Further work to design a better apparatus simulating both types of mechanical factors will lead to a better understanding of the relation between mechanical factors and vascular physiology and pathology.

5.3. **Studies of stress- and strain-responsive elements**

Molecular characterization of the responsive regions of shear- and strain-regulated genes including PAR-1 and the isolation of shear- and strain-activated binding proteins will help to determine the events leading to shear transduction and to altered gene regulation.
Various shear stress response elements (SSREs) have been identified in the promoters of endothelial genes such as PDGF and eNOS. In addition, several transcription factor binding proteins such as NF-KB, TRE-1, and AP-1 have been found crucial for the stress responses as mentioned in chapter 1. Reporter gene studies can also be done to identify the promoter elements necessary for the cyclic strain response and the binding protein elements for this process.

The results of these gene promoter studies may pave the way to the engineering of shear- and strain-responsive gene constructs. These constructs could be introduced into vascular cells by the gene transfer therapy to express anti-inflammatory, anti-proliferative, or anti-thrombotic proteins in regions of either decreased shear stress or elevated cyclic strain where vascular lesions are often observed. For instance, ECs in regions of low shear stress could be modified to express tPA or NO to inhibit atherosclerotic plaque and thrombus formation. In addition, it would be possible to construct two promoter regions into a vector promoter for gene therapy. For example, promoter regions of ET-1 could be attached to those of NO or tPA to express NO or tPA while suppressing ET-1 as this construct is introduced into desired areas of the vascular wall.

5.4. Mechanotransduction and intracellular signal mechanisms

An understanding of mechanotransduction events and signal mechanisms that mediate the gene regulation of shear stress and cyclic strain will allow interventions not only involving specific therapeutic molecules but also altering the upstream mechanical signals. This would prevent undesirable remodeling and produce therapeutic remodeling.
Several potential mechanisms by which the cells sense the mechanical stimuli, shear stress and cyclic strain, and alter gene expression have been discovered. These include ion channels, cytoskeletons, focal adhesion proteins, G-protein coupled receptors, NO, ROS, protein kinases, tyrosine kinases, and MAPKs. Which of these various responses control long-term regulatory (important candidate mechanisms for mechanotransduction) becomes an important question. Targeting the critical signaling steps common to the action of multiple stimuli could also be more useful than inhibition of a single stimulus pathway. Strategies targeting the signaling pathways, therefore, may permit the development of new therapeutic agents for the treatment of vascular diseases. Of interest, too, is the possibility that the new microarray technology could be applied to more effectively screen candidate molecules and gene regulatory elements involved in these processes. In addition, this DNA microarray technique could be applied to investigate the mechanotransduction differences between different mechanical factors such as shear stress and cyclic strain or between different cell types such as macro- and microvascular endothelial cells.

5.5. Effects of mechanical and humoral factors on vascular cells

Although the precise stimulus responsible for VSMC proliferation and migration, leading to the development of restenosis and atherosclerosis, is not known, experimental evidence supports a role for several vasoactive factors including hemodynamic factors, growth factors, and vasoactive substances. Progression of a lesion is most likely due to the influence of complex biochemical and hemodynamic factors directly on ECs and SMCs. Cell proliferation and function are regulated not only by hemodynamic factors
but also by growth factors. In particular, the responses of PAR-1 mRNA levels were upregulated not only by cyclic strain but also by growth factors such as bFGF in this thesis. In addition, PAR-1 expression is synergistically regulated by cyclic strain and growth factor bFGF, indicating the additive effects of hemodynamic factors and biochemical factors in gene alterations and thus the development of vascular diseases. A further understanding of how these factors individually and collectively alter the response of vascular cells offers the hope of allowing intervention to prevent arterial proliferative disorders. Furthermore, studies of vascular responses to hemodynamic factors in the presence of pathologic risk factors such as hypoxia and elevated glucose concentration would further our understanding of the development of atherosclerosis in the presence of systemic risk factors, including diabetes.
Closing remarks

The use of simple in vitro models has yielded new insights into how fluid mechanical forces regulate gene expression in vascular cells, leading to changes in cell functions and proliferation. Particularly in this project, shear stress and cyclic strain differently regulated PAR-1 gene expression in both ECs and VSMCs, leading to changes in cell responses to thrombin. Numerous in vivo and in vitro studies over the last 20 years have yielded immense increases in our knowledge of vascular cell responses to hemodynamic forces. However, the basic question of how the cells sense these mechanical forces and lead to altered regulatory events is not fully understood. A better understanding of the key early events such as second messenger generation, protein phosphorylation, and transcription factor expression involved in the mechanical regulation should allow us to predict how vascular cells respond to mechanical stimuli. This knowledge could contribute to our understanding of the pathogenesis of vascular diseases, thereby enabling better therapeutic strategies such as the design of therapeutic sequences with genes of interest for insertion into vascular cells of interested regions.

It is also particularly interesting to note the many common signaling pathways regulated by shear stress and cyclic strain in vascular cells. Continued studies of the signal mechanisms involved in these responses should provide new insights into which of the various responses observed in cultured cells operate in vivo as regulatory pathways that control long-term structural remodeling in the vascular cells. Finally, an important goal for those who conduct in vitro experiments is to continue improving the models used to better simulate the in vivo physiologic environment, so that vascular biology and events associated with the initiation of vascular diseases can be studied.
References


146. Yano Y. Saito Y. Narumiya S. Sumpio BE. Involvement of rho p21 in cyclic strain-induced tyrosine phosphorylation of focal adhesion kinase (pp125FAK), morphological changes and migration of endothelial cells. *Biochem Biophys Res Commun.* 1996;224:508-515.


APPENDIX A:

Effects of shear stress on VEGF receptor KDR (or flk-1) expression in ECs.

A.

![Bar graph showing KDR expression in HUVECs at different time points and shear stresses.]

B.

![Bar graph showing KDR expression in HMECs at different time points and shear stresses.]

In addition to decreasing PAR-1 expression, shear stress also reduces VEGF receptor KDR mRNA time- and dose-dependently as shown by Northern blot analysis in both HUVECs (A) and HMECs (B). Values are mean ± SEM, n=3-5. Difference between means is statistically significant to those of static controls (*, p < 0.05).
APPENDIX B:

Effects of cyclic strain on VEGF expression in HASMCs.

A.

![Bar graph showing VEGF mRNA fold induction over time (hours) with 20% strain and motion conditions.](image)

B.

![Bar graph showing VEGF production (ng/ml per total protein) over time (hours) with static and 20% strain conditions.](image)

Cyclic strain also induces VEGF mRNA (A) and protein (B) in HASMCs in addition to increasing PAR-1 expression, as shown by Northern blot and ELISA (Cytimmune Inc.). Values are mean ± SEM, n=3-5. Difference between means is statistically significant to those of static controls (*, p < 0.05). Recently, other work has published the findings that cyclic strain induces VEGF expression in smooth muscle cells.