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Mechanisms of the Anti-inflammation Action of Pulsatile Laminar Flow:

Role of AMPK in Epigenetic Modifications

A dissertation submitted in partial satisfaction of the requirements for the Doctor of Philosophy

in

Biomedical Sciences

by

Leona Marie Flores

Committee in Charge:

Professor Shu Chien, Chair
Professor Ronald M. Evans
Professor Christopher K. Glass
Professor Michael Karin
Professor Jason Yuan

2010

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University of California, San Diego

2010

Dedication

This dissertation is dedicated to my parents Herman and Rosario Flores who passed away during my tenure here at UCSD. Their life lessons and maxims continue to guide and inspire me. Never a day goes by when I don't think about them.

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List of Symbols and Abbreviations

ACC	acetyl coenzyme A carboxylase
acet	acetylated
AICAR	5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside
AMPK	AMP-activated protein kinase
CAD	coronary artery disease
ChIP	chromatin immunoprecipitation
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
EC	endothelial cell
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK5	extracellular signal-regulated kinase-5
H3	histone H3
HAT	histone acetylase
HDAC	histone deacetylase
HMT	histone methyltransferase
HUVEC	human umbilical vein endothelial cell
Hz	Hertz
ICAM-1	intercellular adhesion molecule-1
K#	lysine #
KLF2	Krüppel-like factor-2
MCP-1	monocyte chemoattractant protein-1
me	methylated
MEF2	myocyte enhancing factor-2
NCoR	nuclear receptor corepressor
NF-κB	nuclear factor-kappaB
NO	nitric oxide
PCR	polymerase chain reaction

PS	pulsatile laminar shear flow
PTM	posttranslational modification
R	Reynolds number
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase – quantitative PCR
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SELE	E-selectin
SMC	smooth muscle cell
SMRT	silencing mediator of retinoid and thyroid hormone receptors
TM	thrombomodulin
TNFα	tumor necrosis factor alpha
TSS	transcriptional start site
VCAM	vascular cell adhesion molecule

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was my honor to be under his tutelage.

My dearest friend, Dr. Amy Knapp, has kept me healthy through countless miles of cycling, swimming and running as well as innumerable hours of supportive conversations that continues to this day. Finally, I owe my success in completing this degree to my husband, Steve Gregg, who has greatly expanded the definition of “supportive” with his unending source of patience, humor, understanding and love — this degree would not have been possible without him.

Vita

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Wang, Y., **L. Flores**, S. Lu, H. Miao, Y.S. Li, S. Chien (2009) Shear stress regulates the Flk-1/Cbl/PI3K/NF-kappaB pathway via actin and tyrosine kinases. Cell Mol Bioeng. Sep 1;2(3):341-350.

ABSTRACT OF THE DISSERTATION

Mechanisms of the Anti-inflammation Action of Pulsatile Laminar Flow: Role of AMPK in Epigenetic Modifications

by

Leona Marie Flores

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2010

Professor Shu Chien, Chair

Pulsatile laminar flow (PS) mediates various anti-inflammatory and anti-proliferative functions in endothelial cells (ECs) through the upregulation of atheroprotective molecules such as Krüppel-like factor 2 (KLF2) and endothelial nitric oxide synthase (eNOS). However, the mechanisms involved in the repression of inflammatory responses by mechanical stimuli have not been elucidated. In human umbilical vein endothelial cells (HUVECs), 24-hr PS (12 ± 4 dynes/cm^2) inhibited the gene expression of inflammatory and adhesion molecules monocyte chemoattractant protein-1 (MCP-1), vascular

cell adhesion molecule-1 (VCAM) and E-selectin (SELE). Using Compound C, an AMP-activated protein kinase (AMPK) specific inhibitor, I found that the inhibition of gene expression by PS was dependent on the activity of AMPK. In assessing the functional consequences of the PS-inhibition of adhesion molecule expression, I found that PS attenuated monocyte attachment to ECs and that this effect was reversed under Compound C treatment, suggesting the requirement of AMPK in the reduction of monocyte attachment by PS.

Recent studies suggest a key role for epigenetics in the pathogenesis of human disease, especially those involving inflammatory responses, and have established epigenetic pathways as fundamental determinants of endothelial gene expression. In concert with the finding on PS-induced gene repression, I have shown that PS induced the recruitment of the corepressor enzyme histone deacetylase-5 (HDAC5) to the MCP-1 and VCAM proximal promoters and that this recruitment was associated with a histone hypoacetylation status on these promoters. Furthermore, these PS-regulated epigenetic changes were demonstrated to be AMPK-dependent.

In examining the relationship among shear stress, epigenetic dynamics, and inflammatory responses, I have demonstrated that PS exerts anti-inflammatory effects on ECs through downregulation of inflammatory gene expression and functional responses, and via recruitment of corepressors with subsequent histone modifications. Furthermore, I have established that these responses are dependent on the AMPK signaling pathway. This study has

contributed to the understanding of the anti-inflammatory mechanisms in vascular cells, especially on the roles of intracellular mediators and epigenetic elements in the PS-induced downregulation of inflammatory processes, which are implicated in the early development of atherosclerosis.

Chapter 1: Introduction

1.1 Cardiovascular Disease

In the United States, cardiovascular disease (CVD) is the leading cause of death in both men and women, and few topics are of such crucial importance in terms of human morbidity and mortality than disorders of the arterial system and in particular those affecting its luminal interface. The data for specific age groups in 2005 show that CVD was the No. 1 cause of death for people age 75 and older, No. 2 for people ages 25–74, and No. 5 for people ages 15–24 (Lloyd-Jones et al. 2009). The incidence and the consequent cost of CVD are expected to increase, given the rapid rise of risk factors such as diabetes and obesity (CDC 2007; Danaei et al. 2009). Thus, continued and vigorous research into arterial diseases will be invaluable for both clinicians and pathologists who have to deal with the formidable challenge with which these conditions represent.

CVD includes stroke, congenital heart defects, arrhythmias and coronary artery disease (CAD). In some cases, there is a genetic predisposition to developing CVD, yet there are also other potentially modifiable risk factors that contribute to its incidence (Danaei et al. 2009). These include cigarette smoking, high cholesterol levels, hypertension, diabetes, abdominal obesity, a sedentary lifestyle, a diet poor in fruits and vegetables, alcohol overconsumption, and psychosocial indices (Lloyd-Jones et al. 2010).

1.2 Basic Cardiovascular Organization

The cardiovascular system is critically responsible for transporting nutrients and removing gaseous wastes from the body. It consists of the heart, blood vessels, and blood. The different types of blood vessels differ not only with respect to size and location but also anatomical composition and function. In the systemic circulation, arteries transport oxygenated blood away from the heart to the peripheral regions of the body.

Arteries are composed of three coaxial layers: the intima, the media, and the adventitia. The intima is the innermost layer exposed to blood and consists of a monolayer of ECs atop a single basement membrane. The endothelium serves many homeostatic roles, including regulation of vessel tone, tissue perfusion, vascular permeability, blood fluidity, anticoagulant activity, and inflammatory responses (Davies 1995; Chien 2007). The media is the layer underlying the intima and is comprised of mostly smooth muscle cells (SMC) embedded within layers of elastic laminae. These SMCs are responsible for contraction-relaxation responses, usually in response to signals transmitted from the endothelium. The adventitia is the outermost layer made of connective tissue, elastin, and collagen and forms a tough, protective covering. It also often houses the *vasa vasorum*, a vessel bed that supplies larger vessels with nutrients as well as innervation.

1.3 Atherosclerosis

Atherosclerosis is a multifactorial process that is characterized by an

inflammatory response to injury of the endothelium, which causes reshaping of the vessel wall in size and composition causing CAD. It imposes a severe burden on western society and is among the most frequent causes of death worldwide. It is the dominant form of acquired cardiovascular disease and is now recognized as an inflammatory disease (Ross 1999). Atherosclerosis is a chronic progressive disease that occurs principally in large- and medium-sized arteries and results in part from the chronic buildup of fatty material (i.e., low density lipoproteins) within the intimal layer - predominantly in arteries supplying blood to the heart, brain, kidneys, digestive system, or lower extremities. Interestingly, the disease may be present throughout one's lifetime. The earliest type of atherosclerotic lesion, the fatty streak, is common in young children (Napoli et al. 1997). Over time, plaque buildup may restrict blood flow or even occlude the blood vessel entirely, resulting in an infarction of the organ supplied by the vessel. When this occurs in a coronary artery supplying the heart, it results in myocardial infarction or commonly known as heart attack. When this occurs in any of the arteries supplying the brain, it leads to ischemic stroke.

Risk factors, including physical inactivity, obesity, smoking, and a high cholesterol diet, can affect all arteries of the body, yet atherosclerosis tends to localize at branches and bends within the arterial tree. This observation led to the now widely accepted hypothesis that local hemodynamic factors play a critical role in atherogenesis (Caro et al. 1971; Friedman et al. 1981; Zarins et

al. 1983; Chien and Shyy 1998). Hemodynamic loads appear to play equally important roles in the progression of the disease and its devastating end-point – plaque rupture. Atherosclerotic plaques can develop over extended periods of time from initial “fatty streaks” to small obstructions consisting primarily of foam cells, proliferating SMCs, and extra collagen, and to large obstructions that also contain calcium deposits and necrotic debris (Humphrey 2002). It is thought that changes in both wall shear stress and intramural wall stress affect the rate and extent of such structural changes, including a “compensatory” atrophy of medial smooth muscle (which helps reduce the extent of the obstruction) due to stress shielding by a stiff plaque cap. Most important clinically, however, is rupture of the “vulnerable” plaque, resulting in the exposure of highly thrombogenic material to the flowing blood and in the formation of an intraluminal thrombus. Such a thrombus can directly obstruct the lumen or dislodge and travel downstream where it can obstruct a smaller vessel potentially triggering an acute life-threatening event. Understanding plaque rupture requires knowledge of the applied load (hemodynamics) and its action on the structure of the plaque (wall stress), whereas understanding the development and dissolution of a thrombus requires knowledge of the chemomechanical properties of the clot in relation to the hemodynamics. Finally, there is a need for mechanistic studies that relate hemodynamic factors to cell-mediated biochemo-mechanical processes that are responsible for atherosclerosis.

1.4 Hemodynamics

Atherosclerosis is a pathological process resulting from dysfunctions of ECs and SMCs and can be considered as a chronic inflammatory disease that involves EC perturbation, SMC phenotypic changes, and leukocyte invasion (see (Ross 1999) for review). The focal distribution of atherosclerotic lesions in the arterial tree indicates that the vascular dysfunction is attributable, at least in part, to the local hemodynamic forces generated by blood flow. In the lesion-prone areas (e.g. branch points), the wall shear stress is low but its spatial gradient is high, and the flow oscillates back and forth. *In vivo* studies on experimental animals and *in vitro* studies on ECs cultured in flow chambers have shown that shear stress causes dynamic changes in EC structure and function (see (Gimbrone et al. 2000) for review). Genes encoding growth factors, vasoactive substances, adhesion molecules, monocyte chemoattractants, and many other immediate early genes in ECs are modulated by shear stress (see (Davies et al. 1995; Gimbrone et al. 2000) for review). Thoumine et al. showed that shear stress can play a role in atherogenesis by altering the structure and function of ECs, SMCs, and the extracellular matrix (ECM) (Thoumine et al. 1995). Step flow channels have also been used to investigate the effects of flow disturbance on ECs (Truskey et al. 1995; Tardy et al. 1997; Chiu et al. 1998). Previous studies in our laboratory showed that the disturbed flow in the reattachment area, where flow is reciprocating in nature, caused an increase of monocyte adhesion to ECs

(Chiu et al. 2003). It has been found that the expression of EC adhesion molecules and monocyte adhesion to ECs are greater under oscillatory flow than PS flow (Hsiai et al. 2003). These results indicate that hemodynamic forces play important roles in regulating vascular inflammatory responses and that the net forward component of shear stress is a key factor in the flow-modulation of EC functions. Furthermore, it underscores the importance of PS flow in the repression of inflammatory modulators.

Blood flow through the arterial vasculature is pulsatile due to cardiac contraction. Various types of forces are generated on the vessel wall, as illustrated in Figure 1.1 (Papadaki and Eskin, 1997). The normal force exerted on the vessel wall due to the pressure of the blood results in a compressive stress. Changes in pressure during the cardiac cycle cause the periodic stretch of the vessel wall to result in cyclic circumferential stress on the vessel wall. Fluid shear stress is the tractive, tangential force produced by the blood passing along the luminal surface of the endothelium (Fung 1997).

As a first approximation, blood flow can be modeled as a simple laminar flow in a circular cylindrical tube, with the assumptions that blood is an incompressible Newtonian fluid, the flow is well developed, and there is a “no slip” condition at the boundary. These conditions lead to a parabolic velocity profile described by the Hagen-Poiseuille flow, as shown in Figure 1.2. From this solution, one can also obtain the rate of flow Q through a tube, which is the Poiseuille formula (Fung 1997).

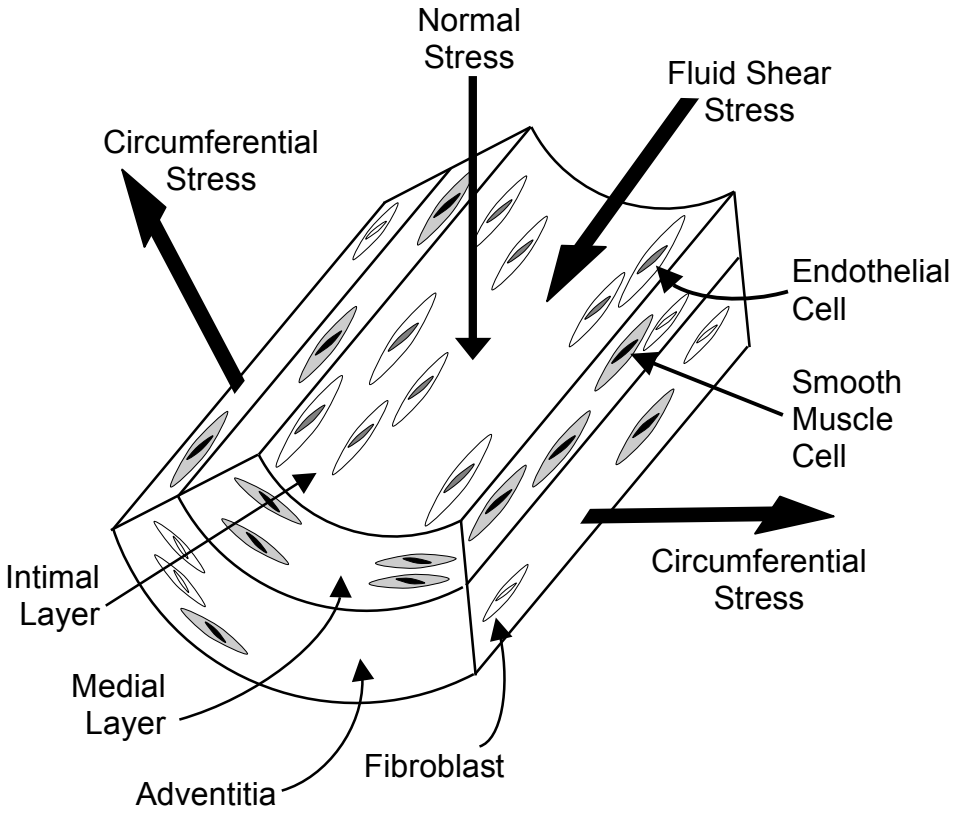
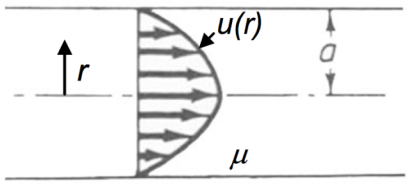


Figure 1.1: Forces acting on the vessel wall. The pump-driven nature of blood flow results in forces that cause cyclic circumferential stress, compressive stress, and fluid shear stress on the elastic vessel wall. (Adapted from (Papadaki and Eskin 1997))



$$u = -\frac{1}{4\mu}(a^2 - r^2)\frac{dp}{dx}$$

$$\dot{Q} = -\frac{\pi a^4}{8\mu}\frac{dp}{dx}$$

Figure 1.2: Hagen-Poiseuille velocity profile in a circular cylinder.

While this model is a convenient way to understand the conceptual relationship between blood flow, properties of blood (viscosity), and vessel size, blood flow patterns can vary in complexity throughout the vasculature. There are relatively uniform, well-developed laminar patterns that occur in the unbranched portions of medium-sized arteries and also complex, disturbed flow patterns at bifurcations, branch points, and curvatures in the vasculature. The disturbed flow patterns involve regions of flow separation, recirculation, and reattachment that generate oscillatory wall shear stress (Frangos et al. 1999).

The importance of flow and shear stress in vascular pathophysiology is shown by the focal development of atherosclerosis in hemodynamically defined areas of the vasculature. Regions of branches, curves, and bifurcations in the arterial tree that experience disturbed flow with unsteady, oscillatory shear stress, typically with a range of ± 5 dynes/cm^2 (± indicating a change in flow direction) and a low net shear stress, are prone to atherosclerotic lesion development (Ross 1999). In contrast, regions of straight arteries that experience steady, unidirectional shear stress, typically on the order of 15 ± 4 dynes/cm^2, are protected from lesion development (Ross 1999; Wang et al. 2006; Yee et al. 2008).

1.5 Shear Stress and Endothelial Cells

The endothelium forms a dynamic interface between the blood and the underlying vessel wall that responds to and transduces both humoral and

biomechanical stimuli. It is well established that differences in local hemodynamic environments result in phenotypically distinct ECs. Under steady, unidirectional or pulsatile shear stress, ECs elongate and align in the direction of flow. This has been demonstrated both *in vivo* and *in vitro* (Levesque and Nerem 1985; Helmlinger et al. 1991; Barbee et al. 1994; Davies 1995). This promotes an atheroprotective phenotype where nitric oxide (NO), a vasodilator, and other atheroprotective molecules are expressed.

It is still a matter of debate on how ECs sense and interpret a mechanical force such as shear stress. The simplest model of mechanotransduction suggests that it occurs when a shear-sensitive receptor is stimulated and the biochemical signal emanates from that point. A more complex, decentralized model suggests that there are multiple types of mechanoreceptors distributed throughout the cell, integrated via the cytoskeleton. Thus, when an input is received, the signal is transduced to other mechanosensors as well, leading to a biochemical response from many locations having an integrated, cumulative effect on the cell (Davies 1995). Proposed mechanosensors include integrins, adherens junctions, potassium channels, receptor tyrosine kinases, and caveolae (Davies et al. 1995; Ohno et al. 1995; Okamoto et al. 1998; Chen et al. 1999). More recently, a group identified a mechanosensory complex upstream of previously identified integrin-mediated response. This complex is comprised of vascular endothelial growth factor receptor-2 (VEGFR2), platelet endothelial cell adhesion

molecule-1 (PECAM-1), and VE-cadherin (Tzima et al. 2005).

1.6 Hypothesis and Objectives

Atherosclerosis is an inflammatory disease that preferentially develops in regions of the arterial vasculature such as branches, curves, and bifurcations that experience low and/or unsteady, oscillating shear stress, as opposed to straight regions that experience higher, steady shear stress. It is known that ECs lining the blood vessel are important modulators and indicators of vascular health. The differences between the atherogenic and atheroprotective areas in the vasculature is due in part to the local hemodynamics and its subsequent effects on gene and protein expressions and functions of these ECs. Pulsatile laminar shear flow (PS) mediates various anti-atherogenic functions in ECs through the upregulation of atheroprotective molecules such as KLF2 and eNOS. However, the equally important mechanisms of the downregulation of inflammatory mediators by PS flow have not been elucidated.

My goal was to investigate the mechanisms by which PS flow confers protection against the early inflammatory events of atherosclerosis. I hypothesized that the flow-dependent protective mechanism occurs through signaling pathways that downregulate inflammatory mediators and cell surface adhesion molecules and that this anti-inflammatory action is mediated through AMPK signaling pathway and subsequent epigenetic modifications (Figure 1.3).

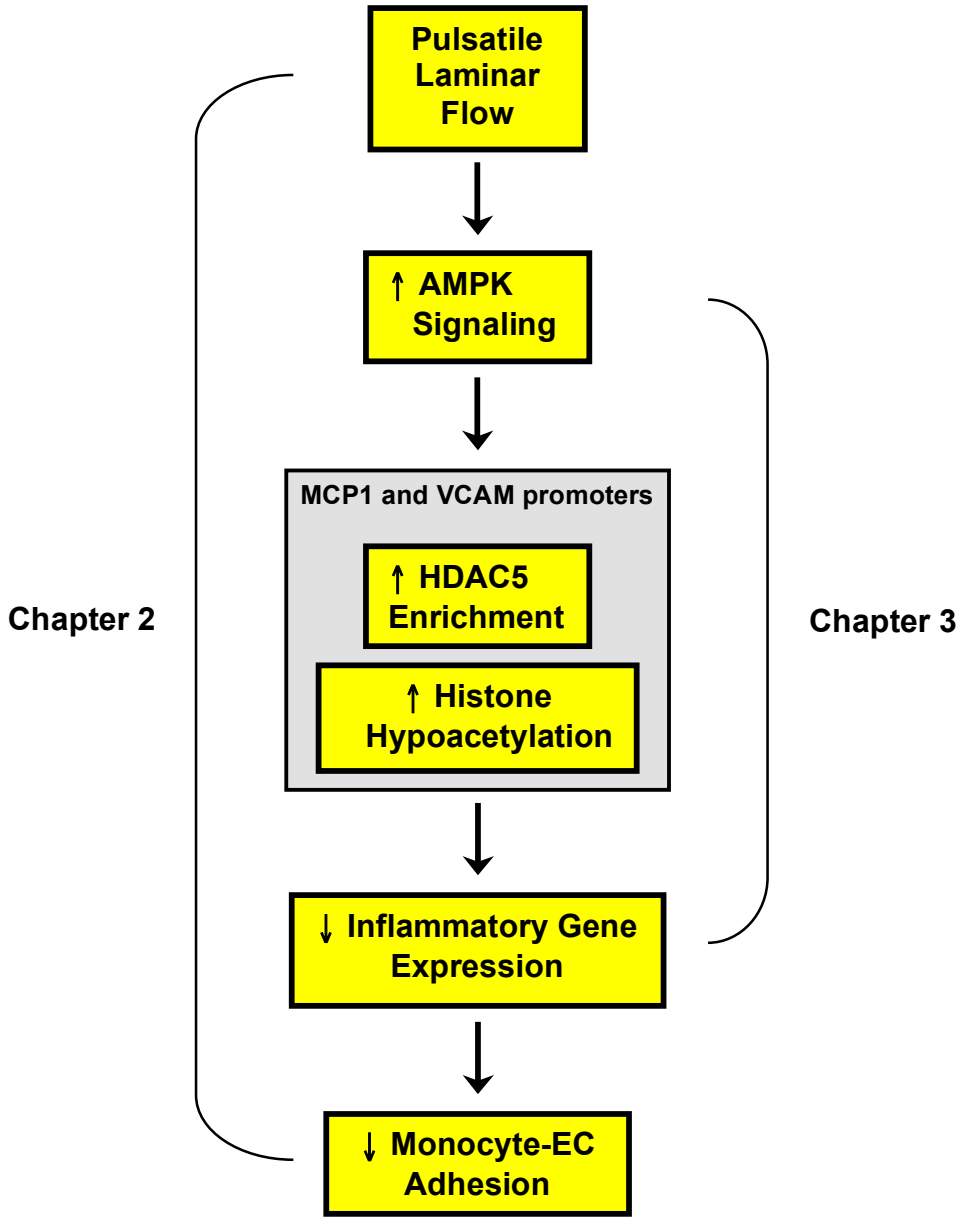


Figure 1.3: Outline of the thesis.

The aim of Chapter 2 was to elucidate the regulatory mechanisms of inflammatory gene repression under flow. Using our *in vitro* parallel-plate flow chamber system, I demonstrated that flow significantly reduced the expression of the inflammatory and adhesion molecule genes MCP-1, VCAM and SELE. By using a pharmacologic agonist of AMPK, I verified that AMPK was sufficient to inhibit MCP-1 and VCAM expression. Previous studies in our lab had demonstrated that AMPK activity was induced by PS flow (Young et al. 2009). By inhibiting AMPK activity, I showed that the flow-induced repression of MCP-1 and VCAM was mediated by AMPK. The presentation of cell surface adhesion molecules constitutes an early inflammatory event of atherogenesis by allowing circulating monocytes to tether to ECs for subsequent ingress into the intimal layer. I demonstrated that the flow-induced repression of VCAM and SELE expression resulted in the attenuation of monocyte adhesion to ECs and that AMPK activity was essential for this inhibitory effect. These results showed that AMPK played a pivotal role in mediating anti-inflammatory functions of PS flow.

Epigenetic mechanisms play an essential role in the transcriptional control of gene expression. In Chapter 3, I explored the epigenetic regulation of inflammatory gene expression as a mechanism by which PS flow inhibits inflammatory responses. Testing my hypothesis that PS-induced downregulation of inflammatory gene expression involves histone modifications associated with transcriptional repression, I demonstrated that

the enrichment of an activator histone H3 mark (H3K9K14acet) in response to flow is significantly decreased at the proximal promoters of MCP-1 and VCAM correlating with repression of gene transcription. Furthermore, I showed that this histone hypoacetylation status is associated with the PS-induced recruitment of a relevant histone deacetylase, HDAC5, to the same promoters. To examine whether AMPK activity is critical for mediating these PS-regulated epigenetic responses, a pharmacologic agonist and inhibitor were used to manipulate the activation of AMPK. Using the AMPK inhibitor Compound C, I established that AMPK regulates PS-depended histone hypoacetylation at the MCP-1 and VCAM proximal promoters. I also confirmed that AMPK activity is both sufficient and necessary for HDAC5 recruitment to these promoters. Forced expression of the corepressor HDAC5 by transient transfection was sufficient to further attenuate flow-inhibition of MCP-1 and VCAM expression; however, HDAC5 was not essential for this inhibition, suggesting the presence of other compensatory corepressors.

Studies conducted in this dissertation have provided a detailed mechanistic exposition of the flow-induced inhibition of inflammatory and adhesion molecule gene expression and EC function in which AMPK plays a critical role. The epigenetic modifications occurring at the MCP-1 and VCAM proximal promoters support a novel mechanism of PS-downregulation of inflammatory gene expression that involves histone modifications and corepressor recruitment tightly associated with transcriptional repression.

Chapter 2: AMPK-mediated anti-inflammatory effects of pulsatile laminar shear flow

2.1 Abstract

It has been well established that a normal functioning endothelium is critical for preventing the onset of atherosclerotic development. The early stage of plaque formation usually begins with an inflamed “activated” ECs where leukocyte adhesion and transmigration occur. These activated ECs display adhesive molecules such as VCAM and SELE on their cell surface, as well as secrete inflammatory chemotactic factors such as MCP-1. In the current study, I showed that PS flow confers protection in the vasculature by repressing MCP-1, VCAM and SELE gene expression. Since AMPK has been shown to be activated by shear flow and possess several anti-inflammatory functions, I investigated the role of AMPK in the flow-induction of anti-inflammatory actions. AMPK reversed the flow-regulated repression of MCP-1, VCAM and SELE indicating that AMPK is a key player in regulating the flow-induced anti-inflammatory responses. In exploring functional consequences of PS flow, I demonstrated that the flow-regulated repression of VCAM and SELE expression resulted in attenuation of monocyte adhesion to ECs and that AMPK activity was essential for this inhibitory effect. These results suggest that long-term PS flow maintains the vascular endothelium in an anti-atherogenic state by way of activation of AMPK, which subsequently modulates target gene expression and functions to exert anti-inflammatory effects.

2.2 Introduction

2.2.a Inflammatory Pathways in Endothelial Cells

Inflammation is a basic pathological mechanism that underlies a variety of diseases. Recent insights into the pathogenesis of atherosclerosis underscore the importance of chronic inflammation in both the initiation and progression of vascular remodeling. Hence, the expression of immunoregulatory molecules by vascular wall components within the atherosclerotic lesions is thought to contribute to the ongoing inflammatory process.

The inflammatory reaction involves the complex interactions between the inflammatory cells (neutrophils, lymphocytes, and monocytes/macrophages) and the vascular cells (ECs and SMCs). The endothelium plays a central role in regulating the inflammatory responses to various forms of stimuli, including cytokine molecules (e.g., TNFα, interleukins), altered wall mechanics, and disturbed hemodynamics (e.g., shear stress level and pattern). Prolonged fluid shear stress on cultured ECs induces the expression of atheroprotective genes and the release of the anti-atherogenic molecule NO. In previous studies, our lab has demonstrated that pulsatile shear stress causes increased KLF2-regulated endothelial gene transcription through processes that involved MEF2 and ERK5 kinases (Young et al. 2009), suggesting a link between the atheroprotective role of pulsatile shear stress and the anti-inflammatory and -proliferative actions of KLF2.

ECs are continually exposed to hemodynamic forces generated by pulsatile blood flow that induce endothelial structural changes, modulate gene expression (Davies 1995) and can potentially influence the nature of the inflammatory response. ECs can regulate inflammatory responses by modulating the expressions of adhesion molecules, cytokines, chemokines, matrix metalloproteinases, and growth factors. Research in vascular biology in the last decade has increased our understanding of the vascular cell responses to inflammatory stimuli and has identified major intracellular inflammatory signaling pathways, particularly the IκB/NF-κB system (Cacicedo et al. 2004; Wang et al. 2004; Kumar et al. 2005; Ewart et al. 2008; Orr et al. 2008). Most *in vitro* experiments have shown that prolonged exposure of ECs to physiological levels of laminar shear stress, as seen *in vivo* in the atheroprotective regions of the arterial tree, have anti-inflammatory and anti-adhesive actions with downregulations of ICAM-1 and VCAM (Tedgui and Mallat 2001).

2.2.b AMP-activated protein kinase (AMPK)

AMPK plays a key role as a master regulator of cellular homeostasis by sensing and regulating the energy status in various cell types. Ubiquitous in distribution, AMPK is activated by numerous physiological and pathological stresses that deplete cellular ATP supplies such as low glucose, hypoxia, ischemia and heat shock (see (Towler and Hardie 2007) for review). Thus, AMPK activation regulates signaling pathways to replenish cellular ATP

supplies. AMPK is a key regulator of skeletal muscle oxidative functions, including metabolic enzyme activities, mitochondrial biogenesis, and angiogenesis, by mediating these processes primarily through alterations in gene expression (Ouchi et al. 2005). In addition, it also stimulates catabolic processes such as fatty acid oxidation and glycolysis via inhibition of acetyl coenzyme A carboxylase (ACC) and activation of phosphofructosekinase-1 (PFK2), respectively. By catalyzing the phosphorylation of ACC-1 and ACC-2, AMPK inhibits the production of malonyl-CoA. Fatty acid synthesis is diminished by a lack of the substrate malonyl-CoA, however, fatty acid oxidation is stimulated due to decreased inhibition by malonyl-CoA on carnitine palmitoyl transferase-1 (CPT-1), which catalyzes the transfer of fatty acids into mitochondria for subsequent β-oxidation (Towler and Hardie 2007). Due to its role as a central regulator of both lipid and glucose metabolism, AMPK is considered to be a key therapeutic target for the treatment of obesity, type II diabetes mellitus, and cancer.

AMPK is a serine/threonine kinase and exists as a heterotrimeric complex composed of a catalytic α subunit and regulatory β and © subunits. Each α and β subunit (α1, α2 and β1, β2) is encoded by a distinct, corresponding gene, whereas the © subunit is encoded by three genes (©1, ©2 and ©3). Of these multiple isoforms, AMPKα1 is the predominant AMPK isoform in ECs (Zou and Wu 2008). While all subunits are necessary for the full activity of AMPK, the binding of AMP to the © subunit allosterically

activates the complex and promotes the phosphorylation of the threonine residue (Thr-172) within the activation domain of the ⟨ subunit by an upstream kinase, the tumor suppressor LKB1 (Carling 2004; Hardie 2004). This phosphorylation is further sustained by an inhibitory effect of AMP on the dephosphorylation at Thr-172 by protein phosphatases (Davies et al. 1995). Several studies indicate that AMPK can be activated in various tissues by adiponectin (Kubota et al. 2007), thrombin (Stahmann et al. 2006), metformin (Shaw et al. 2005) and leptin (Towler and Hardie 2007). Furthermore, calmodulin-dependent protein kinase kinase (CaMKK) is an additional upstream kinase of AMPK (Woods et al. 2005). Activation of AMPK by CaMKK is stimulated by an increase in intracellular calcium ions, which appears to be independent of changes in AMP:ATP ratio (Hurley et al. 2005).

5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) has been used in many studies to assess the effects of AMPK activation on cellular metabolism and function (Dagher et al. 1999; Morrow et al. 2003; Guigas et al. 2006; Prasad et al. 2006; Kim et al. 2008; Li et al. 2008; Gaskin et al. 2009). Upon entering a cell, AICAR is phosphorylated to ZMP, an AMP analog, which allosterically activates both AMPK and an upstream AMPK kinase that causes further activation of AMPK (Jensen et al. 2007; Sun et al. 2007). In HUVECs, the activation of AMPK and inhibition of ACC by AICAR bring about robust alterations in fuel metabolism and energy balance (Dagher et al. 1999).

2.2.c AMPK Signaling in Endothelial Cells

The AMPK pathway is conventionally considered an intracellular fuel gauge and regulator of metabolism. However, recent evidence indicates that it may also be important for EC homeostasis as well as inflammatory processes associated with vascular disease (Dagher et al. 1999; Chen et al. 2009). AMPK activity in ECs can be regulated by stimuli that affect cellular ATP levels, such as hypoxia and the hormone adiponectin (Nagata et al. 2003; Ouchi et al. 2004), but importantly, AMPK has been linked to the phosphorylation and activation of eNOS (Morrow et al. 2003) and mediates the PPARα-dependent suppression of NF-κB activation (Okayasu et al. 2008; Hattori et al. 2009). These AMPK-mediated protective effects in ECs demonstrated by others founded the hypothesis that AMPK plays an important role in the anti-inflammatory action of PS flow. Indeed, the steady laminar flow-induced activation of AMPK downregulates forkhead box O1a (FoxO1a) transcription factor and angiopoietin-2 expressions in ECs thus preventing vascular remodeling (Dixit et al. 2008).

That PS flow induced activation of AMPK and its downstream target substrate ACC (Ser79) was demonstrated by Young et al. (Young et al. 2009). Their studies showed that PS flow (12 ± 4 dynes/cm^2; 1 Hz frequency) increased the phosphorylation of AMPK (Thr172), which peaked at 1 hr and remained elevated for 4 hr. PS flow induced the phosphorylation of ACC (Ser79), and this induction was reversed by using Compound C (CC), a

pharmacological inhibitor of AMPK, thus underscoring the functional relevance of PS-regulated AMPK activation. Their findings confirm that ACC is phosphorylated by AMPK in PS-stimulated ECs and that PS enhances ACC phosphorylation in a time-dependent manner with its kinetics comparable to those of AMPK activation.

These results established a foundation for the further examination of the mechanisms by which PS flow confers a protective phenotype in vascular cells and the role of AMPK in mediating these effects. The incidence of chronic inflammation in both the initiation and progression of vascular remodeling underscores the need to understand the regulation of the inflammatory molecules secreted by the vascular wall components and the mechanism by which prolonged PS flow ameliorates this ongoing inflammatory process.

2.3 Materials and Methods

2.3.a Cell Culture and Pharmacologic Reagents

Pooled human HUVECs were purchased from Cell Applications (San Diego, CA). Cells were cultured on 100-mm diameter petri dishes in medium M199 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Omega, Tarzana, CA), 25% endothelial cell growth medium (Cell Applications, San Diego, CA), 2 mM L-glutamine, 1 unit/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 16.9 units/mL heparin sodium salt (Sigma, St. Louis, MO). Cells were maintained in a humidified 95% air-5% CO_2 incubator at 37°C. HUVEC cultures between passages 5 and 8 were used in all experiments. Pharmacological reagents used to treat cells included AICAR (1 mM for 4 hr at 37°C; Cell Signaling), recombinant tumor necrosis factor-α (TNFα; 10 ng/mL for 30 min at 37°C; Sigma), and 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine (Compound C; 15 μM for 30 min at 37°C; EMD Bioscience, San Diego, CA).

2.3.b Experimental Model

Many *in vitro* devices have been developed and characterized over the past three decades in order to examine EC responses under hemodynamic shear stress. Studies in our lab exclusively utilize the parallel-plate flow chamber for shear stress experiments (Dewey et al. 1981). The parallel-plate flow chamber was first designed by Frangos and colleagues in 1985 (Frangos et al. 1985) to examine the effects of flow on ECs. Several modifications have

since been made to this system in order to impart varying and complex flow profiles. The basic system consists of two critically spaced planes (acrylic chamber and glass slide), between which fluid is driven by a hydrostatic pressure gradient. Cells are cultured on the inner surfaces of glass slides and are subjected to a dragging force from the fluid moving between the planes. Ignoring the small regions affected by edge effects, the flow can be represented by plane Poiseuille flow (Fung 1997). The shear stress τ is steady over the plate and can be calculated as follows where μ is dynamic viscosity, Q is the volumetric flow rate, b is the channel width, and h is the channel height (Equation 1).

$$\tau = \frac{6\mu Q}{bh^2} \quad (1)$$

2.3.c Pulsatile Laminar Shear Flow Experiments

A parallel-plate flow system was used to impose fluid shear stress on cultured ECs but was modified to allow pulsatile flow (Jen et al. 1996). HUVECs were seeded onto glass slides (75x38mm; Corning, Corning, NY), coated for 30 min with collagen type-I (50 μg/mL; BD Biosciences, Bedford, MA), and allowed to grow to >90% confluency. The flow chamber consists of an acrylic base plate (1.0cm x 2.5cm x 5.0cm) with an entrance and exit port through which media is perfused, a 250 μm silicone gasket (SMI, Saginaw, MI) that defines the flow volume, and the slide containing the confluent monolayer of HUVECs. This apparatus was secured together using 2” metal binder clips.

The hydrostatic pressure head (Δh) between an upper and lower reservoir containing circulating media determined the flow rate (Figure 2.1). The circulating media was infused with humidified 5% CO_2-95% air, and the entire flow system was kept at 37°C in a temperature-controlled housing. To this circulating flow setup, a reciprocating syringe pump was connected to impart a sinusoidal component of 1 Hz frequency to simulate the physiologic pulsatile nature of blood flow due to the cardiac cycle. Based on the geometry of the flow chamber, viscosity of the assay medium and pump flow rate, a laminar shear stress with magnitude of 12 dyn/cm^2 (similar to that present in arterial circulation) was imparted on the cellular monolayer. Static, time-matched control cells were kept in a humidified 5% CO_2-95% air incubator at 37°C.

2.3.d Reverse Transcriptase-Quantitative PCR

Total RNA was isolated using the Trizol reagent using a manufacturer-suggested protocol (Invitrogen, Carlsbad, CA). The concentration and quality of RNA were checked by using a spectrophotometer U-2000 (Hitachi, San Diego, CA); ~1 μg/μl at OD 260/280 ≥ 1.8. Reverse transcription was carried out with 1-2 μg of total RNA using the Superscript II reverse transcriptase (Invitrogen) incubated at 42°C for 50 min. The synthesized cDNA was used to perform reverse transcriptase quantitative PCR (RT-qPCR) in triplicate with the iQ SYBR Green supermix (Bio-Rad) on the MyIQ Real-time PCR detection system (Bio-Rad). Results are presented as the amount of mRNA present relative to the untreated or vehicle-treated, time-matched static mRNA

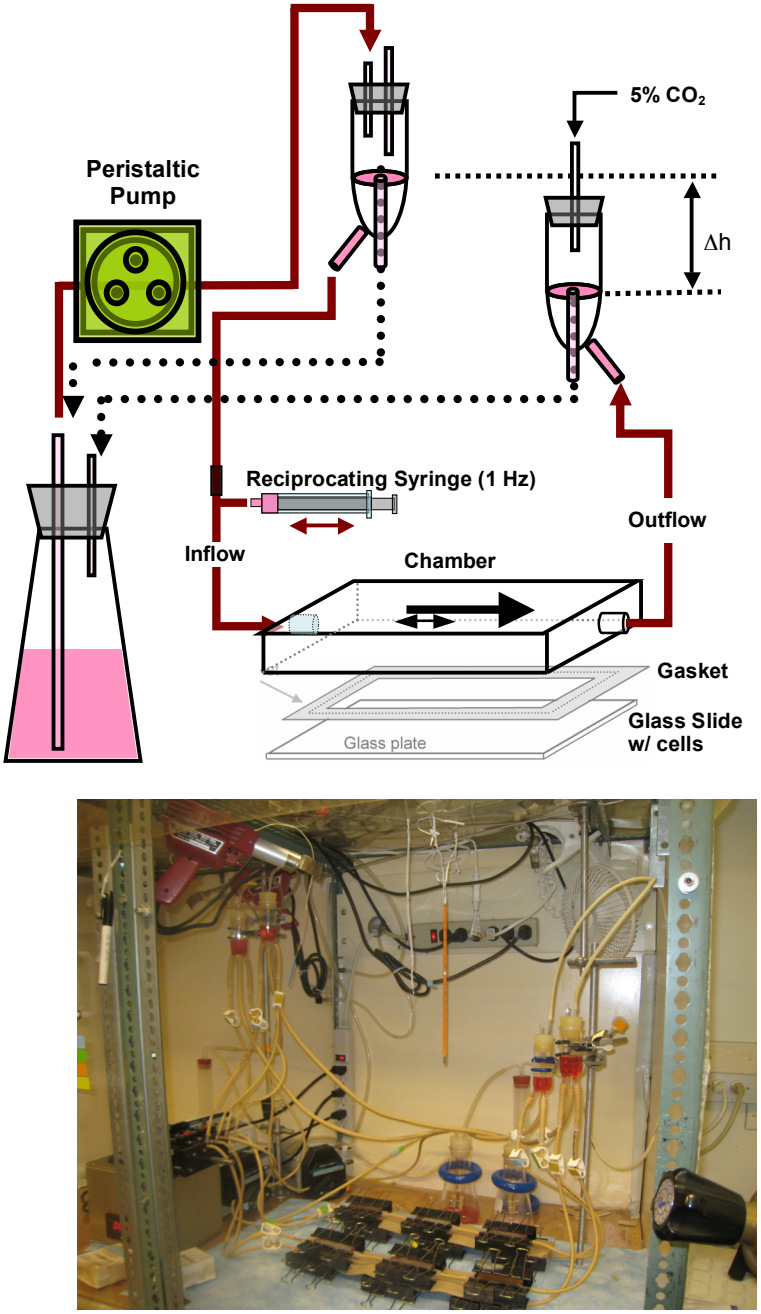


Figure 2.1: Upper panel: schematic of parallel-plate flow chamber system. Lower panel: photographic image of the PS flow system.

normalized with the level of GAPDH mRNA. Specificity of the RT-qPCR reaction was verified with dissociation curve analysis, no-template controls, and no-RT controls. The human primers sequences used were the following (Table 2.1):

Table 2.1: Sequences of the primers used for quantitative RT-qPCR.

	Forward 5'-3'	Reverse 5'-3'
MCP-1	ATAGCAGCCACCTTCATTCC	TGGAATCCTGAACCCACTTC
VCAM	ATGGGAAGGTGACGAATGAG	ATCTCCAGCCTGTCAATGG
SELE	GCTCTGCAGCTCGGACAT	GAAAGTCCAGCTACCAAGGGAAT
HDAC5	CGGAACAAGGAGAAGAGCAAAG	GCTCAGCCACCTTCTGTTTTAG
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG

2.3.e Immunoblotting

At the conclusion of the shearing experiment, cells were washed twice with ice-cold PBS and lysed directly on the slide with a modified radio-immunoprecipitation assay (RIPA) buffer (1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0; Sigma) containing a protease inhibitor cocktail (Roche Complete, EDTA-free Protease Inhibitor Cocktail Tablets, 5 mM sodium fluoride (NaF)) for 20 min on ice. After centrifugation for 10 min at 14,000 RPM (4°C) to remove cellular debris, the protein content of the lysate was determined according to the Bradford method using a spectrophotometer (Hitachi U-2000). Equal amounts (~20 μg) of denatured protein were resolved using 10% SDS-polyacrylamide gels, separated by electrophoresis according

to standard protocols and subsequently immobilized onto nitrocellulose membranes (Bio-Rad) at 4°C. The membranes were blocked with 5% bovine serum albumin (Sigma) followed by incubation with the primary antibody in 1X TBST (Tris-buffered saline, 1% Tween 20) for 1 hr. The antibodies used for immunoblotting (1:500-1000 dilution in TBST) were polyclonal α-tubulin (Sigma), phospho-ACC(Ser79), phospho-AMPKα (Cell Signaling). The bound primary antibodies were detected by using a goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase conjugate (1:2000 dilution in TBST; Santa Cruz Biotechnology). Immunoreactive proteins were visualized by use of an ECL Plus Kit (GE Healthcare, Piscataway, NJ) followed by detection using a charged-coupled device (CCD) imaging system (FluorChem Q; Alpha Innotech, Santa Clara, CA). Densities of the protein bands were quantified by use of ImageJ software (National Institutes of Health), and results were normalized by arbitrarily setting the densitometry of control sample to 1.0.

2.3.f Monocyte Adhesion Assay

Monocytic THP-1 cells were from Dr. Christopher K. Glass (Department of Cellular and Molecular Medicine, UCSD) and grown in RPMI medium 1640 (Gibco) supplemented with 10% fetal bovine serum (Omega). THP-1 cells were concentrated by centrifuging at 400 x *g* for 10 min at room temperature. For adhesion assays, HUVECs were grown to confluence, pretreated with Compound C, AICAR, TNFα, or control vehicle and exposed to either PS or static conditions for 24 hr. The adhesion assay was performed by adding 2.0

mL of the concentrated THP-1 cells (preincubated with calcien-AM at 5 μM; Invitrogen) to the slides for 30 min at 37°C. The unbound cells on the slides were removed by gently washing 3 times with PBS or RPMI, and the monolayers were fixed with 4% paraformaldehyde for 30 min. Fluorescent micrographs of the cells on the slides were taken with Olympus IX70 (magnification: X20; numeric aperture: 0.40; Simple PCI imaging software, Hamamatsu Corporation, Sewickley, PA). The number of adherent cells was determined using an image-processing algorithm that measures fluorescence intensity/area after background noise subtraction set at a user-defined threshold value. The MATLAB-coded program was conceived and written by Dr. Sung Hur. Cell adhesion data were collected from 6 randomly selected optical fields in each slide from 3 independent experiments (n=3).

2.3.g Statistical Analyses

All quantitative data in this study are represented as means ± standard error of the mean (SEM) of three experiments, if not stated otherwise, and calculated using the Data Analysis package of Microsoft Excel software or JMP Statistical Discovery software (SAS Inc., Cary, NC) for post-hoc analysis. Comparisons of results between two groups were performed by using unpaired two-tailed Student *t*-test assuming unequal population variance. *p*-values of <0.05 were considered statistically significant.

2.4 Results

2.4.a Anti-inflammatory responses of pulsatile shear flow

Studies by our lab and others have demonstrated that steady laminar shear flow downregulates the expression of various inflammatory genes including MCP-1 (Shyy et al. 1994; Xing et al. 2001; Yu et al. 2002). To assess if PS would also repress inflammatory gene expression, I exposed HUVECs to 24 hr of PS (12 ± 4 dynes/cm^2 at 1 Hz) or 30 min of TNFα (10 ng/mL) treatment and isolated the total RNA for RT-qPCR analysis. The MCP-1, VCAM and SELE gene expressions were all significantly attenuated below basal levels (Figure 2.2) by 24 hr of PS flow. As a positive control, cytokine treatment induced robust expressions of these inflammatory and adhesion molecule genes. These results show that the application of PS flow using our flow system can effectively repress inflammatory and adhesion molecule gene expressions.

2.4.b AMPK activation attenuates inflammatory and adhesion molecule gene expression

AMPK activation has been shown to inhibit the inflammatory response of palmitate-induced NF-κB expression in HUVECs (Cacicedo et al. 2004). Previous studies carried out by Dagher et al. (Dagher et al. 1999) showed that incubation of HUVECs with 2 mM AICAR caused a 5-fold activation of AMPK, which was accompanied by a 70% decrease in ACC activity, demonstrating the presence of both AMPK and ACC in HUVECs and their responsiveness to AICAR. To determine whether AMPK is upstream to MCP-1 and VCAM in

regulating their gene expressions in HUVECs, I utilized AICAR as a pharmacological activator of AMPK. Time course experiments were performed, and subsequent RT-qPCR analysis revealed that 1 mM AICAR inhibited MCP-1 and VCAM gene expressions after incubation for 4 hr, but not at earlier times (Figure 2.3). These results indicate that AMPK is involved in the regulation of inflammatory and adhesion molecule gene expressions.

2.4.c AMPK signaling participates in flow-induced inhibition of inflammatory and adhesion molecule gene expressions

The data from section 2.4.a demonstrate that inflammatory and adhesion molecule gene expressions were suppressed by PS flow (Figure 2.2). Since PS flow has been shown to stimulate AMPK activity (Young et al. 2009), my finding that AMPK activation causes reduction of MCP-1 and VCAM expressions (Figure 2.3) provides a basis for the hypothesis that PS flow regulates MCP-1, VCAM and SELE expressions via AMPK activation. To investigate the regulatory role of AMPK in PS-induced downregulation of these genes, I assessed the effect of directly inhibiting AMPK activity using Compound C, a pharmacological inhibitor of AMPK, under PS flow using the same protocol as in section 2.4.a. HUVECs were incubated with 15 μM Compound C for 30 min before being subjected to 24-hr PS flow. RNA samples were isolated, and the levels of MCP-1, VCAM and SELE mRNA were determined and quantified by RT-qPCR, with the results normalized by GAPDH. Under static conditions, 15 μM Compound C did not significantly change the basal level of MCP-1 or VCAM gene expressions (data not

shown). Figure 2.4 establishes that the pharmacological inhibition of AMPK significantly blocked the inhibition of MCP-1, VCAM and SELE induced by PS flow and that AMPK activity is crucial in the PS-induced repression of these genes.

2.4.d Monocyte adhesion inhibition by pulsatile flow is dependent upon AMPK activation

Since the studies of Young et al. have shown the ability of PS to activate AMPK in HUVECs (Young et al. 2009), and my results have demonstrated that AMPK mediates the flow-induced gene repression of adhesion molecules, I explored the functional effects of PS and AMPK on monocyte adhesion. In an earlier study, Ewart et al. reported that incubation of human aortic endothelial cells (HAECs) with the AMPK activator AICAR markedly reduced the adhesion of monocyte lineage U937 cells induced by TNFα stimulation in an AMPK-dependent manner (Ewart et al. 2008). Furthermore, prolonged incubation of HAECs with AICAR reduced TNFα-stimulated SELE expression in an AMPK-dependent manner (Ewart et al. 2008). Based on these data and my finding that AICAR was sufficient to reduce adhesion molecule expression (Figure 2.3), I then examined the role of AMPK activity in the PS-modulation of monocyte adhesion. The results in Figure 2.5 show that the monocyte adhesion is significantly reduced by PS flow and that such PS-induced inhibitory effects are abolished by the AMPK inhibitor Compound C (Figure 2.5). Under static conditions, 15 μM Compound C did not significantly change the basal level monocyte adhesion (data not

shown). These findings demonstrate that AMPK mediates the PS-induced suppression on monocyte adhesion.

2.5 Discussion

ECs are the main regulator of vascular wall homeostasis. In response to a variety of injurious stimuli, ECs undergo phenotypical modulation to a non-adaptive state of “endothelial dysfunction”, which is associated with the increased expressions of adhesion molecules and increased synthesis of pro-inflammatory and pro-thrombotic factors. Physiologically, ECs maintain a relaxed vascular tone and also actively regulate vascular permeability to plasma constituents and adhesion of platelets and leukocytes (Ross 1986; Ross 1999; Tedgui and Mallat 2001). Current evidence suggests that endothelial dysfunction occurs early in the process of atherogenesis and contributes to the formation, progression, and complications of the atherosclerotic plaque (Montecucco and Mach 2009).

Shear stress has an established effect on upregulating atheroprotective mediators in ECs (e.g., eNOS, KLF2, and thrombomodulin), thus leading to an anti-atherogenic response (Wang et al. 2006; Young et al. 2009; Wang et al. 2010), but little is known about how shear stress downregulates proinflammatory responses and inhibits the onset of endothelial dysfunction. In this chapter, I investigated the mechanotransduction mechanism by which PS flow represses inflammatory and adhesion molecule expression and endothelial function in terms of monocyte adhesion. A key finding of this study is the identification of AMPK as an upstream and essential player in mediating the anti-inflammatory function of PS flow. Long-term PS flow (12 ± 4 dyn/cm^2)

led to a significant repression of the expressions of both inflammatory and adhesion molecules at the mRNA level, as demonstrated by RT-qPCR analysis (Figure 2.2). This is in concert with the myriad of studies demonstrating that steady laminar shear flow exerts an anti-atherogenic effect on vascular cells (Shyy et al. 1994; Boo and Jo 2003; Chiu et al. 2003; Liu et al. 2004; Heydarkhan-Hagvall et al. 2006; Fisslthaler et al. 2007). PS flow has been shown to repress the gene expressions of inflammatory and adhesion molecules in ECs exposed to 4 hr of laminar flow (Shyy et al. 1994). Since the adaptation of ECs *in vivo* to continuous flow over a longer time period is crucial for appropriate physiological functioning (Garcia-Cardena et al. 2001), I chose the longer time period of 24 hr. Figure 2.2 shows that 24-hr PS flow at 12 ± 4 dynes/cm^2 significantly reduced MCP-1, VCAM and SELE expressions.

Previous studies in our lab have demonstrated that PS induces a sustained activation of AMPK through phosphorylation on its Thr172 site in HUVECs (Young et al. 2009) and that this activation is maintained in ECs exposed to 24-hr PS flow (data not shown). To determine the necessity of AMPK activation for MCP-1 and VCAM repression under PS flow, I first tested whether AMPK activation was sufficient for inhibiting inflammatory gene expression. I chose a pharmacological approach to alter AMPK activity in order to assess biological effects on a rapid time scale. AICAR, a compound that stimulates AMPK activity, is taken up into cells and phosphorylated to form ZMP (Sabina et al. 1985), which mimics the effects of AMP on AMPK

activation (Sullivan et al. 1994). AICAR significantly suppressed MCP-1 and VCAM gene expressions after 4 hr (Figure 2.3), demonstrating that AMPK is sufficient in attenuating MCP-1 and VCAM gene expressions. I then investigated the role of AMPK in PS-induced downregulation of MCP-1, VCAM and SELE by using Compound C, a specific pharmacological inhibitor of AMPK (Zhou et al. 2001). Compound C significantly attenuated PS-induced downregulation of MCP-1, VCAM and SELE (Figure 2.4). These results identified a critical role for AMPK in mediating the PS-induced anti-inflammatory actions.

Monocyte adhesion to extracellular matrix proteins on endothelial surface has been considered as a major early step in the initiation of atherosclerosis (Huo et al. 2000; Vogl-Willis and Edwards 2004). In order to investigate the functional effects of the PS-induced repression of inflammatory and adhesion molecule expressions, I examined the role of AMPK activation on monocyte adhesion. The results showed that monocyte adhesion was significantly reduced by PS flow and that the use of Compound C, an AMPK inhibitor, abrogated this PS-induced inhibitory effect (Figure 2.5). Taken together, the results from this chapter revealed that the PS-induced suppression of inflammatory and adhesion molecule gene expressions has the functional consequence of inhibition of monocyte adhesion and that this inhibitory effect is mediated via activation of the AMPK signaling pathway.

My finding that AMPK plays a pivotal role in mediating the anti-

inflammatory responses of long-term PS flow provides a rational basis for investigating the mechanism of the anti-inflammatory responses induced by PS flow and their modulation by AMPK. Recently, several reports have linked AMPK activation with alterations in endothelial gene expression via the modulation of histone acetylases and deacetylases (HDACs). In the next chapter, I explored the epigenetic regulation of vascular endothelial gene expressions as a mechanism by which PS flow inhibits inflammatory responses.

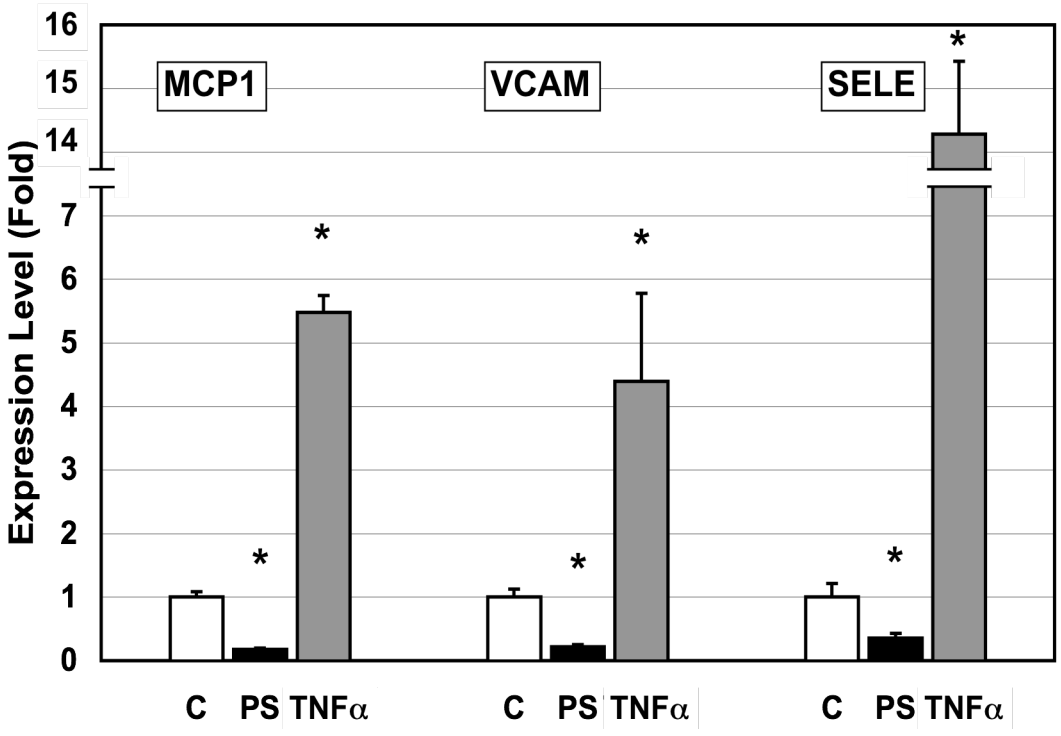


Figure 2.2: Flow-dependent inflammatory and adhesion molecule gene expressions. HUVECs were exposed to PS flow (12 ± 4 dynes/cm^2) for 24 hr (PS), 30 min TNFα (10 ng/mL; TNFα) or left untreated (C). RNA samples were isolated, and the levels of MCP1, VCAM and SELE mRNAs were determined and quantified by RT-qPCR, with the results normalized by GAPDH. The shear results were normalized by the corresponding static control. The data represent mean ± SEM from at least 3 independent experiments. *p<0.05 vs. Control (C).

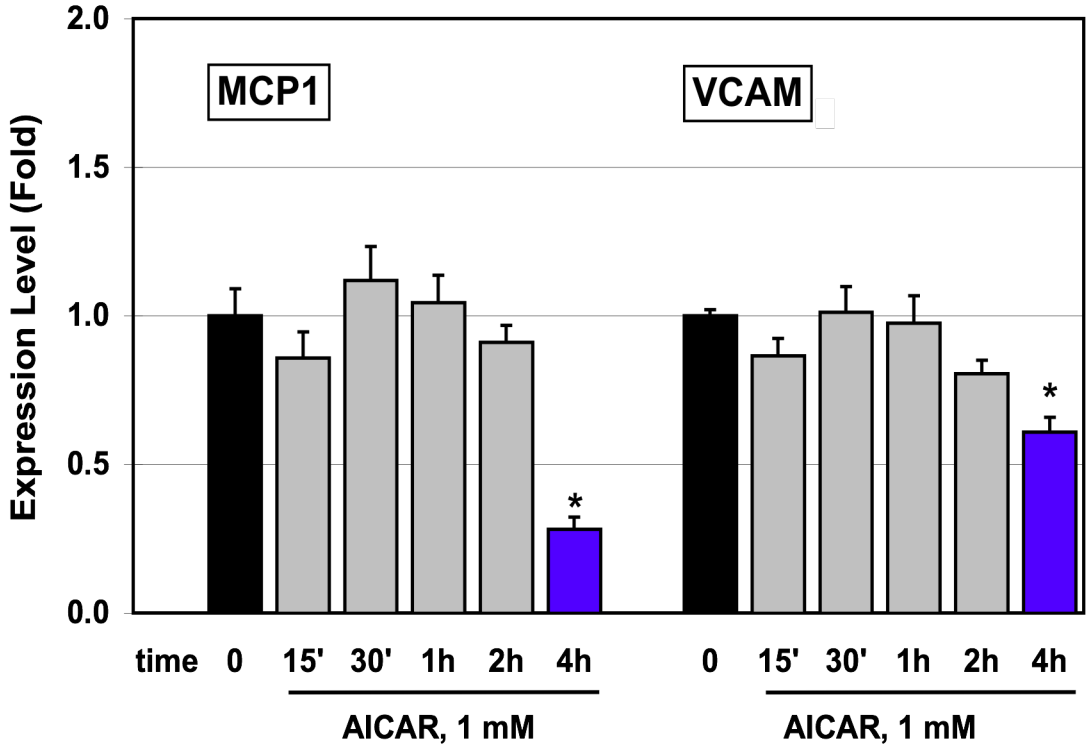


Figure 2.3: Time course of the effects of AICAR treatment in confluent HUVECs on MCP-1 and VCAM gene expression. HUVECs were treated with 1 mM AICAR for the given timepoints, RNA samples were isolated, and the levels of MCP1 and VCAM mRNAs were determined and quantified by RT-qPCR, with the results normalized by GAPDH. At the 4hr time point, AMPK activation attenuated the expressions of MCP1 and VCAM. The data represent mean ± SEM from 3 independent experiments. *$p<0.05$ vs. no treatment.

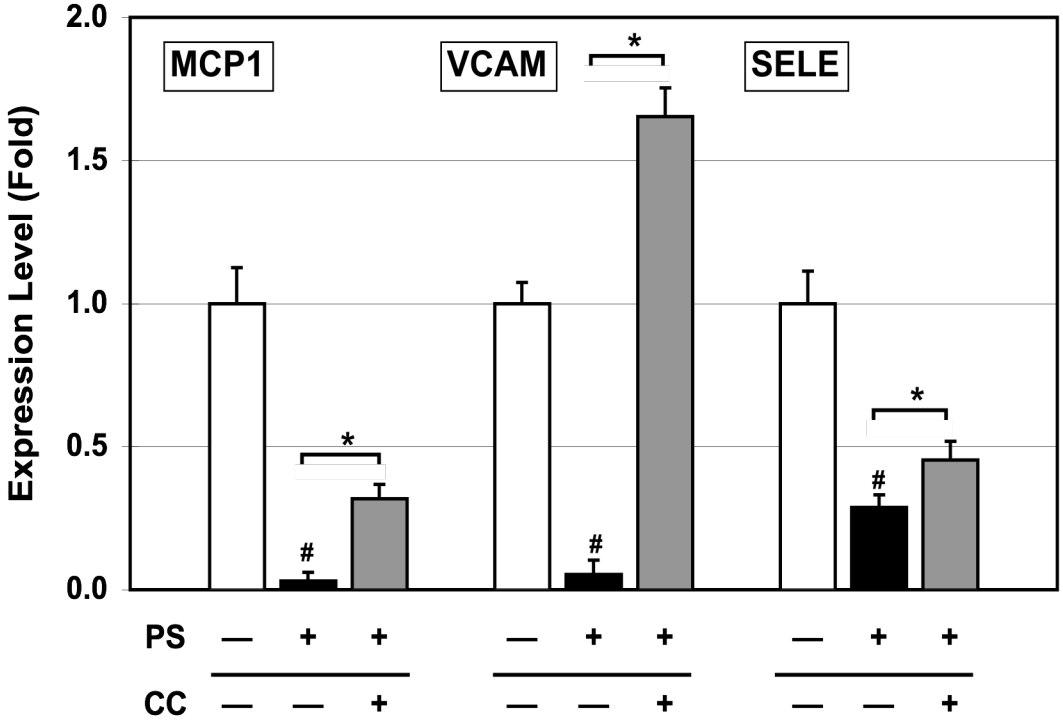


Figure 2.4: AMPK mediates PS-induced repression of MCP1, VCAM and SELE. 15 μM Compound C (CC) was added into the medium of cultured HUVECs for 30 min before the cells were subjected to 24-hr PS flow. RNA samples were isolated, and the levels of MCP1, VCAM and SELE mRNA were quantified by RT-qPCR with the results normalized by GAPDH. The data represent mean ± SEM from 3 independent experiments. # $p<0.05$ vs. no shear, *$p<0.05$.

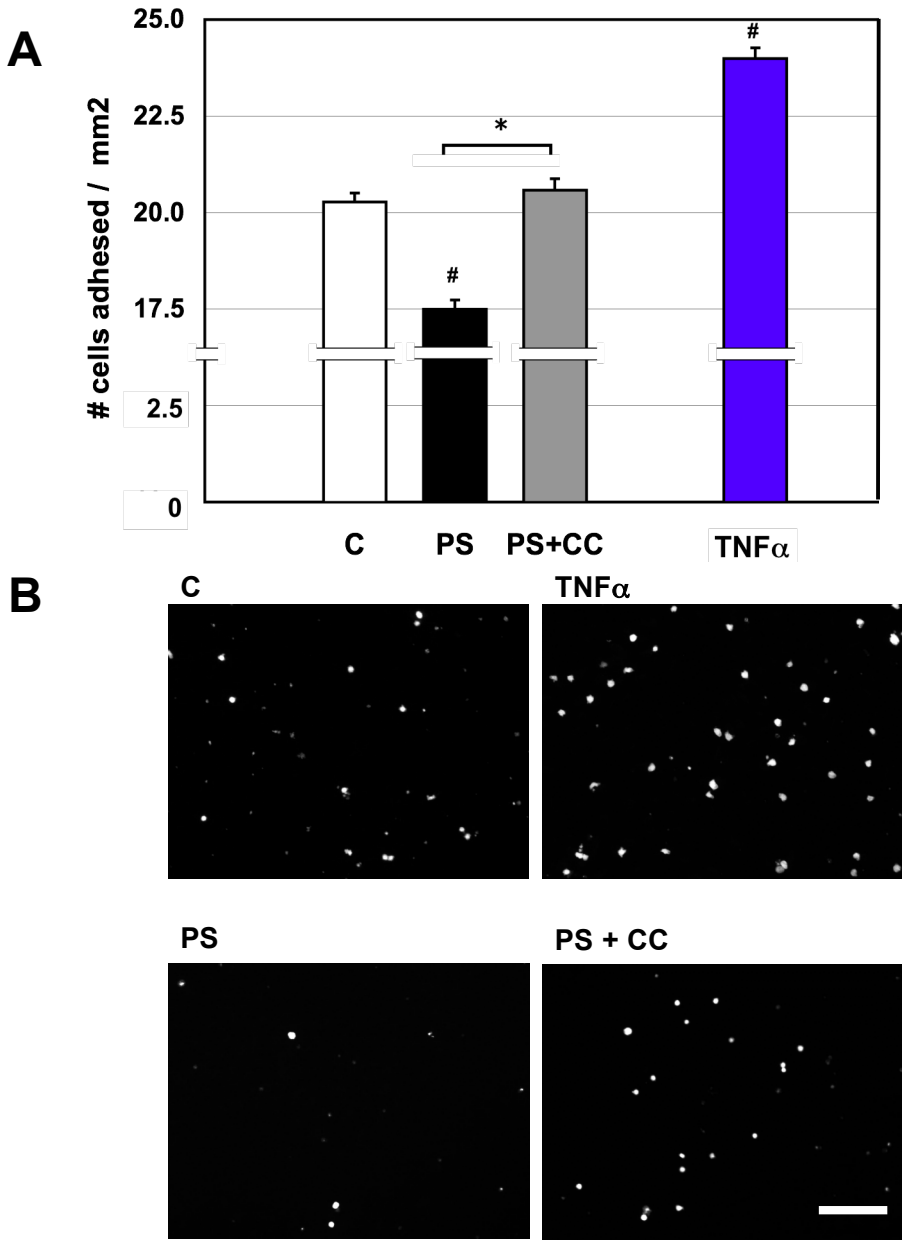


Figure 2.5: Inhibition of monocyte adhesion by PS flow is dependent upon AMPK signaling. 15 μM Compound C (CC) was added into cultured HUVECs for 30 min before cells were subjected to 24-hr PS flow. As a positive control, HUVECs were incubated with 10 ng/ml TNFα for 2 hr. HUVECs were washed thoroughly and overlaid with calcien-AM THP-1 cells for 30 min. (A) THP-1 cell adherence was quantified by image processing software. (B) Representative micrographs. The data represent mean ± SEM from 3 independent experiments using 6 fields. #p<0.05 vs. Control (C), *p<0.05. Scale bar, 100 μm.

Chapter 3: AMPK signaling pathway participates in shear-induced histone modifications mediating anti-inflammatory responses

3.1 Abstract

Epigenetics refers to the study of chromatin-based pathways important in the regulation of gene expression and offers a newer perspective on transcriptional control paradigms in vascular ECs. A growing body of evidence has implicated epigenetic pathways in the control of vascular endothelial gene expression in health and disease. Hence, I proposed that the epigenetic regulation of inflammatory gene expression is a mechanism by which PS flow inhibits inflammatory responses. In this dissertation research, I hypothesized that PS-induced inflammatory gene repression involves corepressor recruitment and histone modifications associated with transcriptional repression. Using chromatin immunoprecipitation protocols, I demonstrated that the residency of an activator histone H3 mark (H3K9K14acet) in response to flow is significantly decreased at the proximal promoters of MCP-1 and VCAM. Histone hypoacetylation changes lead to chromatin condensation and subsequent repression of gene transcription. Furthermore, I showed that this histone hypoacetylation status is associated with the PS-induced enrichment of HDAC5, a class IIa histone deacetylase. To investigate whether AMPK activity mediates these PS-regulated epigenetic responses, pharmacologic agents (an agonist and an inhibitor) were used to manipulate the activation of AMPK. By using the inhibitor, I established that AMPK is crucial for mediating

the PS-induced histone hypoacetylation and HDAC5 recruitment to the MCP-1 and VCAM proximal promoters. Over-expression of the corepressor HDAC5 by transient transfection was sufficient to further attenuate flow-induction of MCP-1 and VCAM repression, but HDAC5 was not essential for this PS-induced inhibition, suggesting the presence of other compensatory corepressors.

Chromatin remodeling induced by histone hypoacetylation and HDAC recruitment is generally associated with transcriptional repression. My results demonstrate the occurrence of epigenetic modifications at the MCP-1 and VCAM proximal promoters and the correlation of such epigenetic changes with the transcriptional repression induced by PS flow. These findings provide evidence for a novel mechanism of PS-induced anti-inflammatory action that involves AMPK-mediated corepressor recruitment and histone modifications.

3.2 Introduction

Broadly defined, epigenetics refers to heritable, chromatin-based mechanisms important in the regulation of gene expression that are independent from DNA coding variability. Epigenetics offers a new perspective on gene regulation that expands the classic *cis*/*trans* paradigm and helps to explain some of its limitations. Central to this model is the concept that the structure of DNA is an effector of gene expression such that identical DNA sequences may demonstrate variable expressivity. By altering the accessibility of chromatin, epigenetic mechanisms play important roles in the transcriptional control of gene expression. These phenomena have been recognized as important permissive and suppressive factors in controlling gene transcription. Two major epigenetic mechanisms are the covalent posttranslational modification (PTM) of histone proteins in chromatin and the methylation of DNA itself. These are regulated by distinct but coupled pathways. The covalent PTMs that decorate histone tails, as well as altering histone density and DNA methylation, can differentially affect chromatin activity in several distinct biological settings (Rea et al. 2000; Jenuwein and Allis 2001; Turner 2002).

The concept of epigenetic regulation is being increasingly recognized as a critical factor in the pathogenesis of atherosclerosis and other key inflammatory response genes. It posits a newer perspective for understanding how gene expression is perturbed in various prevalent diseases of the human

cardiovascular system. Epigenetic pathways have been established as fundamental determinants of endothelial gene expression in health and disease, and importantly, these processes are reversible and may provide an excellent therapeutic target.

3.2.a Chromatin Structure and Histone Modifications

Within the nucleus, chromosomal DNA is tightly associated with proteins, and these interactions form the ordered structure known as chromatin. The individual units that make up chromatin are referred to as nucleosomes, which consist of 147 base pairs of DNA and an octamer of associated core histone proteins (two each of H2A, H2B, H3, and H4) and a linker histone H1. These protein complexes control gene expression partly by constantly packing and unpacking the chromosomal DNA to form heterochromatin or euchromatin, respectively. Heterochromatic domains are in general inaccessible to DNA binding factors and are transcriptionally silent. Euchromatic domains, in contrast, define more accessible and transcriptionally active portions of the genome (Luger et al. 1997; Grewal and Moazed 2003). Core histones are characterized by the presence of a histone fold domain and N-terminal tails. These terminal tails are crucial for the normal functioning of cellular processes, including replication and transcription (Morales and Richard-Foy 2000), because they are targets for PTMs. Histone PTMs can function as epigenetic marks for either active or repressed chromatin (Kouzarides 2002; Fischle et al. 2003; Lachner et al. 2003).

The modulation of chromatin condensation can be achieved by reversible acetylation on the lysine residues of histone tails. In its natural state, negatively charged DNA forms tight electrostatic associations with positively charged histone proteins. This association leads to chromatin compaction and impaired gene expression. However, if the positive charges of the histone proteins are decreased by the addition of an acetyl group (i.e., histone acetylation), the DNA and histones form a more relaxed (open) configuration, which favors gene expression. Other histone PTMs include methylation, phosphorylation, ubiquitylation, glycosylation, ADP-ribosylation, carbonylation and SUMOylation. Many of these modifications can modulate other histones, collectively constituting the “histone code” and are positively or negatively correlated with specific transcriptional states or the specific organization of repressive or open chromatin (Strahl and Allis 2000; Jenuwein and Allis 2001; Turner 2002).

3.2.a.i Histone Acetylation

Histone acetylation modulates transcription in multiple ways. In euchromatin, the best-characterized histone acetylation that is associated with transcriptionally active chromatin occurs on H3 at K9 (lysine 9), K14, K18 and K56 and on H4 at K5, K8, K13 and K16 (Grunstein 1997; Struhl 1998; Berger 2007). Heterochromatic domains that are transcriptionally silent are typically characterized by histone H3 and H4 hypoacetylation (Holbert and Marmorstein 2005). The acetylation reaction involves the transfer of an acetyl group from

acetyl coenzyme A (acetyl-coA) on the ε-amino group of the lysine residue to neutralize the positive charge, thereby relaxing the chromatin structure. This relaxation interferes with the generation of higher-order chromatin structures and increases the accessibility of transcription factors to their target genes thus enhancing transcriptional activation (Shahbazian and Grunstein 2007). Additionally, acetylated histones can serve as binding sites for bromodomain proteins, which often act as transcriptional activators (Kouzarides 2000). The addition or removal of acetyl groups into core histones is a dynamic process controlled by the antagonistic actions of two large families of enzymes — the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). The balance between the actions of HATs and HDACs serves as a key regulatory mechanism for gene expression and governs various inflammatory processes and disease states.

An early suggestion was that histone acetylation reduces the positive charges, thereby relaxing chromatin structure and facilitating access to the DNA for the initiation of transcription. However, the proposed histone code hypothesis suggests that multiple histone modifications act in combination to regulate transcription (Ruthenburg et al. 2007; Shahbazian and Grunstein 2007; Haberland et al. 2009).

3.2.a.ii Histone Deacetylases

Histone deacetylases (HDACs) repress transcription by deacetylating nucleosomal histones and other components of the transcriptional machinery,

thereby promoting chromatin compaction and perturbing protein-protein interactions essential for gene activation. It is clear that HDAC enzymes seldom operate alone. HDAC proteins alone do not possess HDAC activity, but require PTMs and interacting proteins, with various functions such as recruitment, co-repression or chromatin remodeling, to possess activity. Since HDAC proteins cannot bind DNA, they require other proteins for recruitment to appropriate target genes (de Ruijter et al. 2003). HDACs will often homo- or hetero-dimerize with other HDACs and can participate in various repression complexes, including (but not limited to) nuclear receptor corepressor (NCoR), silencing mediator of retinoid and thyroid hormone receptors (SMRT), nucleosome remodeling and deacetylase (NuRD), Sin3 and Co-REST, for full transcriptional repression activity (Cress and Seto 2000; Guenther et al. 2001; Fischle et al. 2002; Zhang et al. 2002).

The HDAC family comprises 18 isoforms and are categorized into four classes: class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, and 9), class III HDACs or sirtuin family (Sirt proteins) and class IV HDAC (HDAC11). HDACs catalyze the reverse reaction of HATs, removing acetyl groups from lysine residues (Wang et al. 2007). The class I HDACs are expressed ubiquitously, while the class II HDACs are expressed in a tissue-enriched manner (reviewed in (McKinsey et al. 2002)). The class I HDACs are smaller than class II HDACs; they are composed almost entirely of an HDAC domain. Class I and class II HDAC proteins possess a conserved HDAC

domain. However, class I HDACs possess deacetylase activity, while class II HDACs do not (Fischle et al. 2001; Fischle et al. 2002). Nevertheless, class II HDACs can recruit class I HDACs and thus gain deacetylase activity through their deacetylase domain (Fischle et al. 2002).

The class IIa HDACs (HDAC4, 5, 7 and 9) have become the focus of intense interest because of their ability to respond to extracellular signals via regulated phosphorylation, which provides a mechanism for linking stimuli at the cell membrane with the genome (Wade 2001) (Ha et al. 2008). Class IIa HDACs have a nuclear export signal (NES) within the catalytic domain and are able to shuttle in and out of the nucleus in a well-regulated process. The shuttling of HDACs 4, 5 and 7 between the cytosol and the nucleus has been studied extensively in differentiating muscle cells, resulting in a clear model (Fischle et al. 2001; Fischle et al. 2002). Upon HDAC phosphorylation by upstream kinases, such as AMPK (McGee et al. 2008), the association of 14-3-3 (an anchor protein) with class II HDACs results in the sequestration of these complexes in the cytoplasm. Loss of this interaction allows HDAC4 and HDAC5 to translocate to the nucleus, where they can interact with other corepressor proteins to repress gene expression (Grozinger and Schreiber 2000; Bertos et al. 2001; Nishino et al. 2008).

3.2.a.iii Histone Methylation

Histone methylation takes place on the side chains of both lysine (K) and arginine (R) residues. Histone methylation is a reversible process that is

catalysed by histone methyltransferases (HMT), whereas, histone demethylation is catalysed by histone demethylases, such as lysine demethylase 1 (LSD1) or Jumanji domain-containing proteins (Shi et al. 2004; Hong et al. 2007). In contrast to histone acetylation, which is tightly associated with activation, the impact of histone lysine methylation on gene expression is dependent on the specific lysine residue, i.e., the modification is not associated with a single output, and the effect on gene expression is context-dependent. Furthermore, these residues can be mono-, di- or tri-methylated, and some histones exhibit permissive and repressive K methylations (Reik 2007). For example, genome-wide profiles of histone methylation show that H3K4 and H3K36 methylations are associated with transcriptionally permissive chromatin, whereas H3K9 and H3K27 methylation are markers of transcriptionally silent chromatin (Barski et al. 2007). Histone lysine methylations are removed by histone demethylases; however, only those enzymes related with demethylase activity on H3 have been identified (Trojer and Reinberg 2006).

3.2.b Epigenetics and Atherosclerosis

Epigenetics provides an attractive explanation how diet, environment and lifestyle may contribute to disease. In principle, epigenetics explains how such external factors can impose aberrant gene expression patterns in an individual's lifetime and even transgenerationally. One of the earliest studies linking DNA methylation to atherosclerosis showed that the extracellular

superoxide dismutase (ec-SOD) gene was hypomethylated in atherosclerotic lesions in rabbits (Laukkanen et al. 1999). The importance of DNA methylation as a contributing factor to the pathogenesis of atherosclerosis is underscored by a study linking global DNA hypermethylation with a predisposition to atherosclerosis (Stenvinkel et al. 2007). Clinical correlation studies suggest that global DNA hypermethylation is strongly associated with surrogate markers of inflammation such as C-reactive protein and increased mortality in chronic kidney disease patients. Importantly, hypermethylation was found to be the strongest independent risk factor for CVD mortality for these patients (Stenvinkel et al. 2007). In the case of CAD, increases in global DNA methylation associated with disease was also identified (Sharma et al. 2008). Furthermore, a positive correlation was found between global DNA methylation and homocysteine levels, a known independent risk factor for CAD (Sharma et al. 2008). This study underscores the potential effect DNA methylation can have with respect to disease development and outcome. However, the specific genes targeted by DNA methylation were not investigated, and thus the precise mechanisms that induce DNA methylase activity remain to be elucidated.

Although DNA methylation is one of the most investigated aspects of epigenetic research, studies are now performed to a greater extent on the specific contribution of histone modifications. In fact, research conducted by Hastings et al. demonstrated a direct link between epigenetic histone

modifications and atherosclerosis. They demonstrated that atheroprone flow induced changes in SMC differentiation markers at the chromatin level that included decreased histone H4 acetylation and serum response factor (SRF) binding at the smooth muscle α-actin promoter region. These epigenetic changes were also associated with differentially regulated phenotypes for ECs and SMCs (Hastings et al. 2007). Interestingly, components relevant for the development of atherosclerosis, such as OxLDL, reduced the expression of HDAC1 and HDAC2. These results were confirmed *in vivo* in that decreased expression of HDAC2 in ECs was found in atherosclerotic plaques of human coronary arteries (Dje N'Guessan et al. 2009). In a similar report, a relative reduction of eNOS expression was found in atherosclerosis-prone regions of the mouse aorta compared to normal mouse aorta (Won et al. 2007). These studies were recapitulated *in vitro* using disturbed flow pattern models. While epigenetic pathways have been confirmed in the cell specificity of eNOS expression (Chan et al. 2004), epigenetic pathways may, in part, determine the susceptibility of different regions of the vascular system to atherosclerosis. Whether epigenetic pathways underlie these differences in eNOS expression is presently under investigation (Teichert et al. 2008; Fish et al. 2009). Collectively, these data show the importance of histone modifications in atherosclerosis.

3.3 Materials and Methods

3.3.a Cell Culture and Pharmacologic Reagents

See section 2.3.a.

3.3.b Pulsatile Laminar Flow Experiments

See section 2.3.c.

3.3.c Plasmids and Transient Transfection

HUVECs were transfected with the wild-type human HDAC5 construct (HDAC5 in pCS4-3myc), which was kindly provided by Suk-Chul Bae (Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju, South Korea). For high-efficiency transfection, primary HUVECs were transfected by a nucleofection technique (Nucleofector Solution; Amaxa Inc., Gaithersburg, MD). HUVECs at passages 4 to 10 were used. Cells (0.5–1 x 10^6) at 90% confluence were trypsinized, pelleted, and resuspended in 100 µL solution for HUVECs (Amaxa Inc.). Cell suspensions were mixed with 4 µg of highly purified plasmid DNA (EndoFree Plasmid Maxi Kit; Qiagen), and the entire suspension was transferred to an Amaxa cuvette. The cells were subjected to electroporation using the nucleofector program A-034 (Nucleofector I Device; Amaxa). Medium EGM2-MV (Clonetics) was then added, and the cells were plated and incubated in 5% CO_2 at 37°C. Transfection efficiency was confirmed by visualization of GFP (in cells transfected with a GFP-encoding plasmid) and found to be approximately 60 to 70% efficient.)

3.3.d Reverse Transcriptase-Quantitative PCR

The procedures and primer sequences for the RT-qPCR were described in section 2.3.d.

3.3.e RNA Interference

HUVECs were reverse transfected with 400 pmol si-HDAC5 (Santa Cruz Biotech) using the siPORT *NeoFX* transfection reagent according to the manufacturer's protocol (Ambion, Austin, TX). Cells were incubated with the siRNA-transfection reagent complex in 2 mL of culture media on a glass slide for 12 hr. Fresh culture media was then added and replaced again at 24 hr, after which cells were used in PS flow experiments.

3.3.f Chromatin Immunoprecipitation

Chromatin was harvested from six 75x38mm slides (~$1.5x10^6$ cells) of passage 5-8 HUVECs after shearing or chemical treatment, as well as untreated controls. Cells were then crosslinked with 1% formaldehyde (Sigma) for 10 min at room temperature with rocking. The crosslinking procedure was quenched with the addition of 0.125M glycine for 10 min at room temperature followed by four washes with ice-cold PBS. The cells were collected and centrifuged at 700 x *g* for 8 min at 4°C, after which the pellet was snap-frozen in liquid nitrogen and stored at -80°C until further use. The frozen cell pellets were thawed on ice and resuspended in lysis buffer (1% NP-40, 1M KOH, pH 7.9, 85 mM KCl, 1 mM EDTA, pH 8.0) with protease inhibitors (Roche Complete, EDTA-free Protease Inhibitor Cocktail Tablets) and 1 mM PMSF on ice for 10 min. After centrifugation (700 x *g* for 5 min at 4°C), nuclear pellets

were resuspended in nuclear lysis buffer (50 mM Tris/HCl, pH 7.4, 1% SDS, 0.5% Empigen BB (Sigma), 10 mM EDTA, pH 8.0) with protease inhibitors (Roche Complete, EDTA-free Protease Inhibitor Cocktail Tablets) and 1 mM PMSF. Isolated nuclei were subjected to 10-sec sonication pulses on ice at a power setting of 13 W (out of 20) on a 60 Sonic Dismembranator (Fisher Scientific, Pittsburgh, PA). The lysates were cleared by centrifugation (16,000 x *g*; 5 min at 4°C). Uniformity of the sonication treatment was confirmed by reversing the crosslinking and running recovered DNA on an agarose gel. Optimal sheared chromatin fragment sizes were targeted to approximately 250-400 bp (Figure 3.1).

Equal aliquots of isolated chromatin (~1.5-2 x10^6 cells) were diluted 1:5 in dilution buffer (20 mM Tris/HCl, pH 7.4, 100 mM Na Cl, 2 mM EDTA, pH 8.0, 0.5% Triton X-100, protease inhibitors (Roche Complete, EDTA-free Protease Inhibitor Cocktail Tablets) and 1 mM PMSF, and subjected to a 4°C overnight immunoprecipitation with various antibodies (1 μg antibody per ~1 x 10^6 cells). The antibodies used for chromatin immunoprecipation were HDAC4, HDAC5 (Santa Cruz Biotech), H3K4me3 (Abcam), and H3K9K14acet (Millepore). The immunoprecipitates were recovered by adding Protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) preblocked with 1 mg/mL salmon sperm to the binding reaction for 1 hr at 4°C. The immunoprecipitated complexes (i.e., the bead–antibody–protein–target DNA sequence complex) were collected and sequentially washed in buffers with increasing NaCl

concentrations (20 mM Tris/HCl, pH 7.4, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150-500 mM NaCl) to remove non-specifically bound chromatin. The protein-DNA complexes were eluted from the beads (0.1M $NaHCO_3$, 1% SDS), and the protein-DNA crosslinks were then reversed (incubation at 65°C overnight) and proteins removed by digestion with Proteinase K (0.5 μg/μL; 37°C for 2 hr; Sigma). Input DNA and DNA associated with specific immunoprecipitates or with negative control immunoglobulin G were purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA), and 2-4 μL were used as a template for qPCR to amplify genomic promoter regions of various genes (Table 3.1).

Table 3.1: Sequences of the genomic primers used for ChIP RT-qPCR.

	Forward 5'-3'	Reverse 5'-3'
MCP-1	TCCTGGAAATCCACAGGATG	GTGCGAGCTTCAGTTTGAGA
VCAM	AGCTTCAGCAGTGAGAGCAA	CAGATACCGCGGAGTGAAAT
SELE	GGGAAAGTTTTTGGATGCCATT	TGTCCACATCCAGTAAAGAGGAAAT

RT-qPCR was performed in triplicate with the iQ SYBR Green supermix (Bio-Rad) on the MyiQ Real-time PCR Detection System (Bio-Rad). Immunoprecipitated DNA was normalized by 5% input DNA and the relative folds of treated samples were obtained by normalization with the appropriate time-matched, untreated static control.

3.3.g HDAC Activity Assay

At the conclusion of the experiment, control, sheared or TNFα-treated HUVECs were washed twice with ice-cold PBS and lysed directly on the slide with a modified radio-immunoprecipitation assay (RIPA) buffer (1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0, 5 mM sodium fluoride) containing a protease inhibitor cocktail (Roche Complete, EDTA-free Protease Inhibitor Cocktail Tablets) for 20 min on ice. After centrifugation for 10 min at 14,000 RPM (4°C) to remove cellular debris, the nuclear extract was separated from the cytoplasmic fraction using a nuclear/cytosol fractionation kit (Biovision, Mountain View, CA). The protein content of each fraction was determined according to the Bradford method using a spectrophotometer (Hitachi U-2000). HDAC activity was assessed with 50 μg of each fraction using a fluorometric HDAC activity assay kit (Abcam). HDAC activity (fluorescence emission at 460 nm) was quantified in a fluorescence plate reader (SpectraMaxM5, Molecular Devices, Sunnyvale, CA) using the software SoftMaxPro 5.2 and expressed as a nuclear/cytoplasmic ratio fold over time-matched controls.

3.3.h Statistical Analyses

See section 2.3.g.

3.4 Results

3.4.a Proximal promoter changes in acetylation of DNA-bound histones under flow

Generally, histone hyperacetylation leads to gene activation or up-regulation, while histone hypoacetylation leads to chromatin condensation and subsequent inactivation or downregulation of genes. Since the expression of MCP-1 and VCAM is repressed under PS flow, I hypothesized that epigenetic changes of histone hypoacetylation associated with transcriptional repression could account for this flow inhibition of expression. Using chromatin immunoprecipitation (ChIP) combined with RT-qPCR, I examined the residency of acetylated histone H3 (H3K9K14cet) across the MCP-1 and VCAM proximal promoters in HUVECs exposed to 24-hr PS flow (12 ± 4 dynes/cm^2) or 30 min TNFα (10 ng/mL). At approximately 200 bp upstream of the transcriptional start site (TSS), both MCP-1 and VCAM promoters showed hypoacetylation of histone H3 under PS flow compared to static control and TNFα-treated HUVECs (Figure 3.2). In ECs under TNFα treatment, these promoters displayed a hyperacetylated status that is strongly associated with transcriptional activation. This PS-induced hypoacetylated status of the MCP-1 and VCAM promoters associated with gene transcriptional inactivation is in keeping with the hypothesized PS-inhibition of these genes.

3.4.b PS induces enrichment of the corepressor HDAC5 to inflammatory proximal promoters

Recruitment of histone deacetylases (HDACs) to a promoter can cause

localized histone deacetylation to result in a compact chromatin structure and gene silencing (for review see (Marmorstein 2001)). As such, HDACs mostly function as transcriptional repressors (Ng and Bird 2000). Thus, I asked whether the PS-induction of histone H3 hypoacetylation on MCP-1 and VCAM proximal promoters involved the recruitment of Class II histone-modifying enzymes (HDAC4 or HDAC5) to the MCP-1 and VCAM promoters. To determine whether HDAC4 or HDAC5 is differentially recruited to the MCP-1 and VCAM promoters, I used ChIP against the respective HDACs on HUVECs exposed to PS or TNFα treatment. While PS flow did not differentially recruit HDAC4 to the MCP-1 or VCAM promoters, treatment of HUVECs with TNFα, a robust stimulator of inflammatory gene transcription, resulted in a significant reduction of HDAC4 recruitment at the VCAM promoter that is linked with VCAM induction (Figure 3.3A). Notably, PS flow significantly induced HDAC5 recruitment to the MCP-1 and VCAM proximal promoters (Figure 3.3B). This recruitment of a relevant modifying enzyme provides an explanation for the flow-induced hypoacetylation status of histone H3 seen on the MCP-1 and VCAM promoters.

3.4.c PS induces global HDAC activity independent of HDAC5 expression levels

Next, I queried whether flow-induced recruitment of HDAC5 is paralleled with an increase in HDAC activity. HUVECs were subjected to PS or TNFα treatment, and the nuclear and cytoplasmic fractions analyzed for HDAC activity using a commercial HDAC fluorescent activity assay kit

according to protocol. Global HDAC activity level was increased under flow compared to basal and cytokine-treated levels (Figure 3.4A). To test whether the PS-induced increase in enzymatic activity is due to increased recruitment of HDAC5 rather than an overall increase in HDAC5 expression, HUVECs were exposed to 24 hr of PS flow or 30 min TNFα treatment, and total RNA was isolated for RT-qPCR analysis. Neither PS nor TNFα treatment significantly altered HDAC5 expression levels (Figure 3.4B). These results demonstrate that the flow-induced HDAC enzymatic activity occurs independently of HDAC5 gene expression and correlates with the increased enrichment of HDAC5 at the MCP-1 and VCAM promoters (Figure 3.3B).

3.4.d Activation of AMPK recruits corepressor HDAC5 to inflammatory promoters

AMPK activation has been shown to be sufficient to inhibit the gene expression of MCP-1 and VCAM (Figure 2.3). To determine whether the AMPK-dependent repression of MCP-1 and VCAM gene expression involves the recruitment of the corepressor HDAC5, I utilized AICAR as a pharmacological activator of AMPK to determine the effects of AMPK activation on the enrichment of the corepressor HDAC5 on MCP-1 and VCAM proximal promoters. HUVECs were incubated with 1 mM AICAR for 4 hr followed by ChIP analysis. HDAC5 was immunoprecipitated from nuclear extracts and segments of promoter DNA amplified by RT-qPCR. The results revealed that 1 mM AICAR is sufficient to recruit the corepressor HDAC5 to both the MCP-1 and VCAM proximal promoters (Figure 3.5). In concert with

the AMPK inhibition of the expressions of these genes, these findings suggest an essential role for AMPK in the regulation of transcriptional repression of inflammatory genes through the recruitment of HDAC5.

3.4.e AMPK-dependent pathway mediates shear stress recruitment of corepressor HDAC5 to inflammatory promoters

The data from sections 3.4.b and 3.4.d demonstrated that both PS and AMPK promote the recruitment of corepressor HDAC5 to the MCP-1 and VCAM proximal promoters, concomitant with repression of inflammatory gene expression. To investigate the role of AMPK in the PS-induced HDAC5 enrichment at MCP-1 and VCAM promoters, I studied the effects of inhibition of AMPK activity using Compound C. HUVECs were incubated with 15 μM Compound C for 30 min before the cells were subjected to 24-hr PS flow and followed by ChIP analysis. HDAC5 was immunoprecipitated from nuclear extracts and segments of promoter DNA amplified by RT-qPCR. The results from subsequent RT-qPCR analysis demonstrates that the pharmacological inhibition of AMPK significantly blocked HDAC5 recruitment by PS flow (Figure 3.6), thus establishing that AMPK activity is essential in the PS-induced enrichment of HDAC5 on the MCP-1 and VCAM proximal promoters.

3.4.f AMPK plays a role in the flow-induction of histone H3 hypoacetylation on inflammatory promoters

The results from section 3.4.e suggest that AMPK activity lies upstream to and mediates the HDAC5 recruitment under flow. To evaluate whether AMPK participates in the flow-induction of histone hypoacetylation, I used the

inhibitor Compound C to inhibit the activation of AMPK. Utilizing the same protocol and conditions set forth in section 3.4.e, the binding of acetylated histone H3 (H3K9K14acet) on MCP-1 and VCAM promoters was assessed via ChIP analysis and DNA amplification of promoter regions. In ECs subjected to 24-hr PS flow, Compound C significantly reversed the hypoacetylation on these promoters (Figure 3.7) suggesting that AMPK mediates the flow-induction of histone hypoacetylation most likely through its concurrent regulation of the deacetylase enzyme HDAC5.

3.4.g Forced expression of HDAC5 further attenuates flow-inhibition of inflammatory gene expression

The results reported above suggest that PS flow induces repression of inflammatory gene expression through the AMPK-mediated recruitment of the HDAC5 transcriptional repressor. To assess whether HDAC5 can modulate inflammatory gene repression in response to PS flow, I transfected HUVECs with expression plasmids encoding myc-tagged, wild-type HDAC5 (myc-HDAC5-wt) for 24 hr before exposure to PS flow (12 ± 4 dynes/cm^2). The expression of HDAC5-wt in static control ECs was markedly increased at both the protein (Western blot; Figure 3.8A) and gene levels (RT-qPCR; Figure 3.8B) by the myc-HDAC5-wt transfection. The forced expression of HDAC5-wt further attenuated MCP-1 and VCAM expression under flow (Figure 3.9A & 3.9B, respectively) demonstrating that HDAC5 plays a significant role in the flow-induced anti-inflammatory actions.

3.4.h Corepressor HDAC5 is not required for flow-induced inhibition of inflammatory gene expression

I next evaluated whether HDAC5 is required for the PS-induced inhibition of inflammatory gene expression. HUVECs were transfected with siRNA oligonucleotides against HDAC5, and exposed to PS flow (24 hr; 12 ± 4 dynes/cm^2) 24 hr after transfection. The knockdown effect on HDAC5 expression in static ECs was assessed by RT-qPCR (Figure 3.10A). Unexpectedly, knockdown of HDAC5 did not derepress the PS-inhibition of MCP-1 or VCAM expression (Figure 3.10B), indicating that while HDAC5 is sufficient for flow inhibition effects (Figure 3.9), it is probably not necessary. However, the HDAC5 knockdown reduced its expression by only less than 40% (Figure 3.10A), therefore, the possibility of HDAC5 being a necessary player cannot be ruled out. It is also possible that there may be other corepressors in the complex which are compensatory or have stronger deacetylase activity.

3.5 Discussion

Epigenetic mechanisms posit that the overall transcriptional output of a cell depends on the coordinated activation or silencing of gene expression by the action of DNA-bound or associated transcriptional activators or repressors. Recent studies suggest a key role for epigenetics in the pathogenesis of human disease, especially those involving inflammatory responses and have established epigenetic pathways as fundamental determinants of EC gene expression (Chan et al. 2004; Sharma et al. 2008; Dje N'Guessan et al. 2009). Although the transcription of several inflammatory genes, including MCP-1 and VCAM, is decreased upon exposure to long-term PS flow, it is not clear what are the underlying mechanisms. The role of coactivator proteins in transcriptional regulation is well established, but the equally important role of corepressor proteins in gene regulation has become apparent only relatively recently. Hence, I investigated the possible biological role of epigenetic mediators in the flow-induced repression of inflammatory genes involved in atherogenesis.

Histone hyperacetylation leads to a more relaxed chromatin structure to enhance transcriptional activation, whereas histone hypoacetylation changes leads to chromatin condensation and subsequent inactivation or downregulation of genes (Shahbazian and Grunstein 2007). I considered whether alterations to the histone code of the MCP-1 and VCAM genes play a role in the downregulation of transcription that occurs following exposure of

ECs to long-term PS flow. Indeed, under 24-hr PS flow, I show that histone H3 is hypoacetylated at lysine residues 9 and 14 (H3K9K14acet) at the MCP-1 and VCAM proximal promoters (Figure 3.2). Another mark of actively transcribed genes includes histone H3 lysine 4 trimethylation (H3K4me3) (Santos-Rosa et al. 2002), and of note, this histone mark has been demonstrated to colocalize with histone H3K14acet (Nakanishi et al. 2008). In this dissertation research, H3K4me3 also shows a significantly decreased enrichment fold at the MCP-1 and VCAM promoters under 24-hr PS flow compared to static control (data not shown). Hence, a specific histone code exists at these regions in ECs that is associated with transcriptional repression. Thus, the reduced acetylation (H3K9K14acet) and methylation of histone H3 (H3K4me3) at the promoters of the MCP-1 and VCAM genes play important parts in this flow-induced repression. My results suggest that PS flow negatively regulates gene expression, in part, through chromatin-based pathways.

As discussed in section 3.2.a.ii, HDAC recruitment to a promoter can result in localized histone hypoacetylation and transcriptional inhibition. In accordance with the finding of PS-induced histone hypoacetylation (Figure 3.2), I demonstrate that HDAC5 is differentially recruited to the MCP-1 and VCAM promoters under PS flow (Figure 3.3B). One possible mechanism by which the HDAC proteins exert their corepressor function is by modifying the chromatin structure through their HDAC activity. I show that the flow-induced

recruitment of HDAC5 is accompanied by a global increase of HDAC activity in the nuclear compartment (Figure 3.4A). This global increase is independent of HDAC5 expression (Figure 3.4B). The PS-induced recruitment of HDAC5 and increased HDAC activity provide a viable rationale for the histone H3 hypoacetylation seen on the MCP-1 and VCAM promoters under flow.

Since AMPK mediates the PS-induced repression of inflammatory gene expression (Figure 2.4), I examined whether AMPK activity was critical for mediating the PS-induced epigenetic responses. A pharmacologic agonist and an inhibitor were used to manipulate the activation of AMPK. Using the AMPK inhibitor Compound C, I establish that AMPK regulates PS-induced histone hypoacetylation at the MCP-1 and VCAM proximal promoters (Figure 3.7). I also confirm that AMPK activity is both sufficient (Figure 3.5) and necessary for HDAC5 recruitment to these promoters (Figure 3.6). My results suggest that AMPK-mediated HDAC5 recruitment and H3 hypoacetylation regulates the flow-inhibition of MCP-1 and VCAM genes. Interestingly, inhibition of AMPK activation not only reverses the PS-induced inhibition of VCAM expression but actually raises the levels to higher than the basal transcription level (Figure 2.4). The reason for this is not clear; it is possible that AMPK is acting in a manner to sequester other endogenous HDAC or corepressor proteins, thus causing the derepression of transcription.

My results demonstrating the AMPK-mediated regulation of HDAC5 recruitment (Figure 3.5) are in contrast to the report by McGee et al. (McGee

et al. 2008), who showed that AMPK upregulated the GLUT4 gene via HDAC5 and that AICAR reduced HDAC5 recruitment to the GLUT4 promoter concomitant with increases in GLUT4 gene expression. In contrast, my results show that in HUVECs, AICAR treatment increases HDAC5 association with both MCP-1 and VCAM promoters (Figure 3.5) concordant with repression of gene expression (Figure 2.3). Whether the difference in cell types accounts for the disparity between the two data sets is not known at present, though it is possible that temporal effects can account for this discrepancy, since the study by McGee et al. was conducted on human primary myotubes under 1 hr AICAR incubation compared to the present study performed on HUVECs under 4 hr AICAR treatment.

Using a flow system analogous to that used in my study, Wang et al. showed that 1-hr fluid shear stress (24 dynes/cm^2) induced HDAC5 phosphorylation and subsequent nuclear export, thus removing the inhibitory effect of HDAC5 on KLF2 expression in HUVECs (Wang et al. 2010). However, in my system, 24-hr PS flow downregulates HDAC5 phosphorylation and retains HDAC5 in the nucleus (data not shown), which would support my hypothesis that PS-induced MCP-1 and VCAM repression is mediated by epigenetic changes. The variance in HDAC5 localization is likely due to the temporal effects of flow, and though the time factor was not investigated here, it would be of great interest to examine these differences more extensively.

HDAC5 has been shown to be involved in the transcriptional repression

of various genes involved in atheroprotection (Kumar et al. 2005) and myogenesis (Lemercier et al. 2000; McKinsey et al. 2000), hence, I postulated that HDAC5 would play a role in PS-induced MCP-1 and VCAM repression. Indeed, the data herein indicate that overexpression of HDAC5 is sufficient to further accentuate the flow-induced inflammatory gene repression (Figure 3.9) demonstrating that HDAC5 does play an important role. Despite previous reports demonstrating HDAC5 involvement in transcriptional repression (Lemercier et al. 2000; McGee et al. 2008), knockdown of HDAC5 did not abrogate the PS-inhibition of MCP-1 or VCAM expression (Figure 3.10) suggesting that HDAC5 may not be essential to the flow-induced anti-inflammatory actions. Conceivably, other corepressor proteins in the complex may possess stronger deacetylase activity. It is of note that the deacetylase domain on class II HDACs lacks enzymatic activity; instead relying on its recruitment of class I HDACs for deacetylase effects (Fischle et al. 2002). Therefore, perhaps HDAC5 relies on complexing with HDAC3 in order to gain the full deacetylase effects on resident histones. In addition, other corepressors known to complex with HDAC5 such as NCoR and SMRT (Fischle et al. 2002) may also play a prominent role in the repressive effects of flow. Thus, additional studies are needed to determine a more comprehensive picture of the corepressor complex recruited to inflammatory gene promoters under PS flow.

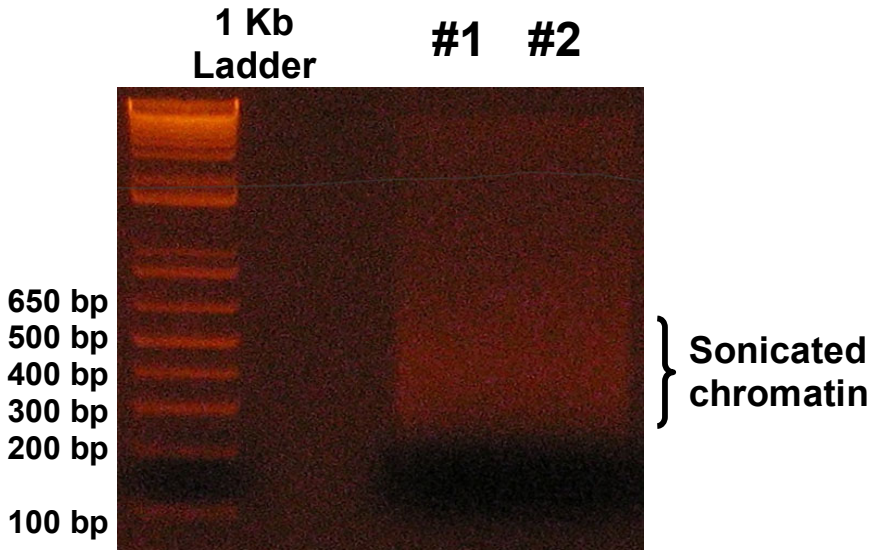


Figure 3.1: DNA agarose gel showing the size of sonicated chromatin of two samples.

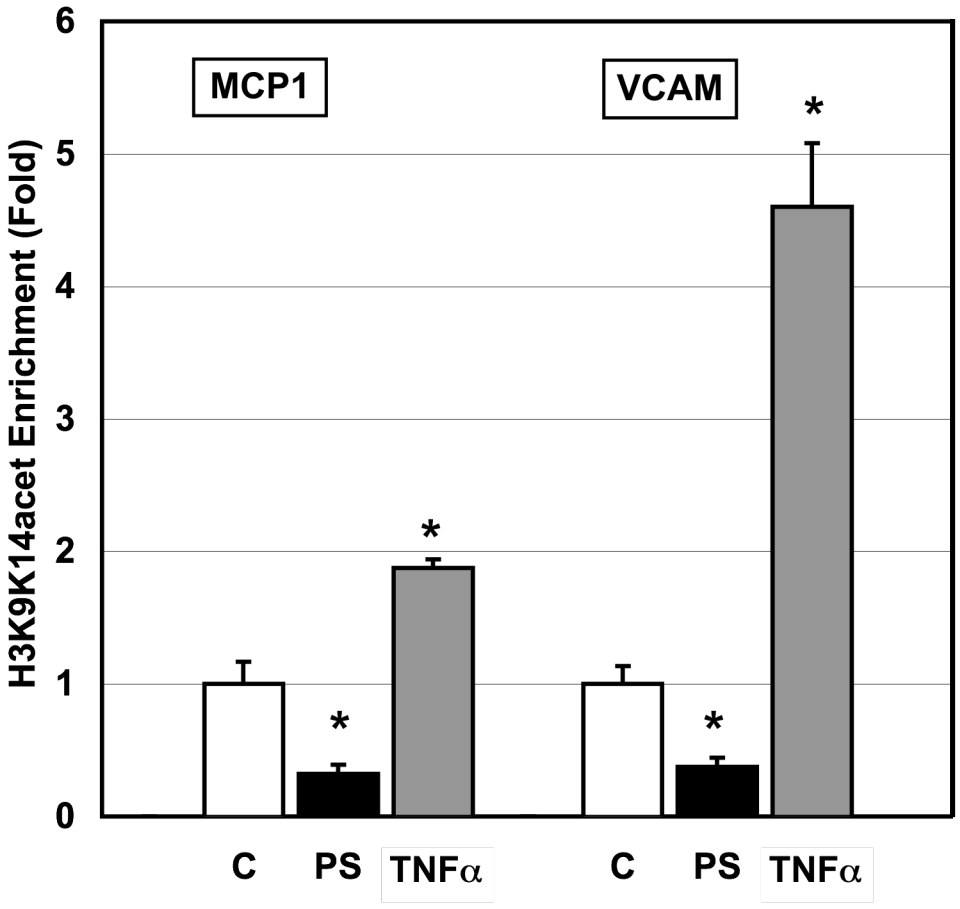


Figure 3.2: Effects of PS and TNFα on acetylated histone 3 (H3K9K14acet) enrichment profile. HUVECs were exposed to PS flow (12 ± 4 dynes/cm^2) for 24 hr (PS), 30 min TNFα (10 ng/mL; TNFα) or left untreated (C). The results show ChIP analysis of H3K9K14acet binding to the MCP1 and VCAM promoters. H3K9K14 was immunoprecipitated from nuclear extracts and segments of promoter DNA were amplified by RT-qPCR. Experimental samples (PS and TNFα) are normalized to corresponding inputs (nuclear extracts before immunoprecipitation) and time-matched static controls and represented as folds over static control (C). n=3, *p<0.05 vs. Control (C).

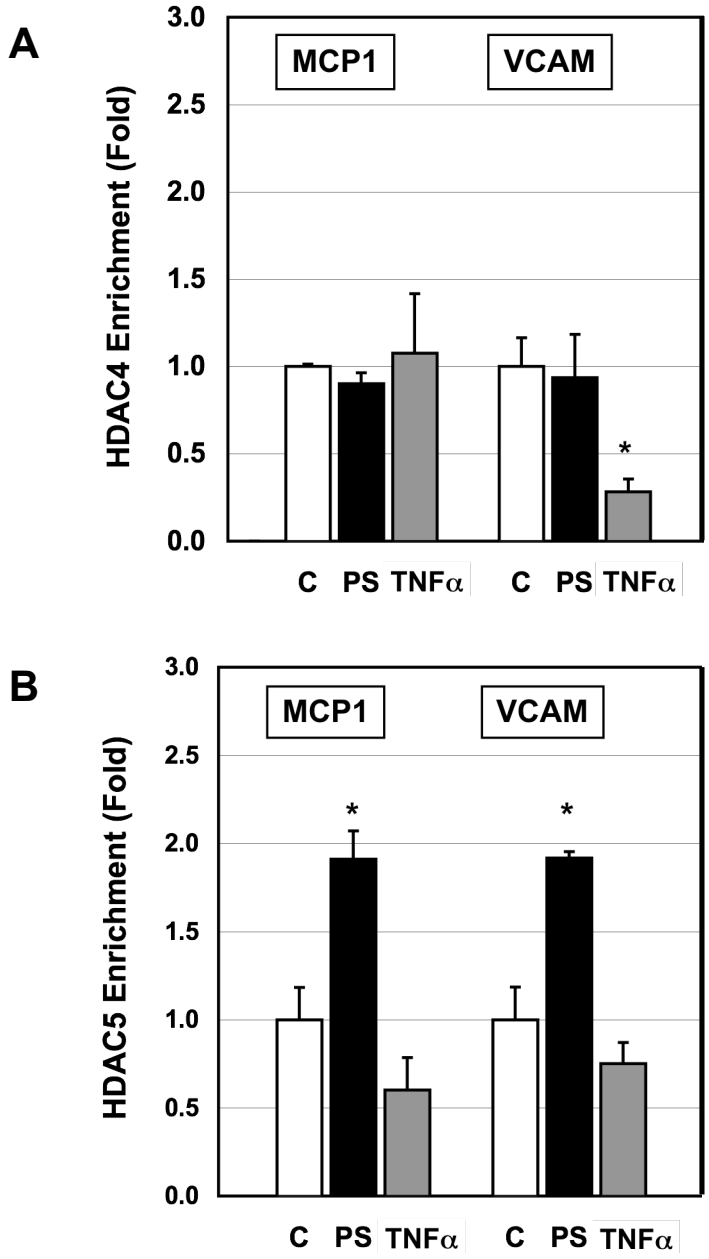


Figure 3.3: Effects of PS and TNFα on HDAC4 and HDAC5 enrichment profiles at inflammatory and adhesive molecule proximal promoters MCP1 and VCAM. HUVECs were exposed to PS flow (12 ± 4 dynes/cm^2) for 24 hr (PS), 30 min TNFα (10 ng/mL; TNFα) or left untreated (C). Panels show ChIP analysis of HDAC4 (A) and HDAC5 (B) binding to the MCP1 and VCAM promoters. HDAC4 and HDAC5 was immunoprecipitated from nuclear extracts and segments of promoter DNA were amplified by RT-qPCR. Experimental samples (PS and TNFα) are normalized to corresponding inputs (nuclear extracts before immunoprecipitation) and time-matched static controls and represented as folds over static control (C). n=3, *p<0.05 vs. Control (C).

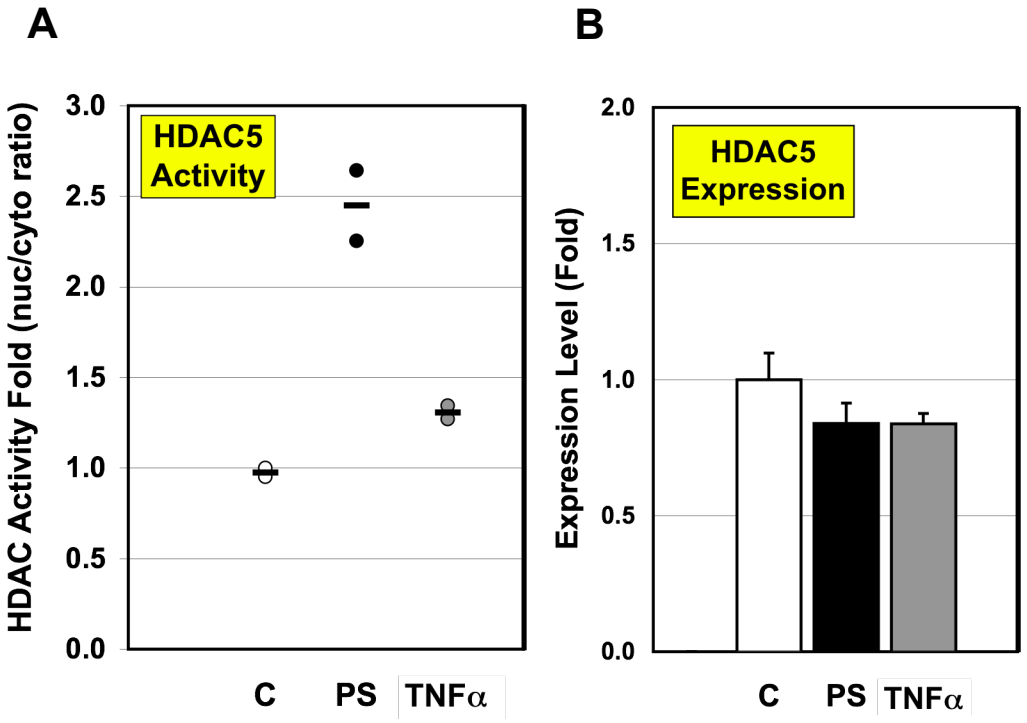


Figure 3.4: Effects of PS and TNFα on HDAC activity and HDAC5 expression profiles. HUVECs were exposed to PS flow (12 ± 4 dynes/cm^2) for 24 hr (PS), 30 min TNFα (10 ng/mL; TNFα) or left untreated (C). (Panel A) 50 μg each of nuclear and cytosolic fractions were isolated and used in an HDAC fluorescence activity assay kit. HDAC activity (fluorescence emission at 460 nm) was quantified using a fluorescence plate reader and expressed as fold over untreated (C). Panel A represents the average of 2 experiments, each performed in triplicate. (Panel B) RNA samples were isolated, and the levels of HDAC5 mRNA were determined and quantified by RT-qPCR with the results normalized by GAPDH. The data represent mean ± SEM from 3 independent experiments.

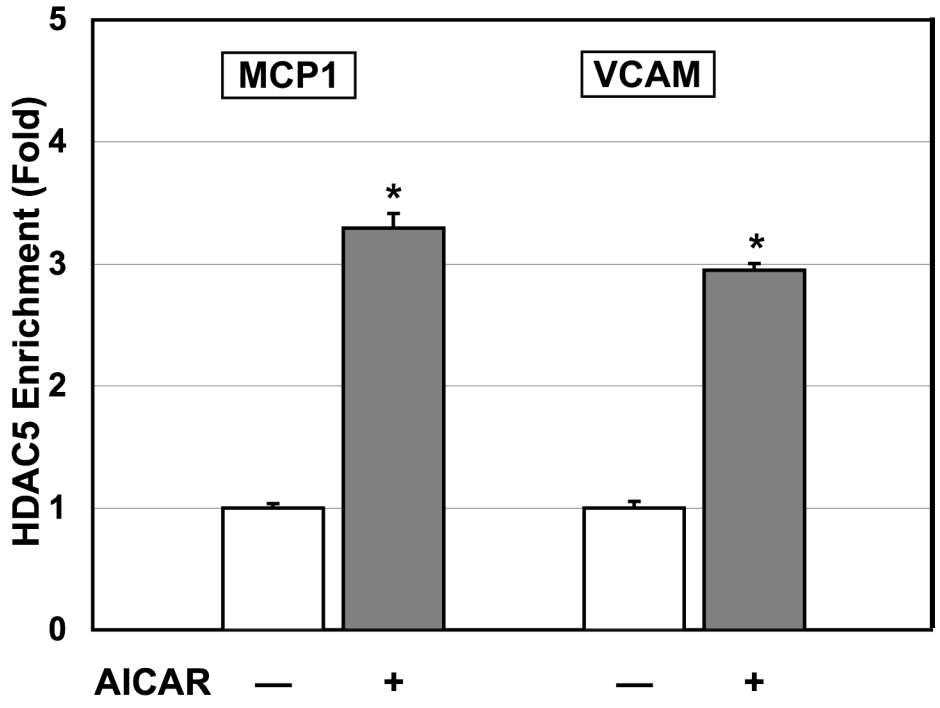


Figure 3.5: AMPK is sufficient to recruit HDAC5 to MCP1 and VCAM proximal promoters. HUVECs were exposed for 4 hr to either 1 mM AICAR (+) or vehicle (—). The results show ChIP analysis of HDAC5 binding to the MCP1 and VCAM promoters. HDAC5 was immunoprecipitated from nuclear extracts and segments of promoter DNA were amplified by RT-qPCR. Experimental samples are normalized to corresponding inputs (nuclear extracts before immunoprecipitation) and time-matched static controls and represented as folds over static control (C). n=3, *p<0.05 vs. vehicle.

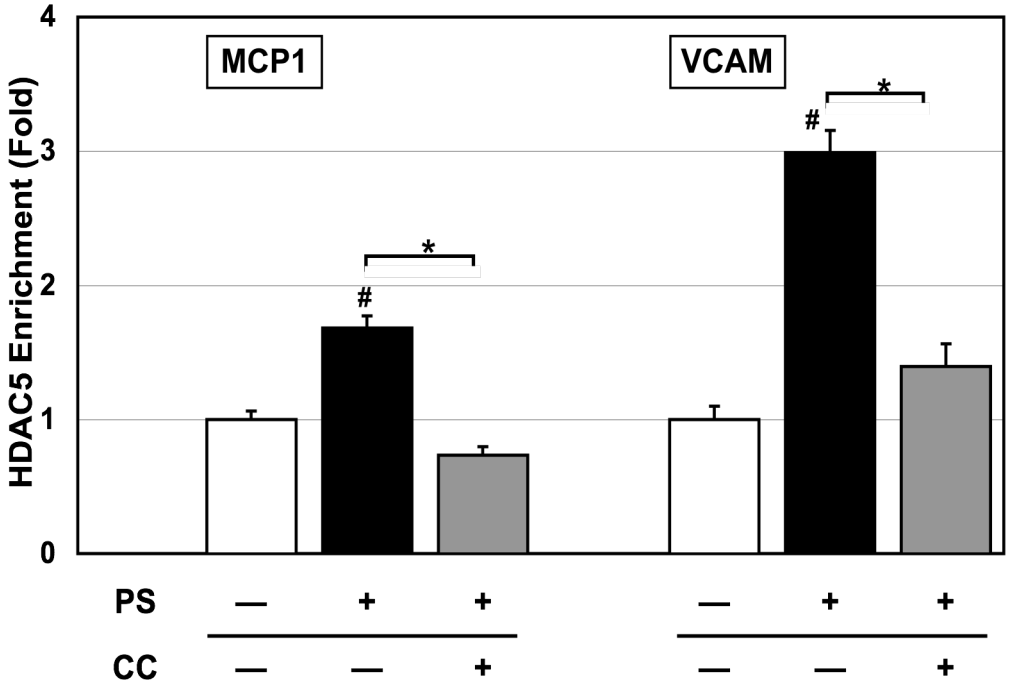


Figure 3.6: AMPK-dependent pathway mediates shear stress recruitment of corepressor HDAC5 to inflammatory promoters. HUVECs were exposed for 30 min to 15 μM Compound C (CC) or vehicle before exposure to PS flow (12 ± 4 dynes/cm^2) or static conditions for 24 hr. The results show ChIP analysis of HDAC5 binding to the MCP1 and VCAM promoters. HDAC5 was immunoprecipitated from nuclear extracts and segments of promoter DNA were amplified by RT-qPCR. Experimental samples are normalized to corresponding inputs (nuclear extracts before immunoprecipitation) and time-matched static controls and are represented as folds over static control. n=3, #p<0.05 vs. no shear,* p<0.05.

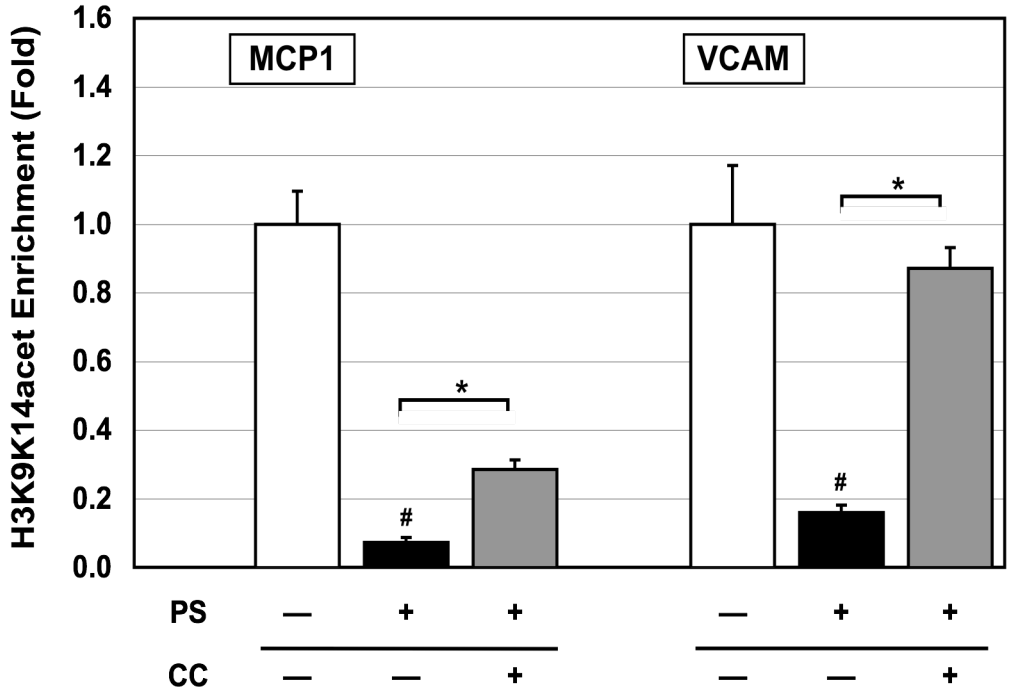


Figure 3.7: AMPK regulates PS-induction of histone hypoacetylation at MCP1 and VCAM proximal promoters. HUVECs were exposed for 30 min to 15 μM Compound C (CC) or vehicle before exposure to PS flow (12 ± 4 dynes/cm^2) or static conditions for 24 hr. The results show ChIP analysis of H3K9K14acet binding to the MCP1 and VCAM promoters. H3K9K14acet was immunoprecipitated from nuclear extracts and segments of promoter DNA were amplified by RT-qPCR. Experimental samples are normalized to corresponding inputs (nuclear extracts before immunoprecipitation) and time-matched static controls and are represented as folds over static control. n=3, #p<0.05 vs. no shear,* p<0.05.

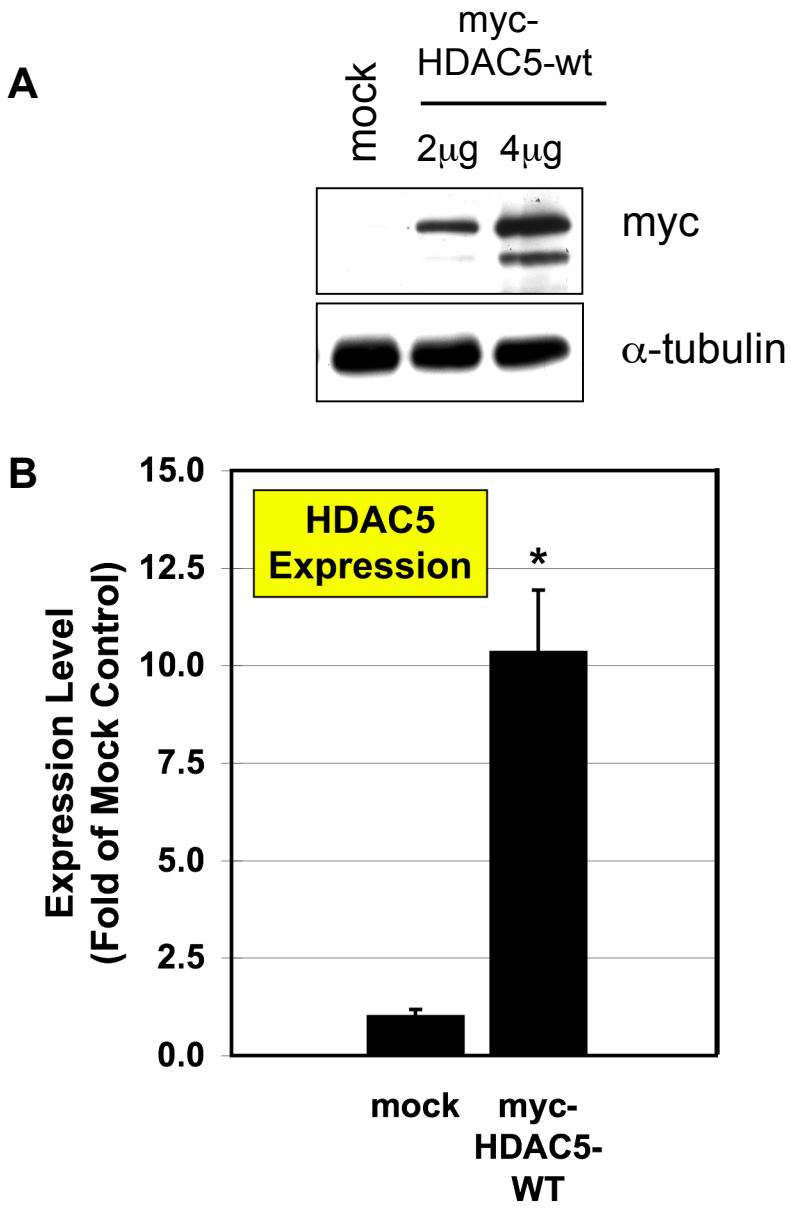


Figure 3.8: Protein and gene expressions of HDAC5 are markedly increased following HDAC5-wt transfection. HUVECs were electroporated with myc-HDAC5-wt or mock constructs for 48 hr. (A) ECs were lysed and separated by SDS-PAGE, and the membranes were probed with antibodies as indicated. (B) RNA samples were isolated, and the levels of MCP1 and VCAM mRNA were determined and quantified by RT-qPCR, with the results normalized by GAPDH.The data represent mean ± SEM from 3 independent experiments. *p<0.05 vs. mock.

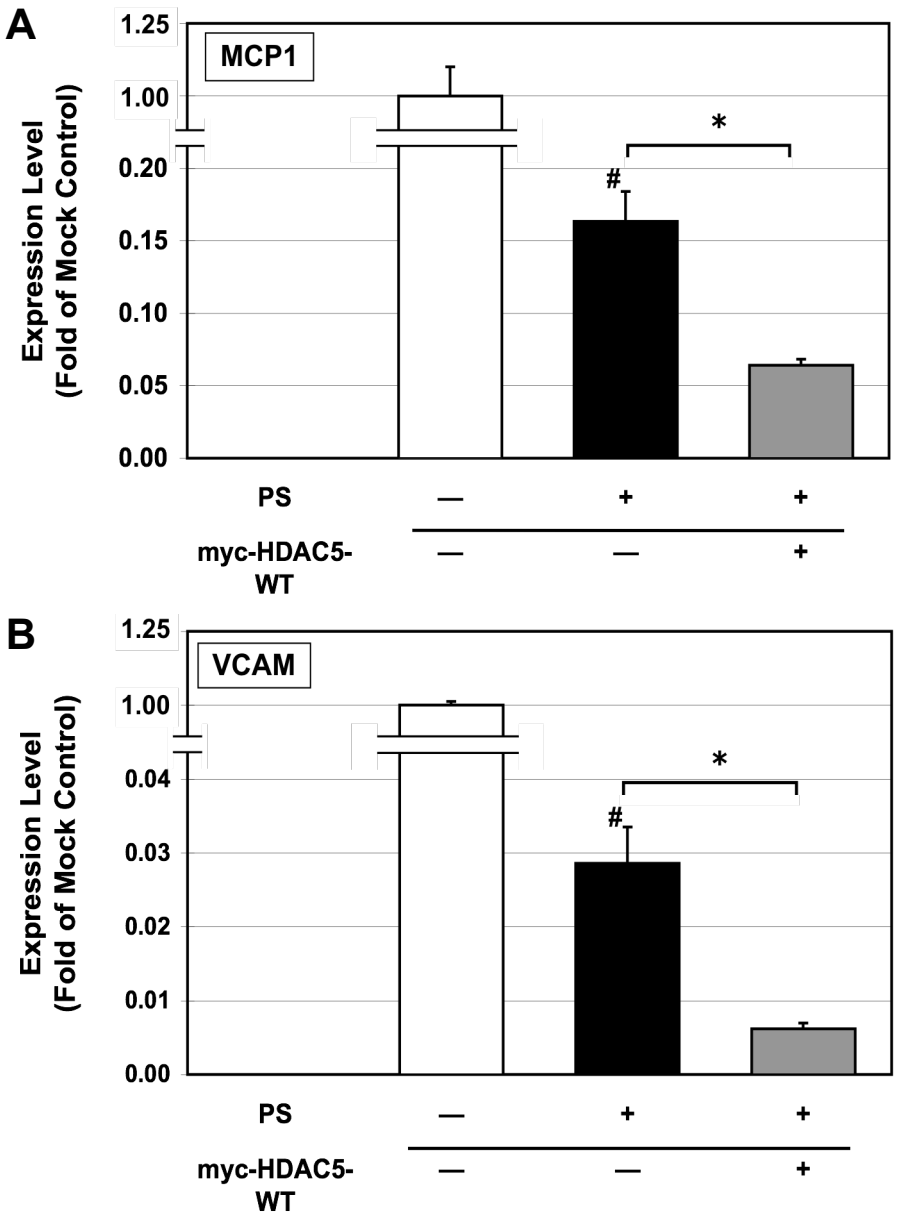


Figure 3.9: HDAC5 overexpression further accentuates the inhibition of MCP1 and VCAM expressions by PS flow. HUVECs were electroporated with myc-HDAC5-wt or mock constructs for 24 hr before exposure to PS flow (12 ± 4 dynes/cm^2) or static conditions for 24 hr. RNA samples were isolated, and the levels of MCP1 (A) and VCAM (B) mRNA were determined and quantified by RT-qPCR with the results normalized by GAPDH. The data represent mean ± SEM from 3 independent experiments. #p<0.05 vs. no shear, *p<0.05.

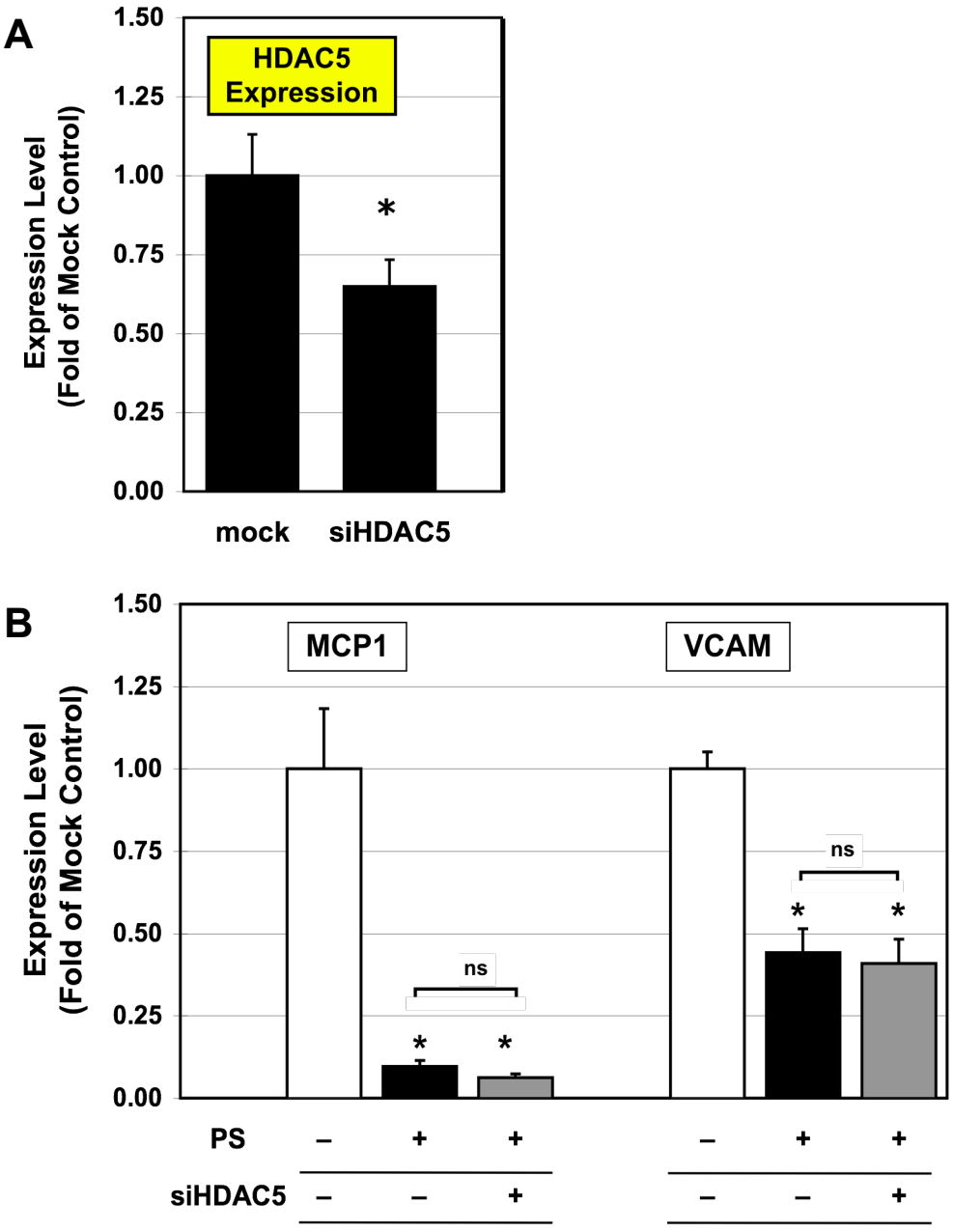


Figure 3.10: HDAC5 knockdown does not affect the PS-induced inhibition of MCP1 or VCAM expression. HUVECs were transfected with 400 pmol siHDAC5 constructs or mock-transfected for 24 hr before exposure to PS flow (12 ± 4 dynes/cm^2) or static conditions for 24 hr. RNA samples were isolated, and the levels of (A) HDAC5 mRNA or (B) MCP1 and VCAM mRNA were determined and quantified by RT-qPCR, with the results normalized by GAPDH. The data represent mean ± SEM from 6 independent experiments. *p<0.05 vs. mock or no shear, ns = not significant.

Chapter 4: Summary and Conclusions

The pathogenesis of atherosclerosis is a complex multifactorial process that involves vascular wall injury or dysfunction and atheroma formation due to local and systemic factors. Shear stress plays an important role in the pathogenesis of the atherosclerotic plaque. Vascular ECs are constantly exposed to fluid shear stress, the dragging force generated by blood flow. Substantial evidence suggests that fluid shear stress regulates vascular homeostasis and focal distribution of atherosclerosis by influencing endothelial gene expression (Berk et al. 2001). Studying the regulation of flow-mediated genes involved in EC function is therefore important for our understanding of flow atheroprotection.

Mechanisms of the anti-inflammatory action by laminar flow include the upregulation of atheroprotective mediators and transcriptional factors such as eNOS, KLF2 and thrombomodulin (TM) (Takada et al. 1994; Ranjan et al. 1995; Dekker et al. 2002). These proteins are potent anti-inflammatory and anticoagulant molecules and are thought to account for a large part for the anti-inflammatory properties of healthy ECs. But apart from the direct eNOS and TM induction by KLF2, the majority of the anti-inflammatory effects by these flow-induced mediators are most likely indirect. So while the upregulation of atheroprotective mediators by flow has been well studied and characterized, the mechanisms involved in the repression of inflammatory responses by mechanical stimuli have not been elucidated and was the focus

of this dissertation.

In this thesis, a systematic approach was used to study the mechanisms that mediate the anti-inflammatory effects of PS flow. I show that AMPK mediates the PS-induced HDAC5 enrichment and histone hypoacetylation at the MCP-1 and VCAM gene promoters. These AMPK-dependent epigenetic modifications are upstream to subsequent PS-induced inhibition of MCP-1 and VCAM gene expression and monocyte adhesion. This work provides a rational basis for investigating the mechanism of the anti-inflammatory responses induced by PS flow and their modulation by AMPK.

Although HDAC5 is known to serve as a negative regulator of the atheroprotective genes KLF2 and eNOS (Kumar et al. 2005; Wang et al. 2010), the present study provides the first demonstration that HDAC5 recruitment to inflammatory gene promoters is affected by PS flow in ECs. The PS-induced repression of MCP-1 and VCAM expression is preceded by chromatin marks that are associated with transcriptional repression. These marks include HDAC5 enrichment and histone H3 hypoacetylation at these proximal promoters. Also, experiments using pharmacologic inhibitors establish that the flow-induced histone hypoacetylation and HDAC5 recruitment are mediated by AMPK.

The results from this study provide further insight into the flow-induced regulation of AMPK activity. AMPK has been shown to regulate leukocyte–EC interactions, EC proliferation and endothelial gene expression including eNOS

and KLF2 expressions (Ewart et al. 2008; Chen et al. 2009; Young et al. 2009). In addition to manipulating the AMPK pathway, I demonstrate that PS flow and TNFα affect HDAC5 recruitment to modulate inflammatory gene expressions. Using a pharmacologic inhibitor, I confirm that HDAC5 recruitment and histone hypoacetylation on MCP-1 and VCAM promoters in response to flow requires AMPK activation. The anti-inflammatory actions mediated by these flow-induced epigenetic changes have functional importance as demonstrated by the inhibition of monocyte adhesion to ECs. My results are in concert with the concept that the gene–environmental interactions relevant for complex diseases such as atherosclerosis in which inflammation, proliferation, and remodeling play an important role, are regulated by epigenetic mechanisms (Curtis et al. 2004; Pons et al. 2009).

A potential future direction resulting from this research is to investigate the key histone methylated marks and their modifying enzymes that are strongly associated with transcriptional repression. H3K9me3 plays significant roles in gene silencing by recruiting repressors and cofactors, including HDACs and heterochromatin protein-1α (HP1α) (Lachner et al. 2003; Shilatifard 2006). In a transcriptional repression paradigm, El Gazzar et al. demonstrated that TNFα gene transcription silencing involved recruitment of HP1α and sustained H3K9 methylation on the TNFα promoter (El Gazzar et al. 2007). Since 24-hr PS flow can inhibit NF-kB activation (unpublished data) and downstream target genes, one might speculate whether the transcriptional

regulation of TNFα responsive genes (e.g., MCP-1 and VCAM) under flow involves chromatin remodeling at these inflammatory promoters that mediate changes through different but overlapping effector mechanisms.

In conclusion, I have identified a novel mechanism regulating the anti-inflammatory function of PS flow. Furthermore, my work shows that AMPK, in association with HDAC5 and histone H3 hypoacetylation, plays an important role in mediating flow-induced MCP-1 and VCAM gene repression and anti-inflammatory function. Since blocking the activity of HDACs has become a primary target for drug therapy in a number of diseases, it is important to understand what effect this may have on genes negatively regulated by flow and on the efficacy of the therapy itself. This study has contributed to the understanding of the anti-inflammatory mechanisms in vascular cells, especially on the roles of intracellular mediators and epigenetic elements in the PS-induced downregulation of inflammatory processes, which are implicated in the early development of atherosclerosis.

References

Barbee, K. A., P. F. Davies and R. Lal (1994). "Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy." Circ Res **74**(1): 163-71.

Barski, A., S. Cuddapah, K. Cui, T. Y. Roh, D. E. Schones, Z. Wang, G. Wei, I. Chepelev and K. Zhao (2007). "High-resolution profiling of histone methylations in the human genome." Cell **129**(4): 823-37.

Berger, S. L. (2007). "The complex language of chromatin regulation during transcription." Nature **447**(7143): 407-12.

Berk, B. C., J. I. Abe, W. Min, J. Surapisitchat and C. Yan (2001). "Endothelial atheroprotective and anti-inflammatory mechanisms." Ann N Y Acad Sci **947**: 93-109; discussion 109-11.

Bertos, N. R., A. H. Wang and X. J. Yang (2001). "Class II histone deacetylases: structure, function, and regulation." Biochem Cell Biol **79**(3): 243-52.

Boo, Y. C. and H. Jo (2003). "Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases." Am J Physiol Cell Physiol **285**(3): C499-508.

Cacicedo, J. M., N. Yagihashi, J. F. Keaney, Jr., N. B. Ruderman and Y. Ido (2004). "AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells." Biochem Biophys Res Commun **324**(4): 1204-9.

Carling, D. (2004). "The AMP-activated protein kinase cascade--a unifying system for energy control." Trends Biochem Sci **29**(1): 18-24.

Caro, C. G., J. M. Fitz-Gerald and R. C. Schroter (1971). "Atheroma and arterial wall shear. Observation, correlation and proposal of a shear dependent mass transfer mechanism for atherogenesis." Proc R Soc Lond B Biol Sci **177**(46): 109-59.

CDC (2007). "Diabetes Data & Trends." http://apps.nccd.cdc.gov/DDTSTRS/

Chan, Y., J. E. Fish, C. D'Abreo, S. Lin, G. B. Robb, A. M. Teichert, F. Karantzoulis-Fegaras, A. Keightley, B. M. Steer and P. A. Marsden (2004). "The cell-specific expression of endothelial nitric-oxide synthase: a role for DNA methylation." J Biol Chem **279**(33): 35087-100.

Chen, K. D., Y. S. Li, M. Kim, S. Li, S. Yuan, S. Chien and J. Y. Shyy (1999). "Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc." J Biol Chem **274**(26): 18393-400.

Chen, Z., I. C. Peng, W. Sun, M. I. Su, P. H. Hsu, Y. Fu, Y. Zhu, K. DeFea, S. Pan, M. D. Tsai and J. Y. Shyy (2009). "AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633." Circ Res **104**(4): 496-505.

Chien, S. (2007). "Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell." Am J Physiol Heart Circ Physiol **292**(3): H1209-24.

Chien, S. and J. Y. Shyy (1998). "Effects of hemodynamic forces on gene expression and signal transduction in endothelial cells." Biol Bull **194**(3): 390-1; discussion 392-3.

Chiu, J. J., L. J. Chen, P. L. Lee, C. I. Lee, L. W. Lo, S. Usami and S. Chien (2003). "Shear stress inhibits adhesion molecule expression in vascular endothelial cells induced by coculture with smooth muscle cells." Blood **101**(7): 2667-74.

Chiu, J. J., D. L. Wang, S. Chien, R. Skalak and S. Usami (1998). "Effects of disturbed flow on endothelial cells." J Biomech Eng **120**(1): 2-8.

Cress, W. D. and E. Seto (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

Curtis, A. M., S. B. Seo, E. J. Westgate, R. D. Rudic, E. M. Smyth, D. Chakravarti, G. A. FitzGerald and P. McNamara (2004). "Histone acetyltransferase-dependent chromatin remodeling and the vascular clock." J Biol Chem **279**(8): 7091-7.

Dagher, Z., N. Ruderman, K. Tornheim and Y. Ido (1999). "The effect of AMP-activated protein kinase and its activator AICAR on the metabolism of human umbilical vein endothelial cells." Biochem Biophys Res Commun **265**(1): 112-5.

Danaei, G., E. L. Ding, D. Mozaffarian, B. Taylor, J. Rehm, C. J. Murray and M. Ezzati (2009). "The preventable causes of death in the United States: comparative risk assessment of dietary, lifestyle, and metabolic risk factors." PLoS Med **6**(4): e1000058.

Danaei, G., A. B. Friedman, S. Oza, C. J. Murray and M. Ezzati (2009). "Diabetes prevalence and diagnosis in US states: analysis of health surveys." Popul Health Metr **7**: 16.

Davies, P. F. (1995). "Flow-mediated endothelial mechanotransduction." Physiol Rev **75**(3): 519-60.

Davies, S. P., N. R. Helps, P. T. Cohen and D. G. Hardie (1995). "5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC." FEBS Lett **377**(3): 421-5.

de Ruijter, A. J., A. H. van Gennip, H. N. Caron, S. Kemp and A. B. van Kuilenburg (2003). "Histone deacetylases (HDACs): characterization of the classical HDAC family." Biochem J **370**(Pt 3): 737-49.

Dekker, R. J., S. van Soest, R. D. Fontijn, S. Salamanca, P. G. de Groot, E. VanBavel, H. Pannekoek and A. J. Horrevoets (2002). "Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2)." Blood **100**(5): 1689-98.

Dewey, C. F., Jr., S. R. Bussolari, M. A. Gimbrone, Jr. and P. F. Davies (1981). "The dynamic response of vascular endothelial cells to fluid shear stress." J Biomech Eng **103**(3): 177-85.

Dixit, M., E. Bess, B. Fisslthaler, F. V. Hartel, T. Noll, R. Busse and I. Fleming (2008). "Shear stress-induced activation of the AMP-activated protein kinase regulates FoxO1a and angiopoietin-2 in endothelial cells." Cardiovasc Res **77**(1): 160-8.

Dje N'Guessan, P., F. Riediger, K. Vardarova, S. Scharf, J. Eitel, B. Opitz, H. Slevogt, W. Weichert, A. C. Hocke, B. Schmeck, N. Suttorp and S. Hippenstiel (2009). "Statins control oxidized LDL-mediated histone modifications and gene expression in cultured human endothelial cells." Arterioscler Thromb Vasc Biol **29**(3): 380-6.

El Gazzar, M., B. K. Yoza, J. Y. Hu, S. L. Cousart and C. E. McCall (2007). "Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance." J Biol Chem **282**(37): 26857-64.

Ewart, M. A., C. F. Kohlhaas and I. P. Salt (2008). "Inhibition of tumor necrosis factor alpha-stimulated monocyte adhesion to human aortic endothelial cells by AMP-activated protein kinase." Arterioscler Thromb Vasc Biol **28**(12): 2255-7.

Fischle, W., F. Dequiedt, M. Fillion, M. J. Hendzel, W. Voelter and E. Verdin (2001). "Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo." J Biol Chem **276**(38): 35826-35.

Fischle, W., F. Dequiedt, M. J. Hendzel, M. G. Guenther, M. A. Lazar, W. Voelter and E. Verdin (2002). "Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR." Mol Cell **9**(1): 45-57.

Fischle, W., Y. Wang and C. D. Allis (2003). "Histone and chromatin cross-talk." Curr Opin Cell Biol **15**(2): 172-83.

Fish, J. E., M. S. Yan, C. C. Matouk, R. St Bernard, J. J. Ho, A. Gavryushova, D. Srivastava and P. A. Marsden (2009). "Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with eviction of promoter histones." J Biol Chem **285**(2): 810-26.

Fisslthaler, B., I. Fleming, B. Keseru, K. Walsh and R. Busse (2007). "Fluid shear stress and NO decrease the activity of the hydroxy-methylglutaryl coenzyme A reductase in endothelial cells via the AMP-activated protein kinase and FoxO1." Circ Res **100**(2): e12-21.

Frangos, J. A., S. G. Eskin, L. V. McIntire and C. L. Ives (1985). "Flow effects on prostacyclin production by cultured human endothelial cells." Science **227**(4693): 1477-9.

Frangos, S. G., V. Gahtan and B. Sumpio (1999). "Localization of atherosclerosis: role of hemodynamics." Arch Surg **134**(10): 1142-9.

Friedman, M. H., G. M. Hutchins, C. B. Bargeron, O. J. Deters and F. F. Mark (1981). "Correlation between intimal thickness and fluid shear in human arteries." Atherosclerosis **39**(3): 425-36.

Fung, Y. C. (1997). Biomechanics: circulation. New York, Springer-Verlag.

Garcia-Cardena, G., J. Comander, K. R. Anderson, B. R. Blackman and M. A. Gimbrone, Jr. (2001). "Biomechanical activation of vascular endothelium as a determinant of its functional phenotype." Proc Natl Acad Sci U S A **98**(8): 4478-85.

Gaskin, F. S., K. Kamada, M. Yusof, W. Durante, G. Gross and R. J. Korthuis (2009). "AICAR preconditioning prevents postischemic leukocyte rolling and adhesion: role of K(ATP) channels and heme oxygenase." Microcirculation **16**(2): 167-76.

Gimbrone, M. A., Jr., J. N. Topper, T. Nagel, K. R. Anderson and G. Garcia-Cardena (2000). "Endothelial dysfunction, hemodynamic forces, and atherogenesis." Ann N Y Acad Sci **902**: 230-9; discussion 239-40.

Grewal, S. I. and D. Moazed (2003). "Heterochromatin and epigenetic control of gene expression." Science **301**(5634): 798-802.

Grozinger, C. M. and S. L. Schreiber (2000). "Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization." Proc Natl Acad Sci U S A **97**(14): 7835-40.

Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." Nature **389**(6649): 349-52.

Guenther, M. G., O. Barak and M. A. Lazar (2001). "The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3." Mol Cell Biol **21**(18): 6091-101.

Guigas, B., L. Bertrand, N. Taleux, M. Foretz, N. Wiernsperger, D. Vertommen, F. Andreelli, B. Viollet and L. Hue (2006). "5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation." Diabetes **55**(4): 865-74.

Ha, C. H., W. Wang, B. S. Jhun, C. Wong, A. Hausser, K. Pfizenmaier, T. A. McKinsey, E. N. Olson and Z. G. Jin (2008). "Protein kinase D-dependent phosphorylation and nuclear export of histone deacetylase 5 mediates vascular endothelial growth factor-induced gene expression and angiogenesis." J Biol Chem **283**(21): 14590-9.

Haberland, M., R. L. Montgomery and E. N. Olson (2009). "The many roles of histone deacetylases in development and physiology: implications for disease and therapy." Nat Rev Genet **10**(1): 32-42.

Hardie, D. G. (2004). "The AMP-activated protein kinase pathway--new players upstream and downstream." J Cell Sci **117**(Pt 23): 5479-87.

Hastings, N. E., M. B. Simmers, O. G. McDonald, B. R. Wamhoff and B. R. Blackman (2007). "Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming." Am J Physiol Cell Physiol **293**(6): C1824-33.

Hattori, Y., K. Suzuki, A. Tomizawa, N. Hirama, T. Okayasu, S. Hattori, H. Satoh, K. Akimoto and K. Kasai (2009). "Cilostazol inhibits cytokine-induced nuclear factor-kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells." Cardiovasc Res **81**(1): 133-9.

Helmlinger, G., R. V. Geiger, S. Schreck and R. M. Nerem (1991). "Effects of pulsatile flow on cultured vascular endothelial cell morphology." J Biomech Eng **113**(2): 123-31.

Heydarkhan-Hagvall, S., S. Chien, S. Nelander, Y. C. Li, S. Yuan, J. Lao, J. H. Haga, I. Lian, P. Nguyen, B. Risberg and Y. S. Li (2006). "DNA microarray study on gene expression profiles in co-cultured endothelial and smooth muscle cells in response to 4- and 24-h shear stress." Mol Cell Biochem **281**(1-2): 1-15.

Holbert, M. A. and R. Marmorstein (2005). "Structure and activity of enzymes that remove histone modifications." Curr Opin Struct Biol **15**(6): 673-80.

Hong, S., Y. W. Cho, L. R. Yu, H. Yu, T. D. Veenstra and K. Ge (2007). "Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases." Proc Natl Acad Sci U S A **104**(47): 18439-44.

Hsiai, T. K., S. K. Cho, P. K. Wong, M. Ing, A. Salazar, A. Sevanian, M. Navab, L. L. Demer and C. M. Ho (2003). "Monocyte recruitment to endothelial cells in response to oscillatory shear stress." Faseb J **17**(12): 1648-57.

Humphrey, J. D. (2002). Cardiovascular Solid Mechanics: Cells, Tissues, and Organs. New York, Springer

Huo, Y., A. Hafezi-Moghadam and K. Ley (2000). "Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions." Circ Res **87**(2): 153-9.

Hurley, R. L., K. A. Anderson, J. M. Franzone, B. E. Kemp, A. R. Means and L. A. Witters (2005). "The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases." J Biol Chem **280**(32): 29060-6.

Jen, C. J., H. M. Li, J. S. Wang, H. I. Chen and S. Usami (1996). "Flow-induced detachment of adherent platelets from fibrinogen-coated surface." Am J Physiol **270**(1 Pt 2): H160-6.

Jensen, T. E., A. J. Rose, S. B. Jorgensen, N. Brandt, P. Schjerling, J. F. Wojtaszewski and E. A. Richter (2007). "Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction." Am J Physiol Endocrinol Metab **292**(5): E1308-17.

Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science **293**(5532): 1074-80.

Kim, J. E., Y. W. Kim, I. K. Lee, J. Y. Kim, Y. J. Kang and S. Y. Park (2008). "AMP-activated protein kinase activation by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) inhibits palmitate-induced endothelial cell apoptosis through reactive oxygen species suppression." J Pharmacol Sci **106**(3): 394-403.

Kouzarides, T. (2000). "Acetylation: a regulatory modification to rival phosphorylation?" Embo J **19**(6): 1176-9.

Kouzarides, T. (2002). "Histone methylation in transcriptional control." Curr Opin Genet Dev **12**(2): 198-209.

Kubota, N., W. Yano, T. Kubota, T. Yamauchi, S. Itoh, H. Kumagai, H. Kozono, I. Takamoto, S. Okamoto, T. Shiuchi, R. Suzuki, H. Satoh, A. Tsuchida, M. Moroi, K. Sugi, T. Noda, H. Ebinuma, Y. Ueta, T. Kondo, E. Araki, O. Ezaki, R. Nagai, K. Tobe, Y. Terauchi, K. Ueki, Y. Minokoshi and T. Kadowaki (2007). "Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake." Cell Metab **6**(1): 55-68.

Kumar, A., Z. Lin, S. SenBanerjee and M. K. Jain (2005). "Tumor necrosis factor alpha-mediated reduction of KLF2 is due to inhibition of MEF2 by NF-kappaB and histone deacetylases." Mol Cell Biol **25**(14): 5893-903.

Lachner, M., R. J. O'Sullivan and T. Jenuwein (2003). "An epigenetic road map for histone lysine methylation." J Cell Sci **116**(Pt 11): 2117-24.

Laukkanen, M. O., S. Mannermaa, M. O. Hiltunen, S. Aittomaki, K. Airenne, J. Janne and S. Yla-Herttuala (1999). "Local hypomethylation in atherosclerosis found in rabbit ec-sod gene." Arterioscler Thromb Vasc Biol **19**(9): 2171-8.

Lemercier, C., A. Verdel, B. Galloo, S. Curtet, M. P. Brocard and S. Khochbin (2000). "mHDA1/HDAC5 histone deacetylase interacts with and represses MEF2A transcriptional activity." J Biol Chem **275**(20): 15594-9.

Levesque, M. J. and R. M. Nerem (1985). "The elongation and orientation of cultured endothelial cells in response to shear stress." J Biomech Eng **107**(4): 341-7.

Li, X., Y. Han, W. Pang, C. Li, X. Xie, J. Y. Shyy and Y. Zhu (2008). "AMP-activated protein kinase promotes the differentiation of endothelial progenitor cells." Arterioscler Thromb Vasc Biol **28**(10): 1789-95.

Liu, Y., Y. Zhu, F. Rannou, T. S. Lee, K. Formentin, L. Zeng, X. Yuan, N. Wang, S. Chien, B. M. Forman and J. Y. Shyy (2004). "Laminar flow activates peroxisome proliferator-activated receptor-gamma in vascular endothelial cells." Circulation **110**(9): 1128-33.

Lloyd-Jones, D., R. Adams, M. Carnethon, G. De Simone, T. B. Ferguson, K. Flegal, E. Ford, K. Furie, A. Go, K. Greenlund, N. Haase, S. Hailpern, M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M. McDermott, J. Meigs, D. Mozaffarian, G. Nichol, C. O'Donnell, V. Roger, W. Rosamond, R. Sacco, P. Sorlie, R. Stafford, J. Steinberger, T. Thom, S. Wasserthiel-Smoller, N. Wong, J. Wylie-Rosett and Y. Hong (2009). "Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee." Circulation **119**(3): 480-6.

Lloyd-Jones, D., R. J. Adams, T. M. Brown, M. Carnethon, S. Dai, G. De Simone, T. B. Ferguson, E. Ford, K. Furie, C. Gillespie, A. Go, K. Greenlund, N. Haase, S. Hailpern, P. M. Ho, V. Howard, B. Kissela, S. Kittner, D. Lackland, L. Lisabeth, A. Marelli, M. M. McDermott, J. Meigs, D. Mozaffarian, M. Mussolino, G. Nichol, V. L. Roger, W. Rosamond, R. Sacco, P. Sorlie, V. L. Roger, T. Thom, S. Wasserthiel-Smoller, N. D. Wong and J. Wylie-Rosett (2010). "Heart disease and stroke statistics--2010 update: a report from the American Heart Association." Circulation **121**(7): e46-e215.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-60.

Marmorstein, R. (2001). "Structure of histone deacetylases: insights into substrate recognition and catalysis." Structure **9**(12): 1127-33.

McGee, S. L., B. J. van Denderen, K. F. Howlett, J. Mollica, J. D. Schertzer, B. E. Kemp and M. Hargreaves (2008). "AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5." Diabetes **57**(4): 860-7.

McKinsey, T. A., C. L. Zhang, J. Lu and E. N. Olson (2000). "Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation." Nature **408**(6808): 106-11.

McKinsey, T. A., C. L. Zhang and E. N. Olson (2002). "Signaling chromatin to make muscle." Curr Opin Cell Biol **14**(6): 763-72.

Montecucco, F. and F. Mach (2009). "Common inflammatory mediators orchestrate pathophysiological processes in rheumatoid arthritis and atherosclerosis." Rheumatology (Oxford) **48**(1): 11-22.

Morales, V. and H. Richard-Foy (2000). "Role of histone N-terminal tails and their acetylation in nucleosome dynamics." Mol Cell Biol **20**(19): 7230-7.

Morrow, V. A., F. Foufelle, J. M. Connell, J. R. Petrie, G. W. Gould and I. P. Salt (2003). "Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells." J Biol Chem **278**(34): 31629-39.

Nagata, D., M. Mogi and K. Walsh (2003). "AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress." J Biol Chem **278**(33): 31000-6.

Nakanishi, S., B. W. Sanderson, K. M. Delventhal, W. D. Bradford, K. Staehling-Hampton and A. Shilatifard (2008). "A comprehensive library of histone mutants identifies nucleosomal residues required for H3K4 methylation." Nat Struct Mol Biol **15**(8): 881-8.

Napoli, C., F. P. D'Armiento, F. P. Mancini, A. Postiglione, J. L. Witztum, G. Palumbo and W. Palinski (1997). "Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions." J Clin Invest **100**(11): 2680-90.

Ng, H. H. and A. Bird (2000). "Histone deacetylases: silencers for hire." Trends Biochem Sci **25**(3): 121-6.

Nishino, T. G., M. Miyazaki, H. Hoshino, Y. Miwa, S. Horinouchi and M. Yoshida (2008). "14-3-3 regulates the nuclear import of class IIa histone deacetylases." Biochem Biophys Res Commun **377**(3): 852-6.

Ohno, M., J. P. Cooke, V. J. Dzau and G. H. Gibbons (1995). "Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production. Modulation by potassium channel blockade." J Clin Invest **95**(3): 1363-9.

Okamoto, T., A. Schlegel, P. E. Scherer and M. P. Lisanti (1998). "Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane." J Biol Chem **273**(10): 5419-22.

Okayasu, T., A. Tomizawa, K. Suzuki, K. Manaka and Y. Hattori (2008). "PPARalpha activators upregulate eNOS activity and inhibit cytokine-induced NF-kappaB activation through AMP-activated protein kinase activation." Life Sci **82**(15-16): 884-91.

Orr, A. W., C. Hahn, B. R. Blackman and M. A. Schwartz (2008). "p21-activated kinase signaling regulates oxidant-dependent NF-kappa B activation by flow." Circ Res **103**(6): 671-9.

Ouchi, N., H. Kobayashi, S. Kihara, M. Kumada, K. Sato, T. Inoue, T. Funahashi and K. Walsh (2004). "Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells." J Biol Chem **279**(2): 1304-9.

Ouchi, N., R. Shibata and K. Walsh (2005). "AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle." Circ Res **96**(8): 838-46.

Papadaki, M. and S. G. Eskin (1997). "Effects of fluid shear stress on gene regulation of vascular cells." Biotechnol Prog **13**(3): 209-21.

Pons, D., F. R. de Vries, P. J. van den Elsen, B. T. Heijmans, P. H. Quax and J. W. Jukema (2009). "Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease." Eur Heart J **30**(3): 266-77.

Prasad, R., S. Giri, N. Nath, I. Singh and A. K. Singh (2006). "5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates experimental autoimmune encephalomyelitis via modulation of endothelial-monocyte interaction." J Neurosci Res **84**(3): 614-25.

Ranjan, V., Z. Xiao and S. L. Diamond (1995). "Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress." Am J Physiol **269**(2 Pt 2): H550-5.

Rea, S., F. Eisenhaber, D. O'Carroll, B. D. Strahl, Z. W. Sun, M. Schmid, S. Opravil, K. Mechtler, C. P. Ponting, C. D. Allis and T. Jenuwein (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature **406**(6796): 593-9.

Reik, W. (2007). "Stability and flexibility of epigenetic gene regulation in mammalian development." Nature **447**(7143): 425-32.

Ross, R. (1986). "The pathogenesis of atherosclerosis--an update." N Engl J Med **314**(8): 488-500.

Ross, R. (1999). "Atherosclerosis--an inflammatory disease." N Engl J Med **340**(2): 115-26.

Ruthenburg, A. J., H. Li, D. J. Patel and C. D. Allis (2007). "Multivalent engagement of chromatin modifications by linked binding modules." Nat Rev Mol Cell Biol **8**(12): 983-94.

Sabina, R. L., D. Patterson and E. W. Holmes (1985). "5-Amino-4-imidazolecarboxamide riboside (Z-riboside) metabolism in eukaryotic cells." J Biol Chem **260**(10): 6107-14.

Santos-Rosa, H., R. Schneider, A. J. Bannister, J. Sherriff, B. E. Bernstein, N. C. Emre, S. L. Schreiber, J. Mellor and T. Kouzarides (2002). "Active genes are tri-methylated at K4 of histone H3." Nature **419**(6905): 407-11.

Shahbazian, M. D. and M. Grunstein (2007). "Functions of site-specific histone acetylation and deacetylation." Annu Rev Biochem **76**: 75-100.

Sharma, P., J. Kumar, G. Garg, A. Kumar, A. Patowary, G. Karthikeyan, L. Ramakrishnan, V. Brahmachari and S. Sengupta (2008). "Detection of altered global DNA methylation in coronary artery disease patients." DNA Cell Biol **27**(7): 357-65.

Shaw, R. J., K. A. Lamia, D. Vasquez, S. H. Koo, N. Bardeesy, R. A. Depinho, M. Montminy and L. C. Cantley (2005). "The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin." Science **310**(5754): 1642-6.

Shi, Y., F. Lan, C. Matson, P. Mulligan, J. R. Whetstine, P. A. Cole, R. A. Casero and Y. Shi (2004). "Histone demethylation mediated by the nuclear amine oxidase homolog LSD1." Cell **119**(7): 941-53.

Shilatifard, A. (2006). "Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression." Annu Rev Biochem **75**: 243-69.

Shyy, Y. J., H. J. Hsieh, S. Usami and S. Chien (1994). "Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium." Proc Natl Acad Sci U S A **91**(11): 4678-82.

Stahmann, N., A. Woods, D. Carling and R. Heller (2006). "Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta." Mol Cell Biol **26**(16): 5933-45.

Stenvinkel, P., M. Karimi, S. Johansson, J. Axelsson, M. Suliman, B. Lindholm, O. Heimburger, P. Barany, A. Alvestrand, L. Nordfors, A. R. Qureshi, T. J. Ekstrom and M. Schalling (2007). "Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease?" J Intern Med **261**(5): 488-99.

Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-5.

Struhl, K. (1998). "Histone acetylation and transcriptional regulatory mechanisms." Genes Dev **12**(5): 599-606.

Sullivan, J. E., K. J. Brocklehurst, A. E. Marley, F. Carey, D. Carling and R. K. Beri (1994). "Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase." FEBS Lett **353**(1): 33-6.

Sun, Y., K. E. Connors and D. Q. Yang (2007). "AICAR induces phosphorylation of AMPK in an ATM-dependent, LKB1-independent manner." Mol Cell Biochem **306**(1-2): 239-45.

Takada, Y., F. Shinkai, S. Kondo, S. Yamamoto, H. Tsuboi, R. Korenaga and J. Ando (1994). "Fluid shear stress increases the expression of thrombomodulin by cultured human endothelial cells." Biochem Biophys Res Commun **205**(2): 1345-52.

Tardy, Y., N. Resnick, T. Nagel, M. A. Gimbrone, Jr. and C. F. Dewey, Jr. (1997). "Shear stress gradients remodel endothelial monolayers in vitro via a cell proliferation-migration-loss cycle." Arterioscler Thromb Vasc Biol **17**(11): 3102-6.

Tedgui, A. and Z. Mallat (2001). "Anti-inflammatory mechanisms in the vascular wall." Circ Res **88**(9): 877-87.

Teichert, A. M., J. A. Scott, G. B. Robb, Y. Q. Zhou, S. N. Zhu, M. Lem, A. Keightley, B. M. Steer, A. C. Schuh, S. L. Adamson, M. I. Cybulsky and P. A. Marsden (2008). "Endothelial nitric oxide synthase gene expression during murine embryogenesis: commencement of expression in the embryo occurs with the establishment of a unidirectional circulatory system." Circ Res **103**(1): 24-33.

Thoumine, O., R. M. Nerem and P. R. Girard (1995). "Changes in organization and composition of the extracellular matrix underlying cultured endothelial cells exposed to laminar steady shear stress." Lab Invest **73**(4): 565-76.

Towler, M. C. and D. G. Hardie (2007). "AMP-activated protein kinase in metabolic control and insulin signaling." Circ Res **100**(3): 328-41.

Trojer, P. and D. Reinberg (2006). "Histone lysine demethylases and their impact on epigenetics." Cell **125**(2): 213-7.

Truskey, G. A., K. M. Barber, T. C. Robey, L. A. Olivier and M. P. Combs (1995). "Characterization of a sudden expansion flow chamber to study the response of endothelium to flow recirculation." J Biomech Eng **117**(2): 203-10.

Turner, B. M. (2002). "Cellular memory and the histone code." Cell **111**(3): 285-91.

Tzima, E., M. Irani-Tehrani, W. B. Kiosses, E. Dejana, D. A. Schultz, B. Engelhardt, G. Cao, H. DeLisser and M. A. Schwartz (2005). "A mechanosensory complex that mediates the endothelial cell response to fluid shear stress." Nature **437**(7057): 426-31.

Vogl-Willis, C. A. and I. J. Edwards (2004). "High glucose-induced alterations in subendothelial matrix perlecan leads to increased monocyte binding." Arterioscler Thromb Vasc Biol **24**(5): 858-63.

Wade, P. A. (2001). "Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin." Hum Mol Genet **10**(7): 693-8.

Wang, G. G., C. D. Allis and P. Chi (2007). "Chromatin remodeling and cancer, Part I: Covalent histone modifications." Trends Mol Med **13**(9): 363-72.

Wang, N., H. Miao, Y. S. Li, P. Zhang, J. H. Haga, Y. Hu, A. Young, S. Yuan, P. Nguyen, C. C. Wu and S. Chien (2006). "Shear stress regulation of Kruppel-like factor 2 expression is flow pattern-specific." Biochem Biophys Res Commun **341**(4): 1244-51.

Wang, W., C. H. Ha, B. S. Jhun, C. Wong, M. K. Jain and Z. G. Jin (2010). "Fluid shear stress stimulates phosphorylation-dependent nuclear export of HDAC5 and mediates expression of KLF2 and eNOS." Blood **115**(14): 2971-9.

Wang, Y., J. Chang, Y. C. Li, Y. S. Li, J. Y. Shyy and S. Chien (2004). "Shear stress and VEGF activate IKK via the Flk-1/Cbl/Akt signaling pathway." Am J Physiol Heart Circ Physiol **286**(2): H685-92.

Won, D., S. N. Zhu, M. Chen, A. M. Teichert, J. E. Fish, C. C. Matouk, M. Bonert, M. Ojha, P. A. Marsden and M. I. Cybulsky (2007). "Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow." Am J Pathol **171**(5): 1691-704.

Woods, A., K. Dickerson, R. Heath, S. P. Hong, M. Momcilovic, S. R. Johnstone, M. Carlson and D. Carling (2005). "Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells." Cell Metab **2**(1): 21-33.

Xing, H., H. Li, L. Ma, W. Zhu and Y. Jin (2001). "[Influence of fluid shear stress on the expression of monocyte chemotactic protein-1 by vascular endothelial cells]." Zhonghua Bing Li Xue Za Zhi **30**(5): 361-4.

Yee, A., K. A. Bosworth, D. E. Conway, S. G. Eskin and L. V. McIntire (2008). "Gene expression of endothelial cells under pulsatile non-reversing vs. steady shear stress; comparison of nitric oxide production." Ann Biomed Eng **36**(4): 571-9.

Young, A., W. Wu, W. Sun, H. B. Larman, N. Wang, Y. S. Li, J. Y. Shyy, S. Chien and G. Garcia-Cardena (2009). "Flow activation of AMP-activated protein kinase in vascular endothelium leads to Kruppel-like factor 2 expression." Arterioscler Thromb Vasc Biol **29**(11): 1902-8.

Yu, H., Y. Zeng, J. Hu and C. Li (2002). "Fluid shear stress induces the secretion of monocyte chemoattractant protein-1 in cultured human umbilical vein endothelial cells." Clin Hemorheol Microcirc **26**(3): 199-207.

Zarins, C. K., D. P. Giddens, B. K. Bharadvaj, V. S. Sottiurai, R. F. Mabon and S. Glagov (1983). "Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress." Circ Res **53**(4): 502-14.

Zhang, J., M. Kalkum, B. T. Chait and R. G. Roeder (2002). "The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2." Mol Cell **9**(3): 611-23.

Zhou, G., R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M. F. Hirshman, L. J. Goodyear and D. E. Moller (2001). "Role of AMP-activated protein kinase in mechanism of metformin action." J Clin Invest **108**(8): 1167-74.

Zou, M. H. and Y. Wu (2008). "AMP-activated protein kinase activation as a strategy for protecting vascular endothelial function." Clin Exp Pharmacol Physiol **35**(5-6): 535-45.