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MECHANISMS OF SHEAR STRESS-MEDIATED ERK1/2 MODULATING SIGNAL TRANSDUCTION PATHWAYS IN ENDOTHELIAL CELLS

by

Oren Traub

A dissertation submitted in partial fulfillment of the requirements for the degree of

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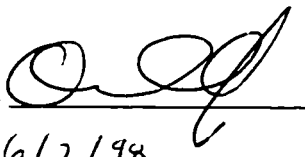
Signature 

Date 6/2/98

University of Washington

Abstract

Mechanisms of Shear Stress-Mediated ERK1/2 Modulating Signal Transduction Pathways in Endothelial Cells

by Oren Traub

Chairperson of the Supervisory Committee:

Professor Bradford C. Berk
Department of Internal Medicine and Department of Pathology

Mechanical forces are important modulators of cellular function in various tissues and are particularly important in the cardiovascular system. The endothelial cell layer, by virtue of its unique location in the vessel wall, is exposed to fluid forces of much greater magnitude than those experienced by other mammalian tissues and thus has developed mechanically-related responses to fluid shear stress. While the effects of shear on endothelial cell function have been well studied, the mechanisms by which endothelial cells sense mechanical stimuli and convert them to biochemical signals are not well characterized. The primary goal of this project is to study the intracellular signal transduction mechanisms activated by fluid shear stress. In this dissertation, we (1) detail the construction and characterize the efficacy of different apparatus to simulate shear stress in vitro on cultured cells; (2) demonstrate that PKC is involved in the shear stress-mediated ERK1/2 activation as well as characterize PKC-ε as the necessary isoform for this signaling pathway; (3) characterize the ion dependency of shear stress-mediated ERK1/2 activation

and show that sodium entry is inhibitory; and (4) identify and characterize mechanosensitive voltage-gated sodium channels in endothelial cells that are involved in the ERK1/2 response to shear stress. The results will allow us to define temporal and functional interactions of endothelial cell signaling mechanisms with shear stress and allow us to characterize their contribution to the modulation of endothelial cell function by hemodynamic forces. This knowledge is important not only to our understanding of the pathogenesis of atherosclerosis but also to a wide variety of biological processes that are modulated by physical forces, such as bone growth, muscle hypertrophy, hair cell sound transduction.

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
AP-1	activator protein-1
BAEC	bovine aortic endothelial cells
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester
BMK-1	big MAP kinase
cDNA	complementary DNA
CHO	Chinese hamster ovary
DAG	diacylglycerol
DNA	deoxyribonucleic acid
EC	endothelial cell
ecNOS	endothelial constitutive nitric oxide synthase
EGF	epidermal growth factor
ERK1/2	extracellular signal regulated kinase
FN	fibronectin
Grb2	growth factor receptor binding protein 2
GTP	guanosine triphosphate
HUVEC	human umbilical vein endothelial cell
ICAM-1	intracellular adhesion molecule
IP_3	inositol trisphosphate
JNK	Jun N-terminal kinase
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase

MCP-1	monocyte chemoattractant peptide 1
MEK-1	MAP and ERK kinase 1
mRNA	messenger RNA
mSOS	mammalian son of sevenless
NO	nitric oxide
PDGF	platelet derived growth factor
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLL	poly-L-lysine
RGD	arginine-glycine-aspartate
RNA	ribonucleic acid
SAPK	stress-activated protein kinase
Shc	Src homology-2 containing proteins
TCF	ternary complex factor
TEY	threonine-glutamate-tyrosine
TGF-β	transforming growth factor b
TNF	tumor necrosis factor
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule

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CHAPTER 1: ENDOTHELIAL RESPONSE TO SHEAR STRESS

INTRODUCTION

Numerous studies suggest that normal functioning of the endothelium is critical in limiting the development of atherosclerosis as illustrated by the correlation between risk factors for atherosclerosis (smoking, high cholesterol, high homocysteine, decreased estrogen, increasing age, and hypertension) and endothelial dysfunction (Ross 1993). Endothelial cells play a critical role in vascular homeostasis by performing many functions. They sense and integrate hemodynamic and hormonal stimuli and effect alterations in vascular function through the secretion of various mediator proteins and molecules (Vanhoutte 1989). As a result of these properties, endothelial cells modulate biological processes related to the blood vessel wall, including regulation of the permeability of plasma lipoproteins, adhesion of leukocytes, and release of pro- and antithrombotic factors, growth factors and vasoactive substances (Rubanyi 1993). Impairment of these endothelial cell-mediated processes has been postulated to play a central role in the pathogenesis of atherosclerosis (Ross 1993).

Just as other tissues have developed mechanisms to detect changes in the physiological conditions to which they are exposed, endothelial cells respond not only to humoral factors in the circulation, but also to the mechanical conditions created by blood flow and the cardiac cycle (Davies 1995). As a result of their unique location, endothelial cells experience three primary mechanical forces: *pressure*, created by the hydrostatic forces of blood within the blood vessel; *circumferential stretch* or *tension*, created as a result of defined intercellular connections between the endothelial cells that exert longitudinal forces

on the cell during vasomotion; and *shear stress*, the dragging frictional force created by blood flow (See Figure I-1). Of these forces, shear stress appears to be a particularly important hemodynamic force since it stimulates the release of vasoactive substances and changes gene expression, cell metabolism, and cell morphology (Davies 1995) .

The nature and magnitude of shear stress plays an important role in long-term maintenance of the structure and function of the blood vessel. The nature of shear stress experienced by endothelial cells is a function of blood flow patterns throughout the vasculature generated by the cardiac cycle. In "linear" areas of the vasculature, blood flows in ordered laminar patterns in a pulsatile fashion dependent on the cardiac cycle and endothelial cells experience *pulsatile* shear stress with fluctuations in magnitude that yields a mean positive shear stress. This flow pattern should be distinguished from the flow pattern which is often used in experimental preparations and which generates a *steady* positive shear stress, being temporally and spatially uniform (while steady shear stress generally stimulates many of the same endothelial cell responses as pulsatile stress, there are some qualitative and quantitative differences (Frangos, Eskin et al. 1985; Hsieh, Li et al. 1993; Helmlinger, Berk et al. 1995)). Cells exposed to positive shear stress undergo reorientation with their longitudinal axis parallel to the direction of blood flow (Flaherty, Pierce et al. 1972; Dewey, Bussolari et al. 1981). This reorientation streamlines the endothelial cell, decreasing the effective resistance and lowering shear stress (Barbee, Mundel et al. 1995), a phenomenon which may or may not be important in terms of adaptation or filtering of shear stress stimuli (Davies 1995). At areas of abrupt curvatures in the vasculature, as in the carotid bifurcation, the laminar flow of blood is disrupted and separated flow patterns result. Specifically, the medial wall of the carotid bulb experiences higher shear stress while the lateral wall experiences recirculation vortices which vary with the cardiac cycle resulting in flow reversal (Ku, Giddens et al. 1985) (See Figure I-2).

Thus, the lateral area of the carotid bulb experiences *oscillatory* shear stress (periodic flow reversal with time-average shear stress approaching zero) and low mean shear stress. As a result of the low magnitude of the time-averaged shear stress, these cells do not reorient (Davies, Remuzzi et al. 1986) and may be exposed to high shear *gradients* (differences in shear stress magnitude on a cell scale) (Davies 1995) as their lack of streamlining yields a membrane topology in opposition to the mean shear vector. Several investigators have demonstrated that endothelial cells may actually be sensitive to the magnitude of the shear gradient (Davies, Remuzzi et al. 1986; DePaola, Gimbrone et al. 1992; Barbee, Davies et al. 1994). Whether these shear gradients or the time-averaged mean shear stress is more critical in terms of atherogenesis remains to be defined. Nevertheless, the significance of these flow patterns is demonstrated by studies that correlate development of atherosclerotic lesions (fatty streaks and small plaques) with areas of the carotid that experience these flow reversals with low time-averaged shear stress (Ku, Giddens et al. 1985; Asakura and Karino 1990). Regions of the carotid bifurcation that experience pulsatile and mean positive shear stress as the result of laminar blood flow patterns, however, were relatively protected from atherosclerosis. Other investigators have confirmed these observations throughout the vasculature (Moore, Xu et al. 1994). The mechanism by which the physical force generated by fluid shear stress is transduced into biological signals remains unclear.

Below we will briefly review the atheroprotective effects of the endothelium that are influenced by shear stress and then discuss several signal transduction mechanisms by which shear stress exerts its beneficial effects on endothelial function.

SHEAR STRESS AND ENDOTHELIAL CELL BIOLOGY: RELEVANCE TO ATHEROSCLEROSIS

The hypothesis that physical injury to the endothelium might precipitate the atherosclerotic process was introduced over two decades ago. More recently this concept has been modified to include biochemical and cellular alterations in endothelial cell function. There is a strong correlation between endothelial cell dysfunction and areas of low mean shear stress and oscillatory flow with flow reversal (Figure I-2). Manifestations of dysfunctional endothelium can be readily observed in certain areas of the arterial tree such as branch points which experience low mean shear stress and flow reversal (Ku, Giddens et al. 1985). These sites demonstrate increased uptake of lipoproteins, increased appearance of leukocyte adhesion molecules on the surface of the endothelial cells and increased leukocyte transmigration. Secretion of chemotactic factors and growth factors causes proliferation of resident monocyte/macrophages as well as smooth muscle cells. Smooth muscle cells synthesize a connective tissue matrix comprised of elastic fibers proteins, collagen and proteoglycans, and the accumulations of lipids and free and esterified cholesterol follows (Ross, Bowen et al. 1990). Recent data suggest that low shear stress and, more importantly, oscillatory flow and flow reversal, are permissive or even causative in this pathogenic process (Davies 1995; Topper, Cai et al. 1996).

In areas downstream of vessel bifurcations, laminar shear stress predominates and the endothelial cells experience pulsatile flow, with shear stress on the order of 10-30 dynes/cm^2 (Davies 1995). The endothelium in these regions maintains circulatory and blood vessel integrity through its ability to regulate several different processes: coagulation, growth of underlying smooth muscle, leukocyte adhesion to and transmigration into the blood vessel wall, and lipoprotein uptake and metabolism (Figure I-3).

COAGULATION

Coagulation stimulates the release of powerful anti-thrombotic agents from endothelial cells. Prostacyclin was the first inhibitor of platelet aggregation shown to be released from endothelial cells upon exposure to shear stress (Frangos, Eskin et al. 1985; Grabowski, Jaffe et al. 1985). Secretion of prostacyclin from endothelial cells is enhanced when the shear stress is pulsatile compared to steady (Frangos, Eskin et al. 1985). Numerous investigators have demonstrated that shear stress is one of the most powerful stimuli for release of the vasodilator, nitric oxide (NO) (Rubanyi, Romero et al. 1986; Busse, Pohl et al. 1989; Vanhoutte 1989), which also possesses strong anti-platelet aggregation properties (Stamler, Mendelsohn et al. 1989). Shear stress can also stimulate release of factors that inactivate the clotting cascade (Davies 1995). Recent studies have shown that shear stress regulates generation of thrombomodulin, which interacts with protein C and protein S to inactivate certain clotting factors. Malek *et al.* (Malek, Jackman et al. 1994) reported that steady shear stress resulted in a small transient increase in thrombomodulin expression but continued exposure to shear resulted in decreased thrombomodulin expression in bovine aortic endothelial cells. However, two other laboratories reported that steady shear stress results in sustained increased thrombomodulin expression in human umbilical vein endothelial cells (Takada, Shinkai et al. 1994; Kawai, Matsumoto et al. 1997). The reason for this disparity in species response is unclear. In addition to the potential effect on thrombomodulin, fluid shear stress has also been shown to stimulate expression of tissue plasminogen activator (tPA) (Diamond, Sharefkin et al. 1990; Malek, Gibbons et al. 1993; Kawai, Matsumoto et al. 1997) and reduce secretion of plasminogen activator inhibitor type-1 (Kawai, Matsumoto et al. 1997). Importantly, endothelial cells exposed to turbulent flow failed to show increases in thrombomodulin and tPA.

LEUKOCYTE ADHESION AND MIGRATION

Endothelial cells regulate leukocyte adhesion and migration of monocytes and leukocytes into the blood vessel wall by secretion of chemotactic factors and expression of cell surface molecules. Intracellular adhesion molecule 1 (ICAM-1) binds β2-integrins on various white blood cell derivatives, while vascular cell adhesion molecule 1 (VCAM-1) mediates adhesion of monocytes to the endothelium (Cybulsky and Gimbrone 1991). VCAM-1 is one of the earliest markers for fatty streaks and is upregulated in areas of the endothelium surrounding atherosclerotic plaque (Cybulsky and Gimbrone 1991). Several investigators have demonstrated an inverse relationship between VCAM-1 expression and shear stress (Ohtsuka, Ando et al. 1993; Ando, Tsuboi et al. 1994; Ando, Tsuboi et al. 1995; Sampath, Kukielka et al. 1995; Tsao, Lewis et al. 1995; Walpola, Gotlieb et al. 1995) which suggests that leukocyte binding should be decreased under conditions of high shear stress. However, other investigators have demonstrated that ICAM-1 expression is upregulated by high shear stress (Nagel, Resnick et al. 1994; Morigi, Zoja et al. 1995; Tsuboi, Ando et al. 1995) and that leukocyte binding after exposure to shear stress is increased (Nagel, Resnick et al. 1994; Morigi, Zoja et al. 1995).

The leukocyte binding experiments described above utilized static cultures of endothelial cells that were exposed to shear stress prior to the binding of leukocytes. However, these experimental conditions do not accurately simulate the physiological conditions of leukocyte binding and the complex interplay between expression of ICAM-1, VCAM-1 and the physical disruption of the leukocyte-endothelial cell interaction by high levels of shear stress (SchmidSchoenbein, Fung et al. 1975; Lawrence, Smith et al. 1990). A study performed by Walpola *et al.* (Walpola, Gotlieb et al. 1995) is helpful in analyzing which of these parameters may be of physiological importance. In this study, shear stress

in rabbit carotid arteries was altered and expression of endothelial cell adhesion molecules as well as leukocyte binding was measured. Low shear stress resulted in VCAM-1 expression 30 times greater than that of control vessels while ICAM-1 expression fell to approximately 30% of control vessels. High levels of shear stress also increased VCAM-1 expression (to 3.5 times that of control vessels) while ICAM expression levels increased (to 1.6 times that of control). Importantly, extensive monocyte adhesion was noted under low shear stress which colocalized to areas of VCAM-1 expression while no monocyte adhesion was noted at high shear conditions, indicating that low mean shear stress promotes leukocyte binding *in vivo* when compared to higher shear stress.

Another key factor in monocyte recruitment is the chemoattractant peptide, MCP-1 (Ross 1993). Shyy *et al.* (Shyy, Lin et al. 1995) showed that MCP-1 expression was transiently increased in HUVEC upon exposure to shear stress. However, MCP-1 gene expression then decreased to basal levels at 4 hr and once gene expression was fully suppressed, it remained so even after static incubation, leading the authors to suggest that MCP-1 expression is likely suppressed in endothelial cells exposed to steady pulsatile shear stress. Thus, it seems probable that for conduit vessels, high shear stress inhibits leukocyte binding and chemoattractant protein expression while low shear stress and flow reversal promotes leukocyte binding and transmigration.

PROLIFERATION

Smooth muscle proliferation is increased in atherosclerotic lesions (Ross 1993) and is likely stimulated by endothelial cell factors that are regulated by shear stress. Kraiss *et al.* (Kraiss, Raines et al. 1993) showed that endothelial PDGF-A mRNA levels and smooth muscle proliferation were increased in areas that experience low blood flow in an arteriovenous fistula model of altered flow in baboons. Endothelin-1, a smooth muscle

mitogen that acts synergistically with PDGF, was shown to be dramatically reduced by exposure to 25 dynes/cm^2 (Sharefkin, Diamond et al. 1991). Nitric oxide (Buga, Gold et al. 1991) and TGF-β (Ohno, Cooke et al. 1995), both inhibitors of vascular smooth muscle cell growth, are secreted by endothelial cells in response to shear stress. Angiotensin II is an important growth factor for vascular smooth muscle and may also be anti-apoptotic (Berk, Duff et al. 1996). Shear stress regulates tissue levels of angiotensin II by virtue of changes in angiotensin-converting enzyme (ACE) expression. Rieder *et al.* (Rieder, Carmona et al. 1997) recently demonstrated that prolonged exposure to shear stress significantly reduced ACE mRNA and activity. With its ability to regulate these disparate smooth muscle cell growth factors, it seems likely that shear stress plays a role in the increased proliferation of smooth muscle seen at areas of low shear stress and flow reversal.

LIPOPROTEINS

Unlike the effects of shear stress on growth factor secretion, the role of shear stress in lipoprotein transport and LDL metabolism is less well defined. Deng *et al.* (Deng, Marois et al. 1995) reported that the concentration of LDL at the surface of canine carotid arteries was inversely related to wall shear stress rate and suggested that increased surface LDL concentration results in an increased rate of lipid infiltration into the blood vessel. This hypothesis is complemented by studies which demonstrate that areas exposed to flow reversal are relatively permeable to macromolecules including LDL and that LDL accumulation within the vascular wall is preferentially localized to these areas of disturbed flow. Berceli *et al.* (Berceli, Warty et al. 1990) reported that the LDL incorporation in the rabbit aorta-iliac bifurcation was elevated in the lateral region that experiences flow reversal versus the medial regions that experiences higher steady shear, while no differences were

present in the transitional or unidirectional zone that experiences relatively steady shear. Other investigators have confirmed these findings in different areas of the vasculature for both rabbits (Schwenke and Carew 1988) and pigs (Fry, Herderick et al. 1993). Mechanistically, it appears that compromised endothelial cell integrity, and hence increased macromolecule permeability, results from high shear gradients that are present in low shear stress/flow reversal conditions (See Weinbaum and Chien (Weinbaum and Chien 1993) for review). A study by Sprague *et al.* (Sprague, Steinbach et al. 1987) showed that ^{125}I-LDL internalization increased in bovine aortic endothelial cells exposed to steady-stress conditions for 24 hr, however this likely reflects an increased metabolic need for LDL under steady shear conditions rather than increased LDL incorporation into the arterial wall (Wiklund, Carew et al. 1985). Additional studies to define the mechanistic details by which LDL accumulation is linked to low shear stress and flow reversal conditions deserves further investigation.

ENDOTHELIAL CELL SURVIVAL

Finally, shear stress may be critical for endothelial cell survival. Early studies performed by Davies *et al.* (Davies, Remuzzi et al. 1986) demonstrated increased endothelial cell turnover in areas that experience turbulent shear stress conditions suggesting compromised endothelial cell integrity under these conditions. Several recent studies report that the lack of shear stress triggers apoptosis in endothelial cells (Dimmeler, Haendeler et al. 1996; Kaiser, Freyberg et al. 1997). Other investigators have demonstrated that shear stress is required for optimal regeneration of an injured endothelium. Vyalov *et al.* (Vyalov, Langille et al. 1996) reported that under low shear stress conditions, endothelial cells on the border of a wound edge failed to maintain contact with neighboring cells and were oriented randomly. Further, the cells spread and migrated

into wound sites more slowly. While steady shear seems to be necessary for endothelial cell integrity, several investigators have demonstrated that steady shear inhibits proliferation of cultured endothelial cells (Levesque, Nerem et al. 1990). Thus, it appears that shear stress acts as an endothelial cell "survival factor" rather than as a "growth" factor.

NITRIC OXIDE: A CRITICAL FACTOR IN SHEAR STRESS-MEDIATED ATHEROPROTECTION

NO appears to be a key mediator of the atheroprotective effects of shear stress on the blood vessel wall. Nitric oxide has been reported to play a role in platelet aggregation and leukocyte binding to the endothelium, in inhibition of vascular smooth muscle tone and growth, and in alteration of lipoprotein metabolism (Vanhoutte and Shimokawa 1989). The ability of shear stress to regulate some of these processes is abrogated by inhibitors of NO production suggesting that shear stress may exert its effects through the release of NO. Further, it has been postulated that the beneficial effects of regular aerobic training, including its anti-atherogenic properties, may be mediated through shear stress-induced increases in NO secretion (Langille, Graham et al. 1991).

NO is produced by a unique enzyme present in the endothelium, termed endothelial constitutive nitric oxide synthase (ecNOS) (Janssens, Shimouchi et al. 1992; Nishida, Harrison et al. 1992; Sessa, Harrison et al. 1992). Shear stress is the most potent physiologic stimulus for NO production in endothelial cells. Rapid increases in NO production are due to post-translational activation of ecNOS while chronic alterations in ecNOS expression are due to changes in gene expression. Experiments by our laboratory and others (Kuchan and Frangos 1994; Fleming, Bauersachs et al. 1997) indicate that two distinct signaling pathways (a Ca^{2+}-dependent and a Ca^{2+}-independent pathway) seem to be involved in rapid shear-mediated increases in NO production (Berk, Corson et al.

1995). We compared NO production in response to the Ca^{2+}-ionophore A23187 with shear stress. While A23187 increased NO production by 3 to 6-fold, shear stress stimulated NO production by 10- to 30-fold above static levels. The initial rapid increase in NO required Ca^{2+}, while the sustained increase in NO production was independent of changes in intracellular Ca^{2+} (Corson, James et al. 1996). Further experiments by our laboratory have demonstrated that ecNOS was phosphorylated in response to shear stress (Corson, James et al. 1996). Although the relationship between ecNOS phosphorylation and NO production is unclear, phosphorylation may regulate the activity of ecNOS. To better understand how shear stress influences ecNOS activity and expression, it will be necessary to identify upstream mediators of ecNOS function which are activated by shear stress, such as protein kinases.

MITOGEN-ACTIVATED PROTEIN KINASES: LIKELY SIGNALING MOLECULES IN THE TRANSDUCTION OF SHEAR STRESS

Several features of the endothelial cell response to shear stress are analogous to receptor-mediated signaling: dependence on G proteins, increase in intracellular calcium, and changes in gene expression (Table I-1). The family of kinases termed mitogen-activated protein (MAP) kinases are potential candidates to mediate some of the effects of shear stress on endothelial cells. MAP kinases are ubiquitously expressed serine/threonine protein kinases that are activated in response to a variety of extracellular stimuli involved in cell growth, transformation, and differentiation. The extracellular signal regulated kinases (ERK1/2), members of the MAP kinase family, have many potential substrates, including other protein kinases (p90rsk, MAPKAP, Raf-1, MEK), transcription factors (c-myc, c-jun, c-fos, p62TCF), enzymes ($cPLA_2$) and cell surface proteins (EGF

receptor), and thus have many effects on cellular physiology and gene expression (Berk, Corson et al. 1995) (Figure I-4).

The pathway for ERK1/2 activation in response to growth factors has been well characterized (Figure I-4). The MAP and ERK kinase (MEK-1) is a dual specificity kinase that phosphorylates ERK1/2 on T-E-Y. MEK-1 is itself regulated by a MAP kinase kinase kinase, one of which has been identified as Raf-1. Raf-1 is activated by translocation to the membrane and association with the small GTP-binding protein, ras. The GTPase activity of ras is regulated by a complex involving Grb2 and mSOS which are recruited and activated by a tyrosine kinase receptor (Pelech and Sanghera 1992).

We have recently reported that ERK1/2 is activated by shear stress in endothelial cells in a time- and force-dependent manner (Tseng, Peterson et al. 1995). Shear stress stimulation of ERK1/2 was unaffected by treatment with the calcium chelator BAPTA-AM, indicating the response was Ca^{2+}-independent. These data, combined with observations that ecNOS contains multiple consensus sites for phosphorylation by a variety of kinases including ERK1/2 (Berk, Corson et al. 1995), make this pathway a likely candidate to participate in the stimulation of sustained NO production in response to shear stress. Additionally, several shear stress-responsive genes contain elements (e.g., AP-1) (Uematsu, Navas et al. 1993; Shyy, Lin et al. 1995) that may be influenced by ERK1/2-mediated phosphorylation of transcription factors (Berk, Corson et al. 1995) such as c-fos, c-jun, and c-myc.

Another member of the MAP kinase family shown to be regulated by shear stress is the stress-activated protein kinase (JNK/SAPK). Two laboratories have shown increases in JNK activity by shear stress, although with varying kinetics (Yi-Shuan, John et al. 1995; Jo, Sipos et al. 1997). Preliminary results in our laboratory show that shear

stress inhibits TNF-stimulated JNK activity in endothelial cells (unpublished observations), a finding consistent with the recently reported ability of shear stress to inhibit endothelial cell apoptosis (Dimmeler, Haendeler et al. 1996; Kaiser, Freyberg et al. 1997). Additional experiments by our laboratory indicate that other members of the MAP kinase family, p38 and BMK-1 (ERK5), are also activated by shear stress in endothelial cells (Lelkes 1998). Experiments to determine the roles of individual MAP kinase family members in endothelial gene expression will be important areas for future research.

UPSTREAM EFFECTORS OF ERK1/2 ACTIVITY

Several reports have shown that shear stress-mediated ERK1/2 activation can be inhibited by agents which may affect the class of enzymes known as protein kinase C (PKC). PKC are well characterized serine/threonine kinases that are activated by a variety of stimuli (Tseng, Peterson et al. 1995). Further, shear stress has been reported to result in increased phospholipase C activity, which should in turn cleaves phosphatidylinositol 4,5-bisphosphate into inositol triphosphate and diacylglycerol. This is important in that diacylglycerol can directly activate PKC, while inositol trisphosphate mobilizes intracellular calcium stores that are needed by some members of the PKC family in order to be activated.

Studies by our laboratory, however, have shown that shear stress-mediated ERK1/2 activation is unchanged by agents that prevent a rise in shear stress-induced calcium entry, such as BAPTA-AM. This does not preclude a role for PKC in the activation of ERK1/2 by shear stress, however, as some PKC isoforms are calcium independent. A classification system for the PKC family separates the different isoforms into distinct classes: the "classical" PKC isoforms, (PKC-α, $-\beta$I, $-\beta$II, -γ) are calcium-

independent and phorbol ester-responsive; the "novel" PKC isoforms (PKC-δ, -ε, -θ, -η) are calcium-independent and phorbol ester-responsive; and the "atypical" PKC isoforms (including ζ, λ/ι), are calcium-independent and phorbol ester-unresponsive. Thus, it is possible that members of the novel or atypical class of PKC isoforms may mediate the ERK1/2 activation in response to shear stress.

POTENTIAL SHEAR STRESS RECEPTORS

A question of great importance in the field of mechanotransduction pertains to the identity of the primary mechanoreceptor(s) responsible for initiating signal transduction. Transduction of mechanical forces in anchorage-dependent cells is due to a combination of force transmission via the cytoskeletal elements and transduction of the physical forces to biochemical signals at mechanotransducer sites (Berk, Corson et al. 1995). Based on the data presented above, the candidate mechanotransducer molecules should be responsive to shear stress over the physiological range and result in the activation of a tyrosine kinases (e.g., c-Src), PKC, and ERK1/2. Due to their interaction with specific signaling molecules already implicated in signal transduction, four candidates have been proposed as likely mechanotransducers; integrin-matrix interactions, specialized membrane microdomains, ion channels, and G proteins (Figure I.5).

In order to sense and transduce signals in response to shear stress, endothelial cells must be anchored to their matrix (Takahashi and Berk 1996). Integrins are ubiquitous α/β heterodimeric transmembrane glycoproteins which act as adhesion receptors involved in the interaction between cells and extracellular matrix. Integrins play an important role in biological processes, including cell adhesion, cell migration, cell growth, tissue organization, blood clotting, inflammation, target recognition by leukocytes, and cell

differentiation (Schwartz, Schaller et al. 1995). Studies performed by Dr. Ingber's group (Ingber 1991; Wang, Butler et al. 1993) using magnetic torsion have demonstrated that integrins are capable of transducing mechanical stimuli to biochemical signals. A recent study by Muller *et al.* (Muller, Chilian et al. 1997) showed that flow-induced vasodilation in coronary arteries, which is mediated by NO release, could be blocked with RGD peptides which compete with the matrix for integrin interactions. Similar attenuation of flow-induced vasodilation was obtained if a blocking antibody against the β3 integrin was employed, supporting the hypothesis that integrins are involved in the mechanotransduction of shear stress. Integrins are also a particularly attractive candidate in that they have been reported to associate with PKC (Wrenn and Herman 1995; Traub, Monia et al. 1997) and c-Src-family tyrosine kinases (Hamasaki, Mimura et al. 1996). Other studies by our laboratory have demonstrated that activation of β1 integrins (the predominant β isoform on endothelial cells) with an activating antibody also stimulated ERK1/2, although at levels less than observed with shear stress (Ishida, Peterson et al. 1996). Further, human endothelial cells showed adhesion-mediated ERK1/2 activation when plated on a matrix of fibronectin, which engages β1 integrins, but showed no ERK1/2 activation when they adhered to matrix consisting of poly-L-lysine (Takahashi and Berk 1996). The relatively small magnitude of ERK1/2 stimulation by integrin activation does not preclude a key role for integrins in shear-mediated ERK1/2 activation; based on the importance of shear stress to endothelial cell function and integrity, it is likely that redundant pathways with different mechanotransducer molecules mediate the full ERK/12 response to shear stress.

Another possible candidate for the transduction of shear stress into biochemical signals are caveolae, specialized domains of the plasma membrane that are rich in cholesterol. Because of their high cholesterol content, caveolae are more rigid than other portions of the plasma membrane. Caveolae are abundant in endothelial cells and have

been implicated in transcytosis, ion movement across the membrane, and signal transduction (Schnitzer, Liu et al. 1995). The principal component of caveolae is a 21-24 kD integral membrane protein called caveolin. Caveolin seems to function as a scaffold for the recruitment and sequestration of signaling molecules. Among signaling molecules known to associate with caveolae are G proteins, c-Src-family tyrosine kinases, ras, PKC, ecNOS (Garcia-Cardena, Oh et al. 1996), shc, Grb2, mSOS, Raf-1, and ERK1/2 (see (Couet, Li et al. 1997)). Caveolae represent an attractive site for mechanotransduction on the basis of their biophysical characteristics and interactions with signaling molecules. Experiments to determine the significance of caveolae and what effect changes in caveolae number may effect in shear stress-mediated signaling should prove an exciting area for future research.

Recent data reported by Dr. Frangos' group (Gudi, Huver et al. 1997) indicate that G proteins may act as primary mechanosensors in endothelial cells. This laboratory showed that treatment of endothelial cells with antisense Gαq oligonucleotides inhibited shear stress-induced ras-GTPase activity, while scrambled oligonucleotide treatment had no effect. Another study reported that treatment of endothelial cells with pertussis toxin prevented shear stress-mediated activation of ERK1/2 (Jo, Sipos et al. 1997), also suggesting that G proteins are activated in response to shear stress. Further, Frangos' group demonstrated that G proteins reconstituted in liposomes, in the absence of protein receptors, showed an increase in activity in response to shear stress (Frangos and Gudi 1997). This shear stress-mediated increase in G protein activity could be attenuated if the lipid bilayer was made more rigid by the addition of cholesterol, a significant finding in the context of caveolae as shear stress signaling domains.

A common mechanism that has evolved to sense changes in mechanical stimuli are the mechanosensitive ion channels. These channels are widely distributed in tissues and participate in processes such as hearing, balance, and reflex contraction of both smooth and skeletal muscle. Endothelial cells exhibit ion channel responses to mechanical forces that are likely to participate in the signaling response to shear stress. Several different mechanosensitive ion channels are present in endothelial cells, including a shear stress-responsive potassium channel, and a stretch-activated calcium channel (Davies 1995). Studies have shown that blockade of mechanosensitive K^+ channels with barium chloride or tetraethylammonium blocked shear-mediated increases in NO production (Uematsu, Ohara et al. 1995) and TGF-β release (Ohno, Cooke et al. 1995), suggesting that transmembrane ion flux and intracellular ion homeostasis are important mediators of the endothelial cell response to shear stress. However, efforts to clone the mechanosensitive K^+ channel from the endothelial cell have not yet been successful.

Based on the demonstrated importance of shear stress to endothelial cell function and integrity, it is likely that each of these putative mechanoreceptors activates intracellular signaling pathways to effect the complete endothelial response to shear stress. Differential coupling of signaling mechanisms and subsequent endothelial cell response to the individual shear stress receptor "subtypes" may provide a flexibility to the endothelial cells in terms of responding to varying types and degrees of shear stress that they cell may encounter.

CONCLUSIONS

Based on the above review, the current dissertation will attempt to characterize the involvement of PKC and mechanosensitive ion channels in shear stress-

mediated ERK1/2 activation. These undertakings are based not only on supporting evidence provided in the literature, but also on the feasibility of determining whether these mechanisms are involved in shear stress-mediated ERK1/2 activation based on the techniques available and time frame allotted.

In the following chapters, we will demonstrate that PKC is involved in the shear stress-mediated ERK1/2 activation as well as characterize which isoform is likely to participate in this signaling transduction pathway. Further, we will characterize and identify mechanosensitive ion channels that are involved in the ERK1/2 response to shear stress.

Table I-1: Endothelial cell responses to shear stress: temporal groupings.

Initiation of Signaling (< 1 minute)

- K+ channel activation
- IP3 and DAG elevation
- cGMP increase
- Calcium increase
- Acute end responses (NO, PGI2 release)

Signaling cascades; Transcription factor activation; Gene regulation (1 minute to 1 hour)

- G protein activation
- MAP kinase signaling
- NFkB activation
- SSRE-dependent gene regulation: (PDGF-B, c-jun)
- bFGF upregulation
- Pinocytosis stimulated

Adaptive responses to new hemodynamic conditions (1-6 hours)

- Reorganization of luminal surface
- Cell alignment
- Completion of cytoskeletal rearrangement
- Increased mechanical stiffness
- Decreased fibronectin synthesis
- Changes of TM expression
- Stimulation of histidine decarboxylase
- Enhanced LDL metabolism
- Induced MHC antigen expression

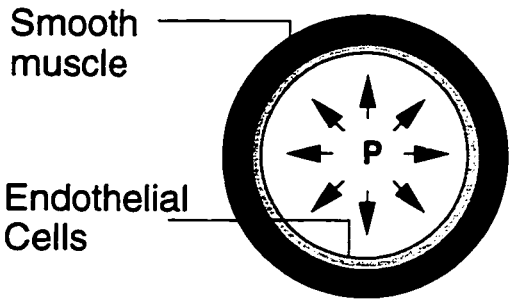
Gene regulation and protein synthesis; Cell-wide adaptive responses (> 6 hours)

- SSRE-dependent gene regulation: (eNOS, tPA, TGF-b, ICAM-1, c-fos, MCP-1)
- Stimulation of HSP-70
- Downregulation of ET-1
- Cytoskeletal rearrangement
- Focal adhesion rearrangement

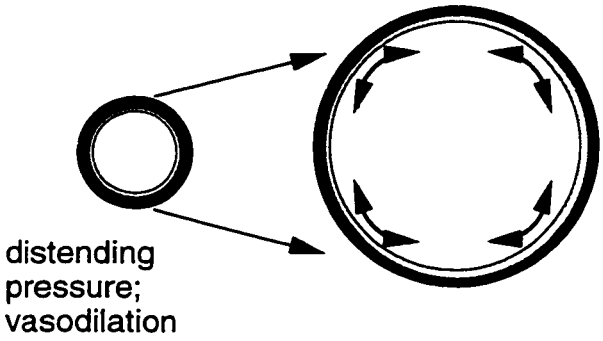
- Transient rearrangement of Golgi

Figure I-1 Physical forces on endothelium include the normal force of pressure exerted by the hydrostatic properties of the blood; circumferential or longitudinal tension exerted by intercellular connections as the blood vessel constricts or dilates; and shear stress, the dragging force created by blood flow over the endothelial cells.

Pressure



Stretch



Shear

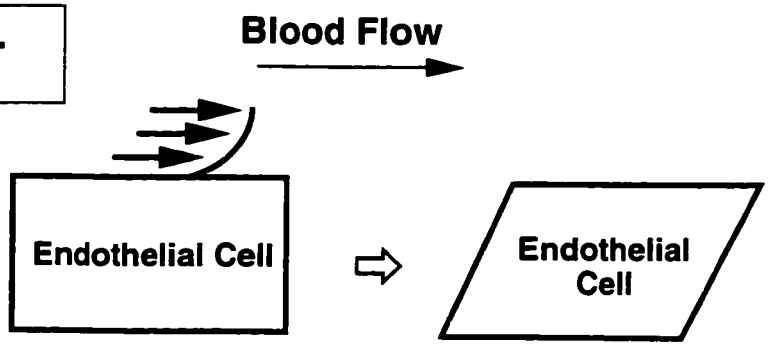


Figure I-2 Carotid bifurcation and atherosclerosis. The medial wall of the carotid bulb experiences higher shear stress while the lateral wall experiences recirculation vortices which vary with the cardiac cycle resulting in flow reversal. The lateral area of the carotid bulb experiences *oscillatory* shear stress (periodic flow reversal with time-average shear stress approaching zero) and low mean shear stress. Endothelial cell dysfunction and atherosclerosis is correlated with the lateral wall (areas of low mean shear stress and oscillatory flow with flow reversal).

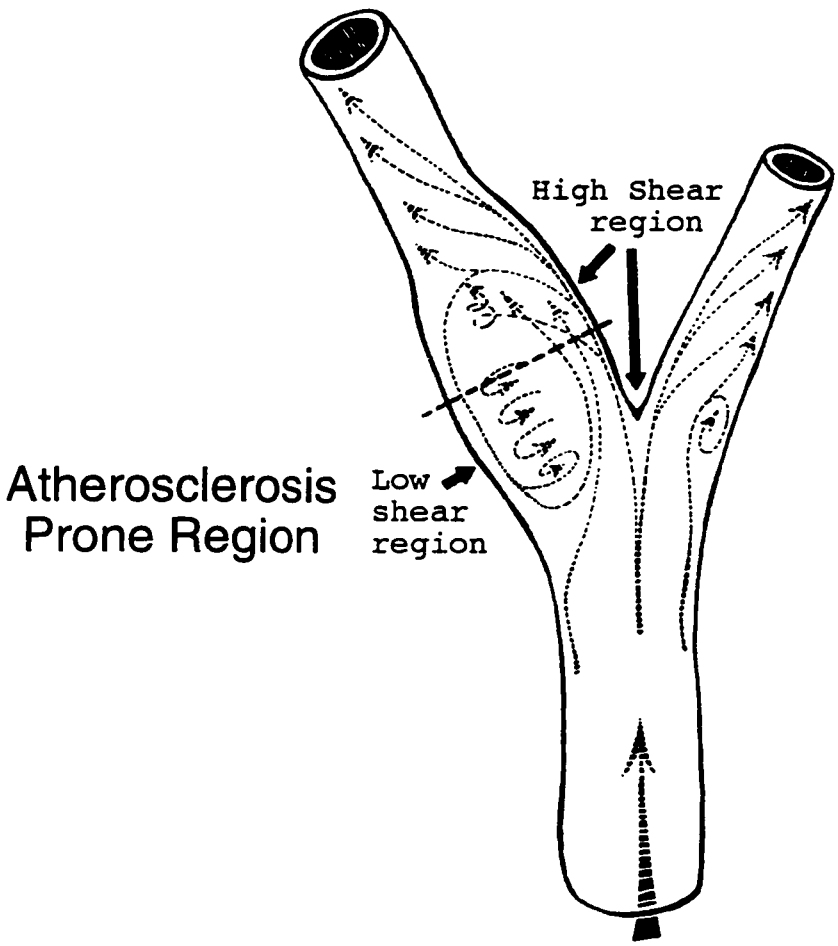
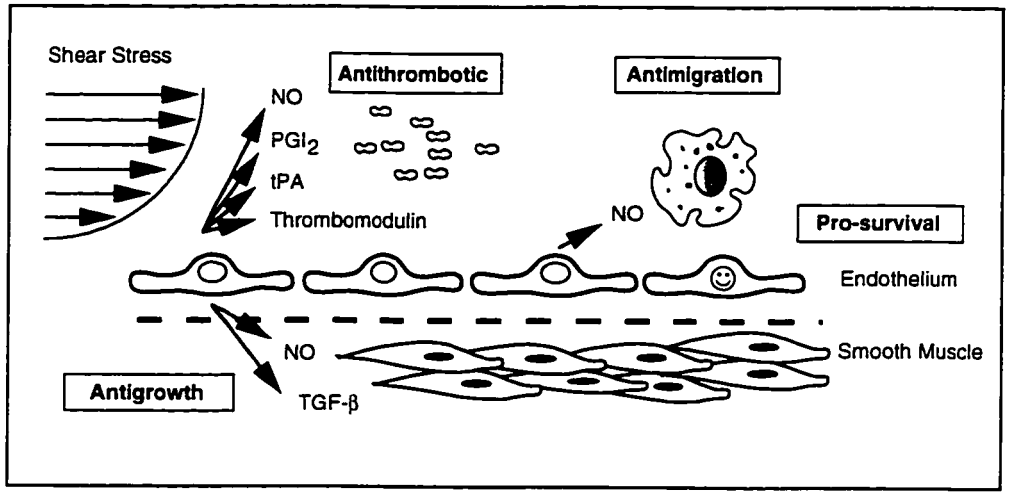

High Shear region
Atherosclerosis Prone Region
Low shear region

Figure I-3 Endothelial cell biology and shear stress. Steady laminar shear stress promotes release of factors from endothelial cells which inhibit coagulation, migration of leukocytes, and smooth muscle proliferation, while simultaneously promoting endothelial cell survival. Conversely, low shear stress and flow reversal shifts the profile of secreted factors and expressed surface molecules to one that favors the opposite effects, thereby contributing to the development of atherosclerosis.

Steady
Laminar
Blood Flow



Flow
Reversal

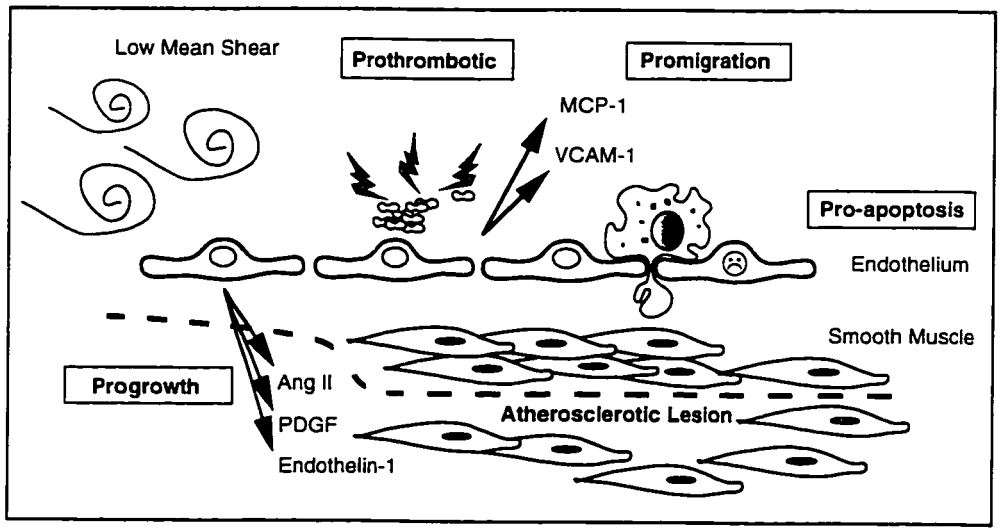


Figure I-4 MAP kinase activation pathways. A common theme in the stimulation of MAP kinase family members is activation by an immediate upstream MAP kinase kinase (MEK) which is, in turn, activated by an immediate upstream MAP kinase kinase (MEKK). Different stimuli activate different signaling pathways leading to individual MAP kinase activation.

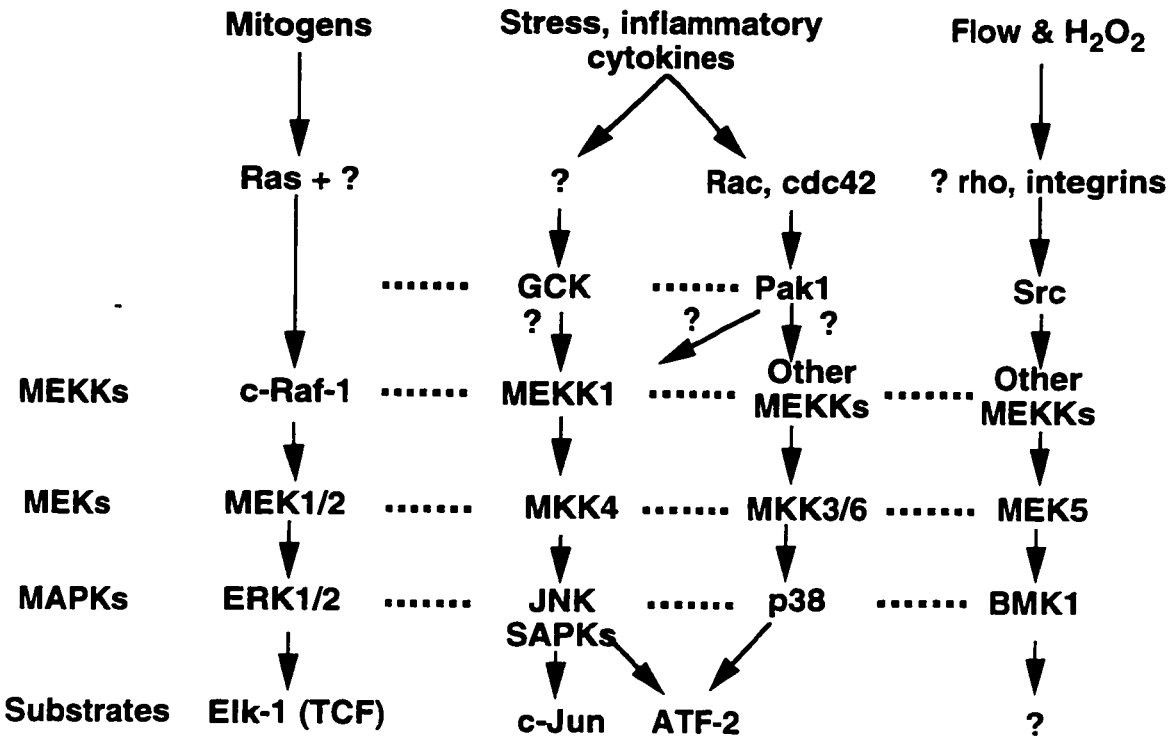
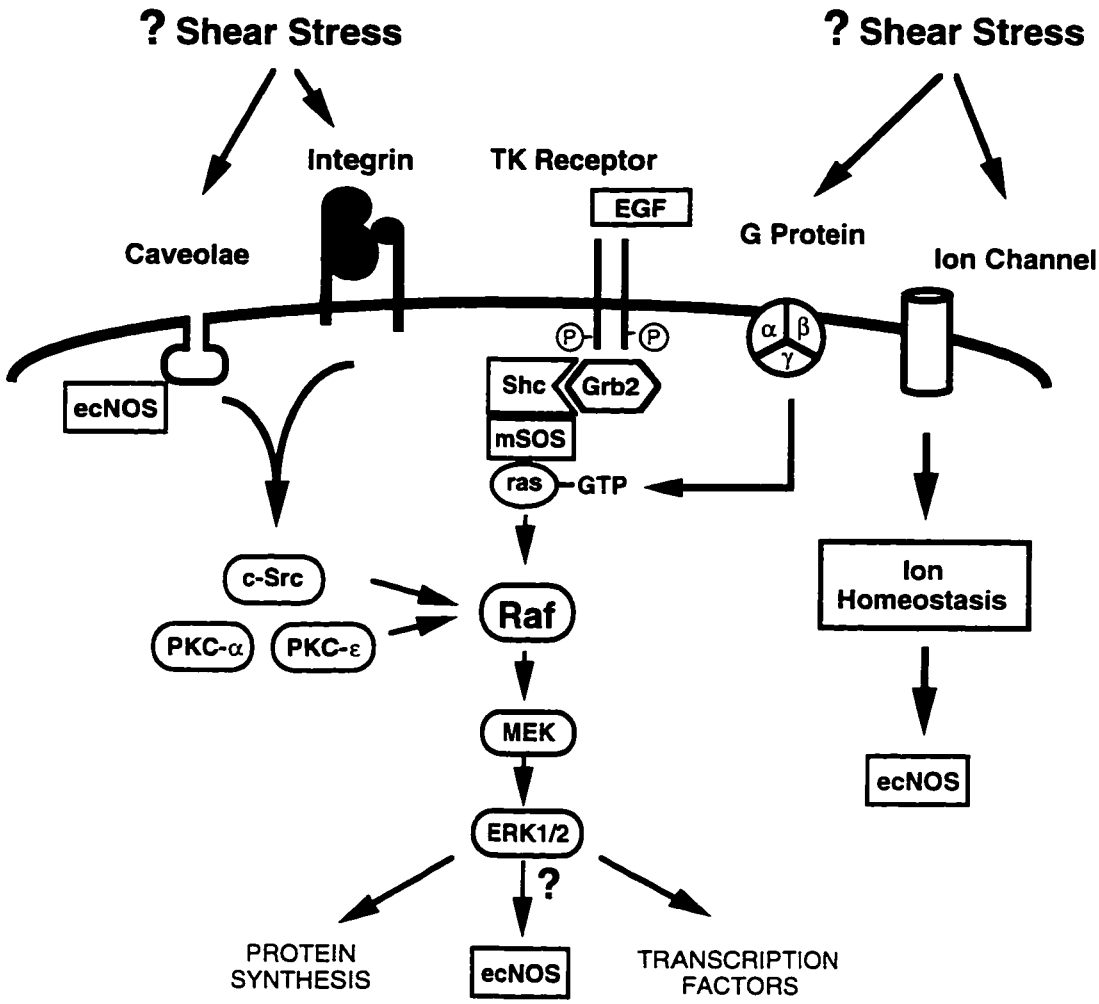

Mitogens
Stress, inflammatory cytokines
Flow & H_2O_2
Ras + ?
?
Rac, cdc42
? rho, integrins
GCK
Pak1
Src
?
?
?
MEKKs
c-Raf-1
MEKK1
Other MEKKs
Other MEKKs
MEKs
MEK1/2
MKK4
MKK3/6
MEK5
MAPKs
ERK1/2
JNK SAPKs
p38
BMK1
Substrates
Elk-1 (TCF)
c-Jun
ATF-2
?

Figure I-5 Proposed model of shear stress-mediated mechanotransduction in endothelial cells. Primary mechanosensors (e.g. integrins, caveolae, G proteins, ion channels) transduce physical stimuli into biochemical signals. Several stimuli serve to activate Raf-1, including tyrosine phosphorylation by c-Src or c-Src-like kinases, serine and threonine phosphorylation by PKC, and GTP-bound ras. Raf-1 activates MEK which in turn activates ERK1/2. Sustained generation of NO may result from the effects of ERK1/2 or through direct effects of mechanosensors (e.g. caveolae) themselves.


? Shear Stress
? Shear Stress
Integrin
TK Receptor
EGF
Caveolae
G Protein
Ion Channel
α
β
γ
P
P
ecNOS
Shc
Grb2
mSOS
ras
GTP
c-Src
PKC-α
PKC-ε
Raf
Ion Homeostasis
MEK
ecNOS
ERK1/2
?
PROTEIN SYNTHESIS
ecNOS
TRANSCRIPTION FACTORS

CHAPTER 2: *IN VITRO* APPLICATION OF SHEAR STRESS TO CULTURED CELLS

INTRODUCTION

Shear stress occurs when a body is subjected to a force tangential to one of its faces while the opposite face is held in a fixed position by a force (e.g., friction, adhesion). If the object if originally a rectangular block, a shear stress results in a shape whose cross-section is a parallelogram (Figure I-1). Thus the shear stress in this simplistic example is defined as:

$$\tau = F / A \qquad (1)$$

where τ is the shear stress equal to the force, F, divided by the area of the face being sheared, A. When speaking of a more complex example of shear stress exerted by a *liquid* passing over an object, other factors must be considered when calculating the magnitude of the shear stress, in particular the fluid dynamic state (Roark 1965; Goldstein 1996; Serway 1996).

If adjacent layers of a viscous fluid flow smoothly over each other, the stable streamline flow is called laminar flow, and the shear stress exerted by the fluid will be unidirectional and summative. However, at sufficiently high velocities or constrained geometry, the fluid flow will change from laminar flow to a highly irregular and random motion of the fluid called turbulent flow. In this fluid dynamic state, the shear stress vectors are often opposing and the mean shear stress exerted over an area can approach zero (in contrast to the high local shear gradients). The fluid velocity at which turbulence occurs depends on the geometry of the medium surrounding the fluid and the fluid

viscosity. Experimentally, it is found that the onset of turbulence is determined by a dimensionless parameter called the Reynolds number (RN) given by:

$$RN = (\rho\ v\ d) / \eta \qquad (2)$$

where ρ is the fluid density, v is the velocity of the fluid, η is the viscosity of the fluid, and d is the geometrical length associated with the fluid flow. For flow through a tube, d would be the diameter of the tube, while for flow around a sphere, d would be the diameter of the sphere. Experiments show that if the Reynolds number is below about 2000, the flow of fluid through a tube is laminar, while turbulence occurs if the Reynolds number is about 3000 (Roark 1965; Goldstein 1996; Serway 1996).

Since relatively little is known about the signal transduction mechanisms by which the physical force of shear stress is transduced into biochemical signals, it is desirable to create a system by which shear stress could be simulated on cells grown in culture. In this manner the parameters that define shear stress can be precisely controlled, while at the same time eliminating other variables that are present in the *in vivo* or *ex vivo* system that respond to and influence changes in shear stress. Using the mathematical formulae presented above as a basis, several different types of apparatus can be constructed in order to simulate shear stress over cultured cells. One apparatus, the parallel plate chamber, is already is use in our laboratory. However, due to technical constraints on the parallel plate chamber (discussed below), we will undertake the construction of a new type of apparatus, the cone and plate viscometer.

The other consideration in determining the effect of shear stress on a biological system is the selection of an appropriate biological endpoint or marker that varies in response to shear stress. The family of mitogen-activated protein kinases (MAPK) are

excellent candidates to serve as biological endpoint as they have been shown to be activated by various stimuli (Pelech and Sanghera 1992), including physical stress (Brewster, de Valoir et al. 1993; Tseng, Peterson et al. 1995; Yamazaki, Komuro et al. 1995), and they have diverse effects on gene expression and cell physiology (Berk, Corson et al. 1995) (Figure I-4). As a result of these properties, it has been postulated that MAPK mediate at least part of the response to shear stress in endothelial cells.

In Chapter I, we discussed the responsivity of different members of the MAPK family to shear stress in endothelial cells. While the response of the members, JNK, p38, and BMK-1 are still being characterized, the activation of ERK1/2 in response to shear stress has been well documented and reproduced by several investigators (Tseng, Peterson et al. 1995; Li, Shyy et al. 1996; Pearce, McIntyre et al. 1996; Jo, Sipos et al. 1997; Traub, Monia et al. 1997). Further, this ERK1/2 activation in response to shear stress is present in widely differing cell types from different developmental origins; in addition to the endothelial cell ERK1/2 response to shear stress, ERK1/2 activation in response to shear stress can also be observed in rat embryonic fibroblasts and Chinese hamster ovary cell lines (Figure II-1), though the response is not as robust when compared to endothelial cells. Using ERK1/2 as a biological endpoint is also useful in that the signaling pathways by which ERK1/2 is activated in response to classical growth factor such as EGF has been well elucidated (Pelech and Sanghera 1992) (Figure I-4, I-5). Thus, EGF is a useful as a positive control for stimulation of ERK1/2 activity through already established signal transduction pathways.

Another advantage in utilizing ERK1/2 activation as a biological endpoint for the response to shear stress is that there exist several methods of determining ERK1/2 activity including an *in vitro* kinase assay, in-gel kinase assay, phosphorylation band-shift

assay (Tseng, Peterson et al. 1995), and quantification of the active, phosphorylated form of ERK1/2 with a phosphospecific antibody (Traub, Monia et al. 1997). Since these methods, particularly Western blotting with the phosphospecific antibody, are relatively simple to perform, ERK1/2 activation in response to shear stress seems an excellent candidate as a biological marker reflecting the endothelial cell response to shear stress.

In the following sections, we will detail construction of a new apparatus for simulating shear stress over cultured cells: the cone and plate viscometer. We will compare the endothelial cell response to shear stress obtained with this apparatus to those obtained with the parallel plate chamber using ERK1/2 activation as our biological endpoint.

MATERIALS AND METHODS

PARALLEL PLATE CHAMBER

Our laboratory has previously reported on the construction of a device to simulate shear stress over cultured cells. This apparatus is a parallel plate chamber (Figure II-2) consisting of a gravity-fed recirculating flow loop system. Gravity-fed medium between two reservoirs is placed in series with a parallel plate flow chamber and a flow meter (Figure II-3). During experiments, the upper reservoir and medium is warmed to 37°C and flow rate through the use of a flow meter. A recirculating pump is connected to pump medium back to the upper reservoir. Fluid medium is a buffered physiological salt solution whose values are, containing in mM, NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 20, pH 7.4. The cell chamber consists of a monolayer of cells grown on tissue culture plastic that is cut to precise dimensions (74 by 36 mm) from a tissue culture dish and a Plexiglas block sandwiching a mylar gasket of known thickness. These items define a cell chamber of known dimensions.

Through a complex series of mathematical derivation discussed elsewhere (Rosenhead 1963) and the use of readings from the flow meter, shear stress applied over cells in this apparatus can be *directly* determined by the formula:

$$\tau = (6 \mu Q) / (h^2 b) \quad (3)$$

where τ = wall shear stress in dynes/cm^2, μ = viscosity of the flow medium (poise), Q = flow rate (mL/sec), b = width of the cell chamber (cm), and h = height of the cell chamber (cm); in the present example, $\mu = 0.006915$; $b = 0.37$ and $h = 0.025$.

We have previously reported that this apparatus is useful is applying defined magnitudes of shear stress over cultured cells, particularly because it provides a precise readout of flow though the system, thereby allowing us to directly calculate the shear stress. However, this apparatus possesses some limitations as well; because of the relatively high volume of flow buffer needed to keep flow constant and free of air bubbles (minimum = 150 mL), large amounts of sometimes expensive drugs are needed in order to achieve sufficient concentration for biological effect. Further, because the tissue culture plates must be cut manually and are not commercially available or engineered, this method is time consuming and prone to leakage if the plates are not cut to exact specifications. The scraping of cells to another dish that is necessary prior to storage also results in a loss of a great deal of protein. These plates must also be submerged in large amounts of cell culture media, presenting problems not only in expense but also when pharmacological or molecular variables (expensive drugs, transfection procedures) are needed prior to the shear stress stimulus. Finally, we have been unable to measure the release of vasoactive mediators that are released from endothelial cells with shear stress, including nitric oxide, when using this apparatus. Finally, because of its large size, this apparatus is relatively

stationary. Because of these constraints, we have undertaken the construction of another apparatus, the cone and plate viscometer.

CONE AND PLATE VISCOMETER

The cone and plate viscometer is an apparatus used to apply shear stress to cultured cells. The apparatus consists of a cone that is inserted into a circular tissue culture dish containing cultured cells and rotated so that the medium within the dish circulates in a laminar fashion and at controlled velocities.

Mathematical Derivation

In deriving the mathematical formula for applied shear stress using this device, it is useful to first examine the effects of a disk and plate apparatus (i.e. a flat surface inserted into the tissue culture dish) (Figure II-4a). When the disk is rotated, a concentric fluid movement occurs in the culture medium in the dish . The endothelial cell monolayer cultured on the bottom of the dish is subjected to the shear stress according to the velocity gradient near the cell surface. As the medium can be regarded as a Newtonian fluid in this case, the induced flow is explicitly expressed by the Navier-Stokes equations in cylindrical coordinates (Nomura, Ishikawa et al. 1988). Movement of the fluid can be expressed where

for r-momentum:

$$\partial u/\partial t + u\partial u/\partial r - v^2/r + w\partial u/\partial z = -(1/\rho)\partial P/\partial r + \nu[\partial^2 u/\partial r^2 + \partial(u/r)/\partial r + \partial^2 u/\partial z^2] \quad (4)$$

for θ-momentum:

$$\partial v/\partial t + (u/r)\partial(rv)/\partial r + w\partial v/\partial z = \nu[\partial^2 v/\partial r^2 + \partial(v/r)/\partial r + \partial^2 v/\partial z^2] \quad (5)$$

for z-momentum:

$$\partial w/\partial t + u\partial w/\partial r + w\partial w/\partial z = -1(1/\rho)\partial P/\partial z + \nu[\partial^2 w/\partial r^2 + (1/r)\partial w/\partial r + \partial^2 w/\partial z^2] \quad (6)$$

and as the equation of continuity:

$$(1/r)\partial(ru)/\partial r + \partial w/\partial z = 0 \quad (7)$$

The coordinate directions (r, θ, z) and corresponding velocities (u, v, w) are defined in Figure II-4a. In addition, the fluid density is ρ, the kinetic viscosity is ν (= μ / ρ) and the pressure is P. The boundary conditions for the velocities u(r,z,t), v(r,z,t) and w(r,z,t) are:

$$\begin{aligned} u(r,0,t) &= 0 & u(r,d,t)&=0 \\ v(r,0,t) &= 0 & v(r,d,t) &= r\omega \\ w(r,0,t) &= 0 & w(r,d,t) &= 0 \end{aligned} \quad (8)$$

where d is the distance between the disk surface and the dish bottom and ω is the angular velocity of the disk rotation. According to Lance *et al.* (Lance and Rogers 1961) it is obvious that when the Reynolds number determined for this geometry:

$$RN = (\omega r^2) / \nu \quad (9)$$

is very low (especially when it is less than 10), the effects of the concentric force in the rotating fluid is negligibly small and therefore the radial and perpendicular velocity components u and w can also be regarded as negligible. Thus, the Navier-Stokes and continuity equations are reduced to a parabolic partial differential equation,

$$\partial v/\partial t = \nu(\partial^2 v/\partial z^2) \quad (10)$$

When the angular velocity ω of the disk rotation is constant ($\partial v/\partial t = 0$) the solution of the above gives

$$v(r,z) = (r\,\omega\,z) / d \qquad (11)$$

and wall shear stress τ exerted in the bottom of the dish (z=0) is obtained as

$$\tau(r) = \mu\, r\, \omega / d \qquad (12)$$

Thus defined, the sole variable is the radius; shear stress increases directly proportional to the increasing radial distance from the center of the disk.

This variability in generated shear stress within a single dish is not desirable for our apparatus, as we wish to have a uniform shear stress applied to the cells. In order to rectify this, the rotating disk can be replaced with a rotating cone (Figure II-4b) with a small, but constant angle (α). Thus we now have two variables, the distance of the cone surface from the dish at each radius as well as the radial distance. However, by basic trigonometric principles, it is known that the ratio of these variables given a constant angle on the cone is constant, and thus:

$$\tan \alpha = d / r \qquad (13)$$

or in other words, d always changes in proportion to r. Equation (12) can thus be altered so that shear stress on the bottom of the dish (z=0) at each radius can now be described as:

$$\tau = (\mu\, \omega) / (\tan \alpha) \qquad (14)$$

and shear stress is now uniform within the cell culture dish at each radius.

However, with the change in geometry that occurs when replacing the disk with a cone, the Reynolds number which dictates fluid dynamic considerations also changes. The fluid dynamic patterns induced by a rotating cone has been previously characterized (Bussolari, Dewey et al. 1982) and the modified Reynolds number defined by:

$$RN_{cone} = (r^2 \omega \rho \alpha^2) / (12 \mu) \quad (15)$$

where laminar flow is obtained when $r < 1$. The range of modified Reynolds numbers obtained using different angle cones are best illustrated graphically for defined shear stress at different radius (Figure II-5). Through the use of the 1° degree angle cone, it is possible to minimize fluid turbulence at the levels of shear stress required for our experiments (12 dynes/cm^2)

Details of construction:

Polyacetal resin (Delrin) cones in two different sizes (for 60 mm and 100 mm Corning tissue culture dishes) were milled with precise angle measurements and attached to a stainless steel shaft which was in turn attached to a BC215GD-AF model motor and 2GD10K gear head purchased from Oriental Motor Co. Torrence, CA (Figure II-6). The motor was wired to a external controller (Model BLD15-AF) with adjustable potentiometer. A step down transformer (120 V to 100 V; Sanyo Model TSD-N0GU) was added and the motor and gear head was placed on an adjustable platform so that the cone could be lowered onto the base containing the cell culture dish. Adjustable set screws were used to obtain reproducible heights from the cell culture dish.

The apparatus (not including external controller) was placed in an adjustable incubator (Robbins Scientific hybridization chamber, Model 1000) set at 37°C for

experiments. External potentiometer reading to angular velocity was measured through visual observation (Figure II-7).

COMMON SHEAR STRESS EXPERIMENT PROTOCOL

Cells were grown in 60 mm or 100 mm Corning circular tissue culture dishes or on 74 cm x 36 cm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Two days after reaching confluence, cells were rinsed free of culture media with HBSS (containing in mM: NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 20, pH 7.4), with 10 mM glucose added. The solution is buffered and pH does not significantly change in either apparatus within the longest time point tested (60 min). The cells were either maintained in static condition or exposed to 12 dynes/cm^2 of fluid shear stress, in a parallel plate chamber or cone and plate viscometer at 37°C. After varying times or magnitude of exposure to fluid shear stress, cells were washed gently with ice-cold PBS (composition, in mM, NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.3) and ERK1/2 activation was determined.

WESTERN BLOT ANALYSIS FOR PHOSPHO-ERK1/2

Cells were washed with PBS and 0.15 mL of modified oncogene science lysis buffer (Na pyrophosphate 50 mM, NaF 50 mM, NaCl 50 mM, EDTA 5 mM, EGTA 5 mM, Na_3VO_4 100 μM, HEPES 10 mM, Triton 0.1%, PMSF 0.5 mM, leupeptin 10 mg/mL, pH 7.4) was added. Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4°C, 14 000 rpm in microfuge). Sample protein concentrations were determined by DC Protein (Biorad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham). To ensure quantitative

transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 hr at room temperature with a commercial blocking buffer (GIBCO BRL). The blots were incubated overnight at 4°C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs) followed by incubation for 1-2 hr with secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized by chemiluminescence (Amersham ECL).

ERK1/2 Kinase Activity Assay

An in-gel kinase assay to measure ERK1/2 kinase activity was performed on cell lysates as described previously. In brief, cells were harvested as described above. Equal amounts of protein (5-10 μg) were separated by SDS-PAGE through a gel containing 0.4 mg/mL myelin basic protein (MBP). The gel was then incubated twice in buffer A (50 mM HEPES, pH 7.4, and 5 mM β-mercaptoethanol) containing 20% isopropyl alcohol for 30 min, once in buffer A for 1 h, twice in buffer A containing 0.04% Tween 20 at 4 °C for 16 h and for 2 h, and once in buffer A containing 100 μM Na_3VO_4, 10 mM $MgCl_2$, 50 μM ATP, and 50 μCi of [γ-32P]ATP for 1 h at 30 °C. The reaction was terminated by washing the gel five to eight times in fixative solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid for 15 min. The gel was dried and subjected to autoradiography, and ERK1/2 kinase activity was measured by densitometry of autoradiogram (in the linear range of film exposure).

Measurement of Nitric Oxide Release

Ozone chemiluminescent determination of NOx products from HUVEC was performed on a Dasibi model 2107 NO-analyzer (Glendale, CA) with a glass reflux chamber containing 40 mM vanadium III chloride in 3 M HCl. Cells were washed twice

with Krebs-HEPES buffer and either incubated for 1 hr in Krebs-HEPES buffer containing 1 μM A23187 or exposed to shear stress of 12 dynes/cm^2 in a parallel plate chamber or cone and plate viscometer. Supernatants were then collected and assayed for NOx products in the chemiluminescent NO-analyzer and quantified using sodium nitrate as a standard.

RESULTS:

FLUID SHEAR STRESS LEADS TO PHOSPHORYLATION AND ACTIVATION OF ERK1/2 IN A FORCE-DEPENDENT MANNER

HUVEC were exposed to shear stress of varying magnitudes for 10 min. Lysates were obtained and either an in-gel kinase assay or Western blotting with phospho-specific ERK1/2 antibodies was performed. Both activity and phosphorylation of ERK1/2 increased in a force-dependent manner (Figure II-8a). These data show activation kinetics similar to those previously reported by our laboratory in both BAEC and HUVEC (Tseng, Peterson et al. 1995). Western blotting with an antibody that detects both the phosphorylated and unphosphorylated forms of ERK1/2 showed no change in whole cell ERK1/2 level with increasing shear stress (not shown). These results demonstrate that ERK1/2 is phosphorylated in response to fluid shear stress in a force-dependent manner and that phosphorylation correlates with ERK1/2 kinase activity (Figure II-8b). Because of the positive correlation between the two techniques and the relative ease of immunoblotting versus an in-gel kinase assay, we employ the phospho-specific ERK1/2 antibody in subsequent experiments as a reflection of activity.

ERK1/2 phosphorylation force-response curves were not significantly different between the two different apparatus tested when the theoretical shear stress generated with the cone and plate viscometer was compared against those results obtained

with actual shear stress measured with the parallel plate chamber flow meter (Figure II-9). This was true independent of the dish size (60 mm or 100 mm) used for the cone and plate apparatus. These results suggest that the theoretical mathematical calculation used for the cone and plate viscometer generate shear stress magnitudes as predicted and indicate that the cone and plate viscometer is useful in generating defined levels of shear stress over cultured cells

SHEAR STRESS STIMULATES ERK1/2 IN A TIME-DEPENDENT MANNER.

Compared with static conditions, fluid shear stress at 12 dynes/cm^2 activated ERK1/2 with a peak at 10 min and return to baseline by 60 min (Figure II-10). These data show activation kinetics similar to those previously reported by our laboratory using other techniques (Tseng, Peterson et al. 1995; Ishida, Peterson et al. 1996). Western blotting with an antibody for ERK1/2 that detects both the phosphorylated and unphosphorylated form of the kinases showed that cellular ERK1/2 levels remained constant throughout the shear stress time-course. These results demonstrate that ERK1/2 is phosphorylated in response to fluid shear stress with time-course similar to receptor agonists, such as thrombin and EGF (Tseng, Peterson et al. 1995) .

Time course kinetics for ERK1/2 phosphorylation using the cone and plate viscometer (Figure II-10) were similar to those results obtained with the parallel plate chamber for early time points (< 30 min). However, levels of pERK1/2 were significantly higher at time points greater than 30 min when compared with results using the parallel plate chamber. One likely explanation for this persistent elevation is that vasoactive mediator released by the endothelium in response to shear stress become concentrated in the relatively low volume (1-4 mL) of media used in the cone and plate viscometer. Many vasoactive mediators are released by endothelial cells in response to shear stress (Davies

1995) and may affect ERK1/2 phosphorylation. These results indicate that the use of the cone and plate viscometer yields results similar to those obtained with the parallel plate chamber for early time points, but not for longer time points.

INCREASED DISTANCE OF THE CONE FROM THE DISH LOWERS ERK1/2 ACTIVATION

Theoretical considerations dictate that as the cone is moved away from the dish, either, (1) turbulent flow will be generated resulting in a mean shear stress close to zero at the dish surface; or (2) if sufficient distance is achieved, an unstirred boundary layer will develop at the bottom of the dish thereby "protecting" the cells from shear stress. In order to determine whether distance of the cone from the dish (cone height) would affect shear stress generated, sequential experiments were performed whereby the rotational velocity of the cone was held constant , but the cone was progressively moved away from the dish and ERK1/2 phosphorylation subsequently measured. ERK1/2 phosphorylation was progressively lower as the cone was moved from a height of 0 μm to 500 μM and then to 1000 μM (Figure II-11). At a 1000 μM height, little difference in ERK1/2 phosphorylation was seen at any of the shear stress magnitudes applied. These results indicate that reproducible cone height is critical in achieving similar levels of shear stress between different samples.

SHEAR STRESS-MEDIATED ERK1/2 PHOSPHORYLATION IS LARGELY UNIFORM AT DIFFERENT RADII IN THE CONE AND PLATE VISCOMETER.

Since the Reynolds number is essentially an experimentally determined parameter (Bussolari, Dewey et al. 1982; Serway 1996), it is necessary to confirm that the response to shear is uniform at each radius and that turbulent flow does not result when testing a new apparatus. In order to determine whether the shear stress distribution is uniform at each radius, sequential experiments were performed where cell were scraped

from the dish just prior to experiments except for defined areas (Figure II-12a). The shear stress was then applied, and the cells were harvested for determination of ERK1/2 phosphorylation. Experiments determined that the ERK1/2 phosphorylation was not significantly different among the different areas tested except for the inner most area (Figure II-12b). It is likely that the increased ERK1/2 phosphorylation in the inner most area results from actual physical deformation of the cell by the cone due to the small distance between the cone and the bottom of the tissue culture dish at the center of the cone. Further, it should be noted that this bias is relatively equal to that reported in the parallel plate chamber due to an "edge turbulence effect" (Geiger 1991). These results confirm mathematical calculations indicating that shear stress is generally uniform, except for the very center of the dish.

SHEAR STRESS GENERATED BY THE CONE AND PLATE VISCOMETER RESULTS IN NITRIC OXIDE RELEASE

Shear stress has been demonstrated to be a potent stimulus for nitric oxide release from endothelial cells (Berk, Corson et al. 1995). Since nitric oxide has a relatively short half life, nitric oxide release is commonly measured through the detection of nitric oxide metabolites. Previously, we have been unable to detect nitric oxide metabolites in the media from the parallel plate chamber apparatus, though this may be due to the relative large volumes needed for this apparatus (>150 mL). Media taken from the cone and plate apparatus after shear stress stimulus showed high levels of nitric oxide metabolites, much greater than those obtained with the calcium ionophore, A23187 (Figure II-13). These results suggest that the cone and plate viscometer is useful for detection of substances released from endothelial cells in response to shear stress, in contrast to the parallel plate chamber

DISCUSSION

The above results indicate that the cone and plate viscometer is useful in simulating shear stress over cultured endothelial cells. When compared against results obtained with the parallel plate chamber, the cone and plate viscometer generated similar results in terms of force-response and early time course (> 30 min). There was a difference in results obtained between the two apparatus in terms of late time points (> 30 min); while the ERK1/2 phosphorylation levels returned to baseline at approximately 30 minutes, the ERK1/2 phosphorylation levels obtained with the cone and plate viscometer remained elevated even at 60 min. The likely explanation for this disparity is that vasomediators released by endothelial cells in response to shear stress are much more concentrated in the lower volume of the cone and plate viscometer medium (1-4 mL) when compared to the volume used in the parallel plate chamber (> 150 mL). Thus, for these longer time points, a medium replacement protocol (Bussolari, Dewey et al. 1982; Topper, Wasserman et al. 1997) (i.e. replace 1 mL of media with fresh media every 10 minutes) may be helpful in determining whether released vasomediators may influence the results and may be helpful in normalizing the results to those obtained with the parallel plate chamber.

It is this same property which makes the cone and plate apparatus very useful in measuring amounts of released vasomediators, such as nitric oxide. We have previously been unable to detect significant levels of nitric oxide when using the parallel plate chamber. Through the use of the cone and plate viscometer, we have been able to detect the presence of nitric oxide metabolites upon exposure of the endothelial cells to shear stress that is much greater than those levels obtained with the calcium ionophore, A23187. This is consistent with reports that have shown that shear stress is a much more potent stimulus for nitric oxide release than A23187 (Berk, Corson et al. 1995).

The other variability in ERK1/2 phosphorylation that occurs with the cone and plate viscometer concerns those cells at the very center of the dish. ERK1/2 phosphorylation levels were significantly greater at the very center of the dish while the remaining portions of the dish showed ERK1/2 phosphorylation levels that were not significantly different from each other. The likely explanation for this is that the endothelial cells at the very center of the dish are physically deformed by the cone itself. Inclusion of these biased set of cells in the end result could be avoided by removing the center circle of cell from the dish via scraping prior to shear stress stimulus.

While the cone and plate viscometer does differ from the parallel plate chamber is some ways, it also provides some very distinct advantages. These include reduced time and expense for each sample, less drug needed to achieve higher concentration, more protein harvested per sample, and less error due to leakage. Conversely, the cone and plate apparatus still provides greater difficulties in terms of fluid hemodynamics (though turbulent flow seems to be at a minimum at the levels of shear stress tested) and due to the fact that flow rate cannot be measured directly as in the parallel plate chamber. However, the use of the parallel plate chamber to compare the ERK1/2 phosphorylation with known amounts of shear stress against those obtained with shear stress levels that are calculated by theoretical mathematics for the cone and plate viscometer are useful in overcoming these difficulties.

Another major finding of these experiments is that the ERK1/2 phosphorylation and activity are excellent biological markers for shear stress responsivity. ERK1/2 phosphorylation and activity varied with shear stress in a force- and time-dependent fashion and measurements of changes in ERK1/2 activation are relatively quick, simple and inexpensive. It should be stated, however, that the use of ERK1/2 activation as

an endpoint is merely one of myriad possible biological markers for shear stress responsivity. Many different signal transduction elements are activated and vasomediators released in response to shear stress (Davies 1995) (Table I-1), each with signal transduction pathways that are likely unique from those that activate ERK1/2. Regardless of this, ERK1/2 possesses all of the desired properties of a biological marker of shear stress responsivity and represents an excellent starting point for the dissection of shear stress-activated signal transduction pathways.

In summary, ERK1/2 is an excellent biological marker for shear stress responsivity based on the following properties: (1) ERK1/2 responds to shear tress in a force- and time-dependent manner; (2) EGF mediated ERK1/2 signal transduction pathways are already elucidated and serve as a excellent positive control; (3) shear mediated ERK1/2 activation is a conserved motif across several cell lines from different developmental origins; (4) measurement of ERK1/2 phosphorylation and/or activity is quick, simple, and relatively inexpensive and does not necessarily require radioactivity; (5) ERK1/2 demonstrates a response to shear stress that is relatively rapid, thus short periods of stimulation are often sufficient for studies characterizing shear stress responsivity.

Figure II-1 ERK1/2 phosphorylation is increased by shear stress in cell types from different developmental origins. HUVEC, BAEC, CHO or rat-1 fibroblasts underwent shear stress of increasing magnitudes for 10 min in a parallel plate chamber. Lysates were separated by SDS-PAGE and Western blotting with anti-phosphospecific-ERK1/2 antibody performed. Shear stress increased ERK1/2 phosphorylation in all cells tested, with the response in endothelial cells being significantly more robust.

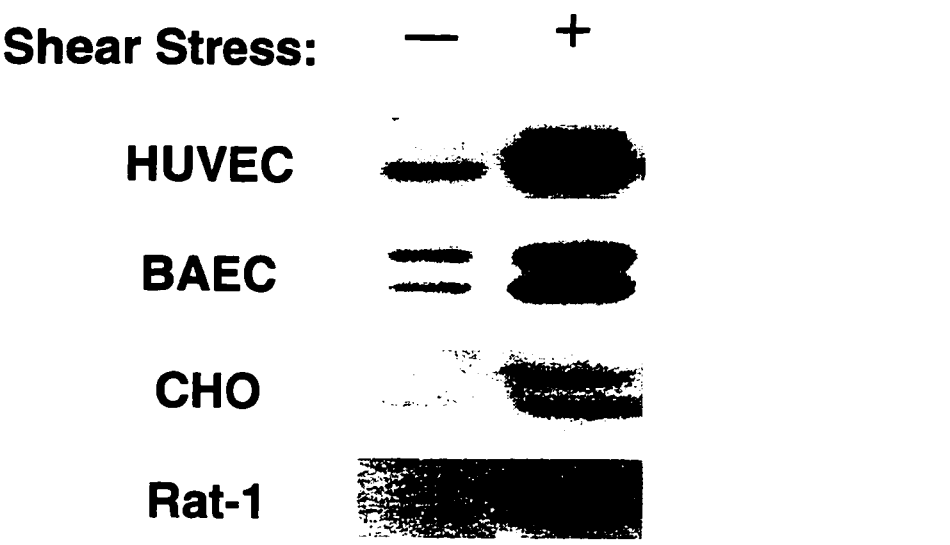

Shear Stress:
—
+
HUVEC
BAEC
CHO
Rat-1
IB: pERK1/2

Figure II-2 Parallel plate chamber for simulating shear stress. The cell chamber consists of a monolayer of cells grown on tissue culture plastic that is cut to precise dimensions (74 by 36 mm) from a tissue culture dish and a Plexiglas block sandwiching a mylar gasket of known thickness. These items define a cell chamber of known dimensions. Shear stress is calculated according to the following formula: $\tau = (6 \mu Q)/(h2b)$ where τ = wall shear stress (dynes/cm2); μ= viscosity of the medium (poise), Q= flow rate (mL/sec); b= width of the cell chamber; h= height of the cell chamber. For the chamber and medium used in these experiments: μ= 0.006915; b= 3.6 cm; and h=0.025 cm.

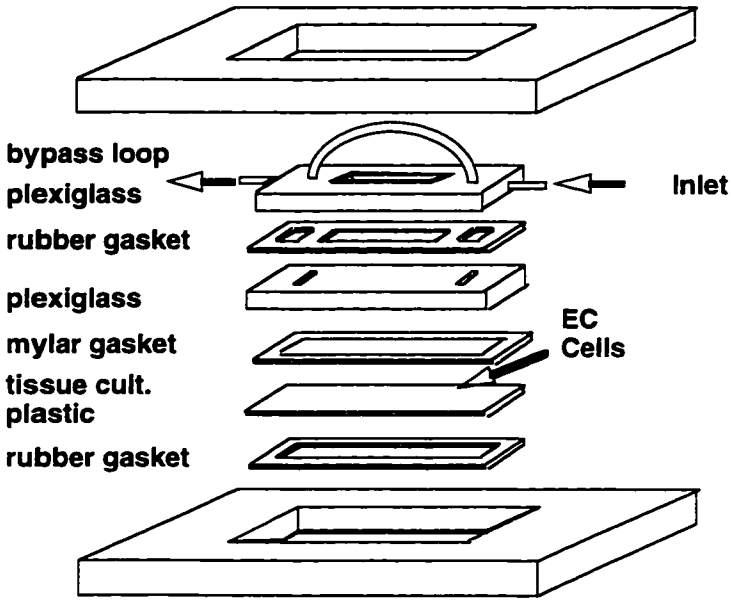

bypass loop
plexiglass
rubber gasket
plexiglass
mylar gasket
tissue cult.
plastic
rubber gasket
Inlet
EC
Cells

Figure II-3 Parallel plate chamber set up. The parallel plate set up consists of a gravity-fed recirculating flow loop system. Gravity-fed medium between two reservoirs is placed in series with a parallel plate flow chamber and a flow meter. During experiments, the upper reservoir and medium is warmed to 37°C and flow rate is measured through the use of a flow meter. A recirculating pump is connected to pump medium back to the upper reservoir. Fluid medium is a buffered physiological salt solution whose values are, containing in mM, NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 20, pH 7.4.

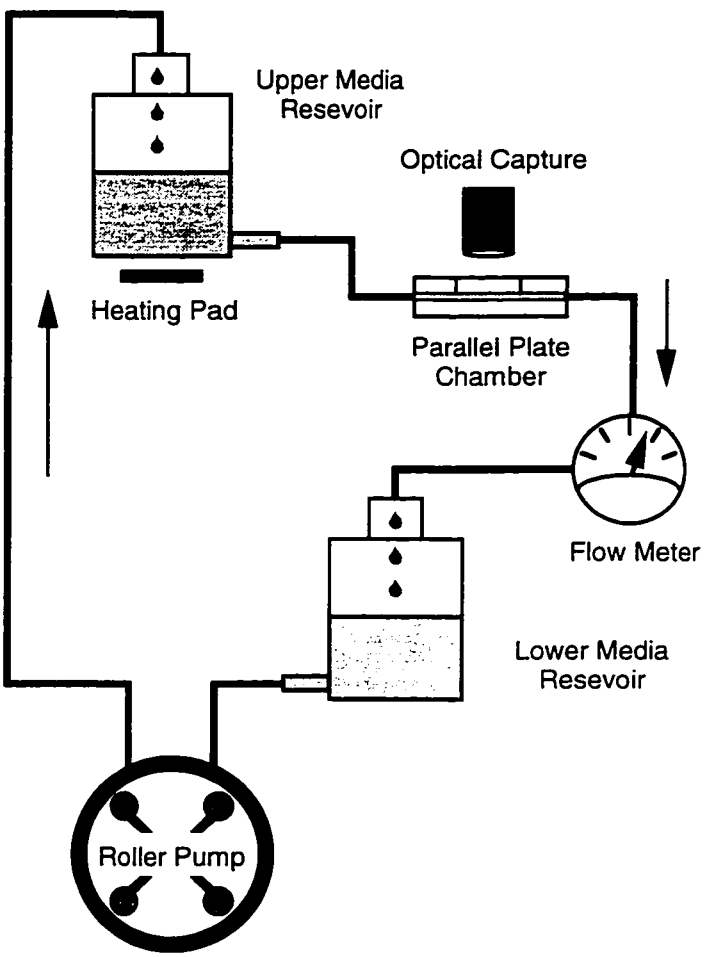
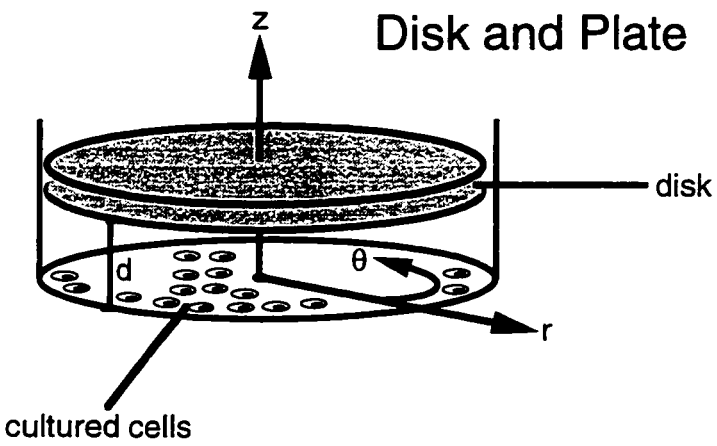

Upper Media
Resevoir
Optical Capture
Heating Pad
Parallel Plate
Chamber
Flow Meter
Lower Media
Resevoir
Roller Pump

Figure II-4 (a) Disk and plate apparatus. When the disk is rotated, a concentric fluid movement occurs in the culture medium in the dish. The endothelial cell monolayer cultured on the bottom of the dish is subjected to the shear stress according to the velocity gradient near the cell surface. As the medium can be regarded as a Newtonian fluid, the induced flow can be expressed by the Navier-Stokes equations in cylindrical coordinates radial [r], angular [θ] and azimuthal [z]. (b) Cone and plate apparatus. In order to achieve uniform shear stress along each radius of the dish, the disk is replaced with a cone with a small, but constant angle. By trigonometric principles, the two variables, d and r, can be replaced with one constant: the trigonometric tangent of the cone angle.

A


z
Disk and Plate
disk
d
θ
r
cultured cells

B

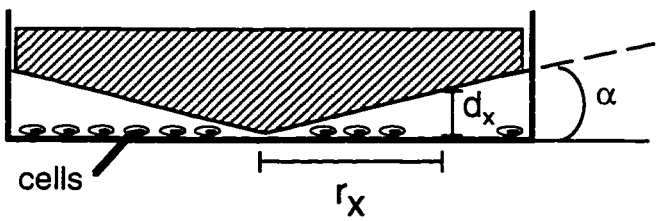

Cone and Plate
α
d_x
cells
r_x

Figure II-5 Calculated Reynolds number ranges for defined levels of shear stress in the cone and plate viscometer using different cone angles. Laminar shear stress predominates when $RN_{cone} < 1$.

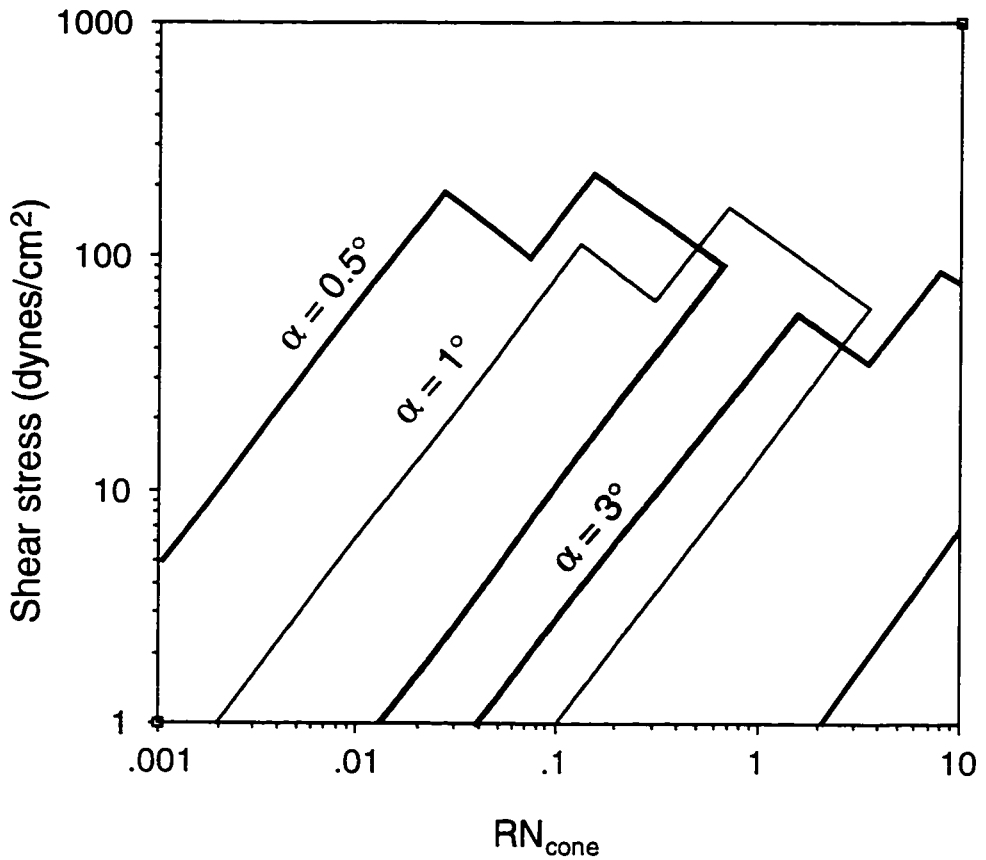

1000
100
10
1
Shear stress (dynes/cm2)
α = 0.5°
α = 1°
α = 3°
.001
.01
.1
1
10
RNcone

Figure II-6 Cone and plate viscometer. Polyacetal resin (Delrin) cones in two different sizes (for 60 mm and 100 mm Corning tissue culture dishes) were milled with precise angle measurements and attached to a stainless steel shaft which was in turn attached to a BC215GD-AF model motor and 2GD10K gear head purchased from Oriental Motor Co. Torrence, CA. The motor was wired to a external controller (Model BLD15-AF) with adjustable potentiometer. A step down transformer (120 V to 100 V; Sanyo Model TSD-N0GU) was added and the motor and gear head was placed on an adjustable platform so that the cone could be lowered onto the base containing the cell culture dish. Adjustable set screws were used to obtain reproducible heights from the cell culture dish. The apparatus (not including external controller) was placed in an adjustable incubator (Robbins Scientific hybridization chamber, Model 1000) set at 37°C for experiments.

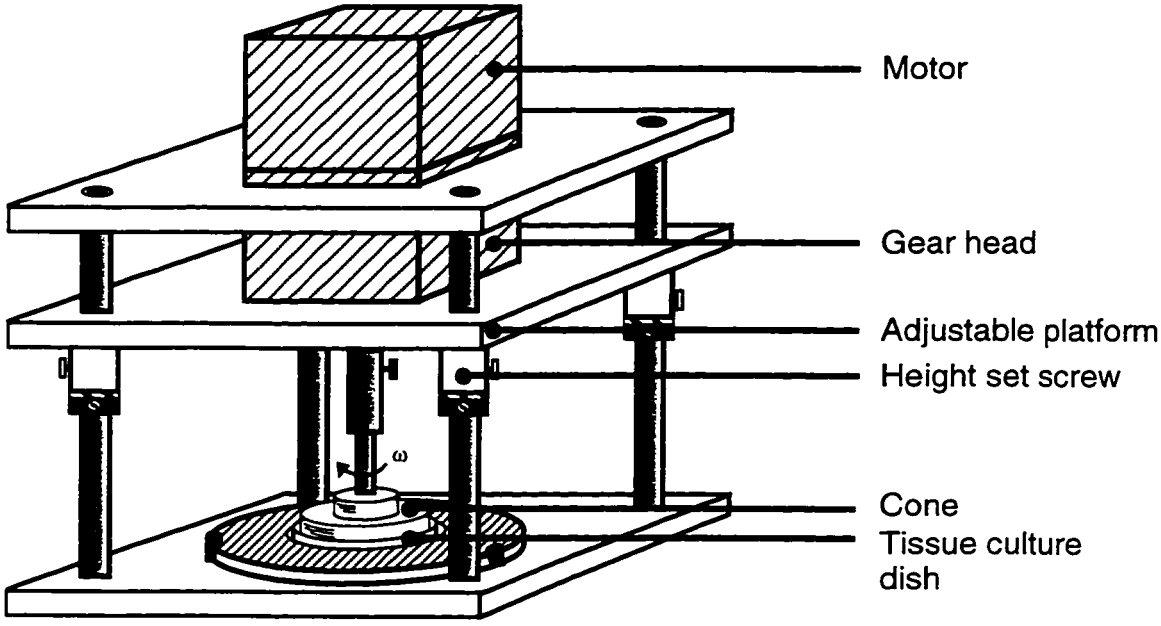

Motor
Gear head
Adjustable platform
Height set screw
ω
Cone
Tissue culture dish

Figure II-7 External potentiometer setting vs. revolutions per minute for the BC215GD-AF model motor and 2GD10K gear head and BLD15-AF Model external controller. RPM were visually observed three times for each potentiometer setting and a linear regression was performed ($r > 0.99$).

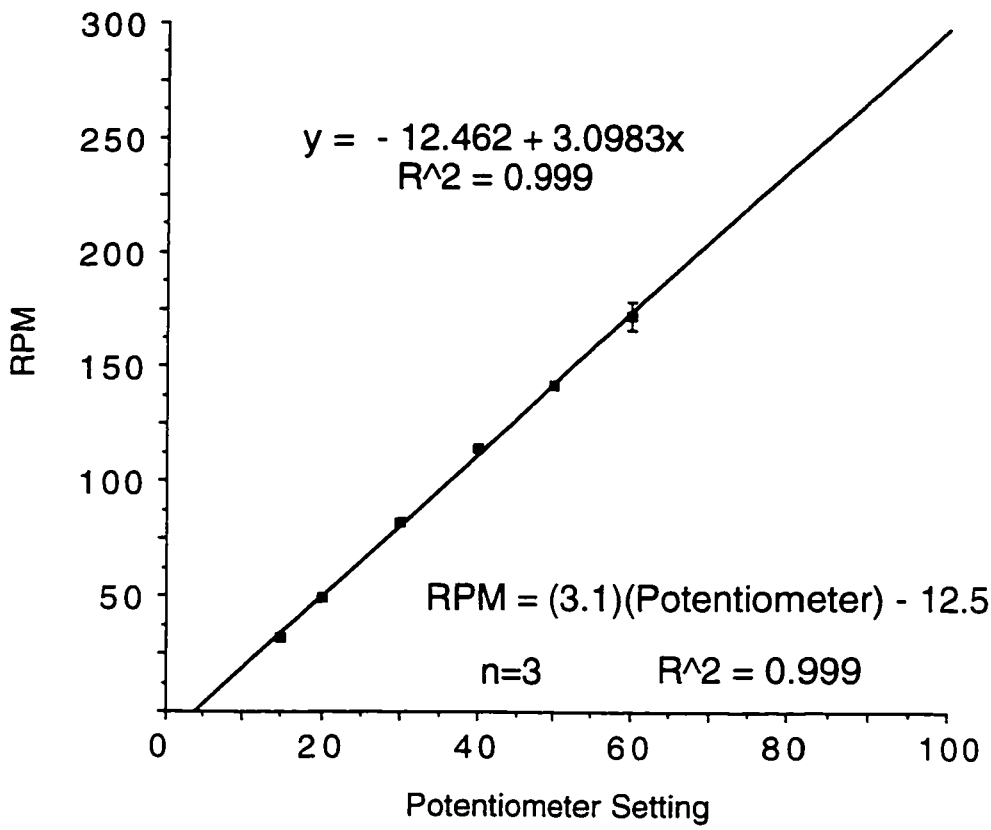
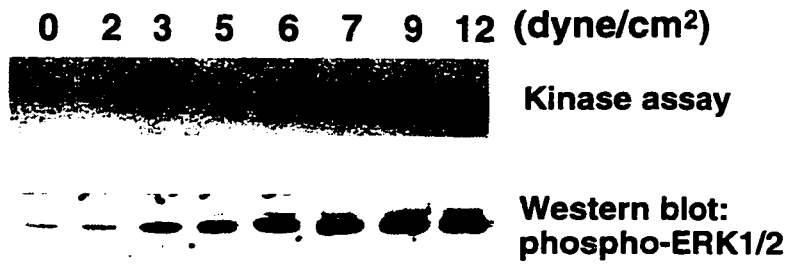

300
250
200
150
100
50
RPM
y = - 12.462 + 3.0983x
R^2 = 0.999
RPM = (3.1)(Potentiometer) - 12.5
n=3
R^2 = 0.999
0
20
40
60
80
100
Potentiometer Setting

Figure II-8 ERK1/2 phosphorylation is directly proportional to ERK1/2 activity. HUVEC underwent shear stress of increasing magnitudes for 10 minutes. (A) Lysates were run on SDS-PAGE containing myelin basic protein. Protein was renatured and incubated with γ-^{32}P-ATP for 1 hour. The gel was dried and autoradiography was performed to measure kinase activity. Lysates were separated by SDS-PAGE and Western blotting with anti-phosphospecific-ERK1/2 antibody performed. ERK1/2 activity and phosphorylation were directly correlated with r = 0.95.

A


0 2 3 5 6 7 9 12 (dyne/cm2)
Kinase assay
Western blot:
phospho-ERK1/2

B

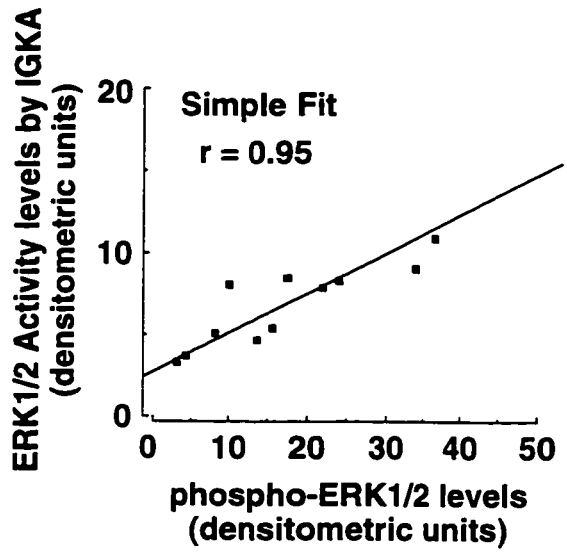

ERK1/2 Activity levels by IGKA
(densitometric units)
20
10
0
Simple Fit
r = 0.95
0 10 20 30 40 50
phospho-ERK1/2 levels
(densitometric units)

Figure II-9 ERK1/2 phosphorylation and activation is increased by shear stress. HUVEC underwent shear stress of increasing magnitudes for 10 minutes in either a parallel plate chamber or a cone and plate viscometer. Lysates were separated by SDS-PAGE and Western blotting with anti-phosphospecific-ERK1/2 antibody performed. Shear stress increased ERK1/2 phosphorylation in a force-dependent manner with no significant difference between the two apparatus tested.

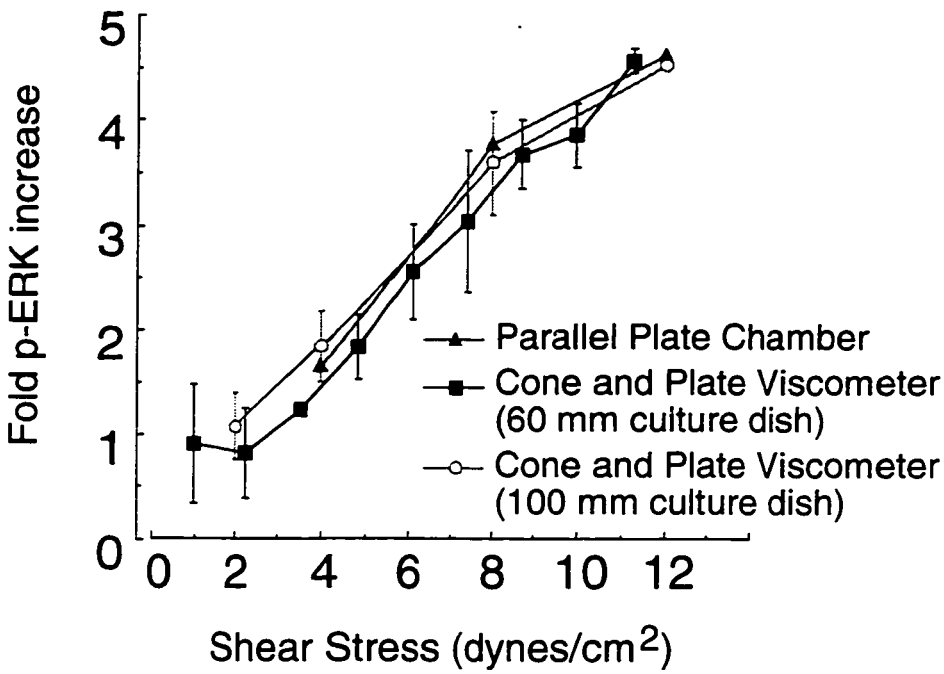

Fold p-ERK increase
0
1
2
3
4
5
0
2
4
6
8
10
12
Shear Stress (dynes/cm2)
Parallel Plate Chamber
Cone and Plate Viscometer
(60 mm culture dish)
Cone and Plate Viscometer
(100 mm culture dish)

Figure II-10 Phosphorylation of ERK1/2 by fluid shear stress assayed by Western blotting: time dependence. EC were washed free of culture medium with HBSS and maintained in static condition or exposed to 12 dynes/cm^2 shear stress in a parallel plate chamber or cone and plate viscometer for varying times. Samples were harvested, protein lysates separated by SDS-PAGE and transferred to nitrocellulose for Western blotting. Time-course for ERK1/2 tyrosine phosphorylation by shear stress. The lower panel demonstrates the whole cell ERK protein levels remain constant throughout the time course.

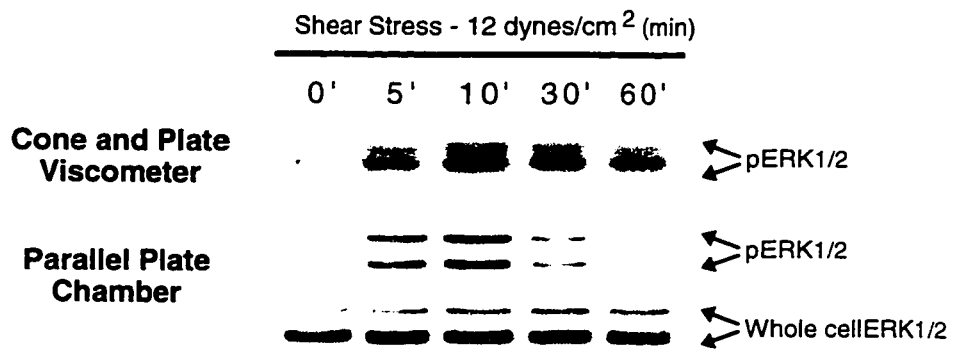

Shear Stress - 12 dynes/cm 2 (min)
0'
5'
10'
30'
60'
Cone and Plate Viscometer
pERK1/2
Parallel Plate Chamber
pERK1/2
Whole cellERK1/2

Figure II-11 Increasing cone distance from dish reduces ERK1/2 phosphorylation. Cone height was increased in sequential experiments and ERK1/2 phosphorylation measured by Western blotting. ERK1/2 phosphorylation was significantly decreased with increasing cone distance, consistent with generation of turbulent flow.

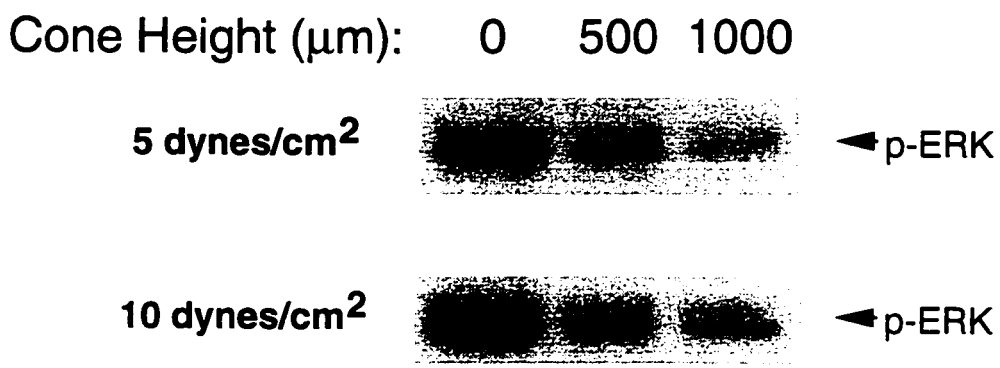
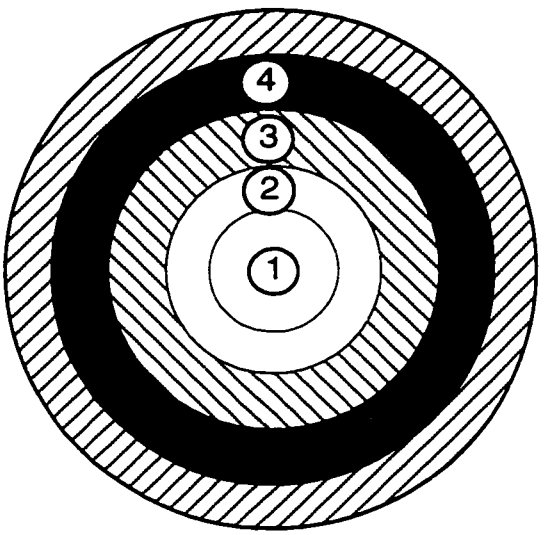

Cone Height (μm): 0 500 1000
5 dynes/cm2
p-ERK
10 dynes/cm2
p-ERK

Figure II-12 ERK1/2 phosphorylation is largely uniform at different radii. (a) Cells were scraped from the dish just prior to experiments to yield defined areas (b) After shear stress stimulus, cells were harvested and ERK1/2 phosphorylation determined by Western blotting. ERK1/2 phosphorylation was not significantly different among the different areas tested except for the inner most area.

A



B

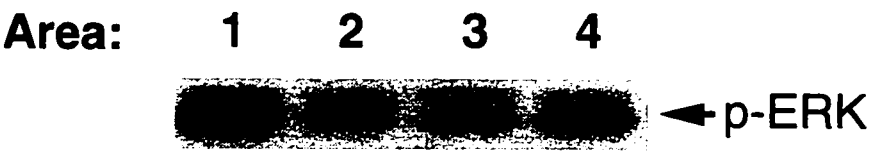


Figure II-13 Measurement of shear stress-mediated nitric oxide release. Medium taken from the cone and plate apparatus after shear stress stimulus showed high levels of nitric oxide metabolites that were significantly greater than those levels obtained with the calcium ionophore, A23187.

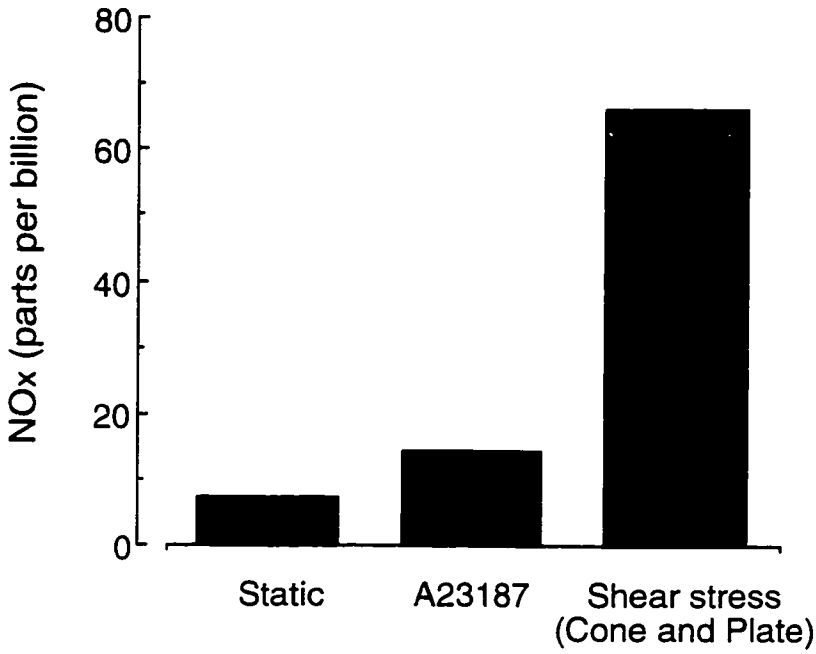

80
60
40
20
0
NOx (parts per billion)
Static
A23187
Shear stress
(Cone and Plate)

Table II-1. Comparison of the Parallel Plate Chamber vs. the Cone and Plate Viscometer in Simulating Shear Stress

	Parallel Plate Chamber	**Cone and Plate Viscometer**
Cost	$1,000	$1,000-$10,000
Media Volume	> 150 mL	1-4 mL
Hemodynamic considerations	simple	complex
ERK1/2 Peak	10 min	10 min
ERK1/2 Duration	baseline level at 30 min	sustained (> 60 min)
Set up time per sample	7 min	2 min
NO measurement	less reliable	reliable
Portable	no	yes
Culture plastic commercially available	no	yes
Technical errors	many	few
Shear Stress determination	mathematical	measured
Experimenter supervision required	yes	no
Easy to sterilize	no	yes

CHAPTER 3: SHEAR STRESS AND PROTEIN KINASE C

INTRODUCTION

As previously discussed, mechanical stimuli are important modulators of cellular function in tissues, particularly in the cardiovascular system (Watson 1991). A key physical force experienced by endothelial cells (EC), by virtue of their unique location in the vessel wall, is fluid shear stress created by blood flow. Changes in fluid shear stress have been shown to release vasoactive mediators such as nitric oxide (Furchgott and Vanhoutte 1989) as well as modulate gene expression such as c-*fos* (Hsieh, Li et al. 1993), PDGF (Mitsumata, Fishel et al. 1993), and ecNOS (Uematsu, Navas et al. 1993). These hemodynamically regulated events may contribute to the pathogenesis of vascular disease as atherosclerotic plaques are preferentially localized to areas of the vascular system that experience low shear and turbulence (Ku, Giddens et al. 1985).

We have also shown in Chapter 2 that two members of the mitogen-activated protein (MAP) kinase family, ERK1 and ERK2, which are known to modulate cell physiology and gene expression in many different ways (Pelech and Sanghera 1992), have been reported to be activated by shear stress in endothelial cells (Tseng, Peterson et al. 1995). However, while growth factor-mediated stimulation of ERK1/2 has been well defined (Lange-Carter, Pleiman et al. 1993), the upstream signaling pathway that leads to activation of ERK1/2 by shear stress remains unexplored.

Shear stress has been shown to activate phospholipase C (Nollert, Eskin et al. 1990), resulting in the cleavage of PIP_2 into inositol 1,4,5-trisphosphate, a calcium-

mobilizing second messenger, and diacylglycerol, an activator of protein kinase C (PKC). Indeed, recent studies have implicated PKC in cellular responses to shear stress, such as endothelin-1 production (Kuchan and Frangos 1993), PDGF expression (Biswas, Abboud et al. 1995) and cytoskeletal reorganization (Girard and Nerem 1993). Previous studies by our laboratory have suggested that PKC is also required for the fluid shear stress-mediated activation of ERK1/2 (Tseng, Peterson et al. 1995). In the current chapter, we investigated the role of PKC in the shear stress-mediated signaling and show that PKC-ε, but not PKC-α or PKC-ζ, is required for ERK1/2 activation by shear stress.

MATERIALS AND METHODS

CELL CULTURE

Bovine aortic EC were isolated from fetal calf aortas and maintained in M199 (GIBCO BRL) supplemented with 10% fetal calf serum. Cells used in experiments were passage <6, as ERK1/2 kinase activation by shear decreased in later passages. For experiments employing antisense oligonucleotides against human PKC isoforms, EC were obtained from human umbilical veins (HUVEC) as previously described (Gimbrone Jr. 1976). Cells at passages between 1 and 3 were grown in RPMI-1640 (GIBCO BRL) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Inc.), heparin (Sigma Chemical Co.) and endothelial cell growth factor (kindly provided by Dr. R. Ross).

SHEAR STRESS EXPERIMENTS

Cells were grown in 74 mm x 36 mm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Two days after reaching confluence, cells were rinsed free of culture media with HBSS (containing in mM: NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 20, pH 7.4), with 10 mM glucose added, and either maintained in

static condition or exposed to 12 dynes/cm^2 of fluid shear stress, in a parallel plate chamber or cone and plate viscometer at 37°C, as described in Chapter 2. After varying times of exposure to fluid shear stress, cells were washed gently with ice-cold PBS (composition, in mM, NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.3) and ERK1/2 activation was determined.

Immunoprecipitation and Western Blot Analysis for ERK1/2 Activation

Cells were washed with PBS and 0.15 mL of RIPA buffer (50 nM NaCl, 50 nM NaF, 50 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 2 mM Na_3VO_4, 0.1% Triton X-100, 0.5 mM PMSF, 10 µg/mL leupeptin, 10 mM HEPES, pH 7.4) was added. Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4°C, 14 000 rpm in microfuge). Sample protein concentrations were determined by DC Protein (Biorad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham). To ensure quantitative transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 hr at room temperature with a commercial blocking buffer (GIBCO BRL). The blots were incubated overnight at 4°C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs; non-specific ERK1 and ERK2 antibodies and PKC isoform-specific antibodies were obtained from Santa-Cruz Biological) followed by incubation for 1-2 hr with secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized by chemiluminescence (Amersham ECL or Pierce ECL).

Separation of Membrane and Cytosolic Fractions for PKC Localization

Cells from one 150 mm culture dish were scraped into 0.75 mL of fractionation lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM benzamidine, 1 mg/mL leupeptin, 50 μg/mL PMSF, 0.1 mg/mL ovalbumin, and 0.1 μg/mL aprotonin, pH 7.4) on ice. After incubation for 5 minutes, cells were disrupted with a Dounce homogenizer (50 strokes) and centrifugation was performed (100,000 x g for 1 hr). The supernatant was saved as the cytosolic fraction. The pellet (membrane fraction) was washed once with lysis buffer, then resuspended in 150 μL of lysis buffer that contained 1% Triton X-100 and was solubilized for 1 hr at 4°C before sonication. Proteins then underwent Western Blot analysis as described above.

PKC Activity Assay by Histone Phosphorylation

Cells from one 150 mm culture dish were scraped into 1.0 mL of assay buffer (150 mM NaCl, 2 nM EDTA, 50 mM Tris-HCl, 1 mM EGTA, 10 μg/mL pepstatin, 10 μg/mL aprotonin, and 10 μg/mL leupeptin with 1% Triton X-100) on ice. Immunoprecipitation and immune complex recovery was performed for PKC-α (using 25 μg/reaction sample), PKC-ε (using 50 μg/reaction sample) and PKC-ζ (using 75 μg/reaction sample) as described above. Protein samples were placed in a reaction mixture containing: 20 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 200 μM $CaCl_2$, cofactor mixed micelle preparation containing diolein (16 μg/mL) and phosphatidylserine (240 μg/mL) in 0.3% Triton X-100 as PKC cofactors, 0.4 mg/mL histone-H1 and γ-^{32}P-ATP. For Ca^{2+}-free experiments, 1.0 mM EGTA was substituted for $CaCl_2$. Also, inhibitors (staurosporine, 10 nM) or activators (PMA, 2 nM; inactive phorbol, 2 nM) were added directly to the bath for some experiments. The mixture was incubated for 10 min at 30°C, at which point the reaction was halted by adding 6X Laemmli buffer. Samples were

subjected to SDS-PAGE under reducing conditions using 15% polyacrylamide, and the gel was allowed to dry overnight. Histone-H1 phosphorylation was detected by autoradiography and quantified by densitometry.

TRANSFECTION PROTOCOL FOR PKC-ISOFORM ANTISENSE OLIGONUCLEOTIDES

HUVEC were grown to 95% confluence in 60 mm tissue culture dishes for transfection. Lipofectin (GIBCO-BRL) was prepared in 0.2 mL OPTI-MEM containing 5 μg lipofectin/μM oligonucleotide and equilibrated for 45 min. A series of antisense oligonucleotides directed against the PKC isoforms were screened and the most active sequences identified. PKC-α: antisense oligonucleotide (GTTCTCGCTGGTGAGTTTCA), scrambled oligonucleotide (GGTTTTACCATCGGTTCTGG); PKC-ε: antisense oligonucleotide (CATGAGGGCCGATGTGACCT), scrambled oligonucleotide (TACGCATAACGCGCTGGTGG); PKC-ζ: antisense oligonucleotide (GACGCACGCGGCCTCACACC), scrambled oligonucleotide (AAGCGCGCACCAGCGCCTCC). Antisense or scrambled phosphorothioate oligonucleotides were prepared at concentrations of 1 μM, 3 μM and 10 μM in 0.2 mL OPTI-MEM and equilibrated for 1 min. The lipofectin and oligonucleotide solutions were then mixed gently and incubated at room temperature for 15 min before being diluted to final volume of 2 mL with OPTI-MEM to give oligonucleotide concentrations of 100 nM, 300 nM and 1000 nM. The cells were washed with sterile PBS and treated with OPTI-MEM/lipofectin/oligonucleotide mixture for 6 hr before being returned to RPMI with 20% fetal calf serum. Cells were harvested 3 days later because preliminary experiments (not shown) determined that antisense PKC oligonucleotides were unable to reduce protein levels when EC were harvested earlier. The three day period following transfection was

necessary to degrade existing PKC, consistent with protein half-lives of PKC proteins reported by other investigators (Woodgett and Hunter 1987).

ADHESION EXPERIMENTS

HUVEC were incubated at 37°C in PBS containing 2 mM EDTA for 5 min and detached from dishes by gentle pipeting as previously described (Takahashi and Berk 1996). The cells were washed three times with RPMI 1640, collected by low speed centrifugation, and resuspended in RPMI 1640 with 0.1% BSA (Sigma Chemical Co.). Approximately 10^6 cells were placed onto 60-mm bacteriologic plastic dishes coated with fibronectin (FN; Sigma Chemical Co.) or poly-L-lysine (PLL; Sigma Chemical CO.) and incubated at 37°C for 10 min. The bacteriological plastic dishes were coated with human FN (10 μg/mL) or PLL (10 μg/mL) for 16 hr at 4°C, and nonspecific binding sites were blocked with 1% heat denatured BSA in PBS for 1 hr at room temperature. Before use, the dishes were rinsed three times with PBS.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM for all experiments that were performed at least three times. Significant differences were determined by Student's t test ($p<0.05$).

RESULTS

FLUID SHEAR STRESS LEADS TO ACTIVATION OF ERK1/2

As shown in Chapter 2, stimulation of ERK1/2 by fluid shear stress was measured by Western blotting with a phosphospecific-ERK antibody. Compared with static conditions, fluid shear stress at 12 dynes/cm^2 activated ERK1/2 with a peak at 10 min and return to baseline by 60 min (Figure II-10). These data show activation kinetics similar

to those previously reported by our laboratory using other techniques (Tseng, Peterson et al. 1995; Ishida, Peterson et al. 1996). Western blotting with an antibody for ERK1/2 that detects both the phosphorylated and unphosphorylated form of the kinases showed that cellular ERK1/2 levels remained constant throughout the shear stress time-course (Figure II-10). These results demonstrate that ERK1/2 is phosphorylated in response to fluid shear stress with time-course similar to receptor agonists, such as thrombin and EGF (Tseng, Peterson et al. 1995) .

ERK1/2 ACTIVATION BY SHEAR STRESS IS PKC-DEPENDENT AND CALCIUM-INDEPENDENT

Several investigators have reported that PKC is activated in response to various mechanical stimuli such as stretch, pressure and shear (Watson 1991). To determine the role of PKC in ERK1/2 activation by shear stress, cells were exposed to 1 μM PDBu for 24 hr prior to shear stress to downregulate PKC. ERK1/2 activation by shear stress was significantly inhibited by PDBu pretreatment, (28±3% of control) as shown by immunoblotting with the ERK1/2 phosphospecific antibody (Figure III-1). Pretreatment with the protein kinase inhibitor, staurosporine (2 nM, 30 min), reduced ERK1/2 activation to 10±6% of control levels. Levels of phosphorylated ERK1/2 in cells maintained in static culture were not changed by either treatment (data not shown). These data suggest that PKC is necessary for the shear stress-mediated activation of ERK1/2.

Mechanical stimuli cause a rapid increase in intracellular calcium concentration (Shen, Luscinskas et al. 1992), and our laboratory has previously reported that shear stress at 12 dynes/cm^2 for 10 min increases intracellular calcium (Geiger, Berk et al. 1992). To determine if the shear stress-mediated increase in intracellular calcium was

necessary for ERK1/2 activation, cells were treated with the Ca^{2+}-chelator BAPTA-AM (75 μM, 30 min), and the shear stress stimulus was performed in a Ca^{2+}-free balanced salt solution supplemented with EDTA (10 mM) in order to inhibit the shear stress-mediated increase in intracellular calcium. Basal levels of ERK1/2 activation (data not shown) and ERK1/2 activation by shear stress was unaffected by pretreatment with BAPTA (Figure III-1), suggesting that a rise on intracellular calcium is not necessary for ERK1/2 activation by fluid shear stress. These results demonstrate that the shear stress-mediated activation of ERK1/2 is PKC-dependent and calcium-independent.

ENDOTHELIAL CELLS EXPRESS SEVERAL DIFFERENT PKC ISOFORMS

At least eleven PKC isoforms have been described, each possessing unique characteristics and perhaps playing different roles in cell signaling. A classification system for the PKC family has emerged that separates the different isoforms into four distinct classes (Newton 1995) (Table III-1). The "classical" PKC isoforms, which include α, βI, βII, and γ, are described as calcium-independent and phorbol ester-responsive enzymes. The second and third class are the "novel" PKC isoforms (including δ, ε, θ, η) and the "atypical" PKC isoforms (including ζ, λ/ι). The novel isoforms lack the calcium-binding domains that are present on the classical isoforms, yet still retain the phorbol ester-binding domains. Hence, the novel isoforms are described as calcium-independent and phorbol ester-responsive PKC isoforms. In contrast, the atypical isoforms lack both the calcium-binding sites as well as the phorbol ester-binding domains and are described as calcium-independent and phorbol ester-unresponsive. The final group, termed "eccentric," contains the recently discovered and little studied PKC-μ isoform. Because ERK1/2 activation by shear stress is phorbol ester-responsive but calcium independent; our results suggest that

some member(s) of the novel class (δ, ε, θ, η) are involved in the signaling pathway that leads to activation of ERK1/2. To determine which PKC isoforms were expressed in EC, we performed Western blotting with isoform-specific antibodies on EC lysates. EC express primarily three PKC isoforms: PKC–α, PKC–ε, and PKC–ζ (Figure III-2) while no significant immunoreactivity was detected for PKC-β, –γ, –δ, –θ, –η, and –λ/ι. Thus, the only member of the novel class present in EC is PKC-ε.

Effect of PDBu treatment on PKC Isoform Expression

Because shear stress-mediated activation of PKC was significantly attenuated by 24 hr pretreatment with PDBu, we determined the time-dependent change in PKC isoform levels (Figure III-3). While brief stimulation with PMA (200 nM, 10 min) had no effect on PKC levels, prolonged exposure of cells to PDBu caused downregulation of PKC–α (100% by 24 hr) and PKC–ε (100% by 12 hr). PKC–ζ levels were unaffected. PDBu treatment had no effect on either cellular ERK1/2 levels (Figure III-3, bottom) or on EGF-mediated ERK1/2 activation (data not shown). These results are consistent with the characteristics of the different PKC-isoforms described above and suggest that PKC–ζ is not involved in ERK1/2 activation by shear stress as it was unaffected by PDBu treatment.

Measurement of PKC Activity by Translocation Assay

To determine whether the PKC isoforms expressed in EC translocate upon cell stimulation, the intracellular localization of the PKC isoforms was determined by centrifugal fractionation, SDS-PAGE separation, and Western blotting (Figure III-4).

Western analysis showed that in the unstimulated state, both PKC–α and PKC–ζ were evenly distributed in the cytosolic and membrane fractions while PKC–ε was localized solely to the membrane fraction. After stimulation with PMA (200 nM, 10 min), PKC–α translocated to the membrane fraction but little difference was observed in the distribution of PKC–ε and PKC-ζ. Since PKC–ε was already localized to the membrane fraction (though whether nuclear or membrane is unknown) and because there was little difference in cellular localization of PKC–ζ in response to PMA, this method of measuring PKC activity would not be useful in determining whether PKC–ε and PKC–ζ are activated by shear stress.

MEASUREMENT OF PKC ACTIVITY BY HISTONE PHOSPHORYLATION

Another method to measure PKC activity is by phosphorylation of a PKC substrate. Since specific substrates for each isoform are not available, we measured the activity of the PKC isoforms by immunoprecipitating each isoform and then performing an immune complex kinase assay with a universal PKC substrate, histone-H1. In this assay, PKC–α activity was inhibited by the addition of staurosporine, exclusion of Ca^{2+} (below basal levels or to basal levels with PMA stimulation; data not shown), or exclusion of both Ca^{2+} and cofactors (diolein and phosphatidylserine, Figure III-5 top). Addition of PMA to the reaction mixture potentiated PKC–α activity. Activity of the PKC–ε isoform was also inhibited by staurosporine and removal of Ca^{2+} and cofactors, but was not affected by removal of Ca^{2+} alone (Figure III-5-middle). Addition of PMA stimulated PKC–ε activity.

The activity of PKC–ζ was not inhibited by staurosporine, removal of Ca^{2+}, or removal of both Ca^{2+} and cofactors (Figure III-5-bottom). Further, PMA added

directly to the assay was unable to stimulate activity. These results confirm that EC PKC–ζ is a calcium-independent and phorbol ester-unresponsive PKC isoform.

Adding PMA to the reaction mixture stimulated activity of PKC–α and PKC–ε, but pretreating cells with PMA prior to immunoprecipitation failed to stimulate PKC activity as measured by histone phosphorylation (see far right, Figure III-5). These results suggests that immunoprecipitation separates PKC from cellular inhibitors and activators that regulate agonist-stimulated activity. In fact, no change in immunoprecipitated PKC activity was noted for any PKC isoform when cells were pretreated with PMA, shear stress or thrombin (data not shown). Therefore, this assay is useful to characterize the effects of calcium, phorbol, and staurosporine *in vitro* on the separate isoforms but is not useful to measure the effects of physiological stimuli on intact cells.

PKC ANTISENSE OLIGONUCLEOTIDES ARE SPECIFIC AND EFFECTIVE

It appears that among the PKC isoforms present in EC, PKC-ε is the most likely isoform to mediate shear stress ERK1/2 signaling. This conclusion is based on the findings in Figures 2,4 and 5 that the PKC isoform is (1) phorbol ester responsive, (2) calcium-independent and (3) inhibited by staurosporine. Further, these data suggest that neither PKC–α nor PKC–ζ is involved in this signaling process as PKC–α is calcium-dependent and PKC–ζ is phorbol ester-unresponsive and resistant to inhibition by staurosporine.

To establish the role of PKC-ε in shear stress-mediated activation of ERK1/2, we decided to inhibit each expressed PKC isoform individually and measure

changes in ERK1/2 activation. Since specific pharmacologic inhibitors of the separate PKC isoforms are currently unavailable, antisense phosphorothioate oligonucleotides and their corresponding scrambled controls for the different PKC isoforms were employed. Antisense oligonucleotides have previously been employed to inhibit expression of PKC-α in mouse and human cell lines in an isoform-specific manner (Dean and McKay 1994; Dean, McKay et al. 1994). HUVEC were transfected with antisense PKC-ε oligonucleotides for 6 hr, and the cells were harvested 3 days later for analysis. Protein levels for PKC–ε were reduced in a concentration dependent manner with reductions of 22±10%, 25±6% and 80±13% at 100, 300 and 1000 nM antisense PKC-ε oligonucleotide, respectively (Figure III-6). Expression of PKC–α and PKC–ζ isoforms was not significantly affected at any concentration of antisense PKC-ε oligonucleotide, indicating that the antisense oligonucleotides were specific for PKC–ε. PKC-ε levels were not affected by treatment with 1000 nM scrambled PKC-ε oligonucleotides demonstrating minimal non-specific effects of the transfection protocol. Similar specificity and efficacy of 1000 nM antisense PKC–α and PKC–ζ oligonucleotides for their corresponding PKC isoforms was observed (data not shown).

ANTISENSE PKC–E OLIGONUCLEOTIDES BLOCK SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION

Several studies have demonstrated that many PKC isoforms are able to activate ERK1/2 in a stimulus-specific manner, including PKC-α, PKC-ε, and PKC-ζ (Clark and Murray 1995; Young, Dickens et al. 1996; Liao, Monia et al. 1997) To determine the effect of inhibiting different PKC isoforms on ERK1/2 activation by shear stress, cells treated with antisense PKC oligonucleotides were maintained in static culture

or exposed to shear stress. Antisense or scrambled PKC-α, -ε, -ζ oligonucleotide treatment did not affect baseline phosphorylation of ERK1/2 (Figure III-7a, left three lanes). Antisense or scrambled PKC–α or –ζ oligonucleotides did not alter the ERK1/2 activation by shear stress. However, in cells treated with antisense PKC–ε oligonucleotides, shear stress-mediated activation of ERK1/2 was completely inhibited (Figure III-7a - far right). Scrambled PKC–ε oligonucleotide treatment had no effect on ERK1/2. Further, antisense PKC–ε oligonucleotides had no effect on bradykinin or EGF-induced ERK1/2 activation demonstrating that ERK1/2 was still capable of being activated through mechanisms independent of PKC-ε (Figure III-7b). Treatment with antisense PKC–ε oligonucleotides inhibited PMA-induced ERK1/2 activation by only 35%. The inability of antisense PKC-ε oligonucleotides to completely inhibit PMA-induced ERK1/2 activation is likely due to PKC isoforms other than PKC-ε than can also activate ERK1/2 in response to PMA.

ANTISENSE PKC–E OLIGONUCLEOTIDES BLOCK ERK1/2 ACTIVATION BY ADHESION TO FIBRONECTIN

We have previously showed that ERK1/2 was activated when EC adhered to fibronectin, but not when cells adhered to poly-L-lysine (PLL) suggesting a role for integrins in ERK1/2 activation in EC (Takahashi and Berk 1996). To determine the role of PKC-ε in these integrin-mediated pathways, we compared activation of ERK1/2 during adherence to PLL and fibronectin in the presence and absence of PKC-ε oligonucleotides (Figure III-8). Adherence to fibronectin for 10 min increased ERK1/2 activity by 4.3-fold compared to cells maintained in suspension. Adherence to PLL for 10 min, in contrast, caused no significant increase in ERK1/2 activity. Antisense PKC-ε oligonucleotides

completely inhibited ERK1/2 activation by fibronectin while scrambled PKC-ε oligonucleotides had minimal inhibitory effect. There were no significant difference in cell morphology or the extent of cell spreading in the presence of antisense PKC-ε oligonucleotides. Thus, PKC-ε appears to be required for integrin-mediated activation in EC. In addition, antisense against PKC-α also inhibited adhesion-mediated ERK1/2 activation while antisense against PKC-ζ had no effect.

DISCUSSION

The major finding of this chapter is that PKC-ε is a component of a mechano-sensitive signal transduction pathway that leads to the activation of ERK1/2 in endothelial cells. Further, this pathway is specific for PKC-ε, as PKC-α and PKC-ζ are not required for the activation of ERK1/2. These results, combined with observations from other investigators that shear stress stimulates changes in cellular physiology and gene expression (Davies 1995), provide evidence that mechanical stimuli can activate signal transduction pathways in a manner similar to conventional agonist-receptor initiated signaling events. These results define a pathway for shear stress-mediated ERK1/2 activation and establish a new function for PKC-ε in EC.

Two upstream mechanisms for the activation of PKC-ε in response to shear stress may be proposed based on previous studies. First, phospholipase C (PLC) is activated by shear stress (Nollert, Eskin et al. 1990), resulting in the cleavage of PIP_2 and generation of inositol 1,4,5-trisphosphate and diacylglycerol (DAG). PKC-ε is similar to the classical PKC isoforms in that it is activated by DAG (Newton 1995); thus, one mechanism for activation of PKC-ε is through shear stress-mediated generation of DAG. Second, other activators of PKC-ε such as phosphatidylinositol(3,4)-bisphosphate and

phosphatidylinositol(3,4,5) trisphosphate, may be increased in EC in response to fluid shear stress. Reports show that both of these phosphoinositides, generated by PI 3-kinase activity, are potent and selective activators of the novel class of PKC isoforms and have little effect on the classical or atypical PKC isoforms (Liscovitch and Cantley 1994). To date, no studies have been published regarding changes in PI 3-kinase activity in response to fluid shear stress. Moriya *et al.* (Moriya, Kazlauskas et al. 1996) reported that both the PI 3-kinase and the PLC pathway can activate PKC-ε in a cell-specific and stimulus-specific manner. The specificity of the PI 3-kinase pathway for the novel PKC isoforms suggests that analysis of PI 3-kinase activity in response to flow will be a fruitful area for future studies.

The plasma membrane sensor or receptor responsible for transducing mechanical stimuli into biochemical signaling events remains undefined. Several molecules have been proposed as candidate mechanotransducers, including integrins and mechanosensitive ion channels such as the inward rectifying K^+ channel and the stretch-activated Ca^{2+} channel (Davies 1995). While it is not known whether mechanosensitive ion channels can affect activation of PKC-ε, integrins have been shown to activate many cellular kinases, including PKC (Vuori and Ruoslahti 1993). Because antisense PKC-ε oligonucleotides completely inhibited adhesion-(i.e. integrin-)induced ERK1/2 activation. the present findings suggest an important role for PKC-ε in integrin-mediated signaling in EC. In two previous studies, we found that integrins played an essential role in activation of ERK1/2 by shear stress in EC (Ishida, Peterson et al. 1996; Takahashi and Berk 1996). Thus, the present data support the concept that integrins participate as mechanoreceptors although additional experiments should be performed to determine which integrins activate PKC-ε specifically .

Although signal transduction events by which PKC-ε activates ERK1/2 remain to be determined, it seems likely that PKC-ε interacts directly with Raf-1 to activate ERK1/2. Activation of ERK1/2 by growth factors has been shown to occur via activation of Ras and subsequent recruitment of Raf-1 to the plasma membrane. Raf-1, in turn, activates MEK-1, the direct upstream activator of ERK1/2 (Pelech and Sanghera 1992). This pathway also appears to be activated in EC by shear stress, since two groups have demonstrated Ras activation by shear (Li, Shyy et al. 1996; Jo, Sipos et al. 1997). We have shown that inhibiting PKC-ε with antisense oligonucleotides completely blocked shear stress-mediated ERK1/2 activation, indicating that PKC-ε directly participates in the signaling cascade leading to ERK1/2 activation (e.g., by its kinase activity), or that the constitutive presence of PKC-ε is required for normal function of some component of the signaling cascade (e.g., by regulating gene expression). While the current data cannot distinguish between these two possibilities, several recent studies suggest that PKC-ε acts directly on Raf-1. Cai *et al.* (Cai, Smola et al. 1997) showed that overexpression of active PKC–ε resulted in increased Raf-1 activity even in the context of dominant negative Ras. These investigators also demonstrated that PKC-ε stimulated Raf-1 in baculovirus-infected Sf9 cells and was able to directly activate Raf *in vitro*. Schaap *et al.* (Schaap, van et al. 1993) also found that overexpression of PKC-ε isoform activated ERK1/2 , but that ERK1/2 activation was not present if PKC-ε was overexpressed in a dominant-negative Raf-1 expressing cell line. A study by Cacace *et al.* (Cacace, Guadagno et al. 1993) demonstrated that PKC-ε acts downstream of Ras but upstream of Raf-1 to induce oncogenic transformation in PC12 cells.

PKC-ε-mediated activation of ERK1/2 is likely to be an important pathway for the EC response to shear stress. ERK1/2 has been shown to phosphorylate and activate

$pp90^{rsk}$ leading to phosphorylation of S6 protein and increased protein synthesis. ERK1/2 is an upstream regulator of Elk-1 and may stimulate ternary complex formation which increases *fos* expression, which has been shown to be induced by shear stress. ERK1/2 may also be involved in regulation of ecNOS based on observations that NO production is regulated by shear stress (Corson, Berk et al. 1993) and that ecNOS has multiple consensus sites for phosphorylation by ERK1/2 (Berk, Corson et al. 1995). Kuchan and Frangos (Kuchan and Frangos 1994) reported that while initial release of NO was Ca^{2+}-dependent, sustained NO production in response to shear was Ca^{2+}-independent. Since the shear stress signaling pathway involving PKC-ε is Ca^{2+}-independent, it may be involved in sustained NO production stimulated by shear stress. Finally, other PKC-ε substrates such as the cytoskeletal proteins, caldesmon (Horowitz, Clement-Chomienne et al. 1996), calponin (Horowitz, Clement-Chomienne et al. 1996) and filamentous actin (Prekeris, Mayhew et al. 1996) may contribute to the rearrangement in EC cytoskeleton induced by long term flow.

The present chapter demonstrates the utility of antisense PKC oligonucleotides to explore the cellular role of specific PKC isoforms. While the traditional pharmacological PKC inhibitors employed to investigate the role of PKC are likely to possess differential affinities for the separate PKC isoforms they are unlikely to be specific for individual PKC isoforms. Indeed, in the present study, staurosporine was an effective inhibitor of PKC-α and PKC-ε, but did not affect PKC-ζ activity. The use of antisense oligonucleotides has been shown to be effective in several studies (Dean and McKay 1994; Dean, McKay et al. 1994) and as demonstrated here can be used to study isoform specific events. In summary, these data are the first to identify a specific PKC isoform required for transduction of mechanical stimuli. Specifically, we showed that PKC-ε, but not PKC-α

or PKC-ζ, serves as a mechanosensitive mediator for activation of ERK1/2 by shear stress in endothelial cells. Further characterization of this signal transduction pathway will yield greater insight into the mechanisms by which mechanical stimuli regulate the vascular wall.

Table III-1. Protein Kinase C Isoform Classification

Group	Isoform	Phorbol-responsive?	Translocation?
Classical	α, β, γ	Yes	Yes
Novel	δ, ε, θ, η	Yes	Yes/No
Atypical	λ/ι, ζ	No	No
Eccentric	μ	No	??

Figure III-1 Effect of PKC-inhibition and Ca^{2+}-chelation on phosphorylation of ERK1/2 by fluid shear stress. Cells were pretreated with vehicle (0.1% DMSO for 24 hours), staurosporine (2 nmol/L for 30 minutes), PDBu (1 μM for 24 hours), or BAPTA-AM (75 μmol/L for 30 minutes) prior to stimulation. EC were washed in HBSS (Ca^{2+}-free HBSS for those treated with BAPTA), and exposed to fluid shear stress of 12 dynes/cm^2 for 10 min (in Ca^{2+}-free buffer supplemented with 10 mM EDTA for those cells treated with BAPTA). Lysates were analyzed by Western blot using a phosphospecific-ERK antibody and quantified by densitometry. Results are expressed as mean ± SEM (n=3). Treatment with staurosporine or PDBu caused significant attenuation of shear stress-induced ERK1/2 activation while BAPTA treatment had no effect ($p<0.05$)

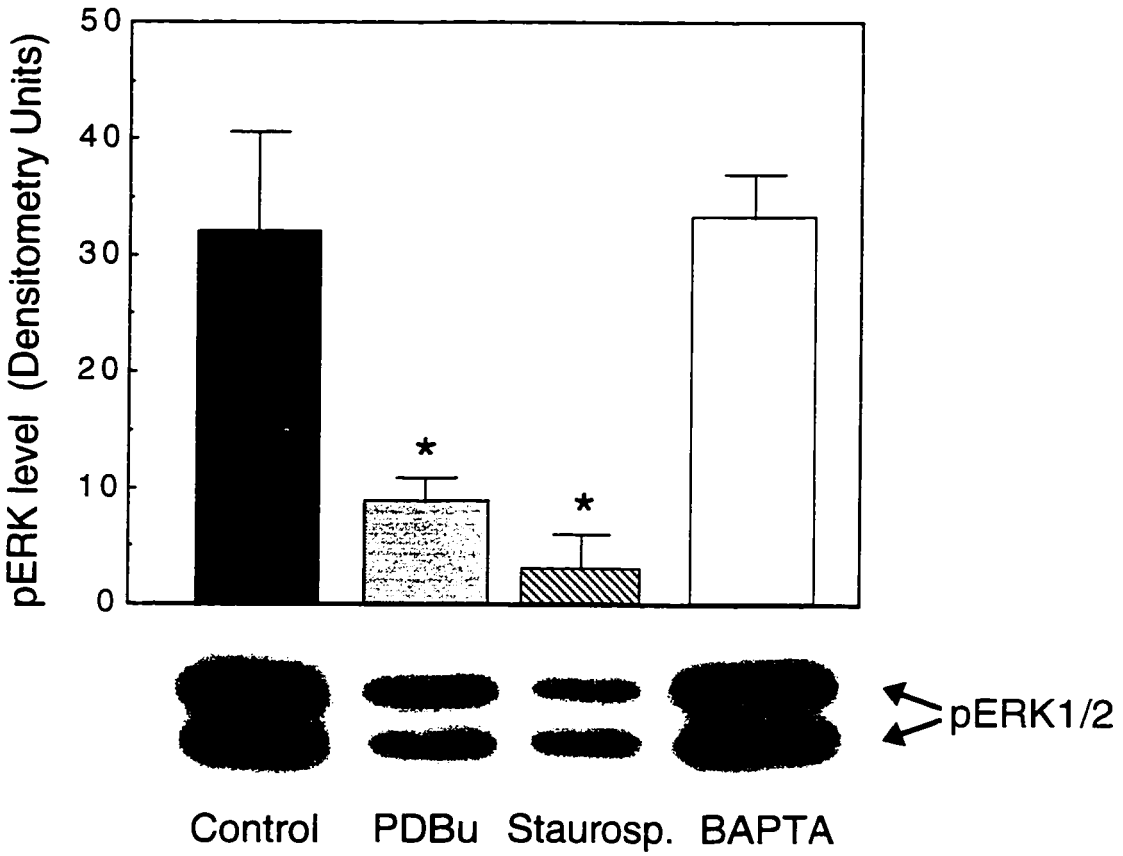

pERK level (Densitometry Units)
50
40
30
20
10
0
*
*
pERK1/2
Control
PDBu
Staurosp.
BAPTA

Figure III-2 PKC isoform-expression in EC. Cells were washed free of culture media with HBSS, and lysates prepared. PKC-isoforms were immunoprecipitated from 100 μg of protein using isoform-specific antibodies and Western blot analysis was performed. No significant immunoreactivity was detected with antibodies to PKC-β, PKC-γ, PKC-δ, PKC-θ, PKC-η, or PKC-λ/ι.

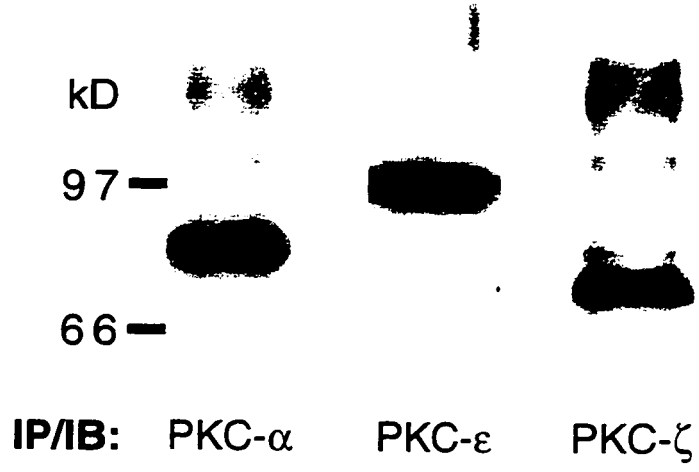

kD
97
66
IP/IB: PKC-α
PKC-ε
PKC-ζ

Figure III-3 Profile of PKC isoform expression after downregulation by PDBu. EC were treated with vehicle (control: DMSO 0.1%, 24 hr), PMA (200 nM for 10 min), or PDBu (1 μM for various times). Cells were washed free of culture media using HBSS and lysates were prepared. Western blot analysis was performed using 25 μg of protein and isoform-specific antibodies for PKC as well as antibodies against total cell ERK1/2.

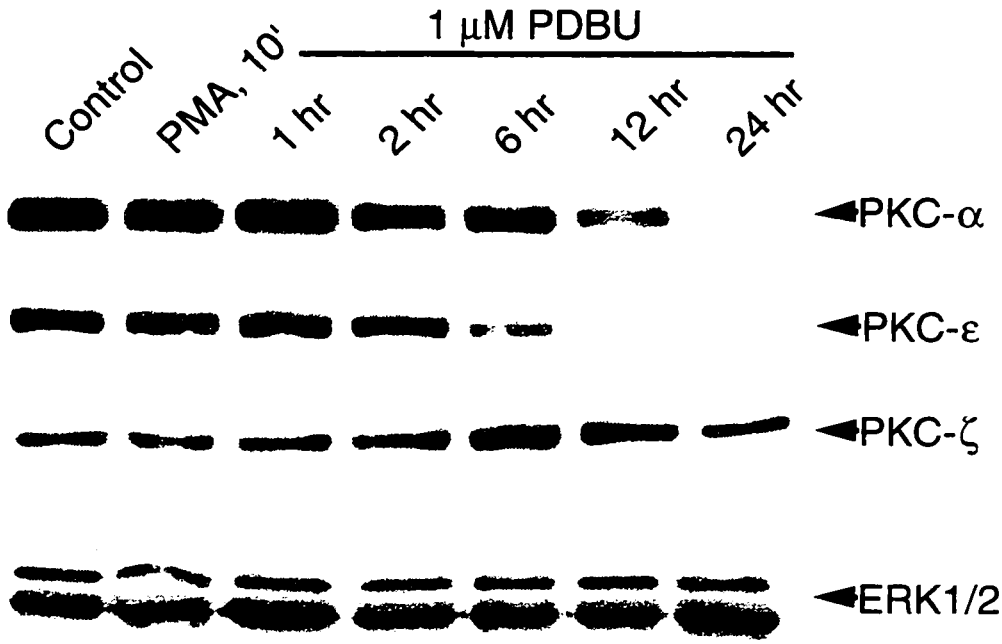

1 μM PDBU
Control
PMA, 10'
1 hr
2 hr
6 hr
12 hr
24 hr
PKC-α
PKC-ε
PKC-ζ
ERK1/2

Figure III-4 Subcellular localization of PKC isoforms in EC assayed by Western blotting. Endothelial cells were pretreated with either vehicle (control: 0.1% DMSO, 10 min) or PMA (200 nM for 10 min) and then washed free of culture media with HBSS. Lysates were prepared, and cytosolic and membrane fractions were purified as described in "Materials and Methods." Western blot analysis was performed using isoform-specific PKC antibodies.

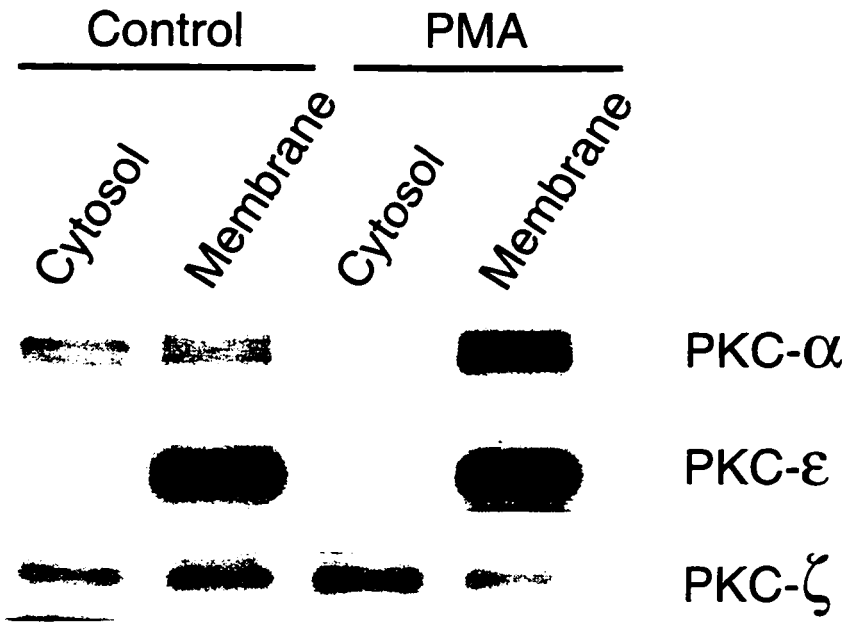

Control
PMA
Cytosol
Membrane
Cytosol
Membrane
PKC-α
PKC-ε
PKC-ζ

Figure III-5 Measurement of PKC isoform activity under varying conditions by histone-H1 phosphorylation assay. EC were washed in HBSS, lysates were prepared using assay-lysis buffer, PKC-isoforms were immunoprecipitated, and equal amounts of protein were assayed in an immune complex kinase reaction (as described in "Materials and Methods") containing histone-H1 and γ-^{32}P-ATP at 30°C for 10 min. For some experiments 2 nM staurosporine, 200 nM PMA, or 200 nM inactive phorbol ester were added directly to the reaction mixture. Proteins were separated by SDS-PAGE and histone phosphorylation determined by autoradiogram and subsequent quantification by densitometry. Cells lysates used in the far right lane (PMA pretreatment) were treated with 200 nM PMA for 10 min prior to cell harvesting and immunoprecipitation.

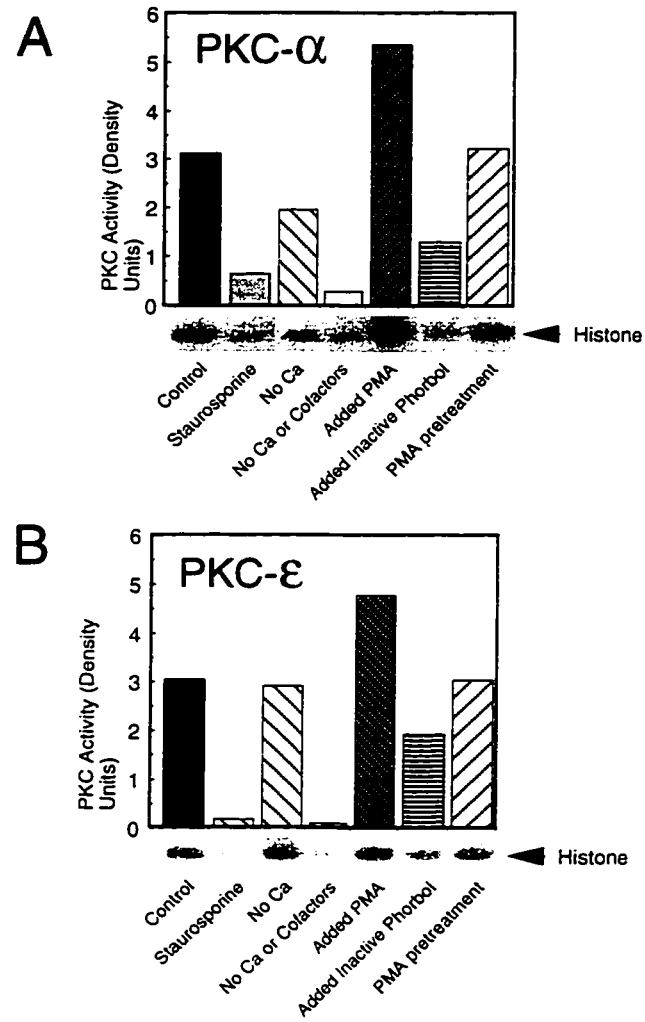

A
PKC-α
PKC Activity (Density Units)
0
1
2
3
4
5
6
Histone
Control
Staurosporine
No Ca
No Ca or Cofactors
Added PMA
Added Inactive Phorbol
PMA pretreatment
B
PKC-ε
PKC Activity (Density Units)
Histone
Control
Staurosporine
No Ca
No Ca or Cofactors
Added PMA
Added Inactive Phorbol
PMA pretreatment
C
PKC-ζ
PKC Activity (Density Units)
Histone
Control
Staurosporine
No Ca
No Ca or Cofactors
Added PMA
Added Inactive Phorbol
PMA pretreatment

Figure III-6 Antisense PKC-ε oligonucleotides specifically inhibit PKC-ε expression. HUVEC were treated with increasing concentrations of antisense oligonucleotides or with 1000 nM scrambled oligonucleotides (as described in "Materials and Methods") for 6 hr before returning the cells to media containing serum. Three days after transfection , cells were washed with HBSS and lysates were prepared for Western blot analysis using 25 μg of protein and PKC isoform-specific antibodies.

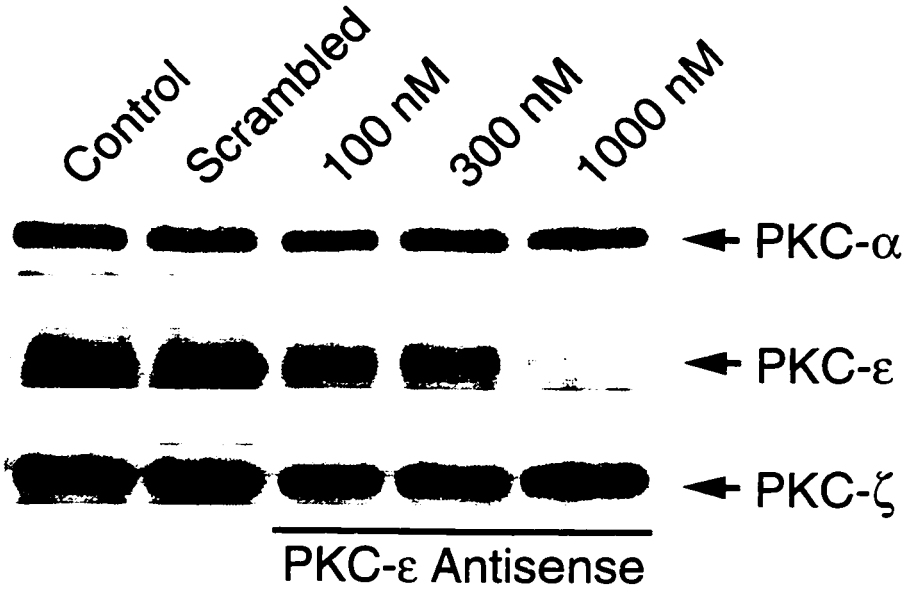
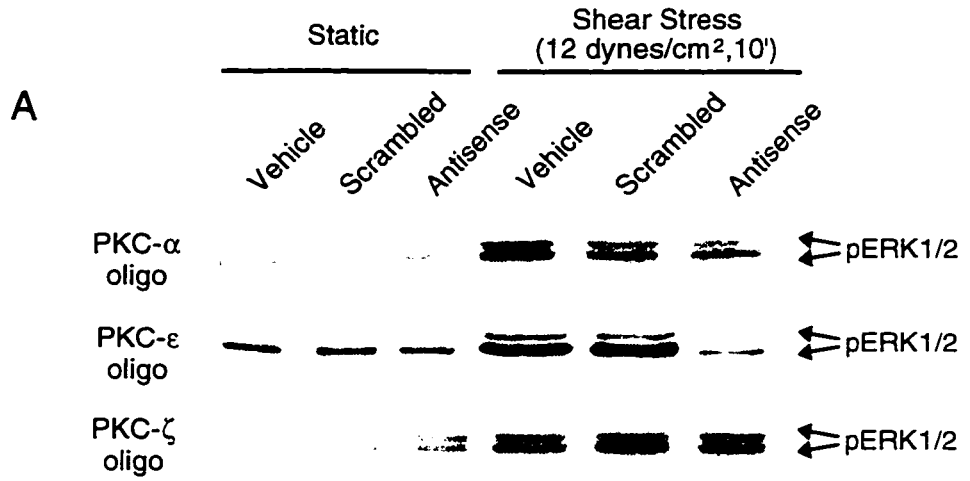

Control
Scrambled
100 nM
300 nM
1000 nM
PKC-α
PKC-ε
PKC-ζ
PKC-ε Antisense

Figure III-7 (A) Antisense PKC-ε oligonucleotides specifically inhibit ERK1/2 activation by shear stress. HUVEC were treated with either 1000 nM antisense oligonucleotides or 1000 nM scrambled oligonucleotides against PKC-α, -ε, and -ζ isoforms for 6 hr before returning the cells to media with serum. Three days after transfection, EC were washed free of culture medium and maintained in static condition or exposed to 12 dynes/cm^2 shear stress for 10 min. Lysates were analyzed by Western blot using phosphospecific-ERK antibody. Western blots are representative from three separate HUVEC preparations. Antisense PKC-ε oligonucleotide treatment completely blocked shear stress mediated ERK1/2 activation while all other treatments did not affect ERK1/2 activation. (B) Effect of antisense PKC-ε oligonucleotides treatment on ERK1/2 activation by various agonists. HUVEC were transfected with antisense PKC-ε oligonucleotides as above. Three days after transfection, EC were either washed free of culture medium and exposed to either PMA (200 nM for 10 min), EGF (100 ng/mL for 5 min), or bradykinin (10 nM for 10 min) or received no treatment (control). Lysates were analyzed by Western blot using a phosphospecific-ERK antibody.


A
Static
Shear Stress
(12 dynes/cm2,10')
Vehicle
Scrambled
Antisense
Vehicle
Scrambled
Antisense
PKC-α
oligo
PKC-ε
oligo
PKC-ζ
oligo
pERK1/2
pERK1/2
pERK1/2

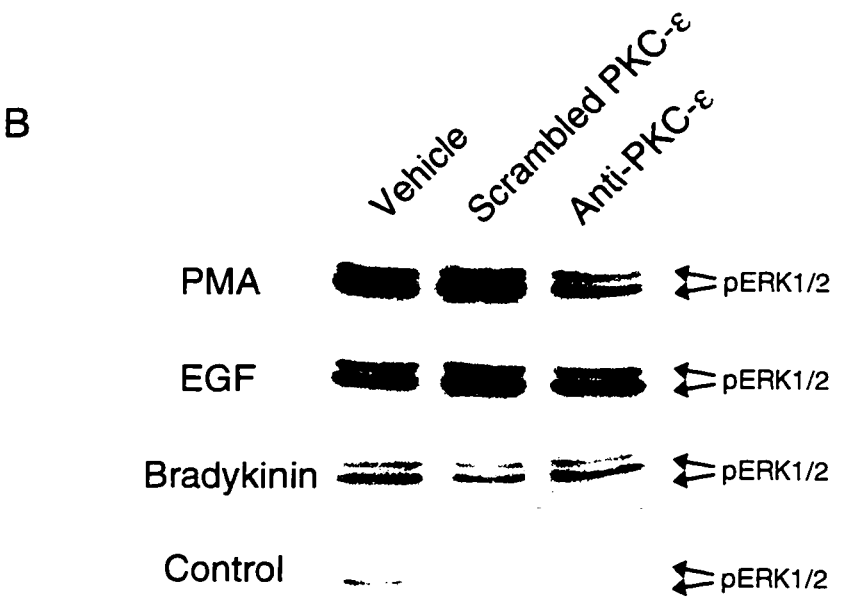
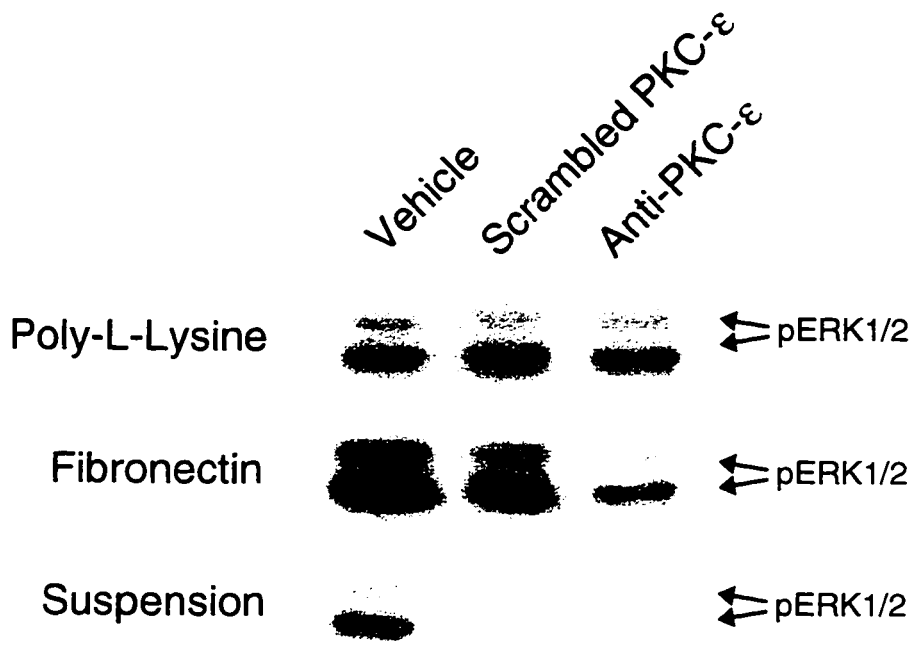

B
Vehicle
Scrambled PKC-ε
Anti-PKC-ε
PMA
EGF
Bradykinin
Control
pERK1/2
pERK1/2
pERK1/2
pERK1/2

Figure III-8 Effect of antisense PKC-ε oligonucleotide treatment on ERK1/2 activation by adhesion. HUVEC were treated with either 1000 nM antisense PKC-ε oligonucleotides or 1000 nM scrambled PKC-ε oligonucleotides for 6 hr before returning the cells to media with serum. Three days after transfection, EC were washed free of culture medium and approximately 10^6 cells were placed onto 60-mm bacteriologic plastic dishes coated with fibronectin or PLL and incubated at 37°C for 10 min or kept in suspension. Lysates were analyzed by Western blot using a phosphospecific-ERK antibody.


Vehicle
Scrambled PKC-ε
Anti-PKC-ε
Poly-L-Lysine
Fibronectin
Suspension
pERK1/2
pERK1/2
pERK1/2

CHAPTER 4: ION DEPENDENCE OF SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION

INTRODUCTION

In the preceding chapters, we have demonstrated that shear stress stimulates signal transduction molecules, including PKC and ERK1/2 in a force- and time-dependent manner, and with kinetics consistent with a classically receptor-mediated process. However, the upstream signaling pathway that leads to activation of ERK1/2 by shear stress remains undefined. Of particular interest are the primary plasma membrane mechanisms by which the physical force of shear stress can be transduced into biochemical signals. Several candidate mechanotransducers have been proposed including G proteins, caveolae, integrins, and mechanosensitive ion channels.

A common mechanism that has evolved to sense changes in mechanical stimuli are the mechanosensitive ion channels (Morris 1990; Davies 1995; Guyton 1996). These channels are widely distributed in tissues and participate in processes such as hearing, balance, touch, and vasoregulation (See Table IV-1). Endothelial cells exhibit ion channel responses to mechanical forces that are likely to participate in the signaling response to shear stress. Several different mechanosensitive ion channels are present in endothelial cells, including a shear-responsive potassium channel, and a stretch-activated calcium channel (Davies 1995). Studies have shown that blockade of mechanosensitive K^+ channels with barium chloride or tetraethylammonium blocked shear stress-mediated increases in NO production (Uematsu, Ohara et al. 1995) and TGF-β release (Ohno,

Cooke et al. 1995), suggesting that transmembrane ion flux and intracellular ion homeostasis are important mediators of the endothelial cell response to shear stress.

The current chapter will determine the contribution of mechanosensitive ion channels to the shear stress-mediated regulation of ERK1/2 activity in endothelial cells through characterization of the ion dependency of this phenomenon. We will demonstrate that intracellular sodium is inhibitory to the activation of ERK1/2 in endothelial cell but that K^+ and Ca^{2+} do not affect the ERK1/2 response.

MATERIALS AND METHODS

CELL CULTURE

Bovine aortic endothelial cells were isolated from fetal calf aortas and maintained in M199 (GIBCO BRL) supplemented with 10% fetal calf serum. Cells used in experiments were passage <6, as ERK1/2 kinase activation decreased in later passages. Human endothelial cells were obtained from human umbilical veins as previously described(Gimbrone Jr. 1976). Cells at passages between 1 and 3 were grown in RPMI-1640 (GIBCO BRL) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Inc.), heparin (Sigma Chemical Co.) and endothelial cell growth factor (kindly provided by Dr. R. Ross, University of Washington). All cells were grown on tissue culture dishes coated with 2.5% gelatin (Sigma Chemical Co.).

SHEAR STRESS EXPERIMENTS

Cells were grown in 74 cm x 36 cm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Two days after reaching confluence, cells were rinsed free of culture media with HBSS (containing in mM: NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$

1.0, HEPES 20, pH 7.4), with 10 mM glucose added, and either maintained in static condition or exposed to 12 dynes/cm^2 of fluid shear stress, in a parallel plate chamber or cone and plate viscometer at 37°C, as described in Chapter 2. After varying times of exposure to fluid shear stress, cells were washed gently with ice-cold PBS (composition, in mM, NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.3) and ERK1/2 activation was determined.

WESTERN BLOT ANALYSIS FOR ERK1/2 ACTIVATION

Cells were washed with PBS and 0.15 mL of buffer oncogene science lysis buffer (Na pyrophosphate 50 mM, NaF 50 mM, NaCl 50 mM, EDTA 5 mM, EGTA 5 mM, Na_3VO_4 100 μM, HEPES 10 mM, Triton 0.1%, PMSF 0.5 mM, leupeptin 10 mg/mL, pH 7.4) was added. Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4°C, 14 000 rpm in microfuge). Sample protein concentrations were determined by DC Protein (Biorad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham). To ensure quantitative transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 hr at room temperature with a commercial blocking buffer (GIBCO BRL). The blots were incubated overnight at 4°C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs; non-specific ERK1 and ERK2 antibodies followed by incubation for 1-2 hr with secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized by chemiluminescence (Amersham ECL).

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. All experiments that were performed at least three times. Significant differences were determined by Student's t test ($p<0.05$). Densitometry was performed using NIH Image 6.0.

RESULTS

SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION IS INDEPENDENT OF AN RISE IN INTRACELLULAR CALCIUM AND INDEPENDENT OF STRETCH-ACTIVATED CALCIUM CHANNELS

Mechanical stimuli cause a rapid increase in intracellular calcium concentration (Shen, Luscinskas et al. 1992), and our laboratory has previously reported that shear stress at 12 dynes/cm^2 for 10 min increases intracellular calcium (Geiger, Berk et al. 1992). To determine if the shear stress-mediated increase in intracellular calcium was necessary for ERK1/2 activation, cells were treated with the Ca^{2+}-chelator BAPTA-AM (75 μM, 30 min), and the shear stress stimulus was performed in a Ca^{2+}-free balanced salt solution supplemented with EDTA (10 mM) in order to inhibit the shear stress-mediated increase in intracellular calcium. Basal levels of ERK1/2 activation (data not shown) and ERK1/2 activation by shear stress was unaffected by pretreatment with BAPTA (Figure III-1), suggesting that calcium is not necessary for ERK1/2 activation by fluid shear stress. These results demonstrate that the shear stress-mediated activation of ERK1/2 is independent of a rise in intracellular calcium.

Stretch-activated calcium channels have been reported to be expressed in endothelial and smooth muscle cells and to mediate the stretch reflex in response to increased pressure. In order to determine whether shear stress-mediated ERK1/2 activation

is dependent on stretch-activated calcium channels, experiments were performed in the presence of the stretch-activated calcium channel antagonist, gadolinium chloride. Increasing concentrations of gadolinium chloride had no effect on shear-stress mediated ERK1/2 activation (Figure IV-1), suggesting that these channels are not necessary for this signal transduction pathway.

SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION IS DEPENDENT ON EXTRACELLULAR Na^+ BUT IS INDEPENDENT OF EXTRACELLULAR K^+, AND Ca^{2+}.

Previous investigators have demonstrated that shear stress-activated K+ channels mediate the release of several vasoactive mediators from endothelial cells (Ohno, Cooke et al. 1995; Uematsu, Ohara et al. 1995). Experiments using an osmolar substitute (n-methyl-d-glucamine) in place of K^+ in the buffer used for shear stress stimulation had no effect on shear stress-mediated ERK1/2 stimulation (Figure IV-2). Increasing amount of the potassium channel blocker, tetraethylammonium, also had no effect on shear stress-mediated ERK1/2 activation (not shown). These results suggest that potassium was also not a necessary factor in shear stress-mediated ERK1/2 activation.

Result studies indicate that a sodium channel mediates mechanosensation in *C. elegens* (Liu, Schrank et al. 1996). Removal of Na^+ and replacement with n-methyl-d-glucamine potentiated the ERK1/2 stimulation (89±10% vs. shear stress in complete buffer) in response to shear stress (12 dynes/cm^2, 10 min) in HUVEC (Figure IV-2), suggesting that Na^+ contributes to the regulation of this signaling cascade and is normally inhibitory to shear stress-mediated ERK1/2 activation. Similar results were obtained when the sodium was replaced with equiosmolar amounts of choline chloride or when the experiments were conducted in BAEC (not shown). A more detailed characterization of this response demonstrated that while no effect on ERK1/2 stimulation by shear stress (12

dynes/cm^2, 10 min) was observed at Na^+ levels greater than 12.5 mM, at Na^+ concentrations of 12.5 mM or less, approximately two-fold potentiation in shear stress-mediated ERK1/2 activation relative to 130 mM Na^+ shear stress condition was observed (Figure IV-3). Further, neither EGF-mediated ERK1/2 activation, nor basal levels of ERK1/2 activation were affected by changes in sodium concentration (Figure IV-3), suggesting that this sodium-dependence is specific for shear stress-mediated ERK1/2 activation.

Effect of Sodium Transport Inhibition on Shear Stress-Mediated ERK1/2 Activation

Sodium is transported across the plasma membrane through specialized transport proteins (Guyton 1996) (Figure IV-4). A sodium gradient is established across the cell membrane by means of the Na^+/K^+-ATPase, which actively transports sodium out of the cell resulting in a negative resting potential within the cell. As a result, Na^+ flows down both a concentration and an electrochemical gradient into the cell when specific sodium channels are activated. Other modulators of Na^+ transport include specialized proteins such as the Na^+/H^+-exchanger, which regulates intracellular pH, the Na^+/Ca^{2+}-exchanger, responsible for homeostasis of intracellular Ca^{2+} concentration, and the $Na^+/K^+/2Cl^-$ cotransporter which helps regulate fluid balance.

In order to determine whether sodium transport mechanisms are involved in shear stress-mediated signaling, we characterized the effect of antagonists of sodium transport mechanisms on shear stress-mediated ERK1/2 activation.

Na^+/K^+-ATPase

Blockade of the Na^+/K^+-ATPase with ouabain, and hence, raising the intracellular levels of sodium (Guyton 1996), resulted in a 62±12% decrease in the shear stress-mediated ERK1/2 activation (Figure IV-5), implying that the intracellular sodium is inhibitory to shear stress-mediated ERK1/2 activation. Ouabain treatment had no effect on EGF-mediated or static levels of phosphorylated ERK1/2.

Na^+/H^+-exchanger

The Na^+/H^+-exchanger is responsible for pH regulation and allows entry of sodium into the cell (Guyton 1996). Blockade of the Na^+/H^+-exchanger with 5-(N-ethyl-N-isopropyl)-amiloride had no effect on the shear stress-mediated ERK1/2 activation (Figure IV-6). 5-(N-ethyl-N-isopropyl)-amiloride treatment had no effect on EGF-mediated or static levels of phosphorylated ERK1/2. These data suggest that sodium entry through this ion pump during shear stress is not significant in terms of ERK1/2 regulation.

Na-K-2Cl cotransporter

The Na-K-2Cl transporter is responsible for volume regulation and allows entry of sodium into the cell (Guyton 1996). Blockade of the Na-K-2Cl cotransporter with the antagonist, bumetanide, had no effect on the shear stress-mediated ERK1/2 activation (Figure IV-7). Bumetanide treatment had no effect on EGF-mediated or static levels of phosphorylated ERK1/2. These data suggest that sodium entry through this ion pump during shear stress is not significant in terms of ERK1/2 regulation.

DISCUSSION

The major findings of this study are that shear stress-mediated ERK1/2 activation is dependent on extracellular Na^+ but not extracellular K^+ or rise in intracellular

Ca^{2+}. Further, this sodium dependence appears specific for shear stress-mediated signal transduction as agents which affect sodium had no effect on baseline ERK1/2 or EGF-induced ERK1/2 activity. Based on the data presented in the current study, we propose a model whereby sodium entry in response to shear inhibits some positive effector molecule upstream of ERK1/2 that is independent of signaling pathways activated by EGF (Figure IV-8). However, since the mechanisms by which shear stress leads to ERK1/2 activation remain to be fully elucidated, it is unclear which upstream signaling molecule(s) may be affected by changes in sodium concentration

Based on the current data, it is unclear exactly how shear stress may affect sodium entry into endothelial cells. It is possible that in addition to shear stress stimulating ERK1/2 activation through some upstream effector, shear stress could lead to opening of some unidentified sodium channel. This would provide both a positive (e.g. MEK activation) and negative stimulus (sodium entry) for ERK1/2 activation in response to shear stress, presumably for finer control of ERK1/2 activation. The motif whereby both a positive and negative stimulus are simultaneously activated in order for precise regulation of an event has been documented in such cases as in mollusks, where stretch-activated K^+ channels exist side by side with stretch-inactivated K^+ channels (Davies 1995). Alternatively, shear stress could close sodium channels, thus reliving a negative stimulus and permitting ERK1/2 activation. The definitive answer to this question could be achieved by patch clamping the channel under shear stress. However, this proves to be technically difficult as the membrane patch is dislodged from the pipet with the application of shear stress.

Numerous studies by Dr. Bevan's group (Bevan 1993; Henrion and Bevan 1995; Henrion and Bevan 1995) document changes in vasomotion with changes in

extracellular sodium that were sometimes independent of changes in calcium. In contrast to our studies, relatively smaller changes in extracellular sodium was sufficient to alter development of myogenic tome in smooth muscle and the authors of these studies postulate that the primary mechanoreceptor(s) are sensitive to extracellular sodium. Other examples of sodium and mechanotransducatory systems include the Pacianian corpuscle (Patton 1989), a mechanically responsive afferent sensor that requires influx of sodium and the degenerin class of sodium channels in *C. elegens*. These degenerins are homologous to channels present in epithelial and various other tissue. Several members of the degenerin family, MEC-4, MEC-10, and unc-105, have recently been described as Na^+ channels that mediate mechanoreception in *C. elegens* and may interact with the matrix (Liu, Schrank et al. 1996).

Several mechanisms by which sodium and/or sodium channels could participate in intracellular signaling leading to endothelial responses such as increased ERK1/2 activity have been proposed (Figure IV-9). These include: 1. Altered Na^+/Ca^{2+} exchange: Although the activation of ERK1/2 was shown to be independent of a rise in calcium levels through the use the calcium chelator BAPTA, localized alterations in Ca^{2+} concentration may still be important for ERK1/2 signaling. Alterations in Na^+ levels can influence the activity of the Na^+/Ca^{2+} exchanger and result in an alteration of calcium-dependent signaling, as has been shown by experiments with ouabain; (2) Regulation of cell volume and cell swelling: Water balance is regulated primarily though Na^+ content. Alterations in net sodium flux across the membrane may result in osmotic stress and cell swelling/shrinkage. Osmotic stress and/or changes in cell volume have been shown to activate MAPKs in a variety of cell types and represents a potential pathway whereby Na^+ could affect ERK1/2 activity. (3) Signaling molecules that interact with a sodium channel; Signaling or scaffolding molecules that interact with the cytoplasmic tail of transmembrane

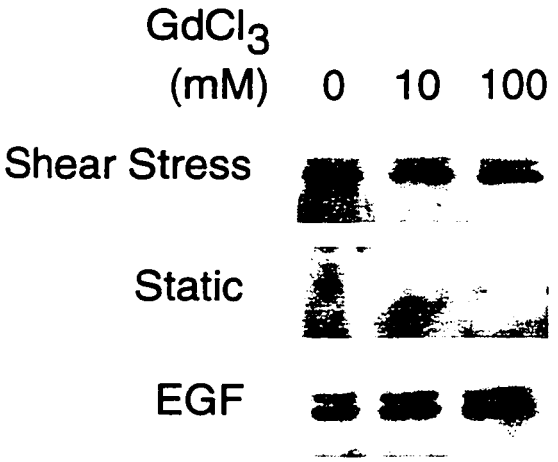
receptor proteins are a common motif in signal transduction. Sodium channels have been shown to interact with cAMP-dependent protein kinases, protein kinase C, and with G proteins through a membrane-delimited pathway. Further, sodium channels are known to interact with substratum proteins including ankyrin, spectrin and actin filaments, which may play some role in intracellular signaling. (4) Na^+-responsive signaling molecules: Another hypothesis postulates the existence of signaling molecules which are directly responsive to Na^+ concentrations despite the relatively high concentrations of Na^+ already present intracellularly. One candidate is PKC; several studies have shown that PKC activity and translocation may be inhibited by Na+ levels (Takeuchi, Hashimoto et al. 1993; Basudev, Romano et al. 1995). Since PKC can regulate Na+ activity through phosphorylation of voltage-gated sodium channels and has been shown to activate ERK1/2 by shear stress (see Chapter 3), it is a good candidate to mediate the inhibitory effect of Na^+. Indeed, another investigator reports the existence of some Na^+-dependent signal transduction element for stimulation of another MAPK, JNK (Kuroki, Minden et al. 1997); the authors report that palytoxin stimulated the sustained activation of both JNK and SEK1 in COS7 and HeLa cells and that this stimulation required extracellular sodium to be present. This is particularly interesting in that it has been hypothesized that ERK1/2 and JNK are reciprocally regulated; (5) one final example of Na+ as a signal transduction element is given by renin secretion. Sodium entry into the macular densa in inhibitory towards renin secretion by an unknown mechanism (Guyton 1996).

In summary these data show that sodium is inhibitory towards shear stress mediated ERK1/2 activation. In the next chapter, we will attempt to identify and clone the sodium channel expressed in HUVEC and characterize their contributions to shear stress mediated ERK1/2 activation.

Table IV-1. Mechanosensitive Ion Channels Mediate Signal Transduction in Various Biological Processes

Process	Stimulus	Channel	Cell Type
Hearing	sound, vibration, tension	Sodium	Hair cell
Blood Pressure	Stretch	Calcium	Smooth muscle; Endothelium; Carotid Baroreceptor
Blood Pressure	Shear	Potassium	Endothelium
Tactile Balance Proprioception Reflex Contraction	Touch Vibration Pressure	Calcium Sodium	Pacianian Corpuscle, free nerve endings, Merkel's discs, Ruffini's endings, Meissner's corpuscles, Krause's corpuscles, muscle spindles, Golgi tendon receptors

Figure IV-1 Shear stress-mediated ERK1/2 activation is independent of stretch-activated calcium channels. EC were exposed to fluid shear stress of 12 dynes/cm^2 for 10 min. Lysates were analyzed by Western blot using a phosphospecific-ERK antibody and quantified by densitometry. Increasing amounts of gadolinium chloride were included in the buffer to block activation of stretch-activated calcium channels. Gadolinium had no effect on static, shear-induced, or EGF-induced ERK1/2 activation.



IB: p-ERK1/2

Figure IV-2 Shear stress-mediated ERK1/2 activation is dependent on extracellular Na^+ but not on extracellular K^+. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in either complete shear stress buffer containing 130 mM Na^+ and 5 mM K^+, or shear stress buffer in which the Na^+ and/or K^+ was replaced with equimolar amounts of n-methyl-d-glucamine or choline chloride. Lysates were obtained and Western blotting with phospho-specific ERK1/2 antibodies were performed. Removal of extracellular Na^+ potentiated shear stress-mediated ERK1/2 activity while removal of K^+ had no effect. Results are expressed as mean ± SEM (n=4).

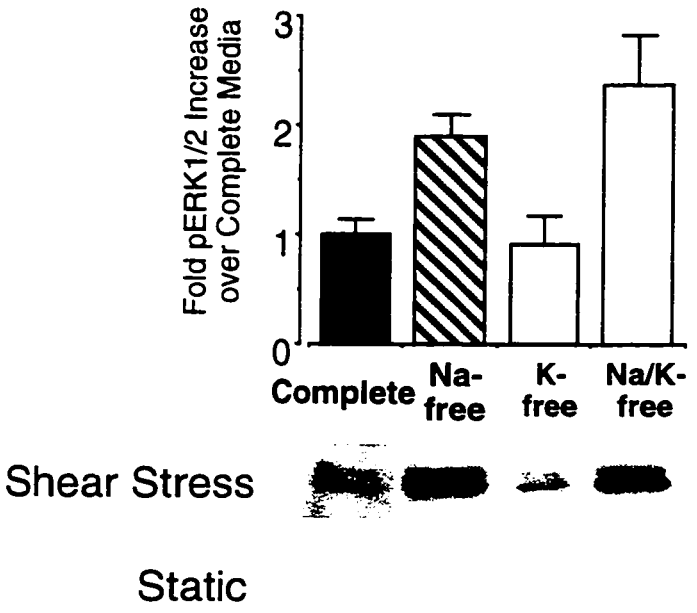

Fold pERK1/2 Increase over Complete Media
3
2
1
0
Complete
Na-free
K-free
Na/K-free
Shear Stress
Static
IB: p-ERK1/2

Figure IV-3 Sodium concentration-response vs. shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in complete shear stress buffer containing varying amounts of sodium that was replaced with n-methyl-d-glucamine. Two-fold potentiation of shear stress-mediated ERK1/2 activity was observed relative to shear stress performed in 130 mM Na^+ buffer when sodium concentration fell below 25 mM. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=4).

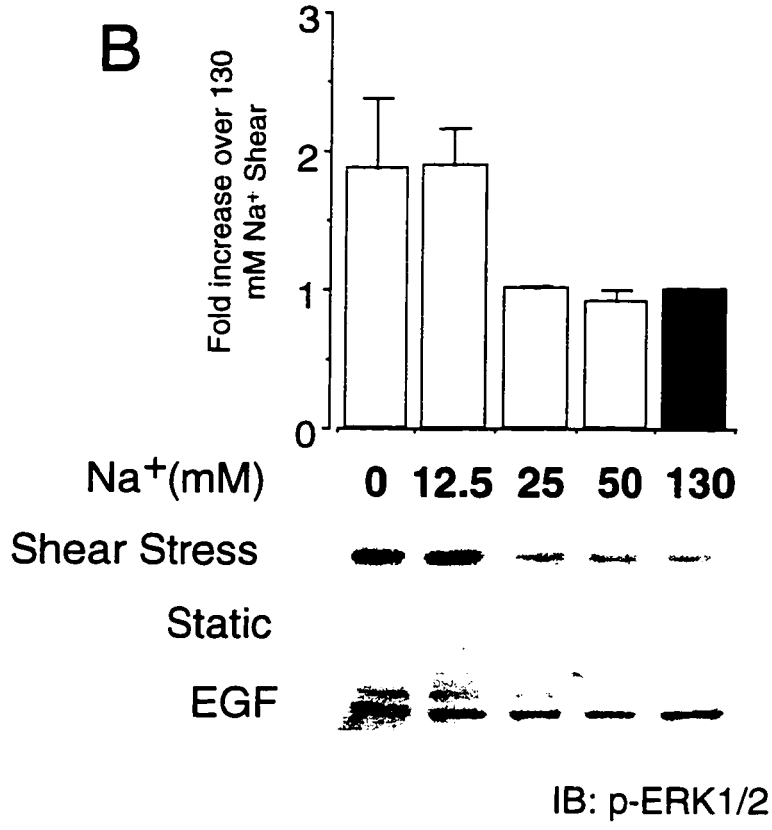

B
Fold increase over 130 mM Na+ Shear
3
2
1
0
Na+(mM)
0 12.5 25 50 130
Shear Stress
Static
EGF
IB: p-ERK1/2

Figure IV-4 Sodium transport mechanisms. A sodium gradient is established across the cell membrane by means of the Na^+/K^+-ATPase, which actively transports sodium out of the cell resulting in a negative resting potential within the cell. Other Na^+ transporters utilize the energy of the sodium gradient for different cellular processes; the Na^+/H^+-exchanger regulates intracellular pH; Na^+/Ca^{2+}-exchanger is responsible for homeostasis of intracellular Ca^{2+} concentration; and the $Na^+/K^+/2Cl^-$ cotransporter is responsible for maintaining cellular fluid balance. Sodium channels, such as the voltage gated sodium channels and epithelial sodium channels allow sodium to flow down its gradient into the cell.

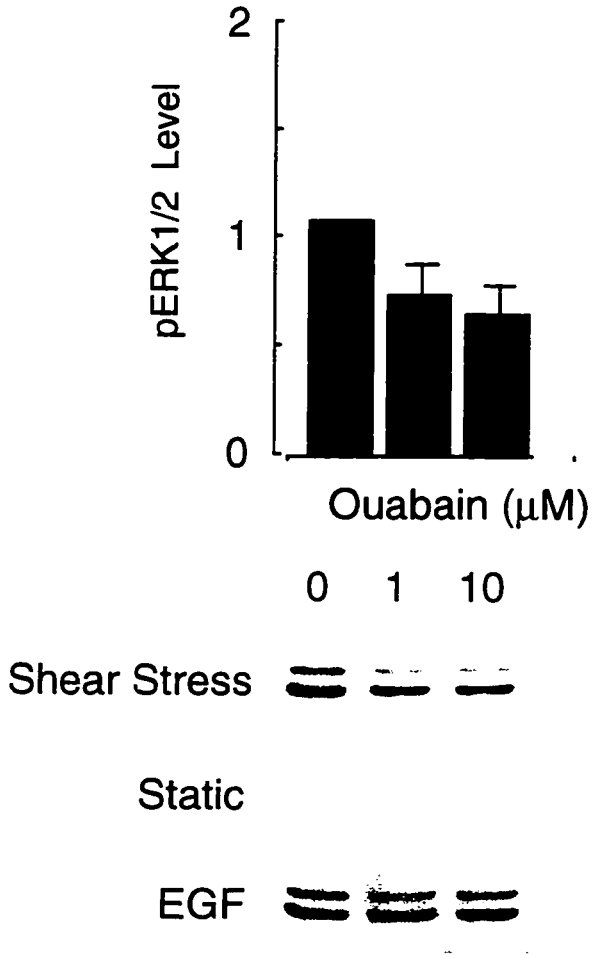

2
1
0
pERK1/2 Level
Ouabain (μM)
0 1 10
Shear Stress
Static
EGF

Figure IV-5 Effect of Na^+/K^+-ATPase inhibition on shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in the presence of increasing concentrations of the Na^+/K^+-ATPase inhibitor, ouabain. Ouabain inhibited shear stress-mediated ERK1/2 activity in a concentration-dependent manner but had no effect on static or EGF-induced levels of phosphorylated ERK1/2. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3).

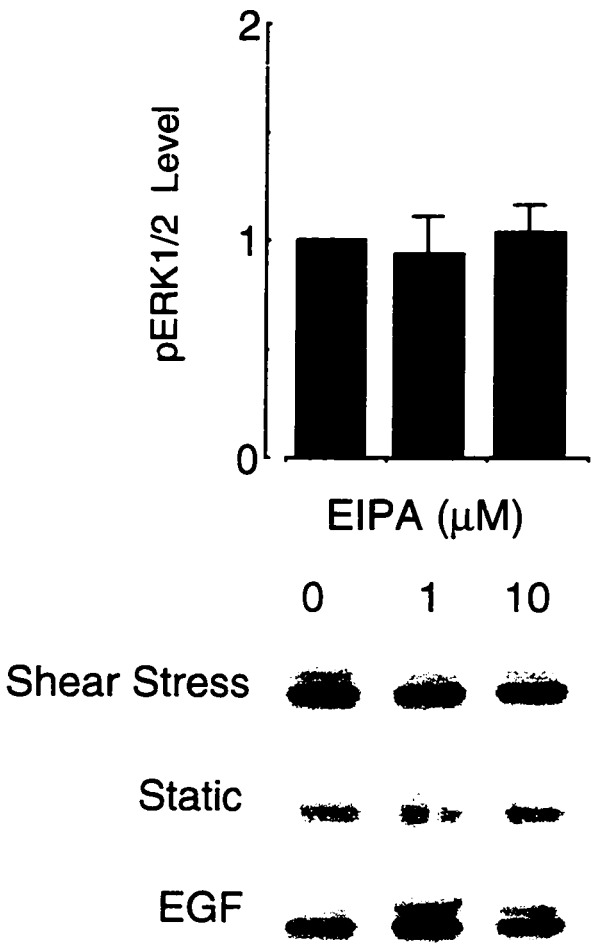

2
1
0
pERK1/2 Level
EIPA (μM)
0
1
10
Shear Stress
Static
EGF

Figure IV-6 Effect of Na^+/H^+ exchanger inhibition on shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in the presence of increasing concentrations of the Na^+/H^+ exchanger inhibitor, EIPA. EIPA had no effect on shear stress-mediated ERK1/2 activity or on static or EGF-induced levels of phosphorylated ERK1/2. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3).

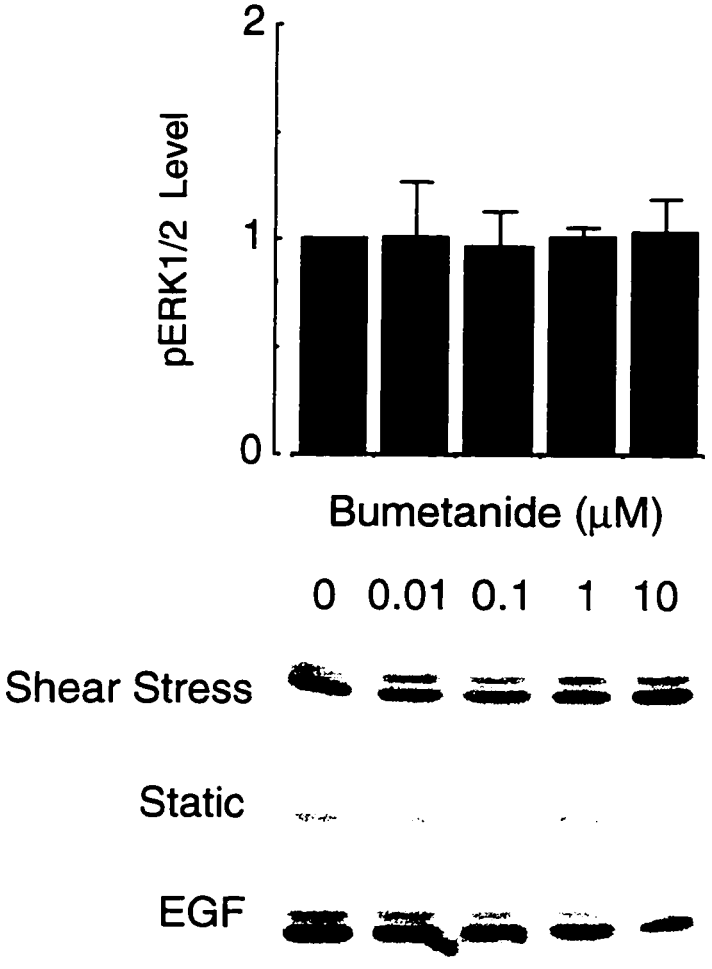

2
1
0
pERK1/2 Level
Bumetanide (μM)
0 0.01 0.1 1 10
Shear Stress
Static
EGF

Figure IV-7 Effect of $Na^+/K^+/2Cl^-$ cotransporter inhibition on shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in the presence of increasing concentrations of the $Na^+/K^+/2Cl^-$ cotransporter, bumetanide. Bumetanide had no effect on shear stress-mediated ERK1/2 activity or on static or EGF-induced levels of phosphorylated ERK1/2. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3)

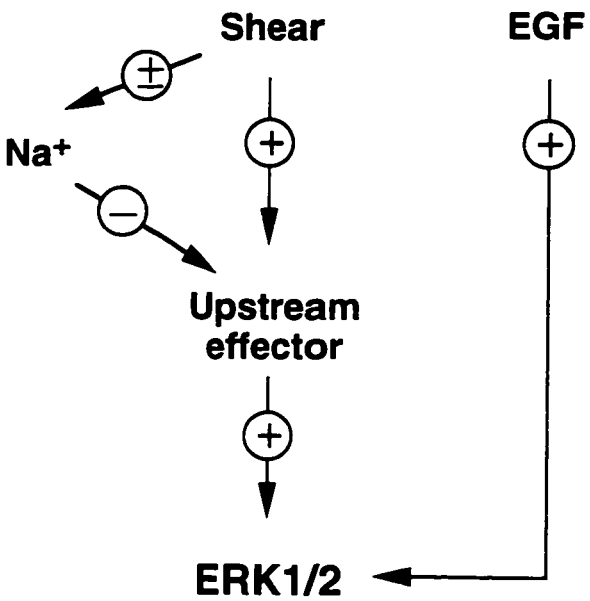
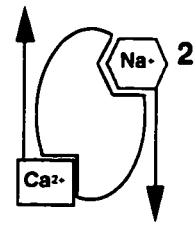

Shear
EGF
Na+
Upstream effector
ERK1/2

Figure IV-8 Model of proposed action of shear stress on sodium concentration and effect on ERK1/2 activity. Shear stress modulates intracellular sodium concentration in HUVEC that regulates intracellular sodium concentration. Sodium acts on upstream ERK1/2 signaling mechanisms to cause inhibition of ERK1/2. This signaling pathway is independent of signaling by such classical growth factors as EGF.

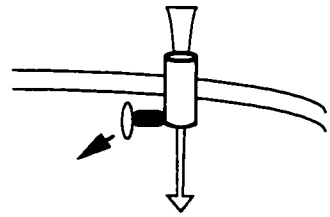
Sodium-Calcium exchange



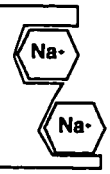
H_2O → Na^+ Na^+ Na^+ Na^+ Na^+

Volume Regulation/ Tensegrity

SIgnaling interactions with the sodium channel



Enzyme

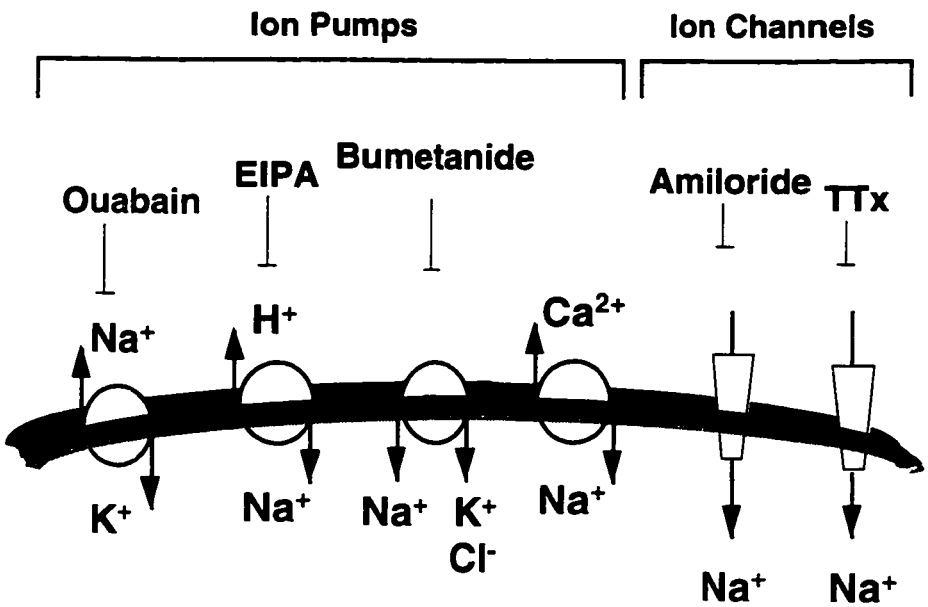


Sodium responsive proteins

Figure IV-9 Sodium as a second messenger. Several mechanisms by which sodium or sodium channels may act as a second messenger in a signal transduction pathway are hypothesized, including Ca2+ regulation, volume regulation, physical interaction of a sodium channel with other signal transduction molecules or the cytoskeleton, and direct interaction of sodium with other signal transduction elements.

Extracellular:

Na^+ = 142 mM
K^+ = 4 mM
Ca^{2+} = 2.4 mM



Intracellular:

Na^+ = 10 mM
K^+ = 140 mM
Ca^{2+} = 0.0001 mM

CHAPTER 5: IDENTIFICATION AND CHARACTERIZATION OF A VOLTAGE-GATED SODIUM CHANNEL INVOLVED IN SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION

INTRODUCTION:

Mechanosensitive ion channels can mediate the transduction of physical stimuli into biochemical signals in various biological processes (See Table IV-1). In the preceding chapter, we have demonstrated that Na^+ is inhibitory to shear stress-mediated ERK1/2 activation and have hypothesized that a mechanosensitive sodium channel may be involved in the regulation of ERK1/2 activation by shear stress.

Sodium ions flow down a concentration and electrochemical gradient into the cell when specific sodium channels are activated. There are at least two different variants of sodium channels in biology. The first are the well characterized voltage-gated sodium channels that are present in nervous and cardiac tissue (Catterall 1995). These channels open in response to cellular depolarization and are critical for generation of action potentials in excitable cells. Another class of channels is the amiloride-sensitive sodium channels (Garty and Palmer 1997). These channels are present in epithelial and various other tissues and are homologous to a groups of proteins in nematodes known as "degenerins". Several members of the degenerin family, MEC-4, MEC-10, and unc-105, have recently been described as Na^+ channels that mediate mechanoreception in *C. elegens* and may interact with the matrix (Liu, Schrank et al. 1996).

Although endothelial cells are largely regarded not to express sodium channels, Gordienko and Tsukahara report the characterization of a channel in endothelial

cells that possesses properties of a voltage-gated sodium channel (Gordienko and Tsukahara 1994). In the following chapter, we attempt to identify sodium channels expressed in endothelial cells and characterize their contribution to shear stress-mediated ERK1/2 activation.

MATERIALS AND METHODS:

CELL CULTURE

Bovine aortic endothelial cells were isolated from fetal calf aortas and maintained in M199 (GIBCO BRL) supplemented with 10% fetal calf serum. Cells used in experiments were passage <6, as ERK1/2 kinase activation decreased in later passages. Human endothelial cells were obtained from human umbilical veins as previously described (Gimbrone Jr. 1976). Cells at passages between 1 and 3 were grown in RPMI-1640 (GIBCO BRL) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Inc.), heparin (Sigma Chemical Co.) and endothelial cell growth factor (kindly provided by Dr. R. Ross, University of Washington). CHO-K1 and CNaIIA-1 cells were obtained from the laboratory of Dr. W. Catterall (University of Washington, Seattle) and maintained in RPMI-1640 supplemented with 5% fetal bovine serum. G418 (200 μg/mL) was added to media for CNaIIA-1 cells. One day prior to the shear stress stimulus, CHO cells were placed in serum containing 0.1% serum in order to reduce baseline ERK1/2 phosphorylation. All cells were grown on tissue culture dishes coated with 2.5% gelatin (Sigma Chemical Co.).

SHEAR STRESS EXPERIMENTS

Cells were grown in 74 cm x 36 cm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Two days after reaching confluence, cells were rinsed

free of culture media with HBSS (containing in mM: NaCl 130, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 20, pH 7.4), with 10 mM glucose added, and either maintained in static condition or exposed to 12 dynes/cm^2 of fluid shear stress, in a parallel plate chamber or cone and plate viscometer at 37°C, as described in Chapter 2. After varying times of exposure to fluid shear stress, cells were washed gently with ice-cold PBS (composition, in mM, NaCl 137, KCl 2.7, Na_2HPO_4 4.3, KH_2PO_4 1.4, pH 7.3) and ERK1/2 activation was determined.

WESTERN BLOT ANALYSIS FOR ERK1/2, PERK1/2, AND SODIUM CHANNELS

Cells were washed with PBS and 0.15 mL of buffer oncogene science lysis buffer (Na pyrophosphate 50 mM, NaF 50 mM, NaCl 50 mM, EDTA 5 mM, EGTA 5 mM, Na_3VO_4 100 μM, HEPES 10 mM, Triton 0.1%, PMSF 0.5 mM, leupeptin 10 mg/mL, pH 7.4) was added. Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4°C, 14 000 rpm in microfuge). Sample protein concentrations were determined by DC Protein (Biorad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham). To ensure quantitative transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 hr at room temperature with a commercial blocking buffer (GIBCO BRL). The blots were incubated overnight at 4°C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs; non-specific ERK1 and ERK2 antibodies; SP-19 anti-voltage-gated sodium channel antibody was provided by Drs. W. Catterall and B. Murphy at the University of Washington; antibodies against the amiloride-sensitive sodium channels were provided by Dr. D. Benos at the University of Alabama) followed by incubation for 1-2 hr with secondary antibody (horseradish peroxidase-

conjugated). Immunoreactive bands were visualized by chemiluminescence (Amersham ECL).

Preparation of Endothelial Cell Membrane Fractions

Cells or tissue were homogenized with fractionation lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM benzamidine, 1 mg/mL leupeptin, 50 µg/mL PMSF, 0.1 mg/mL ovalbumin, and 0.1 µg/mL aprotonin, pH 7.4) on ice. After incubation for 5 minutes, cells were disrupted with a Dounce homogenizer (50 strokes) and centrifugation was performed (100,000 x g for 1 hr). The supernatant was saved as the cytosolic fraction. The pellet (membrane fraction) was washed once with lysis buffer, then resuspended in 150 µL of lysis buffer that contained 1% Triton X-100 and was solubilized for 1 hour at 4°C before sonication. Proteins then underwent Western Blot analysis as described above.

Phosphorylation of Cardiac Na^+ Channel by Protein Kinase A

SP-19-immunoprecipitated Na^+ channel were phosphorylated by incubation at 37°C in 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10 mM MgCl2, 1 mM EGTA, 0.15 µM [γ-32P]ATP (3,000 Ci/mmol), or 100 µM unlabeled ATP, in the presence of 0.25 µM purified catalytic subunit of protein kinase A for 1 hr. Phosphorylation reactions were terminated by heating at 65°C for 3 min in 80 mM Tris-HCl (pH 6.8), 10% glycerol, 10 mM dithiothreitol, and 2% SDS.

Reverse Transcription cDNA Synthesis

mRNA was isolated from P1 HUVEC using the Poly(A)Pure kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. This kit utilizes oligo dT

cellulose to bind mRNA. The oligo dT cellulose is subsequently washed to remove ribosomal RNA, and is then pelleted and mRNA is eluted through a spin column. cDNA was synthesized using Superscript cDNA synthesis kit (GibcoBRL, Gaithersburg, MD). This kit utilizes random hexamers and a reverse transcription enzyme that lacks RNase H activity.

POLYMERASE CHAIN REACTION AND SEQUENCING

Three sets of nested degenerate primers were designed using conserved sequences of the brain, skeletal muscle, and heart voltage gated sodium channel or using conserved sequences of the class of epithelial sodium channels and its homologues (See Figure V-6, Table V-2 for PCR strategy). Degenerate primers were designed for minimal degeneracy, particularly on the 3' end, and were at least 14-mer in length (see Table V-1). Polymerase chain reaction was performed using a GeneAmp PCR system 2000 thermal cycler (Perkin Elmer), Taq polymerase, and the materials provided in the AdvanTAge PCR cloning kit (Clontech, Palo Alto, CA). Final concentrations in the PCR reaction mixture were as follows: primers (1 μM); dNTP (200 μM); MgCl2 (1.5 mM); cDNA (1-2 ng/reaction); taq polymerase (1-2 units/reaction); and reaction buffer as supplied in the kit. A single round of amplification (30 cycles) were initiated by denaturation for 5 min at 96°C followed by 30 s each at 40°C, 72°C, and 96°C. Amplified products were separated by agarose gel electrophoresis separation and visualized by ethidium bromide/UV light.

PCR products were cloned into the pT-Adv vector using T4 DNA ligase (4 U T4 DNA ligase; 50 ng/mL pT-Adv vector, 1 ng PCR product, and ligation buffer provided in kit) for 16 hr at 14°C and then transformed into competent *E. coli* and grown overnight on LB agar plates containing X-gal and IPTG. Colonies were chosen by white/blue color selection and then inoculated into fresh media and grown overnight.

Plasmids were isolated by mini prep (Qiagen MiniPrep Spin) and inserts sequenced (ABI PRISM Dye Terminator Cycle Sequencing Kit, Perkin Elmer, Foster City, CA) at the Department of Pharmacology Sequencing Core (University of Washington, Seattle, WA). Insert sequences were compared against those in the Genbank database using the BLAST and Swissprot search algorithms.

NORTHERN BLOT:

The insert of interest was restricted from purified plasmids using EcoRI and isolated by agarose gel electrophoresis separation and subsequently gel purified (GeneClean kit, Bio101, CA). The insert was labeled using α-^{32}P-CTP and random primers DNA labeling system (Life Technologies, Gaithersburg, MD) and free dNTP removed by G50 spin columns. The oligonucleotide probe was hybridized to a membrane containing cDNA from multiple human tissues (Clontech, Palo Alto, CA). Briefly, the membrane was prehybridized with hybridization solution (ExpressHyb solution, Clontech) for 30 min at 37°C. The oligonucleotide probe (30 ng/mL, 2 x 107 cpm/mL) was added in a volume of 5 mL of hybridization solution and was incubated with the membrane in Robbins Scientific Hybridization Rotor Chamber for 1 hr at 37°C. The blot was rinsed with an SSC/SDS mixture until radioactivity was no longer detected in the waste liquid. The blot was covered with plastic and exposed to X-ray film at -70°C for 24 hr.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. All experiments that were performed at least three times. Significant differences were determined by Student's t test ($p<0.05$). Densitometry was performed using NIH Image 6.0.

RESULTS:

HUVEC LYSATES SHOW IMMUNOREACTIVITY AGAINST THE SP-19 ANTIBODY

In order to determine if HUVEC express voltage-gated sodium channels. Western blotting was performed using an antibody (SP-19) directed against the highly conserved inactivation region of the voltage-gated sodium channel alpha subunit. Western blotting with the SP-19 antibody showed immunoreaction of whole cell fractions of BAEC and HUVEC at a molecular weight identical to that of purified voltage-gated sodium channel alpha subunit (Catterall 1995) (Figure V-1a). Similar results were observed with lysates from rat brain and rat heart, but not from rat kidney. Immunoreactivity was enhanced in membrane fractions from HUVEC and BAEC cells indicating that this protein is localized to the membrane fraction. HUVEC showed at least 2 different immunoreactive bands at slightly different molecular weight suggesting that more than one subtype of voltage-gated sodium channels may be expressed. Further, SP-19 immunoprecipitates from HUVEC and BAEC showed phosphorylation by protein kinase consistent with PKA phosphorylation of voltage-gated sodium channels reported by other laboratories (Catterall 1995) (Figure V-1b).

Immunoreactivity when blotting with an antibody against the amiloride-sensitive sodium channel was not detected, though this may be due to technical problems with the antibody itself as only very weak immunoreactivity was seen with positive controls (not shown).

SINGLE CHANNEL/WHOLE CELL RECORDING OF VOLTAGE-GATED SODIUM CHANNEL FROM HUVEC

SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION IS MODULATED BY TETRODOTOXIN AND VERATRIDINE

In order to determine whether voltage-gated sodium channels participate in the shear stress-mediated ERK1/2 activation in endothelial cells, we characterized the effect of agonists and antagonists of voltage-gated sodium channels on the shear stress-mediated ERK1/2 activation. The voltage-sensitive sodium channel antagonist, tetrodotoxin, reproducibly potentiated the shear stress-mediated ERK1/2 activation (12 dynes/cm^2, 10 min) to a magnitude similar to extracellular Na^+ withdrawal (approximately two-fold; Figure V-2), again suggesting that sodium entry through voltage-gated sodium channels is inhibitory to ERK1/2 activation by shear stress, and that sodium is entering through voltage-gated sodium channels during shear stress. Conversely, the voltage-gated sodium channel agonist, veratridine, attenuated the shear stress-mediated ERK1/2 activation in a concentration-dependent manner (Figure V-3). Neither tetrodotoxin nor veratridine had any effect on static or EGF-mediated pERK1/2 levels indicating that this effect on voltage-gated sodium channels is specific for shear stress-mediated ERK1/2 activation. Amiloride had no effect on shear stress-mediated ERK1/2 activation (not shown). These data are consistent with the hypothesis that sodium entry during shear stress is inhibitory to shear stress-mediated ERK1/2 activation and that voltage-sensitive Na^+ channels participate in the regulation of ERK1/2 activation in response to shear stress.

EXPRESSION OF RAT BRAIN VOLTAGE-GATED SODIUM CHANNELS IN CHO CELLS INHIBITS SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION.

In order to determine whether voltage-gated sodium channels could inhibit shear stress-mediated ERK1/2 activation, Chinese hamster ovary (CHO) cells transfected with a cDNA encoding the alpha subunit of rat brain type IIA Na^+ channel (CNaIIA-1 cells), also referred to in the literature as SCN2a (Burgess, Kohrman et al. 1995), and the

corresponding wild type cell line (CHO-K1) were obtained from Dr. W. Catterall at the University of Washington. Upon exposure to shear stress of 12 dynes/cm^2 for 10 min, Western blot analysis revealed that ERK1/2 was phosphorylated in CHO-K1 cells, indicating that the cell were shear stress-responsive. CNaIIA-1 cells that underwent an identical stimulus, however, failed to show ERK1/2 stimulation in response to shear stress indicating that expression of voltage-gated sodium channels could inhibit shear stress-mediated ERK1/2 activation (Figure V-4). Whole cell ERK1/2 levels of both cell types were not significantly different between the two cell lines.

Importantly, removal of Na^+ from the buffer in which the shear stress experiment was performed or inclusion of 100 nM tetrodotoxin restored the shear stress response in CNaIIA-1 cells while veratridine had no effect (Figure V-5), indicating that Na^+ influx through voltage-gated sodium channels was inhibiting shear stress-mediated ERK1/2 activation.

IDENTIFICATION OF VOLTAGE-GATED SODIUM CHANNEL mRNA IN ENDOTHELIAL CELLS BY POLYMERASE CHAIN REACTION

Because removal or sodium or the use of antagonists and agonists of voltage gated sodium channel alter the shear stress-mediated ERK1/2 activation in endothelial cells, and because transfection of CHO cells to express voltage gated sodium channel inhibits shear stress-mediated ERK1/2 activation, we wished to identify the voltage-gated sodium channel potentially expressed in endothelial cells.

Sequential sets of nested degenerate PCR primers were designed against conserved sequences in the voltage gated sodium channel (Figure V-6). These primers were used for sequential PCR reactions using cDNA synthesized from P1 HUVEC as a

template. The first round of PCR yielded a smear of PCR fragment length as predicted while the second reaction yielded four bands (including a band of the predicted length for the voltage gated sodium channels) and the third reaction yielded two bands (approximately the predicted length for the voltage gated sodium channel) (Figure V-7). After subcloning and transformation, twenty positive colonies were selected at random for sequencing. Eighteen of these colonies were homologous to rat and mouse SCN8a voltage gated sodium channel (89% nucleotide homology, 97% amino acid homology). Comparison of our clone sequence with the human SCN8a currently being cloned at the laboratory of Dr. M. Meisler (University of Michigan) revealed 99% nucleotide homology and 100% amino acid homology. Two clones were homologous (100% nucleotide, 100% amino acid) to human SCN4a skeletal muscle voltage gated sodium channel. Northern blots using the SCN8a PCR fragment obtained from our endothelial cells showed high levels of expression of the clone in human brain but not in human cardiac or skeletal muscle, consistent with reports by Dr. Meisler (Figure V-8).

The degenerate PCR protocol for the amiloride-sensitive sodium channel yielded clones with no open reading frames. Design of other sets of degenerate primers against the amiloride-sensitive sodium channels may be needed to determine if endothelial cells express these channels.

These data demonstrate for the first time that endothelial cells express voltage gated sodium channels.

PROTEIN KINASE C-INDUCED ERK1/2 ACTIVATION IS POTENTIATED BY SODIUM REMOVAL

Several investigators report that PKC activity and translocation may be affected by intracellular sodium levels. In order to determine whether PKC may be affected by intracellular sodium levels, CHO cells were stimulated with PMA (1 μM, 10 min) in complete and sodium-free buffer and ERK1/2 phosphorylation was measured by Western blotting. PMA-induced ERK1/2 activation was significantly increased in sodium-free buffer when compared with complete buffer (Figure V-9). This suggests that some element between phorbol ester and ERK1/2, perhaps PKC itself, is sensitive to levels of intracellular sodium and may mediate the inhibitory action of sodium on shear stress-mediated ERK1/2 activation.

EXTRACELLULAR MATRIX ALTERS THE ABILITY OF VOLTAGE-GATED SODIUM CHANNELS TO AFFECT SHEAR STRESS-MEDIATED ERK1/2 ACTIVATION

Salter *et al.* (Salter, Robb et al. 1997) have reported that mechanical-induced depolarization in bone cells involved an integrin-mediated activation of voltage-gated sodium channels. In order to determine whether integrins could affect the ability of voltage gated sodium channels to inhibit shear stress-mediated ERK1/2 activation, CNaIIA-I and CHOK-1 cells were grown on either collagen (Type I), gelatin, or no matrix. The cells were exposed to shear stress (12 dynes/cm^2, 10 min) and ERK1/2 phosphorylation measured. As before, when cells were grown on collagen or gelatin, CHOK-I cells responded to shear stress with elevation in ERK1/2 phosphorylation while CNaIIA-I cells did not. However, if the tissue culture dishes were not coated with matrix, both CHOK-I and CNaIIA-I cells show an elevation in ERK1/2 activity in response to shear stress (Figure V-10). These data indicate that the cellular interaction with the matrix maybe

important for voltage-gated sodium channel activation and the process may involve integrins or perhaps actual binding of the voltage gated sodium channel to matrix itself.

DISCUSSION

The major findings of this study are: (1) endothelial cells express an SP-19 reactive protein with molecular weight and PKA phosphorylation consistent with a voltage-gated sodium channel; (2) shear stress-mediated ERK1/2 activation can be altered by the voltage-gated sodium channel effectors, tetrodotoxin and veratridine and (3) transfection of a shear stress responsive cell line with a voltage-gated sodium channel blocks shear stress-mediated ERK1/2 activation which is reversed by sodium withdrawal, tetrodotoxin treatment, or removal of extracellular matrix (4) endothelial cells express the SCN4a and SCN8a voltage gated sodium channels; and (5) phorbol-induced ERK1/2 activation is inhibited by sodium. Further, this sodium channel dependence appears specific for shear stress-mediated signal transduction as agents which affect sodium or voltage-gated sodium channel haven no effect on baseline ERK1/2 or EGF-induced ERK1/2 activity.

This is the first study to document expression of voltage-gated sodium channels in endothelial cells, but is supported by a study demonstrating patch clamping of an ion channel that appears to be a voltage-gated sodium channel in HUVEC (Gordienko and Tsukahara 1994). These results demonstrate that sodium entry through voltage-gated sodium channels are inhibitory towards shear stress-mediated ERK1/2 activation and that mechanosensitive sodium channels play a role in the transduction of the physical force of shear stress into biochemical signals. Based on the data presented in the current study, we propose a model whereby activity of a voltage-gated sodium channel in response to shear

inhibits some positive effector molecule (possibly PKC) upstream of ERK1/2 that is independent of signaling pathways activated by EGF (Figure V-11).

Based on the current data, it is unclear exactly how shear stress may affect voltage-gated sodium channels in endothelial cells. Voltage-gated sodium channels can exist in three different states (Patton 1989). At low resting potentials, voltage gated sodium channels exist in a resting state in which they are relatively impermeable to Na^+ entry. With depolarization, these sodium channels change conformation and Na^+ entry occurs. As the cell reaches a higher transmembrane potential, the channel enters a closed, inactivated state and the channel is refractory to further stimulus until it is reactivated at low transmembrane potentials. Endothelial cells have been reported to have a high resting potential (Gordienko and Tsukahara 1994) and have been shown to undergo hyperpolarization upon exposure to shear stress by opening a shear stress-responsive K^+ channel (Uematsu, Navas et al. 1993; Ohno, Cooke et al. 1995). It is possible that this hyperpolarization could lead to reactivation of inactivated channels, thus leading to opening sodium channels. This would provide both a positive and negative stimulus for ERK1/2 activation with shear stress, perhaps for finer control of the phenomenon.

Alternatively, hyperpolarization could shut off persistent sodium channels (a class of sodium channels that do not inactivate at high membrane potential and thus remain open and permeable to sodium (Catterall 1995) thus relieving a negative stimulus and allowing ERK1/2 to be phosphorylated. However, removal of extracellular K^+ and introduction of the K^+ channel blocker, TEA, had no effect on shear stress-mediated ERK1/2 activation suggesting that the voltage change mediated by this channel is not involved in voltage gated sodium channel regulation by shear stress. The definitive answer to this question could be achieved by patch clamping the channel under shear conditions.

However, this proves to be technically difficult as the membrane patch is dislodged from the pipet under these conditions.

There are other mechanisms aside from voltage changes which may regulate the activity of these channel and hence sodium entry during shear stress. Catterall and colleagues (Li, West et al. 1993; Ma, Li et al. 1994) have demonstrated that activity of the voltage-gated sodium channels could be altered by intracellular signal transduction pathways including G proteins, PKC, and PKA. This is particularly interesting in that shear stress-mediated ERK1/2 activation has been shown to require G proteins (Tseng, Peterson et al. 1995) and PKC (Traub, Monia et al. 1997) (Chapter 3). PKC has been shown to inhibit Na^+ entry through voltage-gated sodium channels by phosphorylation of the channel itself (Li, West et al. 1993), and thus activation of PKC could reduce inhibitory inward sodium currents in addition to stimulating Raf-1 which leads to ERK1/2 activation. Several other investigators have reported that PKC may be also inhibited by intracellular sodium (Takeuchi, Hashimoto et al. 1993; Basudev, Romano et al. 1995), a finding consistent with data presented in this chapter. Thus, by reducing inward sodium flux, a feed forward mechanism may be established whereby PKC activity is potentiated, resulting in greater Raf-1 phosphorylation and, ultimately, greater ERK1/2 activation.

Another potential link between mechanotransduction by shear stress and voltage gated sodium channel activation is provided by a study by Salter *et al.* (Salter, Robb et al. 1997). In this study, the authors demonstrated that human bone cells showed membrane depolarization that involved tetrodotoxin-sensitive sodium channels. Further, this depolarization could be inhibited by antibodies against alpha V, beta 1, and beta 5 integrins. Integrins have been shown to activate PKC-α (Wrenn and Herman 1995) and PKC-ε (Chun, Auer et al. 1997), as well as contribute to shear stress-mediated ERK1/2

activation (Ishida, Peterson et al. 1996). These observation are consistent with data presented in this chapter that removal of extracellular matrix (and hence removing integrin activation) abolishes the ability of voltage-gated sodium channel to inhibit shear stress-mediated ERK1/2 activation. Alternatively, the voltage gated sodium channel may possess extracellular matrix-binding domains and interact with the matrix directly, thus influencing its conformation with physical stress.

In summary, these data show that sodium entry through voltage-gated sodium channel in HUVEC is inhibitory towards shear stress mediated ERK1/2 activation. In addition to its relevance to the transduction of shear stress and ultimately to atherogenesis, this finding may have significance for disease states such as hypertension where it has been shown that there exist differences in mechanosensitive ion channel expression (Hoyer, Kohler et al. 1997). In the future, experiments to characterize the regulation of this mechanosensitive sodium channel (i.e., PKC involvement, interactions with the matrix) and the effect of sodium on components of shear stress-mediated signal transduction (PKC-ε) should prove exciting areas for further investigation.

Table V-1. Degenerate Primers for PCR of Voltage Gated Sodium Channel

	Primer
Sense 1 (S1)	5'-ATIGAYAAYTTYAA-3'
Sense 2 (S2)	5'-AAYATHTTYGAYTT-3'
Antisense 1 (AS1)	5'GGRTTICCRCARTC-3'
Antisense 2 (AS2)	5'-AAIGTYTCRAARTT-3'
Antisense 3 (AS3)	5'-ACRTAIGCRAARTT-3'

Table V-2. Strategy for PCR Amplification of Voltage Gated Sodium Channel from Endothelial Cells

Reaction	Template	Primer 1	Primer 2	Predicted Size (bp)
A	HUVEC cDNA	S1	AS1	828
B	Reaction A Product	S2	AS2	324
C	Reaction B Product	S2	AS3	273

Table V-3. Mammalian Voltage Gated Sodium Channel Alpha Genes

	Chromos. Location	**Major sites of expression**	**Genetic syndrome**
SCN1a	2q24	Brain and spinal cord	
SCN2a	2q23-24.1	Brain and spinal cord	
SCN3a	2q24	Brain and spinal cord	
SCN4a	17q23-25	Skeletal Muscle	HKPP, Paramyotonia Congenita
SCN5a	3p21	Cardiac muscle	Long QT
SCN6a	2q21-23	Heart and Uterus	
SCN7a	-	Glia	
SCN8a	12q13	Brain and spinal cord	

Figure V-1 Endothelial cells express voltage-gated sodium channels. Several tissue were isolated and homogenized and for some tissue, membrane fractions were prepared by high-speed centrifugation (100,000 x g, 1 hr). (a) Samples were separated by 5% polyacrylamide gel electrophoresis and Western blotting was performed with SP-19 antibody against voltage-gated sodium channel inactivation region provided by Dr. W. Catterall. (b) Immunoprecipitation was performed with SP-19 antibody on bovine aortic endothelial cells and HUVEC. Immunoprecipitates were incubated with protein kinase A and γ-^{32}P-ATP for 1 hr, and separated by electrophoresis and autoradiography performed. Endothelial cells express SP-19 immunoreactive proteins that are phosphorylated by protein kinase A and migrate to ~250 kD under electrophoresis, consistent with their identity as voltage-gated sodium channels.

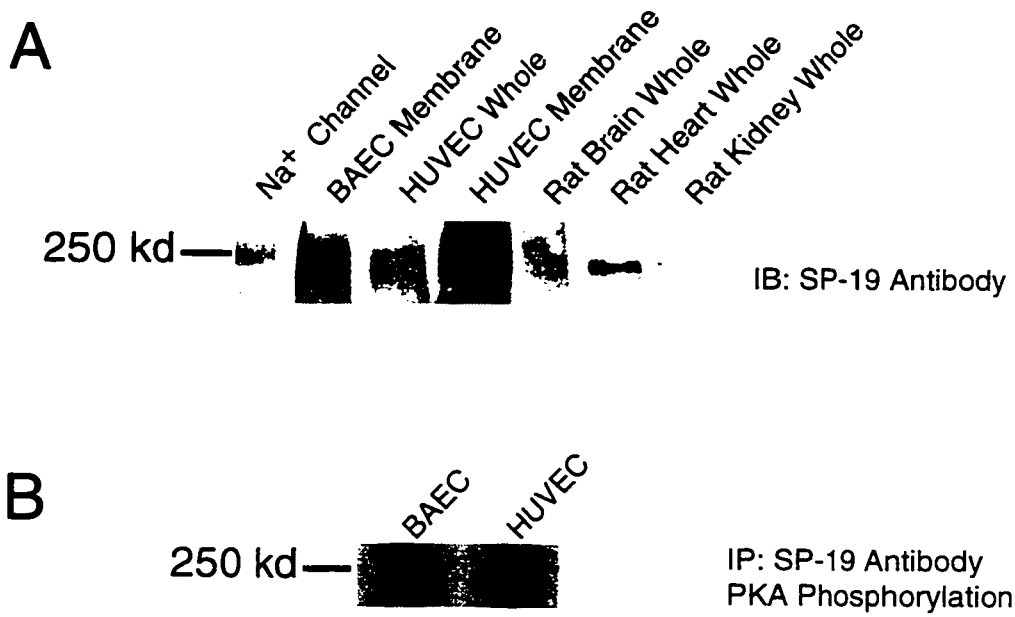

A
Na+ Channel
BAEC Membrane
HUVEC Whole
HUVEC Membrane
Rat Brain Whole
Rat Heart Whole
Rat Kidney Whole
250 kd
IB: SP-19 Antibody
B
BAEC
HUVEC
250 kd
IP: SP-19 Antibody
PKA Phosphorylation

Figure V-2 Effect of voltage-gated sodium channel antagonists on shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in the presence of increasing concentrations of the voltage-gated sodium channel inhibitor, tetrodotoxin. Tetrodotoxin potentiated shear stress-mediated ERK1/2 activity to a magnitude similar to that of extracellular sodium withdrawal. Tetrodotoxin treatment did not have any effect on static or EGF-induced levels of phosphorylated ERK1/2. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3).

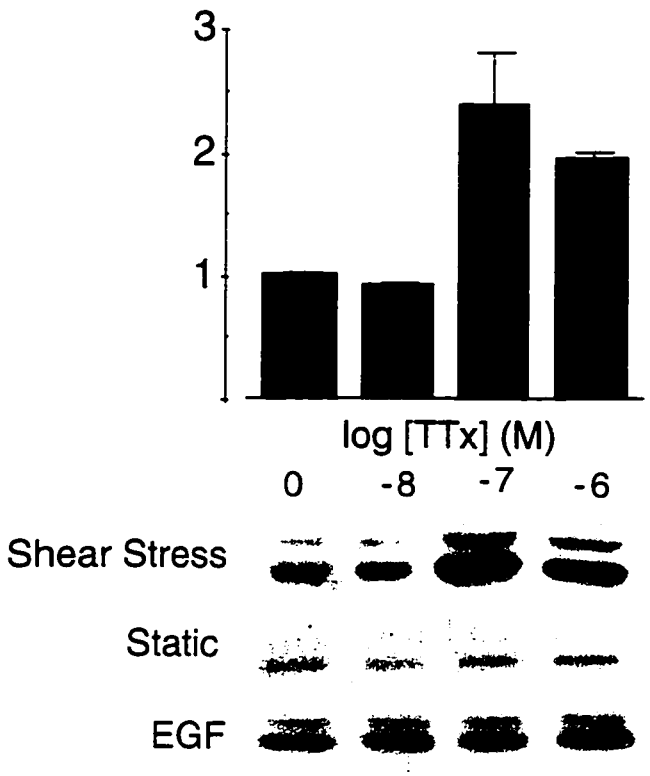

3
2
1
log [TTx] (M)
0
-8
-7
-6
Shear Stress
Static
EGF

Figure V-3 Effect of voltage-gated sodium channel agonist on shear stress-mediated ERK1/2 activation. HUVEC underwent shear stress of 12 dynes/cm^2 for 10 minutes in the presence of increasing concentrations of the voltage-gated sodium channel activator, veratridine. Veratridine attenuated shear stress-mediated ERK1/2 activity in a concentration dependent manner but had no effect on static or EGF-induced levels of phosphorylated ERK1/2. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3).

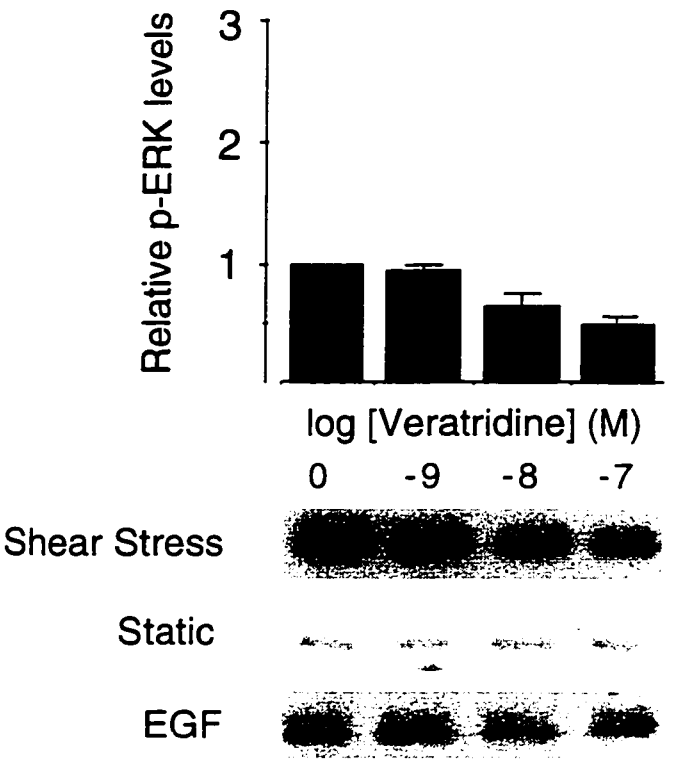

Relative p-ERK levels
3
2
1
log [Veratridine] (M)
0
-9
-8
-7
Shear Stress
Static
EGF

Figure V-4 Expression of voltage-gated sodium channels in CHO cells inhibits shear stress-mediated ERK1/2 activation. Wild-type Chinese hamster ovary cells (CHO-K1) and CHO cells transfected to express rat brain voltage-gated sodium channel IIA (CNaIIA-1) were subjected to shear stress and pERK1/2 levels measured. (a) CHO-K1 cells showed activation of ERK1/2 with shear stress while CNaIIA-I cells did not. Whole cell ERK1/2 levels were similar between the two cells lines. Bar graphs show summary for shear stress data. Results are expressed as mean ± SEM (n=3)

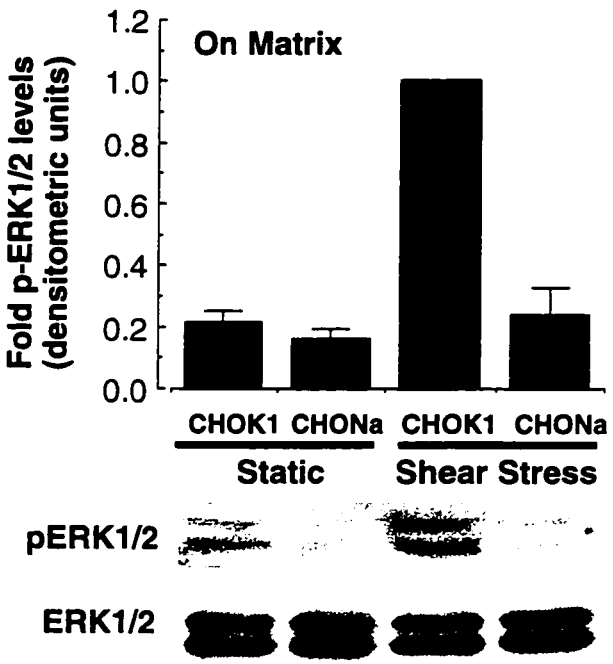

On Matrix
Fold p-ERK1/2 levels (densitometric units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CHOK1 CHONa CHOK1 CHONa
Static
Shear Stress
pERK1/2
ERK1/2

Figure V-5 Expression of voltage-gated sodium channels in CHO cells inhibits shear stress-mediated ERK1/2 activation. Removal of Na^+ from the buffer or inclusion of 100 nM tetrodotoxin restored the shear stress response in CNaIIA-1 cells.

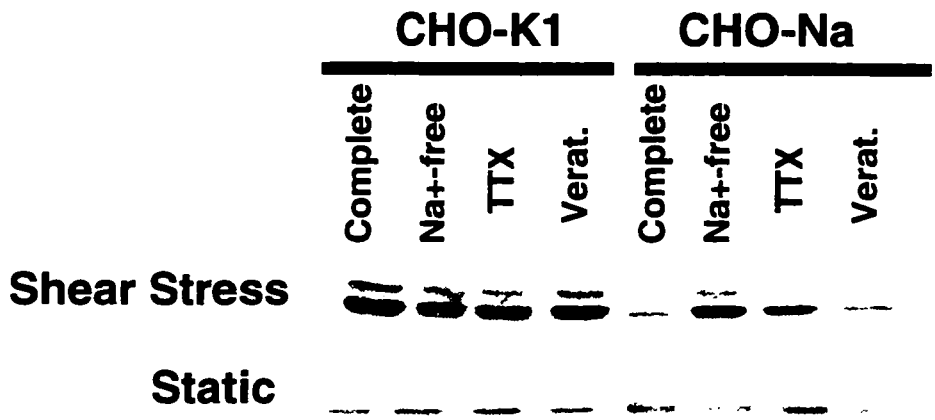
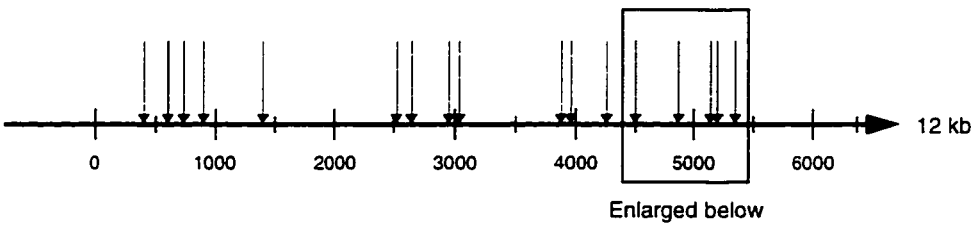

CHO-K1
CHO-Na
Complete
Na+-free
TTX
Verat.
Complete
Na+-free
TTX
Verat.
Shear Stress
Static

Figure V-6 Strategy for PCR amplification of voltage gated sodium channel from endothelial cells. Degenerate primers were designed against conserved sequences of the voltage gated sodium channel family. Three sets of nested primer PCR reactions were performed in order to obtain more specificity with the use of the degenerate primers.

Conserved regions of Brain, Cardiac, and Skeletal Muscle
Voltage Gated Sodium Channels:



PCR strategy:

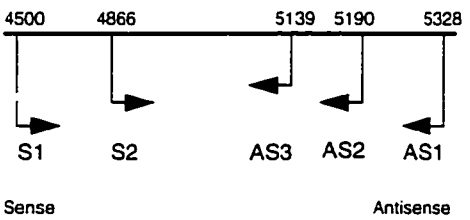
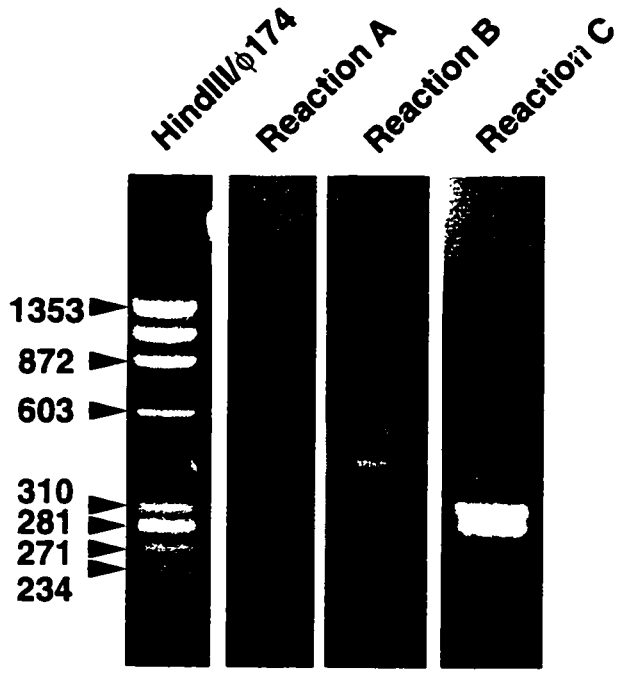


Figure V-7 PCR amplification of voltage gated sodium channels from endothelial cells. Three sets of nested degenerate primer reactions were performed using the PCR product of the previous reaction as the template for the next reaction. The final reaction displays two bands, one of which is the predicted 273 bp size. The PCR products were subcloned, and transformed into *E. coli*. *E. coli* were grown overnight, plasmids isolated by MiniPrep, and PCR insert sequenced. Sequencing determined that the 273 bp fragment was homologous to SCN8a voltage gated sodium channel and the 300+ bp sequence was homologous to SCN4a skeletal muscle voltage gated sodium channel.



90% of clones

273 bp product (100% homology to human SCN8a)

ACATAGGCAAAATTGGACATCCCAAAAATGGAGAAGATGAACATGACCAGGAAGAGCAGAAGGCCGATGTTGAACAGGGCAGGCAAGGAC
ATCATTAAGGCAAAGAGCAGGGTACGAATCCCTTTGGCGCCTTTGATCAGACGCAAGATGCGCCCAATACGGGCCAATCGGATGACTCGG
AATAGGGTTGGGGAAACAAAGTATTTCTCAATTATATCTGCCAGGAACATTCCCACAATGGAGAGGATAACTACCACGAAGTCGAAGATG
TTA

10% of clones

317 bp product (100% homology to human SCN4a)

AAGGTTTCGAAGTTGAACATATCATCTATGCCCGACTCCTTCTTGACGTAGGCAAAGTTGGACATGCCGAAGATGGAGTAGATGAACATG
ACCAGGAAGAGGAGGAGGCCGATGTTGAAGAGGGCAGGCAGCGACATCATGAGGGCGAACAGCAGCGTCCGGATGCCCTTGGCCCCGCGG
ATCAGCCGCAGGACACGCCCAATCCGCGCCAGGCGGATCACACGGAACAGCGTGGGTGACACGAAGTACCTCTGGATCAGGTCAGAGAGG
GCAAGGCCCACAATGGACAGGATAACGACCACGAATCAAAGATGTTA

Figure V-8 SCN8a PCR fragment obtained from endothelial cells was used to probe a human multiple tissue blot. High levels of expression were present in human brain but not in human cardiac or skeletal muscle.

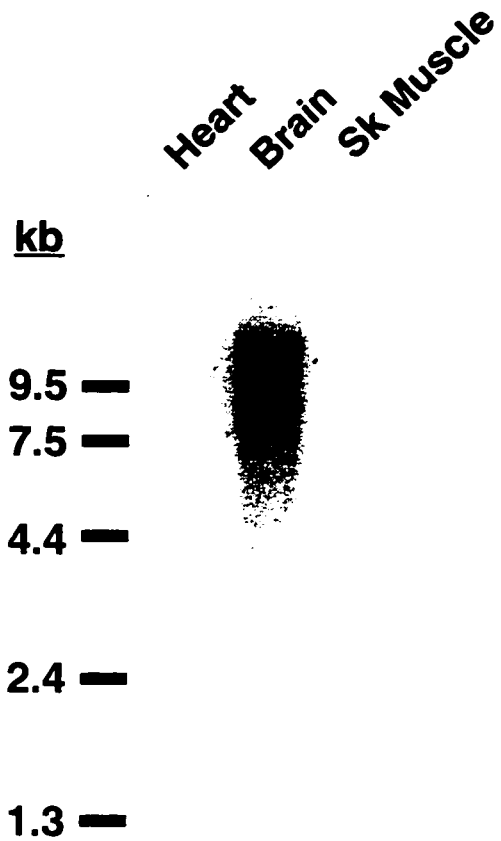

Heart
Brain
Sk Muscle
kb
9.5
7.5
4.4
2.4
1.3

Figure V-9 HUVEC were stimulated with PMA (1 μM, 10 min) in complete buffer or sodium-free buffer and ERK1/2 phosphorylation was measured by Western blotting. ERK1/2 phosphorylation levels were significantly higher in those cells stimulated in sodium-free buffer relative to those stimulated in complete buffer.

Complete
Na+-free
TTX
Verat.
Static
PMA

Figure V-10 CNaIIA-I and CHOK-1 cells were grown on either collagen (Type I), gelatin, or no matrix. After reaching confluence, cells were stimulated with 12 dynes/cm^2 of shear stress for 10 min and ERK1/2 phosphorylation was measured by Western blotting. CHOK-1 cells showed a response to shear stress with or without matrix. CNaIIA-I cells did not show a response to shear stress when grown on matrix, but the response was restored if cells were grown on no matrix.

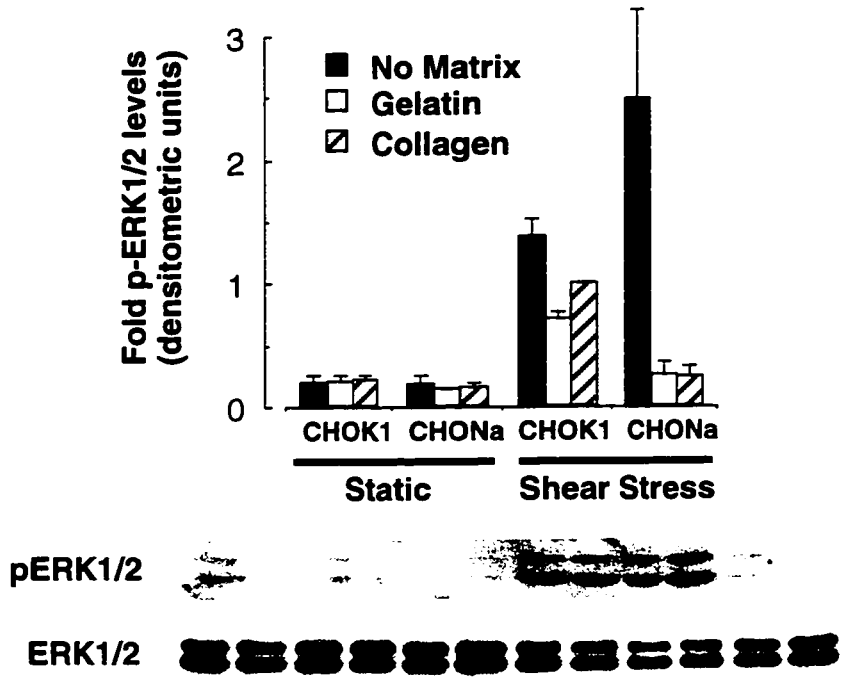
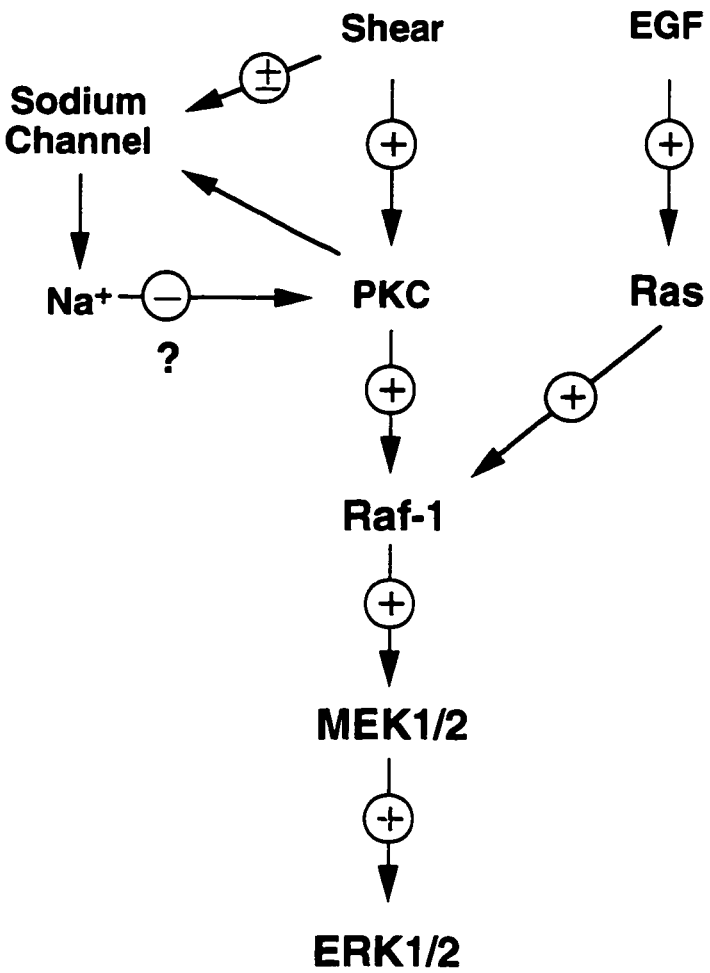

Fold p-ERK1/2 levels
(densitometric units)
3
2
1
0
No Matrix
Gelatin
Collagen
CHOK1
CHONa
CHOK1
CHONa
Static
Shear Stress
pERK1/2
ERK1/2

Figure V-11 Model of proposed action of shear stress on sodium channels and sodium concentration and effect on ERK1/2 activity. Shear stress modulates the activity of a putative voltage-gated sodium channel in HUVEC that regulates intracellular sodium concentration. Sodium or some other effector connected to the sodium channel acts on upstream ERK1/2 signaling mechanisms to cause inhibition of ERK1/2. This signaling pathway is independent of signaling by such classical growth factors as EGF.


Shear
EGF
Sodium
Channel
Na+
?
PKC
Ras
Raf-1
MEK1/2
ERK1/2

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Published journal papers:

1.Watts SW, Traub O, Lamb FS, Myers JH, Webb RC: Effect of ramipril on α-receptor-mediated oscillatory contractions in tail artery of hypertensive rats. *Eur J Pharmacol.* 242:245-253, 1993.

2.Traub O, Webb RC: Angiotensin converting enzyme inhibition during development alters calcium regulation in adult hypertensive rats. *Journal of Pharmacology and Experimental Therapeutics* 257(3):1503-1508, 1993.

3.Watts SW, Traub O, Webb RC: Effects of ramipril on contractile oscillations in arteries from genetically hypertensive rats. *Clin Exp Hypertens.*16(6):881-98, 1994.

4.Traub O, Van Bibber R: Role of nitric oxide in insulin-dependent diabetes related vascular complications.*Western Journal of Medicine.* 162(5):439-445, 1995.

5.Van Bibber D, Traub O, Kroll K, Feigl EO. Norepinephrine-induced coronary vasodilation is mediated by the endothelial nitric oxide pathway. *Am J Physiol.* 37(5):H1973-H1981, 1995.

6.Traub O, Lloyd MC, Webb RC: Long-term effects of brief antihypertensive treatment on systolic blood pressure and vascular reactivity in young genetically hypertensive rats. *Cardiovasc Drugs Ther.* 9(3): 421-9, 1995.

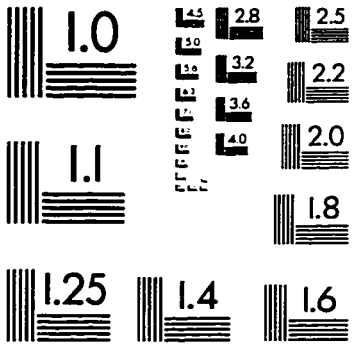
7.Tostes RCA, Traub O, Bendhack LM, Webb RC: Sarcoplasmic reticulum Ca(2+) uptake is not decreased in aorta from deoxycorticosterone acetate hypertensive rats: functional assesment with cyclopiazonic acid. *Can J Physiol Pharmacol.* 73:1536-1545, 1995.

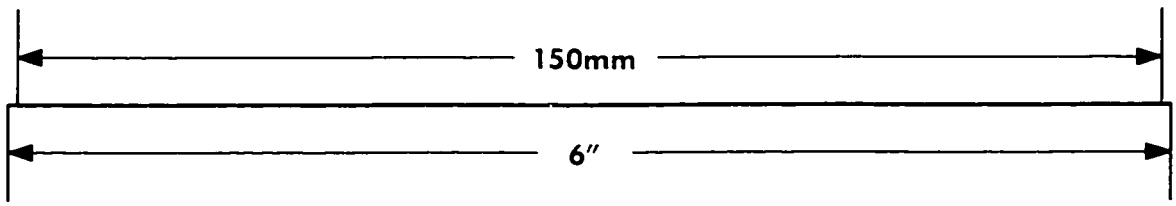
8. Takahashi M, Ishida T, Traub O, Corson MA, Berk BC: Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J Vasc Res.* 34(3):212-9, 1997.

9. Traub O, Monia BP, Dean NM, Berk BC: PKC-epsilon is required for mechano-sensitive activation of ERK1/2 in endothelial cells. *J Biol Chem* 272(50):31251-7, 1997.

10. Traub O, Berk BC: Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arter Thromb Vasc Biol* 17(5): 677-685, 1998

IMAGE EVALUATION
TEST TARGET (QA-3)





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