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Characterization of the human monocyte endothelin B (ETB)
receptor: Functional coupling of ETB receptor to nitric oxide synthase
activation.

by

Jonathan M. King

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of
the requirements for the degree of Doctor of Philosophy, The City University of
New York.

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Abstract

Characterization of the human monocyte endothelin B (ETB) receptor: Functional coupling of ETB receptor to nitric oxide synthase activation.

by

Jonathan M. King

Advisor: Professor Harold I. Magazine

Endothelin-1 (ET-1) is a peptide hormone which exerts diverse actions over numerous physiological mechanisms including cardiovascular hemodynamics; neurotransmission or neuromodulation; muscle contractility; and control of cell proliferation. Human monocytes have the capacity to produce both endothelin-1 (ET-1) and nitric oxide (NO), yet the role of these mediators in monocyte function remains unclear.

Employing a molecular and pharmacological approach we have provided direct evidence that human peripheral blood monocytes (PBM) and the human monocytic cell line THP-1, bind ET-1 and express ET_B receptors. Utilizing an NO selective amperometric probe we have provided the first direct demonstration of NO release in human PBM in response to ET-1 stimulation. This coupled to immunocytochemical analysis of NO synthase (NOS) isoforms in THP-1 cells we have determined that the monocyte ET_B receptor is functionally coupled to activation of constitutive NOS (ecNOS).

Exposure of PBM to ET-1 markedly reduced adhesion of these cells to human saphenous vein whereas PBM adhesion in the presence of BQ-788, an

ET_B selective antagonist, was restored to that of control levels. These data demonstrate that PBM interactions with the vascular wall can be reduced by autocrine production of NO and suggest that PBM ET_B receptors may attenuate monocyte activity at sites of inflammation.

Accumulation of monocytes at sites of vascular injury may have profound hemodynamic effects. We have demonstrated that adherent monocytes augment the vascular contractile potency of ET-1. Increased ET-1 induced vascular tone in the presence of adherent monocytes may contribute to elevated blood pressure in patients suffering from hypertension. Activation of monocytes with proinflammatory cytokines resulted in expression of inducible NOS (iNOS) and enhanced monocyte adhesion to the vascular wall. ET-1 stimulation of activated monocytes did not attenuate their adhesion indicating that the monocyte ET_B receptor is functionally coupled to eNOS but not iNOS activation. Monocyte ET_B receptor-coupled NO is likely to modulate their adhesion and activity at sites of vascular injury. The inability of ET-1 stimulation to modulate adhesion of activated monocytes may be detrimental and contribute to the progression of vascular disease.

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I would like to express a debt of gratitude to my graduate advisor Dr. Harold I. Magazine. Dr. Magazine's support provided an environment in which I could develop skills essential for scientific inquiry. Through a collaborative effort we advanced a project examining human monocyte cell biology at sites of vascular injury. The research aided in resolving a debate pertaining to human monocyte cell biology and nitric oxide production. The experience of researching under Dr. Magazine's guidance has been beneficial and rewarding. I am honored to be Dr. Magazine's first doctoral student and value his support and friendship.

To my colleagues at the Queens College Biology Department, I am grateful for your assistance and encouragement. Specifically, I am honored to have worked with Kamal D. Srivastava an exceptional doctoral candidate whose technical assistance and intellectual contributions greatly impacted the research. I would like to thank two extremely talented undergraduates Babak Souferian and Greg Wagner for their contributions. Finally, I consider it a privilege to have worked with Marie Ball whose support was invaluable.

I am grateful to my graduate advisory committee for their diligence and direction. Especially Dr. George B. Stefano for his overall enthusiasm and unique scientific perspective and insight. To my family and friends I deeply touched and indebted by your constant love and support. Finally, to my wife Linda, words cannot express my love, gratitude and respect for your unwavering support and encouragement throughout my entire graduate career.

TABLE OF CONTENTS:

INTRODUCTION:

ENDOTHELIN	2
ENDOTHELIN RECEPTORS	11
THE BIOLOGY OF NITRIC OXIDE	18
NITRIC OXIDE SYNTHASES	24
HUMAN MONOCYTES PRODUCE NITRIC OXIDE	27
MONOCYTE RESPONSE TO ACUTE INFLAMMATION	28
SMOOTH MUSCLE CONTRACTION	34
CARDIOVASCULAR DISEASE	38
CHAPTER ONE:	41

EVALUATE ENDOTHELIN RECEPTOR SUBTYPE EXPRESSION IN HUMAN MONOCYTES.

OBJECTIVE:	41
MATERIALS AND METHODS:	42
RESULTS:	46
CONCLUSIONS:	49
CHAPTER TWO:	59

DETERMINE WHETHER THE MONOCYTE ET_B RECEPTOR IS FUNCTIONALLY COUPLED TO NITRIC OXIDE SYNTHASE.

OBJECTIVE:	59
METHODS:	60

RESULTS:.....	63
CONCLUSIONS:.....	65
CHAPTER THREE:.....	73
EVALUATE THE BIOLOGICAL IMPACT OF ET-1 INDUCED NO IN HUMAN MONOCYTES.	
OBJECTIVE:	73
METHODS:	74
RESULTS.....	79
CONCLUSIONS:.....	82
CHAPTER FOUR:	96
EXAMINE MONOCYTE ACTIVATION STATE ON ET-1 COUPLED NO RELEASE.	
OBJECTIVE:	96
METHODS:	98
RESULTS:.....	100
REITERATION:.....	114
BIBLIOGRAPHY:	118

INDEX OF TABLES:

Table 1: Amino acid sequences of human endothelins.....	2
Table 2: Pharmacological actions of endothelins.....	10
Table 3: Comparison of nitric oxide synthases.	26
Table 4: Major recognition molecules of the innate immune system expressed on monocytes or macrophages.....	30
Table 5: Nitric Oxide production by human PBM and monocytic cell lines.....	64
Table 6: Modulation of THP-1 adhesion to rat aortic rings.....	81

INDEX OF FIGURES:

Figure 1: The structure of ET-1.	4
Figure 2: Monocyte interactions at sites of vascular injury.....	37
Figure 3: Endothelin Receptor Expression by Monoblastic Cell Lines.	53
Figure 4: Confocal image of THP-1 with biotinylated ET-1.	54
Figure 5: ET-1 and ET-3 Inhibition of ¹²⁵ I-labeled ET-1 binding to THP-1 Cells.	55
Figure 6: Effect of PMA stimulation on ET receptor expression by monocytic cells.	56
Figure 7: Evaluation of ET _B receptor mRNA expression by RT-PCR.	57
Figure 8: Induction of NO Release from PBM.	68
Figure 9: Induction of NO release in monocytic cell lines.	69
Figure 10: Induction of NO release in monocytic cells in response to ionomycin.....	70
Figure 11: Immunocytochemistry of THP-1 ecNOS expression.....	71
Figure 12: Adhesion of monoblastic cell line to fibronectin.	86
Figure 13: Adhesion of monoblastic cell line to fibronectin.	87
Figure 14: PBM adhesion to human saphenous vein is modulated by ET-1.....	88
Figure 15: The Vascular Contractile Potency of Endothelin-1 is Increased in the Presence of Monocytic Cells.....	89
Figure 16: Effect of Monocytic Cells on Endothelin-1 Induced Contraction of Rat Aorta.....	90
Figure 17: Effect of Monocytic Cell Number on Endothelin-1 Induced Contraction of Rat Aorta.	91
Figure 18: Effect of Monocytic Cells on Agonist Induced Contraction of Rat Aorta.....	92
Figure 19: Relaxation of Rat Aorta following Contraction to Endothelin-1.....	93
Figure 20: Stimulation of aortic smooth muscle by THP-1 cells.....	94

Figure 21: iNOS-Mediated Nitric Oxide Release from human monocytes.	106
Figure 22: Monocyte cell conformation in the presence and absence of IFN γ	107
Figure 23: The effect of cytokine activation on monocyte adhesion to human saphenous vein.	108
Figure 24: Immunocytochemistry of cytokine stimulated THP-1 NOS expression	109
Figure 25: Confocal image of invertebrate immunocyte labeled with biotinylated ET-1.....	110
Figure 26: Invertebrate immunocyte response to ET-1 stimulation.....	111
Figure 27: Image analysis of immunocyte response to ET-1.....	112
Figure 28: Image analysis of immunocyte response to ET-1.....	113
Figure 29: Proposed Mechanism of Modulation of Adhesion in Monocytes by ET-1 coupled NO release.....	116
Figure 30: Proposed mechanism of monocyte accumulation during episodes of disease.....	117

Introduction

ENDOTHELIN

Endothelin-1 (ET-1) was first isolated from the culture supernatant of porcine aortic endothelial cells (EC) recognized as a potent vasoconstrictor (Yanagisawa, M. et al. 1988). ET-1 is a 21 amino acid peptide hormone which has attracted much attention within the last decade, nearly one thousand references annually since 1991. Peptide hormones regulate diverse physiological processes such as cardiovascular hemodynamics; neurotransmission or neuromodulation; contractility of skeletal, smooth, or cardiac muscle; and control of cell proliferation (Simonson, M.S. et al. 1991). Peptide hormones have also been implicated in playing a critical role in the development of numerous pathophysiological conditions such as atherosclerosis, hypertension, and oncogenesis (Simonson, M.S. et al. 1991). The understanding that peptide hormones play a vital role in regulation of homeostasis has resulted in intensive research to elucidate the mechanisms by which they function.

Structure and Biosynthesis

Endothelin (ET) is a family of 21-amino acid peptides that potently induce smooth muscle contraction. Southern blot analysis of human genomic DNA revealed the existence of three distinct genes, which encode the peptides; ET-1, ET-2, and ET-3 (Rubanyi, G.M. et al. 1991). ET-2 differs from ET-1 by only two amino acids and functions in a similar manner to ET-1. ET-3 differs from ET-1 by six amino acids conferring selectivity for the various ET receptor subtypes (Table

1). The ET peptides contain two disulfide bonds Cys-1 and Cys-15 and Cys-3 and Cys-11 giving the peptide a hairpin loop configuration (Figure 1) (Inoue, A. et al. 1989; Nakajima, K. et al. 1989; Cody, W.L. et al. 1991). Deletion of Trp-21 markedly reduces ET-1 potency, whereas deletion of residues 17-21 causes a complete loss of activity (Simonson, M.S. et al. 1990). These experiments have demonstrated that the hydrophobic C terminus and the loop configuration are essential to full biological activity (Watanabe, T.X. et al. 1991; Krystek, S.R.J. et al. 1991; Simonson, M.S. et al. 1991).

A.A.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ET-1	<u>Cys</u>	Ser	<u>Cys</u>	Ser	Ser	Leu	Met	Asp	Lys	Glu	<u>Cys</u>	Val	Tyr	Phe	<u>Cys</u>	His	Leu	Asp	Ile	Ile	Trp
ET-2	<u>Cys</u>	Ser	<u>Cys</u>	Ser	Ser	Trp	Leu	Asp	Lys	Glu	<u>Cys</u>	Val	Tyr	Phe	<u>Cys</u>	His	Leu	Asp	Ile	Ile	Trp
ET-3	<u>Cys</u>	Thr	<u>Cys</u>	Phe	Thr	Tyr	Lys	Asp	Lys	Glu	<u>Cys</u>	Val	Tyr	Tyr	<u>Cys</u>	His	Leu	Asp	Ile	Ile	Trp

Table 1: Amino acid sequences of human endothelins.

ET-1 is primarily produced by EC (Hexum, T.D. et al. 1990; Kohno, M. et al. 1990; Anggard, E.E. 1990) although additional cell types are capable of ET-1 production, most notably: human peripheral blood monocytes (PBM) (Chanez, P. et al. 1996; Ehrenreich, H. et al. 1990), polymorphonuclear leukocytes (Sessa, W.C. et al. 1991), macrophages (Ehrenreich, H. et al. 1990), and vascular smooth muscle (VSM) (Luscher, T.F. 1992). Glomerular epithelial cells (Kasinath, B.S. et al. 1992), mesangial cells (Clozel, M. et al. 1993) Clara cells (Laporte, L. et al. 1996) breast cancer cells (Schrey, M.P. et al. 1992), hepatic cells (Bluhm, R.E. et al. 1993), keratinocytes (Imokawa, G. et al. 1992; Yohn, J.J.

et al. 1993), cardiac myocytes (Suzuki, T. et al. 1993), epicardial mesothelial cells (Eid, H. et al. 1994), and astrocytes (Ehrenreich, H. et al. 1991) have all been reported to synthesize endothelin. Treatment of EC with a variety of agents including; thrombin, angiotensin II, vasopressin, interleukin-1, transforming growth factor β , tumor necrosis factor α , and shear stress from blood flow has been shown to induce preproendothelin-1 mRNA (Sakurai, T. et al. 1991; Masaki, T. et al. 1991). In addition, phorbol ester treatment induces ET-1 production suggesting protein kinase C (PKC) activation may be involved in this mechanism (Kuchan, M.J. et al. 1993).

The biosynthesis of ET-1 arises by proteolytic processing of preproendothelin-1 (200 amino acids) to big ET-1 (39 amino acids) by dibasic pair-specific endopeptidases and carboxypeptidases (Rubanyi, G.M. et al. 1991). Big ET-1 is cleaved at Trp-21 - Val-22 by ET converting enzyme to form mature ET-1 (Simonson, M.S. et al. 1990). Phosphoramidon a neutral metalloproteinase inhibitor blocks the conversion of big ET-1 to mature ET-1 (Wu-Yong, J.R. et al. 1993). Both big ET-1 and ET-1 are secreted into the extracellular space, the absence of secretory granules in EC suggests that big ET-1 and ET-1 are secreted through a consecutive pathway (Harrison, V.J. et al. 1995). However, data observed in rat aorta suggest that thrombin stimulation results in a rapid release of ET-1 that is inhibited in the presence of phosphoramidon (Magazine, H.I. et al. 1996c).

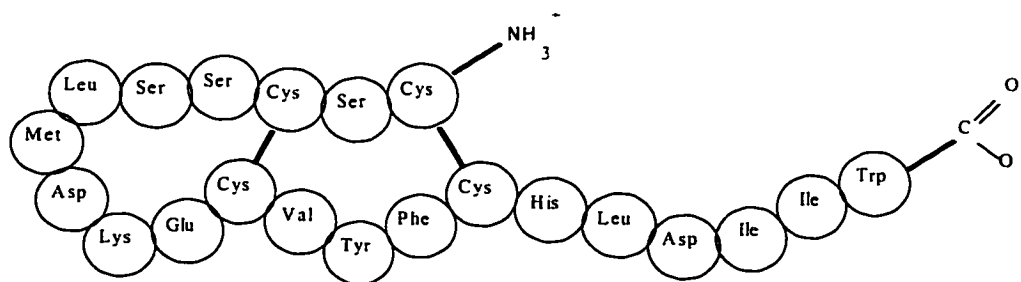


Figure 1: The structure of ET-1.

The diagram depicts an ET-1 molecule, the two disulfide bonds between Cys-1 and Cys-15 and Cys-3 and Cys-11 give the peptide a hairpin loop configuration. The hydrophobic C terminus and the two disulfide bonds are essential for full biological activity of ET-1.

Mitogenic Impact of ET

Endothelin has been shown to possess mitogenic properties in a number of systems in addition to its hemodynamic properties. It has been demonstrated that ET-1 promotes mitogenesis in VSM (Glassberg, M.K. et al. 1994; Fujitani, Y. et al. 1995), fibroblasts (Cambrey, A.D. et al. 1994; Patel, K.V. et al. 1995; Takuwa, N. et al. 1989), and melanocytes (Swope, V.B. et al. 1995). ET-1 has been shown to induce tyrosine phosphorylation in a dose and time dependent manner as did platelet-derived growth factor (PDGF) (Battistini, B. et al. 1993). ET-1 produced a rapid and transient increase in *c-fos* and *c-myc* mRNA in rat VSM cells and increased ³H thymidine incorporation into DNA (Alberts, G.F. et al. 1994). The ET_A receptor subtype antagonist BQ-123 inhibited tyrosine phosphorylation but was not blocked by pertussis toxin indicating that ET-1 is involved in mitogenic signaling (Simonson, M.S. et al. 1993). Stimulation of the ET_B receptor was found to activate three distinct mitogen-activated protein kinase signal transduction pathways, the extracellular regulated kinase 2, c-Jun N-terminal kinase 1, and p38 kinase. The cytoplasmic tail of the endothelin B receptor was found to be required for activation of these kinases in addition to calcium mobilization (Aquila, E. et al. 1996). Activation of multiple mitogen-activated protein kinases may contribute to the biological variability observed in ET-1 stimulation.

Further analysis of ET-1's mitogenic properties revealed that PKC and protein tyrosine kinase (PTK) are involved. Experiments which inhibited and

depleted PKC demonstrated that PKC was necessary but not sufficient for mitogenic signaling (Simonson, M.S. et al. 1993). ET-1 increased tyrosine phosphorylation of cellular proteins that was blocked by preincubation with the PTK inhibitor herbimycin A. Furthermore, herbimycin A attenuated c-fos induction, AP-1 DNA binding, and transcription directed by an AP-1 cis-element in response to ET-1 (Simonson, M.S. et al. 1993). Transcription of protooncogenes c-fos and c-jun is enhanced by ET-1, indicating that ET-1 may provide a mitogenic signal (Simonson, M.S. et al. 1992). ET-1 induced mobilization of intracellular calcium contributes to fos/jun activation, additionally calcium channel antagonists, nifedipine or verapamil, inhibited ET-1 mediated mitogenesis in VSM (Pribnow, D. et al. 1992). This strongly suggests that an increase in intracellular calcium is required for ET-1 mitogenesis. Endothelin has also been shown to potentate the mitogenic activity of PDGF in VSM (Weissberg, P.L. et al. 1990; Irons, C.E. et al. 1996), mesangial cells (Kohno, M. et al. 1994; Schramek, H. et al. 1995), and osteoblast cells (Schvartz, I. et al. 1992). ET-1 is comitogenic with several mitogens on vascular smooth muscle, however the synergism is limited to only a 3 fold increase in growth (Battistini, B. et al. 1993).

Accumulation of PBM at sites of vascular injury may promote proliferation of vascular wall components through autocrine ET-1 synthesis. ET-1's mitogenic and hemodynamic properties may contribute to decreased luminal diameter and hypertension. Elevation of plasma ET-1 is observed during the progression of vascular disease as well as diverse pathological sequelae, such as diabetes and

cancer that impair vascular function. Increased plasma ET-1 levels may promote autocrine release of ET-1 from PBM initiating a positive feedback amplification loop. The impact of ET-1 release by PBM on blood pressure and mitogenesis of the VSM has yet to be explored.

Neurogenic Impact of ET

Endothelins have exerted a regulatory role in a variety of tissues including heart, lung, nervous, endocrine, kidney, liver, reproductive and gastrointestinal (Table 2) (Simonson, M.S. et al. 1990). Considering the previous information endothelins have emerged as an important family of signal molecules, in addition their presence in diverse organisms, e.g., earthworm *Eisenia foetida* (Hasegawa, K. et al. 1991) suggests they have been conserved. ET-1 immunoreactivity has been identified in numerous neural tissues such as spinal cord, brain, peripheral ganglion cells (Hemsen, A. et al. 1991; Inagaki, H. et al. 1991) posterior pituitary (Yoshizawa, T. et al. 1990). ET signaling in astrocytes indicates that ET may be involved in transcriptional regulation of nerve growth factor through the induction of c-fos. This data suggests ET may modulate glial proliferation and neurotrophic activity during brain development or wound healing (Ladenheim, R.G. et al. 1993). Furthermore, ET-1 has been demonstrated to increase dopamine release in rat striatum soundly implicating its role as a neuropeptide (Horie, K. et al. 1995). Microglia with an endothelin B receptor subtype aggregate in rat hippocampus CA1 subfields following transient forebrain ischemia (Yamashita, K. et al. 1994). These studies strongly indicate that ET acts to regulate neuronal

processes, further elaboration of endothelins as neuroregulatory signal molecules have been documented in a recent review compiled by our group (Liu, Y. et al. 1996). Neurogenic induced elevation of vascular tone has been associated with progression of vascular disease suggesting that ET may influence vascular disease through complex interaction with the nervous, vascular and inflammatory systems (Cleland, J.G. et al. 1991; Shepherd J.T. 1995).

Hemodynamic Impact

Intravenous bolus injection of ET-1 into anesthetized or conscious animals triggers a biphasic blood pressure response; an initial and transient depressor response is followed by a sustained pressor response in systemic arterial blood pressure (Rubanyi, G.M. et al. 1991). The depressor response corresponds to the release of nitric oxide (NO) and prostacyclin from the endothelium. The duration of the pressor response is typically one to three hours making ET-1 the most potent vasoconstrictor known (Simonson, M.S. et al. 1991). Administration of ET receptor antagonists attenuates the systemic blood pressure responses to ET-1 substantiating its role in blood pressure homeostasis. Additionally, circulating plasma levels of ET-1 but not ET-2 or ET-3 are markedly increased in numerous diseases such as atherosclerosis (Lerman, A. et al. 1991), hypertension (Schiffrin, E.L. 1995), cardiogenic shock (Cernacek, P. et al. 1989), hypercholesterolemia (Arendt, R.M. et al. 1990), pulmonary hypertension (Stewart, D.J. et al. 1991), diabetes (Haak, T. et al. 1993), and some metastatic

cancers (Ishibashi, M. et al. 1994; Schrey, M.P. et al. 1992). The increased levels of plasma ET-1 may contribute to postischemic myocardial injury and hypertension (Simonson, M.S. et al. 1991). The diverse distribution of ET-1 strongly implicates a critical role for this peptide in physiological and pathophysiological episodes. Thus the following studies used the ET-1 isoform primarily because of the unique relationship between ET-1 and cardiovascular disease.

<u>Tissue or Organ</u>	<u>Effect</u>
Blood Vessel	Vasoconstriction and vasodilation Release of prostanoids and NO Mitogenic for endothelial and smooth muscle cells
Heart	Positive inotropic and chronotropic effects Atrial natriuretic peptide release
Airway muscle	Constriction
Kidney Juxtaglomerular apparatus Nephron Mesangial cell	Vasoconstriction of renal artery Inhibition of renin release Diuresis and natriuresis Mitogen for mesangial cells
Adrenal Gland Cortical glomerulosa cells Medullary chromaffin cells	Release of aldosterone Stimulates catecholamine release
Liver	Glycogenolysis
Central Nervous System Spinal cord Pituitary	Stimulate release of substance P Increase release of LH and FSH Inhibit prolactin release
Uterus	Constriction
Neutrophil	Enhance fMLP superoxide generation
Eye	Increase intraocular pressure

Table 2: Pharmacological actions of endothelins.

ENDOTHELIN RECEPTORS

The tissue distribution of ET receptors resembles the pattern of ET biosynthesis suggesting that the peptide may act as a local hormone. Saturable, specific binding sites for radiolabeled ET have been identified in numerous tissues including lung, kidney, heart, intestine and brain. The density of ET binding is highest in the lung and heart (Simonson, M.S. et al. 1990). The literature suggests that ET is rapidly cleared from circulation predominantly by the lung ET_B receptor (Fukuroda, T. et al. 1994). Neural localization of ET binding sites indicate that ET may function as a neurotransmitter or neuromodulator (Liu, Y. et al. 1996). Presently, two ET receptors have been cloned from mammalian sources and have been pharmacologically classified by their selectivity for the ET isoforms: ET_A receptor is selective for ET-1 over ET-2 and ET-3, ET_B receptor is nonselective for the ET isoforms (Lin, H.Y. et al. 1991).

ET_A and ET_B receptors are members of the heptahelical G protein-coupled receptor family (Elshourbagy, N.A. et al. 1993), consisting of 427 and 442 amino acid residues, respectively. The receptors have 58.9% amino acid sequence identity overall, with the greatest sequence divergence in the extracellular domains (Elshourbagy, N.A. et al. 1993). Approximately 90% amino acid sequence identity is conserved from rat and porcine ET receptors. Ligand-receptor binding results in G-protein mediated activation of phospholipase C and A₂ (Eguchi, S. et al. 1993; Schramek, H. et al. 1994) and subsequent increase in

inositol trisphosphate and elevation of intracellular free calcium (Rapoport, R.M. et al. 1990; Xuan, Y.T. et al. 1991).

ET Receptor Signal Transduction Pathways

The activity of phospholipase C increases rapidly after activation of ET receptors; this results in the production of diacylglycerol (DAG) which remains in the plasma membrane and activates PKC. Activation of phospholipase C also cleaves phosphatidylinositol 4,5-bisphosphate forming the water soluble inositol 1,4,5-trisphosphate which mediates an increase intracellular free calcium (Berridge, M.J. 1993). The calcium signal in response to ET is biphasic, first there is a spike of intracellular calcium (5 sec) followed by a sustained phase of elevated cytosolic free calcium (20 min) (Simonson, M.S. et al. 1991). A variety of inositol phosphates may be formed depending on the specific phosphatases/kinases present in the target cell (Simonson, M.S. et al. 1990). ET-1 promotes calcium entry by receptor-gated calcium channels (Taylor, C.W. et al. 1992) and modulates the activity of L-type voltage-operated calcium channels in VSM (Simonson, M.S. et al. 1991).

A recent study indicates that ET-1 and its receptor colocalize to caveolae where calcium channels, inositol 1,4,5-trisphosphate receptors, and heterotrimeric GTP-binding proteins are enriched. This suggests that ET-1 intracellular signaling takes place in caveolae and signaling persists after receptor internalization accounting for the sustained elevation on intracellular calcium following stimulation (Chun, M. et al. 1994; Lisanti, M.P. et al. 1994).

PKC activation is a mechanism that may function as a negative-feedback signal for calcium signaling in response to ET stimulation (Resink, T.J. et al. 1990). Activation of PKC with phorbol esters attenuates calcium signaling by ET peptides (Simonson, M.S. et al. 1991). Three major mechanisms are involved in the inhibitory effect of PKC on calcium signaling: 1) inhibition of phosphatidylinositol 4,5-bisphosphate hydrolysis reducing IP_3 accumulation, 2) activation of calcium pumps resulting in removal cytosolic calcium, and 3) stimulation of inositol (1,4,5) trisphosphate- 5-phosphatase (Simonson, M.S. et al. 1990), the involvement of these mechanisms are not yet fully defined.

The receptors have an N-terminal signal sequence, and the cytoplasmic C-terminal tail contains a number of serine/threonine residues which may serve as phosphorylation sites for protein kinases (Battistini, B. et al. 1993). ET receptor vasoactivity is tissue specific both vasoconstriction and vasodilation are mediated by the ET receptor. Classically, the ET_B receptor is found in abundance on EC and is linked to vasodilation through the release of NO, the ET_A receptor is coupled to vasoconstriction and is abundant on VSM (Eguchi, S. et al. 1993). There is a minor population of ET_B receptors present on vascular smooth muscle, which have been suggested to mediate contraction (Sumner, M.J. et al. 1992; Shetty, S.S. et al. 1993; LaDouceur, D.M. et al. 1993).

ET Receptors and Heterotrimeric G proteins

ET receptor subtypes are coupled to adenylate cyclase via different G-proteins in the vasculature (Eguchi, S. et al. 1993), this variability of G-protein

coupling allows cell specific response to ET-1. Endothelial cell ET_B receptors are coupled to a pertussis toxin sensitive pathway. Pertussis toxin catalyzes the ADP-ribosylation of most G_i α subunits, this inhibits ET induced phosphoinositide hydrolysis, calcium transience, NO production and cyclic GMP (cGMP) accumulation in EC (Hirata, Y. et al. 1993). In contrast, cholera toxin but not pertussis toxin treatment inhibited phospholipase C mediated events in VSM cells (Eguchi, S. et al. 1993). Cholera toxin catalyzes the ADP-ribosylation of G_s α indicating G-protein coupling in cell specific (Hepler, J.R. et al. 1992; Bounancin, F. et al. 1993).

Stimulation of either ET_A or ET_B receptors results in a rapid response including phosphatidylinositol hydrolysis and arachidonic acid release in transfected Chinese hamster ovary (CHO) cells. However, the two receptors displayed different responses in the cyclic AMP transduction cascades. ET_A mediated the accumulation of cyclic AMP formation which was partially inhibited by pertussis toxin, whereas ET_B displayed an inhibitory action on the forskolin-stimulated cyclic AMP accumulation which was abolished by pertussis toxin treatment (Aramori, I. et al. 1992). Pharmacological evidence with the use of bacterial toxins indicated that the opposite effects were due to the selective coupling of ET_A with G_s α and ET_B with G_i α . Chimeric endothelin receptors indicated that intracellular loops II and III are the major determinant of the selective coupling of ET_A with G_s α and ET_B with G_i α (Takagi, Y. et al. 1995). In rat hepatocytes ET-1 inhibits the calcium extrusion pump which was reversed by

antibody blockage of $G_s \alpha$, while antibody blockage of $G_q \alpha$ antagonized ET-1 phospholipase C activation (Jouneaux, C. et al. 1994). Differential interaction between G-protein α subunits with a variety of G-protein β and γ subunits provides a possible mechanism for signal specificity through this ubiquitous signal transduction pathway.

Receptor Desensitization

Heterologous desensitization and resensitization was investigated in ET_A mRNA that was microinjected into *Xenopus laevis* oocytes. The study indicated that ET_A receptors desensitized within 5 minutes yet had a prolonged resensitization period which lasts up to 2 hours (Cyr, C. et al. 1993). The ET_B , but not ET_A , receptor possesses a consensus amino acid sequence for possible phosphorylation by protein kinase A (PKA). Both arginine vasopressin (AVP) and forskolin reduced ET-1 binding sites suggesting heterologous desensitization may be caused by a cAMP-dependent mechanism (Takemoto, F. et al. 1995). We have recently demonstrated that ET_B receptor mediated endothelial cell NO production could be impaired by repeated ET-1 stimulation yet these cells retained the ability to produce NO to other agonists such as acetylcholine. Since NO has beneficial hemodynamic and anti-thrombogenic effects, ET_B receptor desensitization may facilitate cellular attachment and augmentation of vascular tone in patients with vascular disease.

Ligand - Receptor Interactions

It has been established that the complex formed with ET and its receptor is extremely stable, and release or exchange of the ligand from the receptor is very slow under physiological conditions. Both ET_A and ET_B receptors form a stable complex, however the ET_B receptor-ligand complex is exceptionally stable (Takasuka, T. et al. 1994), the ligand does not dissociate from the ET_B receptor in the presence of acidic conditions or 2% SDS. These conditions would normally disrupt the secondary and tertiary structure of most proteins (Takasuka, T. et al. 1994). It has been suggested that ET-1 may bind to the receptor by disulfide interchange accounting for the irreversible nature of ET-1 receptor binding (Spinella, M.J. et al. 1993). These observations make ET receptor antagonism problematic. Using excess unlabeled ET or ET receptor specific antagonism will not displace bound labeled ET. In addition, preincubation with receptor antagonists will only inhibit for a limited duration due to their relatively high dissociation rates in comparison with ET's irreversible binding characteristics.

Recently a number of ET receptor selective antagonists have been developed. Several antagonists have either selectivity for ET_A (BQ-123, BQ-1610) (Hay, D.W. 1992) or ET_B (BQ-3020, BQ-788) (Ishikawa, K. et al. 1994) receptors whereas others are non-selective (PD142894) (Sakamoto, A. et al. 1993). Additionally, IRL-1620 has proved to be a useful selective ET_B receptor agonist (Karaki, H. et al. 1993). Dr. Magazine developed a biotinylated ET-1 ligand which has proved useful for evaluation of ET receptor populations (Magazine, H.I. et al. 1991).

We have provided the first direct evidence for the expression of ET receptors by monocytes by demonstrating specific binding of ^{125}I -labeled ET-1 to human PBM (Magazine, H.I. et al. 1994). Examination of the human monocytic cell lines THP-1 and U937 suggested that the THP-1 cell line express an ET receptor whereas no specific binding of ^{125}I -labeled ET-1 was observed in U937 cell line. In addition, phorbol ester treatment to promote differentiation of U937 cells was without effect on inducing ET receptor expression. Employing pharmacological and molecular techniques we have identified the receptor to be consistent with the ET_B subtype (King, J.M. et al. 1997).

THE BIOLOGY OF NITRIC OXIDE

Nitric oxide (NO) has recently been discovered to be involved in the signaling pathways of a number systems for instance, endothelium-dependent relaxation, neurotransmission in the central and peripheral nervous systems, and activated macrophage cytotoxicity (Sessa, W.C. 1994). NO is quite promiscuous in terms of potential targets with which it can interact. However, the majority of its physiological effects appear to be mediated by activation of soluble guanylate cyclase (Billiar, T.R. et al. 1992). The biological action of NO on vascular biology, immunology and neurobiology has resulted in the convergence of these disciplines to study the mechanism of NO biosynthesis.

NO is lipophilic and diffuses freely across cell membranes where it directly stimulates soluble guanylate cyclase and subsequently the formation of 3'5'-cyclic guanosine monophosphate (cGMP) (Billiar, T.R. et al. 1992; Stamler, J. et al. 1992). The biological half-life of NO is limited to several seconds due to the availability of proteins and metal ions with which it can react. NO released appears not to be in a polarized fashion and thus, can induce cGMP accumulation in neighboring endothelial cells, the underlying VSM, and in circulating immune cells (Stamler, J. et al. 1992). Accumulation of cGMP in VSM initiates a sequence of events that result in dephosphorylation of myosin light chain kinase and vascular relaxation (Stamler, J. et al. 1992). Subsequently NO increases endothelial cell permeability allowing solutes to permeate the intima (Feng, Q. et al. 1990). These NO mediated events are crucial at sites of

inflammation where accumulation of cytokines and growth factors are necessary to promote immunosurveillance and tissue repair.

NO Regulation

NO inhibits its own production through a feedback mechanism. Regulation of NO production may be achieved by modulation of cofactor availability or synthesis required for enzyme activation (Lyons, C.R. 1995). Other data suggests that NO production markedly reduces interferon- γ (IFN- γ) synthesis, thus reducing the concentration of a proinflammatory mediator. Also cytokines interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor β (TGF β) have all been implicated in inhibition of NO synthesis (Finder, J. et al. 1995; Lyons, C.R. 1995). There appears to be a high level of redundancy in signals which induce and inhibit NO production. The balance of these signals during infection modulate the role, if any, NO will have during the immune response. The efficient elimination of intracellular pathogens requires NO production, however excessive NO production may lead to pathophysiological disorders. Examination of the regulatory mechanisms which control NO production are critical for inhibition and development of disease. A greater understanding of these mechanisms may potentially lead to more strategic therapy for inflammatory diseases control and prevention.

Cytotoxic Aspects of NO Release

Antimicrobial activity is a major function of NO. It has been demonstrated that NO production is partially responsible for resistance in both leishmaniasis

and malaria. NO rapidly reacts with O_2 forming peroxynitrite ($OONO^\cdot$), a species with reactivity equaling the highly toxic hydroxyl radical ($OH^\cdot$). In order to kill intracellular parasites host cells use NO whose effects are both cytotoxic and cytostatic. Interaction of NO with oxygen results in peroxidation and cytolysis of parasites. Additionally, NO induces protein nitrosylation resulting in enzyme inhibition and cytostasis in the host cell which potentially is reversible (Bastian, N.R. et al. 1994).

Numerous studies suggest that NO acts as a tissue-damaging molecule during episodes of inflammation. For example, NO production has also been implicated progression various inflammatory diseases. Mice treated with streptozocin develop diabetes, however short term treatment of mice with an nitric oxide synthase (NOS) inhibitor after completion of streptozocin administration prevents the development of diabetes (Lukic, M.L. et al. 1991). In addition graft-vs.-host reaction in mice can be abolished by administration of NOS inhibitors which reduce inflammatory cell mediated damage and tolerated transplantation (Garside, P. et al. 1992).

Protective aspects of NO Release

In contrast, other data suggest that NO biosynthesis may have protective functions under some conditions (Lyons, C.R. 1995; Bastian, N.R. et al. 1994). Mice injected with lipopolysaccharide (LPS) develop hepatic damage in a dose dependent manner, administration of L-arginine analogs that inhibit NOS resulted in an elevation of hepatic damage (Harbrecht, B.G. et al. 1992). The

investigators reported that NO decreased hepatic protein synthesis (Billiar, T.R. et al. 1989) and mitochondrial respiration (Stadler, J. et al. 1991) suggesting that NO mediated its protective effects through these mechanisms. In addition NO generation may also be protective due to inhibition of platelet aggregation and leukocyte adhesion (Vane, J.R. et al. 1992a; Provost, P. et al. 1994; Lopez-Farre, A. et al. 1995). NO inhibited cytokine activation of EC by selectively reducing adhesion receptor expression and proinflammatory cytokine production (De Caterina, R. et al. 1995; Vane, J.R. et al. 1992b).

Recent studies indicate that NO stimulates ADP ribosylation of actin which inhibits cytoskeletal assembly and adherence. In addition NO stimulates the ADP-ribosylation of at least three proteins (Mw = 28, 33, and 39 Kd) in the mouse macrophage cell line ANA-1. Cholera toxin only ADP-ribosylated the 28 and 33 Kd cytosolic proteins. This data suggests that autocrine and paracrine effects of NO may include ADP-ribosylation of various proteins which is likely to alter cell behavior (Sheffler, L.A. et al. 1995).

Methods of NO Detection

The available methods for detection and measurement of NO have greatly improved within the last few years. Initially, NO detection was studied by measuring vascular relaxation to acetylcholine (Ach) in epinephrine contracted vessels (Lin, L. et al. 1991; Davies, M.G. et al. 1993). Prostacyclin (Moncada, S. et al. 1979) and endothelium-derived hyperpolarizing factor (Feletou, M. et al. 1988) were also demonstrated to be released by blood vessels in response to

Ach suggesting that Ach-induced vascular relaxation is the summed effect of the release of several dilator mediators. The introduction of L-arginine analogs and inhibitors of cyclic GMP accumulation have proved beneficial in the study of NO release. However, inhibitor specificity is potentially problematic (Koller, A. et al. 1993) therefore data inhibiting NO release or cyclic GMP accumulation must be interpreted as suggestive evidence for NO function.

Presently, there are several assays available which indirectly measure NO release. The most prevalent systems measure nitrite accumulation or the conversion of ^3H -L-arginine to ^3H -L-citrulline which is correlated to NO synthase activity. The nitrite accumulation assay does not discriminate between nitrite formed due to the reaction of NO with O_2 and other intracellular sources of nitrite production resulting in an inflated estimation of NO release. The citrulline assay which produces highly quantitative results may be flawed, a recent report suggests that citrulline may be converted back to arginine and reutilized for NO release (Hecker, M. et al. 1990). Therefore levels of NO release measured by citrulline accumulation may be grossly underestimated. These assays which are used extensively for measurement of NO release are not amenable for evaluation of NO release in real time.

We have employed the use of an NO-selective amperometric probe which permits the real-time measurement of NO gas in biological buffers. The tip of the amperometric probe is covered with a polymeric porphyrin coated membrane that catalyzes oxidation of NO ($\text{NO}^* - \text{e}^- \rightarrow \text{NO}^+$) and in doing so generates

electrical current (Malinski, T. et al. 1992). The membrane is relatively impermeable to anions and is hydrophobic allowing selectivity for NO. The probe is insensitive to other agents commonly present in biological specimens including CO₂/CO, methanol, arginine, peroxides and the membrane coating excludes NO₂⁻ providing exquisite NO selectivity (Magazine, H.I. 1995a).

NITRIC OXIDE SYNTHASES

Three genetically distinct NOS isoforms have been identified as reviewed by Magazine, H.I. (1995a). These include two constitutive isoforms known as: endothelial NOS (ecNOS) that modulates vascular tone; neuronal NOS (nNOS) that modulates synaptic plasticity (Bastian, N.R. et al. 1994); and an inducible isoform (iNOS) identified from inflammatory cells utilized during pathogen neutralization (Weinberg, J.B. et al. 1995). Approximately 50% amino acid identity is shared between the NOS proteins, and the calcium dependent forms (ecNOS and nNOS) share the highest degree of identity (60%). There is also a high degree of identity across species ranging from 80 - 94% (Sessa, W.C. 1994). Classically, the constitutive isoforms are considered low output calcium dependent enzymes, ecNOS functions to maintain a non-thrombogenic surface and modulate vascular tone (Bastian, N.R. et al. 1994). Previous reports have assessed the release of NO by unstimulated EC to be in the pM range and following stimulation a modest increase in NO release is observed (Moncada, S. et al. 1991; Moncada, S. et al. 1993). It is generally accepted that iNOS is a high output calcium independent enzyme, and upon cytokine stimulation can aid in the inflammatory response (Bastian, N.R. et al. 1994) or participate in the pathogenesis of various human diseases (Kilborn, R.G. et al. 1991; Hoffman, R.A. et al. 1990; Corbett, J.A. et al. 1991). Use of the NO selective amperometric probe that permits direct assessment of NO generation in real time suggests that ecNOS and iNOS produce similar maximal levels of NO

(Magazine, H.I. 1995a). Our data suggests that the duration of NO release from ecNOS is subject to tight regulation (King, J.M. et al. 1997; Magazine, H.I. et al. 1995b; Magazine, H.I. et al. 1996c; Magazine, H.I. et al. 1996a) whereas iNOS remains in a tonic state of activation independent of calcium levels (Cho, H.J. et al. 1992). The difference in duration of NO release can be employed to provide suggestive evidence for ecNOS vs. iNOS catalyzed NO release.

Rapid increases in intercellular calcium promotes interaction between calmodulin and ecNOS resulting in NO generation in the presence of L-arginine. Calmodulin binding by ecNOS quenches intrinsic protein fluorescence, this suggests that the interaction between calmodulin and ecNOS results in a conformational change in the enzyme which may provide more efficient electron transfer during oxidative reactions (Abu-Soud, H.M. et al. 1993). It has been demonstrated recently that iNOS requires calmodulin binding for activation. Calmodulin binds iNOS with much higher affinity than ecNOS and remains bound without high levels of intercellular calcium accounting for the enzymes relative lack of calcium sensitivity (Cho, H.J. et al. 1992). Additional cofactors required for activity of all three isoforms of NOS include: flavin mononucleotide, flavin adenine dinucleotide, nicotinamide adenine dinucleotide phosphate (NADPH), heme (iron-protoporphyrin IX), and tetrahydrobiopterin. Thus, NO production may be regulated by modulation of cofactor availability or synthesis (Lyons, C.R. 1995). Tetrahydrobiopterin availability is limiting in some tissues (Gross, S.S. et al. 1992) others have shown that iron availability markedly alters iNOS transcription (Kubrina, L.N. et al. 1993; Weiss, G. et al. 1994). However,

recent reports suggest that NO may regulate its own production by interacting with heme (iron-protoporphyrin IX) cofactor (Bastian, N.R. et al. 1994; Magaziner, H.I. 1995a).

Examination of the amino termini revealed that ecNOS contains a consensus sequence for N-myristoylation which is a rare cotranslational modification used for protein localization. The importance of this modification has yet to be fully realized, however it may be involved in NO regulation with respect to shear stress vs. hormone-induced release from the endothelium (Sessa, W.C. 1994).

NOS isoform	Cell Type	Protein Mr (deduced)	Ca ²⁺ dependence
Cytokine inducible (iNOS)	Macrophage	130,556	-
Endothelial (ecNOS)	Endothelial Cells	133,000	+++
Neuronal (nNOS)	Cerebellum	161,037	+++

Table 3: Comparison of nitric oxide synthases.

HUMAN MONOCYTES PRODUCE NITRIC OXIDE.

Recent reports demonstrate that human endothelial cell ET_B receptors are coupled to cNOS activation (Tsukahara, H. et al. 1994), which has been associated with maintenance of a non-thrombogenic surface. In addition NO production by monocytes suggests that autocrine production of NO may contribute to the modulation of cellular adhesion (Magazine, H.I. et al. 1995b). NO production by monocytes of rat and mouse has been clearly demonstrated (Severn, A. et al. 1992; Lyons, C.R. et al. 1992; Langrehr, J.M. et al. 1993), whereas the capacity of human monocytes to produce NO has been the subject of intense debate (Schneemann, M. et al. 1993; Sakal, N. et al. 1993; Denis, M. 1994). Several groups have recently demonstrated that human PBM express ecNOS and can be induced to express iNOS upon cytokine stimulation (Reiling, N. et al. 1994; Weinberg, J.B. et al. 1995; Miles, A.M. et al. 1995). In addition the NO-selective amperometric probe has allowed direct measurement of NO in real time (Magazine, H.I. 1995a) and provides definitive evidence for NO release by human PBM (Magazine, H.I. et al. 1995b). In this regard, morphine stimulation of human monocytes resulted in cell rounding and inactivation that was directly attributed to NO release (Magazine, H.I. et al. 1995b).

MONOCYTE RESPONSE TO ACUTE INFLAMMATION

Monocyte recruitment to sites of injury or infection is controlled by molecular signals. Categorization of these molecular cues provides a general scheme during an inflammatory episode. Initially, chemoattractants are important for leukocyte recruitment and activation of integrin adhesiveness. Followed by rapid increase in expression of specific adhesion proteins and receptors which promote the accumulation of PBM.

PBM express a functional thrombin receptor (Fisker, S. et al. 1992; Goodnough, L.T. et al. 1982) and receptor activation has been demonstrated to stimulate monocyte chemotaxis (Bar-Shavit, R. et al. 1983; Bar-Shavit, R. et al. 1983) and mitogenesis (Bar-Shavit, R. et al. 1986). In this regard, thrombin activation following tissue injury has been demonstrated to promote cellular adhesion by stimulating the expression of cellular adhesion proteins (Van der Wal, A.C. et al. 1992). Thrombin also triggers an upregulation in ET-1 biosynthesis and secretion from EC (Maemura, K. et al. 1992) and aortic rings (Kohno, M. et al. 1990) 24 hours following stimulation. Recently we demonstrated that thrombin receptor activation results in a substantial release of ET-1 five min following exposure to rat aortic rings (Magazine, H.I. et al. 1996c).

Innate Immunity and Monocytes

PBM are an essential component of an individuals innate immune system. Monocytes participate in immunosurveillance, a variety of external stimuli may result in rapid differentiation of monocytes to activated macrophages. The

process of monocyte activation has been extensively studied and numerous factors contribute to the overall state of monocyte activation. Monocytes have receptors capable of recognizing a wide variety of microbial products (Table 4), generally stimulation of these receptors increases the activation state of monocytes (Locksley, R.M. et al. 1996). An activated monocyte may recruit lymphocytes and present antigen, kill pathogens through the release of oxygen radicals and NO, phagocytose pathogens and cellular debris, and produce a variety of cytokines and growth factors which influence the inflammatory composition and tissue repair process. Numerous diseases have an inflammatory component which is largely unregulated and appears to contribute to the progression of disease. It is essential to understand mechanisms contributing to monocyte activation and suppression so that relevant therapies may be developed.

Cytokines and NO Regulation

Targeted gene disruptions have enhanced the study of cytokines and their receptors in murine macrophage function (Gordon, S. et al. 1995). Gene disruption of IFN γ led to susceptibility to mycobacterial infection and a reduction in NO production, reactive oxygen intermediates and major histocompatibility class II expression (Dalton, D.K. et al. 1993). Additionally, mycobacterial infection in mice with disrupted IFN γ receptor was lethal (Huang, S. et al. 1993). Cytokines IL-4 and IL-10 along with TGF β have been implicated in inhibition of NO synthesis (Finder, J. et al. 1995; Lyons, C.R. 1995). Gene disruption of these

products has yielded interesting data concerning their relationship to IFN γ production. IFN γ production was reduced in mice with IL-4 gene disruption after nematode infection whereas there was a fivefold increase in mice with IL-10 gene disruption (Gordon, S. et al. 1995). In addition gene disruption of TGF β also resulted in increased IFN γ production (Kulkarni, A.B. et al. 1993). IL-4 inhibition of NO may be mediated directly through inhibition of IFN γ production whereas IL-10 and TGF β may inhibit NO through a more global suppression of inflammatory responsiveness.

Molecule	Ligands	Function
Scavenger receptors, Types I, II, and MARCO	Bacterial and yeast cell walls	Clearance of LPS and microbes
Lipopolysaccharide receptor (CD14)	LPS; a variety of microbial cell wall components	Clearance of microbes, proinflammatory cytokine
Complement receptors (CD35)	C3B, C4b	Enhances C3b and C4b cleavage
CD11b, CD18	iC3b, LPS, fibrinogen	Adhesion; LPS clearance
Mannose receptors	Multiple carbohydrates	Potentially targets antigens to class II-loading compartments

Table 4: Major recognition molecules of the innate immune system expressed on monocytes or macrophages.

Monocytes and Adhesion Molecules

Selectins are rapidly induced to the luminal surface of EC in response to inflammatory signals. Selectin ligands are lectins on the surface of monocytic cells which interact with the endothelium (Springer, T.A. 1990) and are responsible for initial capture and rolling of monocytes. Selectin interactions provide time to stabilize integrin interactions and allow the monocyte to extravasate into the basement membranes (Larsen, G.R. et al. 1992). Extravasation allows monocytes to penetrate basement membranes and access tissues. Monocytes express the ligands for P-selectin and E-selectin, also the monocyte expresses L-selectin which is rapidly shed into a soluble form.

Integrins are heterodimeric molecules containing non-covalently associated α and β subunits (Springer, T.A. 1990) which mediate cell-substratum and cell-cell adhesion. The CD11/CD18 heterodimer is expressed on monocytes, CD18 is the common β_2 subunit which can interact with three different α subunits CD11a (LFA-1), CD11b (Mac-1), or CD11c (p150,95) (Carlos, T.M. et al. 1990). CD11/CD18 binds to intracellular adhesion molecule-1 (ICAM-1) (CD45), ICAM-2, or ICAM-3 which are members of the immunoglobulin superfamily. Monocytes extravasation between EC junctions in response to inflammatory cues is dependent on ICAM-1/LFA-1 interaction. Individuals with leukocyte adhesion deficiency (LAD) lack the β_2 integrin subunit and their monocytes are unable to traverse the endothelium at sites of infection (Auger & Ross. 1992).

The monocyte Mac-1 receptor binds fibrinogen and a complement protein (C3bi). Monocytes express very late activation antigen (VLA-4) a $\beta 1$ integrin which functions both as a cell and matrix receptor whose ligands are fibronectin (Bauvois, B. et al. 1992) and vascular adhesion molecule-1 (VCAM-1) (Springer, T.A. 1994). Integrin binding is dependent on divalent ions and several integrins can be inhibited using the tripeptide Arg - Gly - Asp(RGD) (Springer, T.A. 1990).

Integrin signaling pathways interact with other receptor pathways to enhance or dampen signals elicited by each receptor. A GTPase (Rho) appears to play a key role in mediating crosstalk between integrin and growth factor receptor pathways (Clark, E.A. et al. 1995). The involvement of integrin signaling in modulating vascular tone has not been explored.

ICAM is expressed primarily on the endothelium however monocytes also express ICAM and its expression is markedly increased in the presence of inflammatory mediators (i.e. thrombin) (Davies, M.G. et al. 1993). Monocytes and EC gap junctions constitutively express CD31 (PECAM-1, platelet endothelial cell adhesion molecule) which may exhibit homotypic binding and play a role in extravasation (Muller, W.A. et al. 1993).

Regulation of cell adhesion molecules during the inflammatory episodes are crucial for appropriate responsiveness. Generally, selectins permit inflammatory cells to interact with the endothelium and allow stabilization of additional cell adhesion molecules. The integrins and immunoglobulin superfamily are required for inflammatory cell extravasation to sites of injury.

Generation of NO is a mechanism that potentially decreases adhesive interactions at acute sites of inflammation. We have demonstrated that both EC and monocytes release NO upon ET-1 stimulation through ET_B receptor activation.

Although NO release induced by ET_B receptor activation may impair monocyte adhesion, ET_B receptor activation may also be capable of promoting monocyte recruitment. In this regard, release of chemotactic factors such as interleukin-6 by ET-1-stimulated monocytes has been recently reported (McMillen, M.A. et al. 1995), suggesting that stimulation of monocytes with ET-1 may contribute to the recruitment and activation of inflammatory cells. NO has been demonstrated to inactivate cytokine stimulated cells and impair chemotaxis (Stefano, G.B. et al. 1995c; Makman, M.H. et al. 1995) suggesting that stimulatory and inhibitory signal molecules released in response to ET_B receptor activation may provide endogenous regulation of localized inflammatory cell responses.

SMOOTH MUSCLE CONTRACTION

The VSM intrinsic ability to remain in a sustained contractile state in the absence of neural and humoral influence is referred to as vascular tone (D'Angelo, G. et al. 1994). Recently, the endothelium has been identified as an important mediator of vascular tone by its ability to produce both relaxing and contracting factors (Hathaway, D.R. et al. 1992). ET-1 induced contraction of VSM is augmented in the presence of human PBM and THP-1 cells (Magazine, H.I. et al. 1994), suggesting that monocytes are capable of altering vascular tone. The major players in smooth muscle contraction have been identified, however the exact mechanism regulating the contractile response of smooth muscle remains unsolved (Jiang, H. et al. 1994).

Contractile proteins in smooth muscle are actin and myosin as in striated muscle. The ratio of thin to thick filaments is 13-15 to 1 in smooth muscle whereas 6 to 1 in striated muscle. Smooth and striated muscle generates equivalent amounts of force which is remarkable considering smooth muscle has one-fifth the amount of myosin (Jiang, H. et al. 1994) and uses 1% of the adenosine triphosphate (ATP) compared to skeletal muscle (Morano, I.L. 1992). Smooth muscle functions in a manner similar to striated muscle, the sliding filament theory for development of tension applies to both striated and smooth muscle (Murphy, R.A. 1994).

The myosin molecule consists of two heavy chains (MHC 200 Kd) and four light chains (MLC 16 - 28 Kd). The carboxyl termini of the MHC form the

helical portion while the amino termini form the globular region of the molecule, the MHC contain actin and ATP-binding sites. The MLC non-covalently associate with globular region of the MHC (Morano, I.L. 1992) and appear to have regulatory functions.

It is generally accepted that smooth muscle contraction is dependent on intracellular free calcium. The main sources of free calcium are from the extracellular space and sarcoplasmic reticulum (SR) (Jiang, H. et al. 1994). Phosphorylation of the MLC 20 Kd subunit at serine 19 by myosin light chain kinase (MLCK) results in myosin - actin interaction, crossbridge cycling and a development of force (Stull, J.T. et al. 1993). In resting smooth muscle MLCK is inactive, however following stimulation, intracellular calcium accumulation and binds calmodulin (CaM). The calcium/CaM complex interacts with MLCK resulting in activation (Walsh, M.P. 1994) and smooth muscle contraction.

In addition calcium/CaM activates calmodulin kinase II (CaMKII) which can phosphorylate MLCK causing desensitization of the contractile response. MLC phosphatase, which dephosphorylates MLC serine 19, and sequestration of free calcium promote smooth muscle relaxation (Walsh, M.P. 1994). Vasodilatory compounds secreted following ET-1 stimulation may also play a role in modulating vascular tone.

Monocyte recruitment to sites of vascular injury and their interactions with VSM may have profound effects on hemodynamics and participate in the development on numerous cardiovascular disorders. Specifically, in atherosclerosis monocytes accumulation may stimulate vasospasm through

release of vasoactive compounds such as ET-1 and NO (Figure 2). In addition monocyte production of growth factors may promote thrombosis formation.

CARDIOVASCULAR DISEASE

Cardiovascular disease remains the primary cause of death in the United States and Western Europe, and atherosclerosis, the leading cause of cerebral and myocardial infarction, accounts for the majority of these deaths (Joossens, J.V. 1980; Gifford, R.W.J. 1993). EC injury is an initiating event in the development of atherosclerosis. Atherogenesis occurs at sites where the endothelial layer is chronically injured by oxidized lipids, turbulent blood flow or mechanical agitation (i.e. balloon angioplasty) (Davies, M.G. et al. 1993). In response to inflammatory mediators the earliest lesions contain monocytes that infiltrated the wound which differentiate into lipid-rich macrophages (foam cells) and proliferating smooth muscle cells. As the disease advances fibrous plaques composed of smooth muscle cells and foam cells project into the lumen altering blood flow (Ross, R. 1993). Complications that accompany advanced atheromatous plaque include: ulceration; thrombosis; medial thinning, which leads to aneurysm; and calcification. Ischemia and infarction are clinically the most common consequences of atherosclerosis (Parums. 1992).

Under normal circumstances the endothelium is non-thrombotic and maintains vascular tone through the release of various vasoactive mediators (Mantovani, A. et al. 1992). In the disease state the endothelium becomes pro-thrombotic, highly permeable, and promotes smooth muscle proliferation (Hajjar, D.P. et al. 1992). Diseased blood vessels have altered vascular reactivity to vasoactive mediators. The role of oxidized-low density lipoproteins (ox-LDL) in

atherosclerosis is being extensively investigated. Endothelium dependent relaxation is inhibited by ox-LDL and ET-1 production increases in the presence of ox-LDL (Luscher, T.F. 1993). The potential consequence of increased ox-LDL are alterations in vascular tone resulting in vasospasm and ischemia. The significance of elevated plasma levels of ET-1 is not yet fully understood however, our data suggests that ET-1 stimulated monocytes has substantial effect on VSM reactivity and autologous production of NO upon ET-1 stimulation is a potentially important immunomodulator mechanism.

CHAPTER ONE:

EVALUATE ENDOTHELIN RECEPTOR SUBTYPE EXPRESSION IN HUMAN MONOCYTES.

OBJECTIVE:	41
MATERIALS AND METHODS:.....	42
RESULTS:.....	46
<i>Endothelin receptor expression by human monoblastic cell lines.</i>	46
<i>ET_B receptor mRNA expression by monoblastic cell lines.</i>	47
CONCLUSIONS:	49

Chapter One:

Evaluate endothelin receptor subtype expression in human monocytes.

OBJECTIVE:

We have demonstrated specific binding sites for ^{125}I -labeled ET-1 on human PBM (Magazine, H.I. et al. 1994; McMillen, M.A. et al. 1995). In addition, other research groups have identified specific binding sites for ^{125}I -labeled ET-1 on alveolar (Millul, V. et al. 1991) and thioglycollate-activated peritoneal macrophages (Kishino, J. et al. 1991) yet the endothelin receptor subtype expressed by these monocytic cells has not been rigorously evaluated. We employed a pharmacological and molecular approach to determine which ET receptor subtype was expressed by monocytes.

Examination of ^{125}I -labeled ET-1 binding to the human monocyte cell lines THP-1 and U937 indicated that the THP-1 cell line express an ET receptor whereas no specific binding of ^{125}I -labeled ET-1 was observed in U937 cells. Furthermore, an ET_B receptor antagonist potently abrogated ^{125}I -labeled ET-1 binding to THP-1 cells suggesting ET_B receptor expression. Evaluation of ET_B mRNA expression in THP-1 and U937 cells confirmed the radioligand binding data. The pharmacological and molecular data indicate that human monocytes express an endothelin receptor that is consistent with the ET_B subtype.

MATERIALS AND METHODS:

a. Materials: ^{125}I -labeled ET-1 was obtained from DuPont-New England Nuclear (Boston, MA). ET-1, ET-3, BQ-123 and BQ-788 were obtained from Peninsula Laboratories Inc. (Belmont, CA). Physiological saline solution (PSS) consisting of [130 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO_4 , 1.18 mM KH_2PO_4 , 14.9 mM NaHCO_3 , 10 mM 4-[2-Hydroxyethyl]-1-piperazineethane-sulfonic acid (HEPES), 5.5 mM Dextrose, 0.03 μM Ethylenediaminetetraacetic acid, and 2.5 mM CaCl_2] from Scientific Products (McGraw Park, IL). RPMI-1640, FBS, Hanks' Balanced Salts (HBS), penicillin-streptomycin solution, albumin-Bovine Fraction V (BSA), sodium azide, N ω -Nitro-L-Arginine (L-NNA), phorbol-12-myristate-13-acetate (PMA), endotoxin kit were purchased from Sigma Chemical Co. (St. Louis, MO) and fibronectin was from Boehringer Mannheim (Indianapolis, IN). IRL-1620 was synthesized in the laboratory using an Advanced ChemTech Model 90 Peptide Synthesizer (Louisville, KY) employing standard solid phase methods and purified by C18 column chromatography as previously described (Magazine, H.I. et al. 1996a; Magazine, H.I. et al. 1996b).

b. Blood Monocytes and Cell Cultures: PBM were isolated by Ficoll-density gradient centrifugation from freshly donated blood purchased from the Long Island Blood Services (Melville, NY) (Magazine, H.I. et al. 1995b). Human monocytic cell-lines THP-1 and U937 were obtained from American Type Culture Collection (Rockville, MD) and were maintained in RPMI-1640 supplemented with 10% FBS, 10 μM 2-Mercaptoethanol, and 100 Units/ml penicillin and 100

mg/ml streptomycin as previously described (Magazine, H.I. et al. 1994). Cells were harvested by centrifugation and washed extensively with RPMI-1640 prior to use in assays. All reagents, glassware and cell cultures were free of lipopolysaccharide, as determined by the litmus amebocyte assay. Cell viability was determined by trypan blue exclusion and was consistently >95%. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated and propagated exactly as previously reported (Tsukahara, H. et al. 1994).

c. Endothelin-1 Radioligand Binding Assay: Monocytic cells were diluted to a cell concentration of 2×10^6 cells/ml in RPMI-1640 media and incubated at 37°C with 5% CO₂ for 16 hours prior to use. THP-1 cells were washed extensively with Hanks Balanced Salt (HBS) consisting of [137 mM NaCl, 5.4 mM KCl, 0.4 mM MgSO₄, 0.44 mM KH₂PO₄, 0.33 mM Na₂HPO₄ and 1.25 mM CaCl₂] and resuspended in HBS supplemented with 0.1% BSA and 10 mM sodium azide just prior to use in assays. Competitors (100 nM): ET receptor antagonists, ET_A selective (BQ-123) or ET_B selective (BQ-788), were added 10 min prior to addition of 100 pM ¹²⁵I-labeled ET-1 to 2×10^6 cells in 12 X 75 mm polypropylene tubes. Binding assays were performed at 24°C for 2 hours, unbound ¹²⁵I-labeled ET-1 was removed by washing 5 times with ice-cold HBS using a Brandel Harvester (Gaithersburg, MD), followed by transfer of cells into polypropylene tubes and ¹²⁵I-labeled ET-1 binding was measured using an Wallac Inc. CliniGamma counter (Gaithersburg, MD). Internalization of ¹²⁵I-labeled ET-1 has been determined to be negligible under these conditions (Magazine, H.I. et al. 1994). Specific binding was defined as total binding minus non-specific binding.

Non-specific binding was considered to be counts remaining in the presence of 1 nM ET-1 and was approximately 10% of the total bound counts. Samples were assayed in triplicate and SEM was calculated.

d. Confocal imaging of ET receptor: Monocytes were allowed to adhere to glass slides for 30 min in PSS followed by fixation in 1:1 methanol acetone (v/v) for 10 min at -20°C. Subsequently, slides were washed three times PSS. Nonspecific binding was blocked using PSS supplemented with 1 mg/ml BSA for 60 min at room temperature. Antagonists or excess unlabeled ET-1 was added to slide 10 min prior to addition of 300 pM biotinylated ET-1. Following a 60 min incubation with biotinylated ET-1 slides were washed three times in PSS supplemented with 1 mg/ml BSA then incubated with 1 µg/ml streptavidin-fluorescein conjugate (Pierce, Rockford, IL) for 60 min at room temperature. Slides were then washed five times in PSS then coverslips were attached using anti-fade mounting media. Slides were stored at -20°C until confocal fluorescent analysis (Meridian Instruments, MI).

e. Evaluation of ET_B receptor mRNA expression by RT-PCR: Total cellular RNA from individual cells was isolated by solubilization in 6M guanidine hydrochloride and serial ethanol precipitation, followed by phenol/chloroform extraction as previously described (Tsukahara, H. et al. 1994; Mirza, H. et al. 1996). Evaluation for ET_B receptor mRNA was completed by reverse transcriptase-polymerase chain reaction (RT-PCR) using oligonucleotide primers synthesized on an Applied Biosystems single-channel synthesizer (Foster City, CA) designed

to encompass the entire open reading frame. Forward primer 5'-GCAGCATGCAGCCGCCTCCAAGTCT-3' corresponded to nucleotides 5 → 20, and reverse primer 5'-TCAAGATGAGCTGTATTTATTACT-3' corresponded to nucleotides 1329 → 1305. The integrity of the RNA was confirmed using primers specific for the functional thrombin receptor, known to be expressed by cells of the monocytic lineage (Mirza, H. et al. 1996). Forward primer 5'-AAGCAACAAATGCCACCTTAGA-3' corresponded to nucleotides 319 → 341, and reverse primer 5'-GCTGATCTTAAAGGGGAGCACAGA-3' corresponded to nucleotide positions 704 → 680. Synthesis of cDNA transcripts was completed by incubation of approximately 1 µg of total RNA with 10 units of avian myeloblastosis virus (AVM) reverse transcriptase for 1 hour at 41°C in 50 mM Tris, pH 8.3, 50 mM KCl, 8 mM MgCl₂, 10 mM dithiotreitol, using 0.06 A₂₆₀ units of a 14-mer oligo(dT) primer. Ten microliters of the reverse transcriptase reaction was then adjusted to PCR buffer conditions, and PCR was completed utilizing a 1.5-minute denaturation step at 94°C, a 1-minute annealing step at 55°C, and a 3-minute primer extension step at 72°C, consisting of 35 cycles in a Coy thermocycler (Ann Arbor, MI), as previously described (Tsukahara, H. et al. 1994). Products were analyzed by size fractionation in a 1% ethidium-stained agarose gel, and the authenticity of the PCR product was confirmed by diagnostic restriction endonuclease analysis.

RESULTS:

Endothelin Receptor Expression by Human Monoblastic Cell Lines.

Human PBM, human monoblastic cell lines, THP-1 and U937 were tested for specific binding for ET-1. Both PBM and THP-1 expressed specific binding sites for ^{125}I -labeled ET-1, whereas the U937 cell line failed to bind ET-1 (Magazine, H.I. et al. 1994). In addition treatment of U937 cells with 10 nM PMA a phorbol esters for 24 hours failed to induce the expression of ET receptors (Figure 6). Binding of ^{125}I -labeled ET-1 to THP-1 was evaluated in the absence and presence of ET receptor subtype specific antagonists to evaluate ET receptor subtype expression (Figure 3). Specific binding site for ^{125}I -labeled ET-1 were identified on THP-1 cells with maximal binding of 18.67 ± 0.45 fmole/ 10^6 cells. In the presence of 100 nM BQ-123 maximal binding was 14.80 ± 1.31 fmole/ 10^6 cells. In contrast, competition with 100 nM BQ-788 markedly reduced maximal binding to 0.28 ± 0.94 fmole/ 10^6 cells. Specific binding of ^{125}I -labeled ET-1 was reduced markedly in the presence of the ET_B selective ET receptor antagonist, BQ-788, whereas the ET_A selective antagonist, BQ-123 was relatively insensitive at displacing ^{125}I -labeled ET-1 binding. These data coupled to previous reports that demonstrate that a low dose of either ET-1 or ET-3 potently abrogate ^{125}I -labeled ET-1 binding to macrophages (Kishino, J. et al. 1991) or THP-1 cells (Magazine, H.I. et al. 1994) suggest predominant expression of ET_B receptors by monocytic cells (Figure 5).

To corroborate the ^{125}I -labeled ET-1 binding data we used confocal imaging of the monocyte ET receptor. The biotinylated ET-1 ligand was developed for evaluation of ET receptor populations (Magazine, H.I. et al. 1991). Labeling cells with biotinylated ET-1 followed by streptavidin-fluorescein allows visualization of ET receptors using standard fluorescent microscopic techniques. Competition of biotinylated ET-1 with receptor antagonists or unlabeled ET-1 demonstrates specific binding and indicates receptor subtype expression (Figure 4) whereas determination of the fluorescent intensity permits quantitation of ET receptor expression. THP-1 cells were labeled with 300 pM biotinylated ET-1 in the absence and presence of 30 nM ET-1 (Figure 4), followed by 1 $\mu\text{g/ml}$ streptavidin-fluorescein. The presence of 30 nM ET-1 markedly reduced fluorescence (panel B) indicating THP-1 cells bound biotinylated ET-1 which was inhibited by excess ET-1. In addition BQ-788 but not BQ-123 inhibited biotinylated ET-1 binding (not shown) suggesting predominant expression of ET_B receptors by human monocytes.

ET_B Receptor mRNA Expression by Monoblastic Cell Lines.

Expression of ET_B receptor mRNA was evaluated by RT-PCR analysis of THP-1 and U937 cells. We have previously demonstrated that ET_B receptors are expressed by HUVEC using oligonucleotide primers encompassing the ET_B open reading frame and reverse transcriptase polymerase chain reaction (RT-PCR) of total cellular RNA (Tsukahara, H. et al. 1994). RT-PCR of THP-1 total cellular RNA demonstrated the presence of a single amplified product of approximately

1350 base pair as indicated by molecular size fractionation in an ethidium-stained agarose gel (Figure 7). The amplification product obtained from HUVEC total cellular RNA as well as that obtained using the cloned ET_B receptor cDNA (Tsukahara, H. et al. 1994) was indistinguishable from that obtained from THP-1 cells. These data when taken together with ET_B receptor antagonist inhibition of ¹²⁵I-labeled ET-1 binding to THP-1 cells (Figure 3) indicates that ET_B receptors are expressed by the THP-1 cells line. Furthermore, these data are consistent with previous reports that have demonstrated the presence of ET-1 binding sites on PBM (Magazine, H.I. et al. 1994). In contrast, no amplification product was demonstrated in total cellular RNA from U937 cells (Figure 7), consistent with the inability of these cells to bind ¹²⁵I-labeled ET-1. In order to demonstrate the RNA was not degraded, thrombin receptor mRNA was amplified and both THP-1 and U937 express a thrombin receptor consistent with their monoblastic lineage.

CONCLUSIONS:

Pharmacological and molecular protocols were utilized to classify the human monocyte endothelin receptor. Pharmacological classification of ET receptors can be performed by analysis ET receptor binding profiles. The ET_A receptor subtype has been demonstrated to bind ET-1 with greater affinity than ET-3 whereas the ET_B receptor binds ET-1 and ET-3 with similar affinity. We have demonstrated that monocytes bind ¹²⁵I-labeled ET-1 and ¹²⁵I-labeled ET-3 with similar affinity (Magazine, H.I. et al. 1994). In addition, ¹²⁵I-labeled ET-1 was displaced with similar efficacy by ET-1 and ET-3 suggesting predominant expression of ET_B receptor subtype (Figure 5).

The development of highly specific ET receptor antagonists have proved useful in determining ET receptor subtype expression. Antagonists have been developed that have selectivity for ET_A (BQ-123) (Hay, D.W. 1992) or ET_B (BQ-788) (Ishikawa, K. et al. 1994) receptors whereas others are non-selective (PD142894) (Sakamoto, A. et al. 1993). We have demonstrated specific binding sites for ¹²⁵I-labeled ET-1 on monocytes that were inhibited by both BQ-788 and PD142894 but not by BQ-123 (Figure 3). These data suggest that monocytes express an ET_B type receptor.

Expression of ET_B receptor mRNA was evaluated by RT-PCR analysis of THP-1 and U937 cells. Using oligonucleotide primers designed to encompass the entire ET_B open reading frame we have demonstrated that ET_B receptors are expressed by HUVEC's (Tsukahara, H. et al. 1994). Furthermore, we have

demonstrated ET_B receptor mRNA expression in a human monocyte cell line, THP-1 (Figure 7). The RT-PCR analysis of the THP-1 cell line are consistent with our ET receptor binding data. The expression of ET_B receptor mRNA taken together with the selective inhibition of ¹²⁵I-labeled ET-1 receptor binding in the presence of ET_B receptor but not ET_A receptor antagonists strongly indicates that THP-1 cells express an ET_B receptor.

In contrast to THP-1 cells, ET_B receptor mRNA expression was not detected in U937 cells consistent with work that failed to demonstrate specific binding of ¹²⁵I-labeled ET-1 to the cell line (Magazine, H.I. et al. 1994). Additionally, PMA treatment which is used to activate monocytes failed to induce the expression of ET receptors by U937 cells (Figure 6). These data demonstrate that PBM and the cell line THP-1 appear similar and express functional ET_B receptors. The monocytic cell line, U937 is well characterized and in many assay systems functions in a manner similar to that of PBM (Anderson, C.L. 1982; Conkling, P.R. et al. 1988). Thus, the failure of U937 cells to express ET_B receptors provides a model system for evaluation of the role of ET_B receptors in modulation of monocyte function.

The monocyte ET_B receptor may be partially regulated by its rapid downregulation after ET-1 stimulation. It has recently been suggested that the ET_B receptor is subject to homologous desensitization through a PKA dependent pathway. It has been suggested that the ET_B receptor has compensated for the rapid internalization through irreversible binding of ET-1 to the ET_B receptor and remains associated with its receptor following internalization into caveolae. In

this manner ET-1 signaling may be enhanced as demonstrated by an elevated concentration of calcium channels, inositol 1,4,5-trisphosphate receptors, and heterotrimeric GTP-binding proteins in caveolae.

Studies have demonstrated that PBM (Chanez, P. et al. 1996; Ehrenreich, H. et al. 1990), polymorphonuclear leukocytes (Sessa, W.C. et al. 1991), and macrophages (Ehrenreich, H. et al. 1990) synthesize ET-1 and upon PKC activation increase ET-1 production 6-10 fold (Ehrenreich, H. et al. 1990). The mechanisms of ET receptor activation are similar to many other G-coupled protein receptors, wherein phospholipase C activation results in inositol phosphate turnover and in a rapid influx of extracellular calcium. Monocytes may produce ET-1 through an autocrine loop, whereby ET_B receptor stimulation activates PKC promoting ET-1 biosynthesis. Elevated plasma ET-1 concentrations is common in patients with a variety of vascular disease (Lerman, A. et al. 1991; Schiffrin, E.L. 1995; Cernacek, P. et al. 1989) and thus, monocyte derived ET-1 may contribute to the pathogenic sequelae through overproduction of ET-1.

Substantial interest and effort has been designated in the accumulation of experimental data pertaining to location of ET-1 production, ET receptor expression and ET-1 signal transduction pathways. Although many agonists can produce similar signal transduction responses (e.g. calcium transience) in monocytes, this report is unique in regards to the function of the monocyte ET_B receptor at sites of acute inflammation. We have identified and examined the monocyte ET_B receptor and have focused on issues pertaining to monocyte

behavior and cell biology in response to ET-1 stimulation at sites of vascular injury.

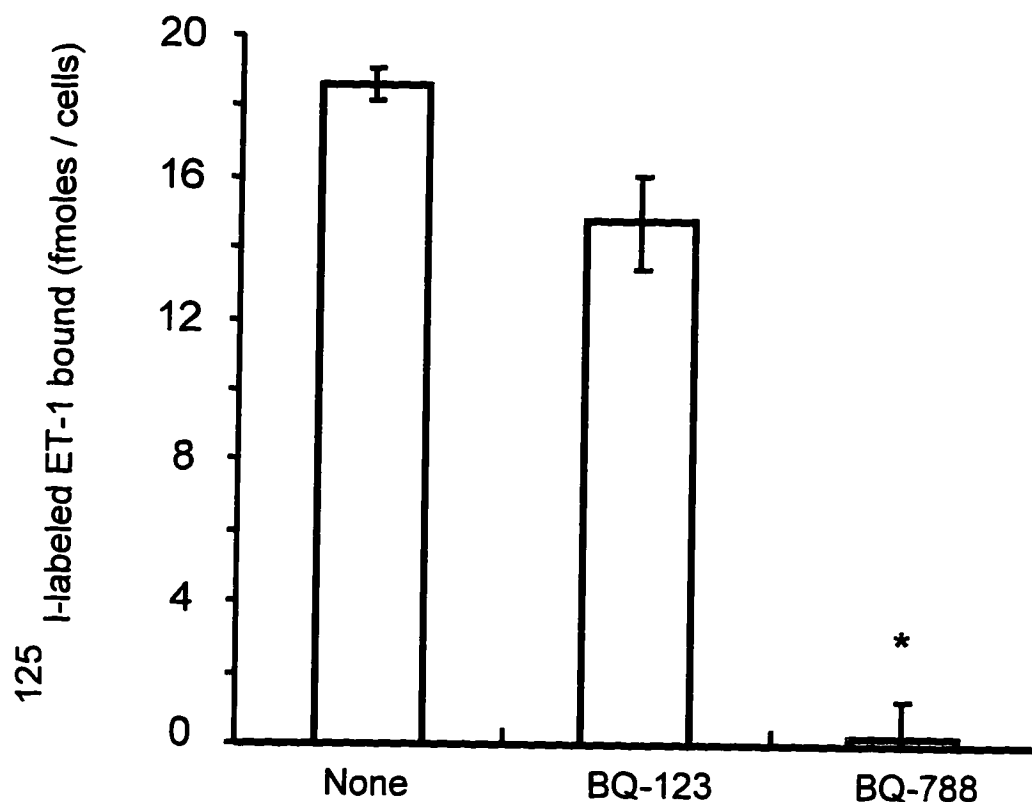


Figure 3: Endothelin Receptor Expression by Monoblastic Cell Lines.

Binding of 100 pM ¹²⁵I-labeled ET-1 to 2x10⁶ THP-1 cells was evaluated in the absence or presence of 100 nM BQ-123 or ET_B selective, BQ-788 ET receptor antagonists. Total binding of ¹²⁵I-labeled ET-1 in the absence of competitor was approximately 2000 cpm with non specific binding (cpm remaining in the presence of 1 nM ET-1) representing approximately 10% of the total cpm. Samples assayed in triplicate and the ET_B receptor antagonist BQ-788 significantly abrogated binding ($P < 0.05$, Student's *t* test). This is a representative of n=3 experiments.

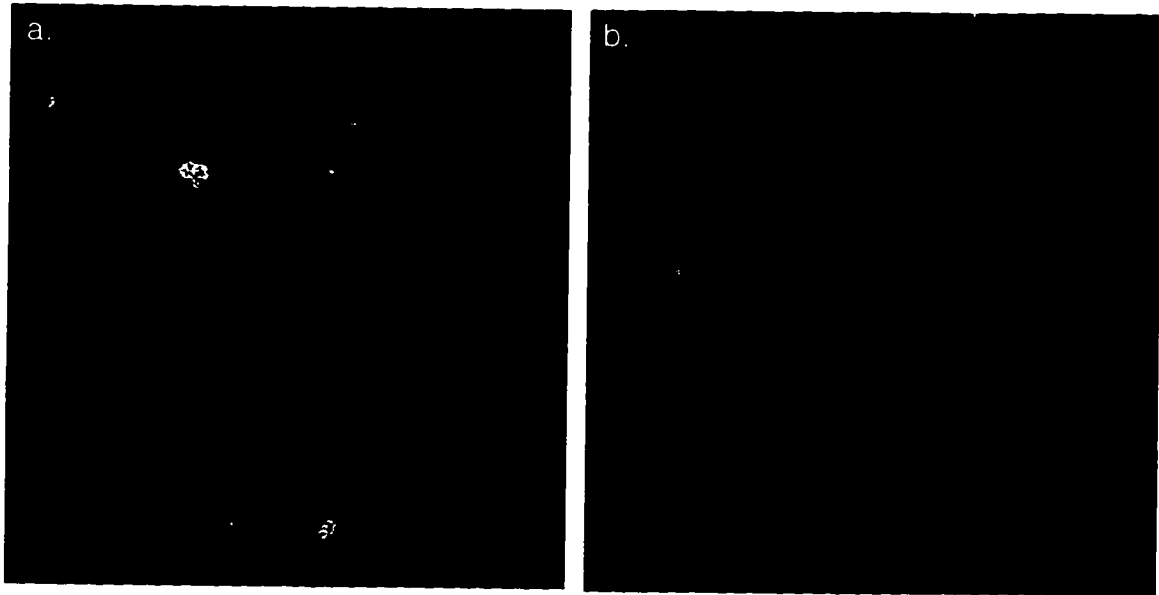


Figure 4: Confocal image of THP-1 with biotinylated ET-1.

Fluorescent images (400x) of fixed THP-1 cells: the cells were labeled with 300 pM biotinylated ET-1 for 1 hour, in the absence and presence of 30 nM ET-1. Subsequently, cells were labeled with 1 $\mu\text{g/ml}$ streptavidin-fluorescein conjugate. Panel (A) represents fluorescence in the presence of biotinylated ET-1 alone, Panel (B) 100 fold excess unlabeled ET-1 was added in the presence of biotinylated ET-1. Fluorescence was visualized using confocal microscopy.

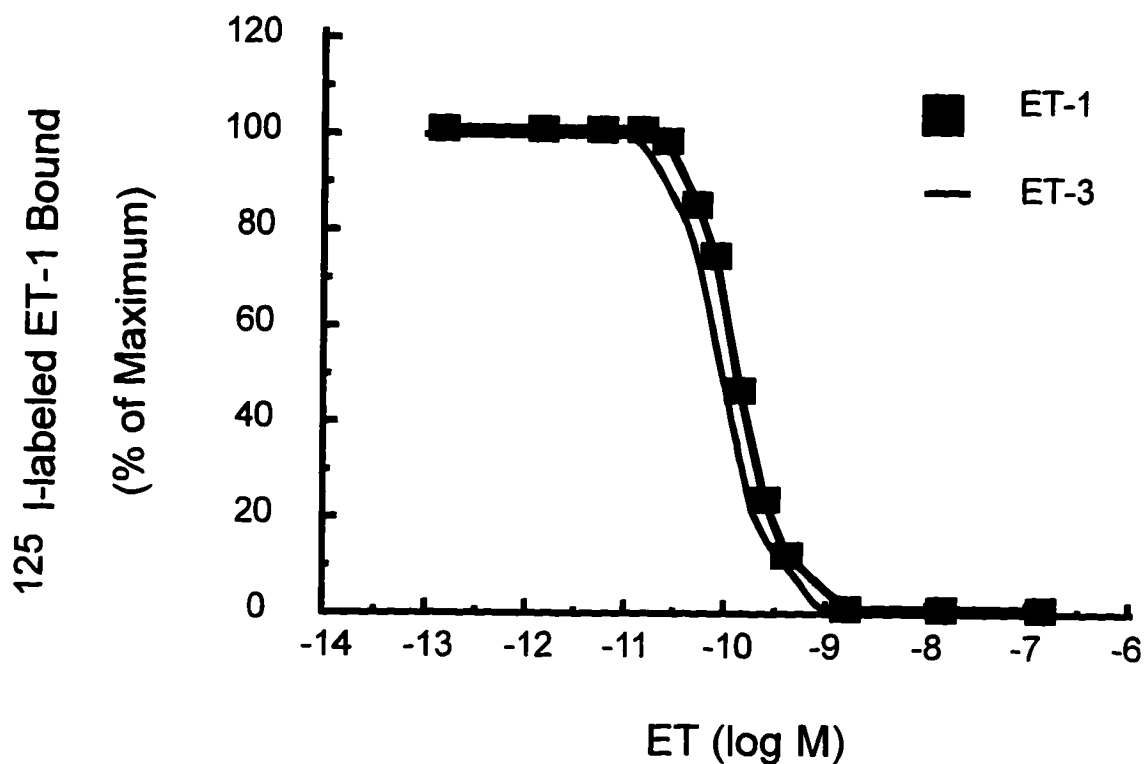


Figure 5: ET-1 and ET-3 Inhibition of ^{125}I -labeled ET-1 binding to THP-1 Cells.

^{125}I -labeled ET-1 (50 pM) binding to 2×10^6 THP-1 cells in the presence of increasing concentrations of ET-1 or ET-3 was determined. Binding of ^{125}I -labeled ET-1 in the absence of inhibitor was approximately 6000 cpm and mean values of sample in triplicate are expressed as percent of binding in the absence of competitor. The competition of ET-1 relative to ET-3 is consistent with predominant expression of ET_B receptors.

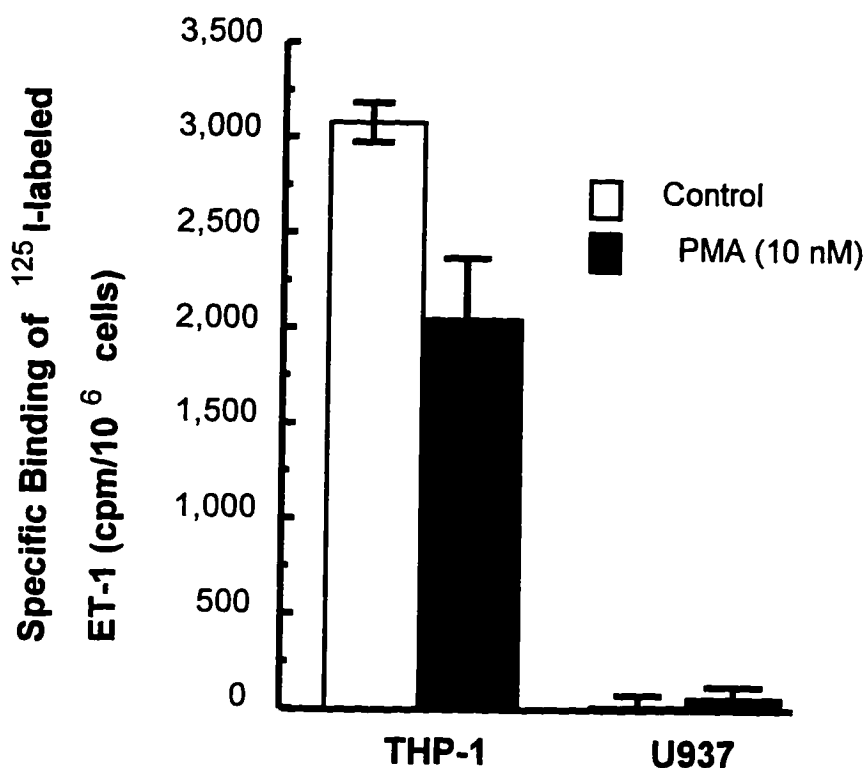


Figure 6: Effect of PMA stimulation on ET receptor expression by monocytic cells.

Examination of ^{125}I -labeled ET-1 (50pM) binding to 2×10^6 THP-1 or U937 cells. THP-1 and U937 were either untreated or treated for 24 hours at 37°C in the presence of 10 nM PMA. U937 cells failed to bind ^{125}I -labeled ET-1 and differentiation with 10 nM PMA was without effect on ET receptor expression. Mean values of triplicate samples, in addition 48 hour PMA stimulation was without effect data not shown.

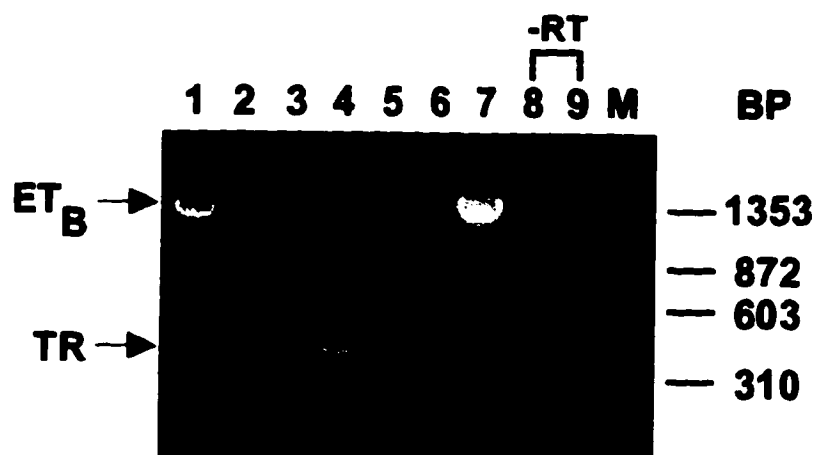


Figure 7: Evaluation of ET_B receptor mRNA expression by RT-PCR.

Total cellular RNA from HUVEC, THP-1 and U937 was isolated and used for RT-PCR as outlined in *Materials and Methods*. RT-PCR using ET_B receptor-specific primers and agarose gel electrophoresis documented a discrete product of the predicted size in HUVEC (Lane 1) and THP-1 cells (Lane 2), but not U937 cells (Lane 3). The integrity of the RNA was established by amplification of the identical samples with thrombin receptor-specific primers (Lanes 4 -6). Lane 7 demonstrates the predicted product size using the ET_B receptor cDNA as a control, and Lanes 8 and 9 demonstrate endothelial RNA controls without reverse transcriptase, using ET_B receptor- or thrombin receptor-specific primers, respectively. Size markers (M) corresponding to PhiX174-Hae III-digested DNA are shown to the right.

CHAPTER TWO:

TO DETERMINE WHETHER THE MONOCYTE ET_B RECEPTOR IS FUNCTIONALLY COUPLED TO NITRIC OXIDE SYNTHASE.

OBJECTIVE:	59
METHODS:	60
RESULTS.....	63
<i>a. Direct demonstration that the human monocyte ET_B receptor is functionally coupled to NO release.</i>	<i>63</i>
<i>b. Determine the isoform of nitric oxide synthase that is coupled to the human monocyte ET_B receptor.....</i>	<i>63</i>
CONCLUSIONS:	65

Chapter Two:

Determine whether the monocyte ET_B receptor is functionally coupled to nitric oxide synthase.

OBJECTIVE:

We have demonstrated that the ET_B receptor in HUVEC's was functionally coupled to ecNOS. There has been an intense debate on whether human monocytes express ecNOS and can produce NO. The NO-selective amperometric probe is a newly described technology that permits the direct measurement of NO release in real time. With the use of the NO-selective amperometric probe we have provided the first direct demonstration that human PBM as well as monocytic cell lines THP-1 and U937 release NO. Furthermore we have demonstrated that PBM and THP-1 release NO in response to ET-1 stimulation whereas U937 did not release NO. These data coupled to the ET-1 binding data suggest that in PBM and THP-1 the monocyte ET_B receptor is functionally coupled to NOS activation. Immunocytochemical analysis of THP-1 cells exhibited positive staining using an anti-human ecNOS antibody. Detection of ecNOS using immunocytochemistry coupled to the NO release data strongly suggests that ET-1 induced NO release is coupled to ecNOS rather than iNOS due to the dependence of the enzyme on extracellular calcium and the tightly regulated release of NO.

METHODS:

- a. Blood Monocytes and Cell culture: as described in Chapter One.
- b. Direct Measurement of NO Release: NO release from ET-1-stimulated monocytic cells was measured directly using an NO-specific amperometric probe (World Precision Instruments, Sarasota, FL) exactly as described (Magazine, H.I. 1995a; Magazine, H.I. et al. 1995b; Magazine, H.I. et al. 1996c; Magazine, H.I. et al. 1996b). Calibration of the probe was achieved by addition of potassium nitrite to a solution of potassium iodide resulting in the liberation of a known concentration of NO gas in accordance with the equation: $2\text{KNO}_2 + 2\text{KI} + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{NO} + \text{I}_2 + 2\text{H}_2\text{O} + \text{K}_2\text{SO}_4$ as previously reported (Magazine, H.I. 1995a). Briefly, monocytic cells, 5×10^5 - 1×10^6 were suspended in 1 ml PSS and baseline levels of NO release were determined by evaluation of NO concentration for 10 min. and subtracted from NO release obtained following agonist (ET-1 or IRL-1620) stimulation. Receptor antagonists (BQ-123 or BQ-788) were added 15 min. prior to stimulation with agonists. Cells were stimulated with 10 nM ET-1 in the absence and presence of 1 μM receptor antagonists and the concentration of NO gas in solution was measured in real-time with computer data acquisition at a sampling rate of 1/sec (Magazine, H.I. et al. 1995b; Magazine, H.I. 1995a).
- c. Immunohistochemical analysis of ecNOS: THP-1 cells were fixed to glass slides treated with Vectabond (Vector Laboratories Inc., Burlingame, CA) to enhance THP-1 adhesion using 4% formaldehyde prepared in Dulbecco's

phosphate buffered saline (PBS) (Sigma, MO) for 10 min followed by five washing steps in PBS. The fixed THP-1 cells were permeabilized in PBS supplemented with 0.1% saponin (Sigma, MO) and 4 mg/ml BSA for 10 min (BSA-sap-PBS). Slides were then incubated with 1 μ g/ml Anti-ecNOS (Transduction Laboratories, KY) diluted in BSA-sap-PBS for thirty min at room temperature followed by five washes in BSA-sap-PBS. Slides were then incubated with 1 μ g/ml Anti-mouse IgG conjugated to rhodamine (Pierce, IL) in BSA-sap-PBS for thirty min at room temperature followed by five washes in PBS. Repeat cell fixation and five washes in PBS then mount coverlips using anti-fade mounting media. Cell fluorescence was imaged using confocal microscopy (Meridian Instruments, MI)

RESULTS:

a. Direct demonstration that the human monocyte ET_B receptor is functionally coupled to NO release.

NO release from PBM was determined in real time using an NO-selective amperometric probe as extensively described (Magazine, H.I. et al. 1995b; Magazine, H.I. et al. 1996c; Magazine, H.I. 1995a; Tsukahara, H. et al. 1993). Stimulation of PBM with 10 nM ET-1 resulted in an marked increase in NO concentration detected by the probe within 1-2 min. following stimulation which remained elevated over baseline for approximately 10 min. (Figure 8a). Maximal release of NO from ET-1 stimulated PBM (36.7 ± 1.1 nM) was not altered by preincubation with the ET_A selective antagonist, BQ-123 (Figure 8b). In contrast, ET-1 stimulated NO release was abrogated in the presence of the ET_B selective antagonist, BQ-788 (Figure 8c), suggesting coupling of the ET_B receptor to NO release in PBM. Maximal production of NO by THP-1 cells was similar to that of PBM as was the ability of BQ-788 but not BQ-123 to block the production of NO following stimulation of the cell line with ET-1 (Table 5). The capacity of the ET_B receptor specific agonist, IRL-1620, to stimulate NO release in THP-1 cells substantiated the role of ET_B receptors in stimulation of NO following ET_B receptor activation (Table 5). In contrast to PBM and THP-1 cells, ET-1 or IRL-1620 treatment of U937 cells failed to stimulate an increase in NO release over baseline levels (Table 5)(Figure 9). Treatment of THP-1 cells, as well as U937 cells with 1 μ M ionomycin resulted in a marked increase in NO release (Table

5)(Figure 10), indicating that both cell lines are capable of NO production. These data when taken together indicate that PBM and THP-1 release increased levels of NO in response to ET_B receptor activation.

b. Determine the isoform of nitric oxide synthase that is coupled to the human monocyte ET_B receptor.

The NO synthase family can be divided into two broad categories based on the requirement for extracellular calcium. The constitutive isoforms are dependent on extracellular calcium whereas the inducible enzyme binds calmodulin tightly even at basal levels of calcium and thus is insensitive to changes in calcium concentration. This coupled with selective inhibition of iNOS using aminoguanidine (Bang, A. et al. 1996; Wolff, D.J. et al. 1995) provides support for iNOS or ecNOS activation. Both THP-1 and PBM require extracellular calcium for NOS activation (data not shown), in addition, examination of NO release indicates that the monocyte NOS is tightly regulated following agonist stimulation (Figure 9) consistent with ecNOS utilization. Preincubation of THP-1 with the iNOS selective inhibitor aminoguanidine was without effect in inhibiting ET-1 induced NO release. Incubation of THP-1 cells in the absence (panel A) and presence of ecNOS antibody (panel B) demonstrates that THP-1 cells were positively stain for ecNOS using immunocytochemical analysis (Figure 11).

Agonist	Antagonist	Nitric Oxide, nM		
		PBM	THP-1	U937
ET-1	--	36.7 ± 1.1	36.6 ± 4.3	1.1 ± 2.3
	BQ-788	7.23 ± 4.3	10.1 ± 2.7	nd
	BQ-123	29.5 ± 3.5	34.1 ± 3.9	nd
IRL-1620	--	nd	29.3 ± 1.2	3.0 ± 0.8
Ionomycin	--	32.1 ± 8.7	30.9 ± 1.8	26.2 ± 2.0

Table 5: Nitric Oxide production by human PBM and monocytic cell lines.

Direct measurement of NO production in response to 10 nM ET-1 or the ET_B receptor agonist, IRL-1620 was evaluated from 5x10⁵ PBM or human monocytic cell lines, THP-1 or U937 as described in *Materials and Methods*. The capacity of the cells to produce NO was evaluated using the 1 μM ionomycin.

CONCLUSIONS:

There has been an intense debate on whether human monocytes express ecNOS. A recent study of mRNA expression demonstrated that human PBM as well as the cell lines used in this report, express mRNA for ecNOS and may be induced to express iNOS (Reiling, N. et al. 1994). We have previously demonstrated that ET_B receptors are functionally coupled to ecNOS activation in HUVEC's (Tsukahara, H. et al. 1994). In addition, stimulation of Chinese hamster ovary cells transfected with ecNOS and either ET_A or ET_B receptor cDNA demonstrated that the ET_B but not the ET_A receptor is functionally coupled to ecNOS activation (Tsukahara, H. et al. 1994). Based on these findings we proceeded to investigate whether the human monocyte ET_B receptor was functionally coupled to ecNOS activation.

The techniques available for detection and measurement of NO gas have dramatically improved markedly in the last several years. The development of the NO-selective amperometric probe permits direct measurement of NO in real-time. Techniques which measure either nitrite or citrulline accumulation are indirect estimations of NO release. These assays have indicated that ecNOS activity was capable of producing only pM concentrations of NO. Analysis of NOS activity in real-time has been beneficial in describing the magnitude and duration of NO release. We have demonstrated using the NO-selective amperometric probe that ecNOS is capable of producing nanomolar concentrations of NO, a value similar to the maximal production observed

following iNOS activation. Real-time analysis demonstrated that ecNOS was activated for several minutes upon ET-1 stimulation and then rapidly returned to basal levels. Thus our data suggests that ecNOS produces similar levels of NO to iNOS. However ecNOS releases a rapid burst of NO whereas iNOS maintains NO production for extended periods without decrement.

Production of increased levels of NO by ET-1 stimulated PBM and THP-1 cells for a maximum of only 10 min is indicative of ecNOS activation and consistent with functional coupling of ET_B receptors to ecNOS activation as demonstrated previously in EC (Tsukahara, H. et al. 1994). In addition THP-1 cells stained positive using a anti-human ecNOS specific monoclonal antibody. When taken together these data suggest that coupling of the ET_B receptor to ecNOS activation and NO release associated with ET_B receptor activation appears to be conserved among cells that express the receptor. U937 cells are capable of NO production (Reiling, N. et al. 1994) as evidenced by copious production of NO in response to ionomycin treatment. However, U937 cells failed to release increased levels of NO following ET-1 or IRL-1620 stimulation, suggesting that the inability of ET-1 and IRL-1620 to stimulate NO production is most likely due to a lack of ET receptor expression.

Monocyte NO production may be important for modulation of adhesion receptor expression or proinflammatory cytokine production (De Caterina, R. et al. 1995). It has been shown that endothelial cell derived NO is important for maintenance of non-thrombogenic surface. Unregulated monocyte adhesion may have pronounced hemodynamic effects through production of ET-1 and other

vasoactive compounds. In addition, monocyte growth factor and cytokine production may produce smooth muscle cell proliferation in the vascular wall contributing to decrease luminal diameter and increase blood pressure. Potentially, unregulated monocyte adhesion could play a role in amplification of cardiovascular disease by directly damaging vascular tissue, releasing vasoactive compounds and promotion of vascular wall proliferation. Both endothelial cell and PBM NO may be important for maintenance of a non-thrombogenic surface. At sites of vascular injury, impairment or focal loss of EC may elevate PBM derived NO to a preeminent role in modulation of the inflammatory response.

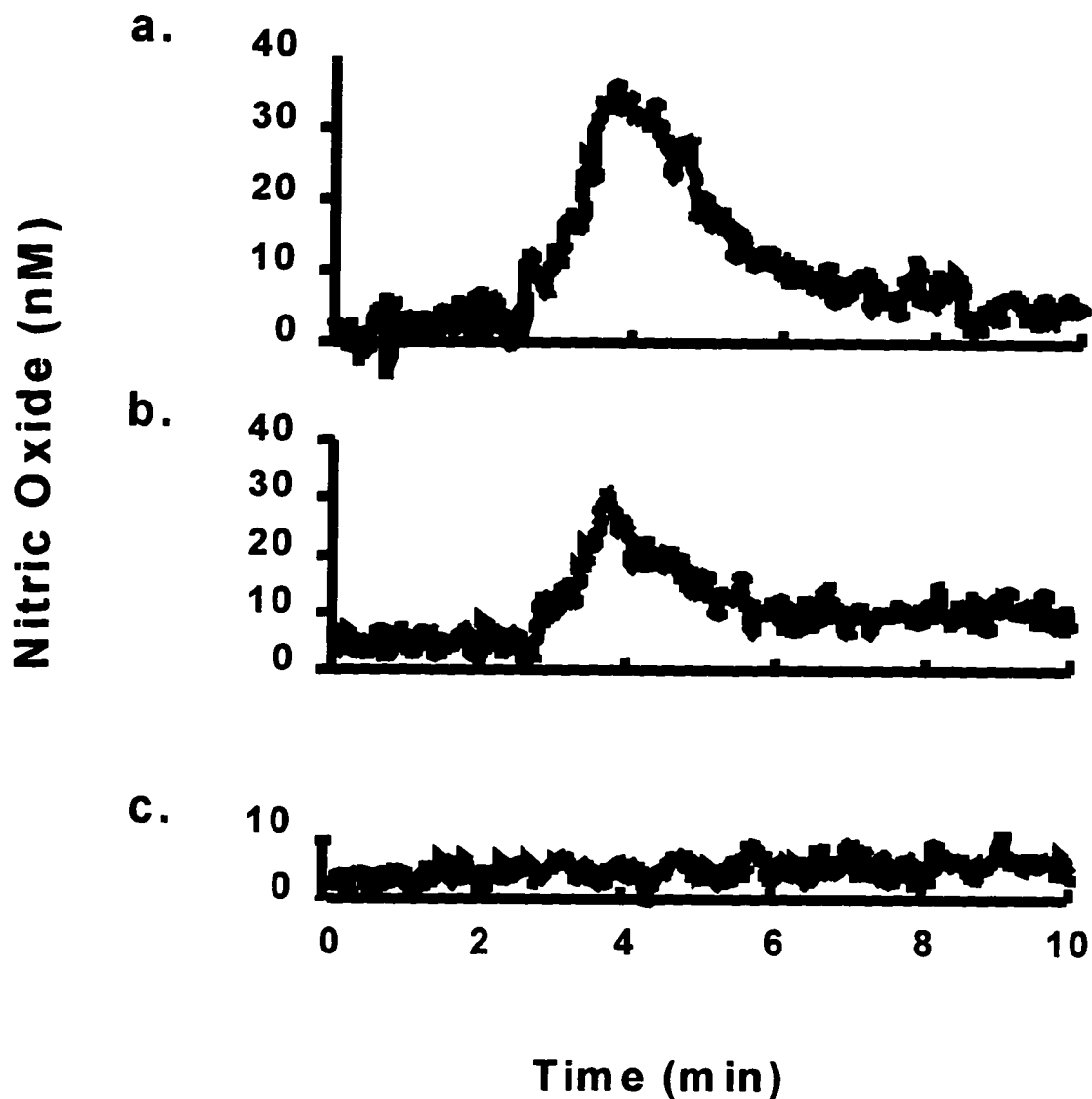


Figure 8: Induction of NO Release from PBM.

To evaluate coupling of ET receptors to NO release, 5×10^5 PBM stimulated with 100 nM ET-1 in the absence (Panel A) or presence of BQ-123 (Panel B) or BQ-788 (Panel C) and NO concentration in solution was measured in real time using an NO-selective amperometric probe (World Precision Instruments, Sarasota, FL). Data shown is a representative tracing of $n = 4$ experiments.

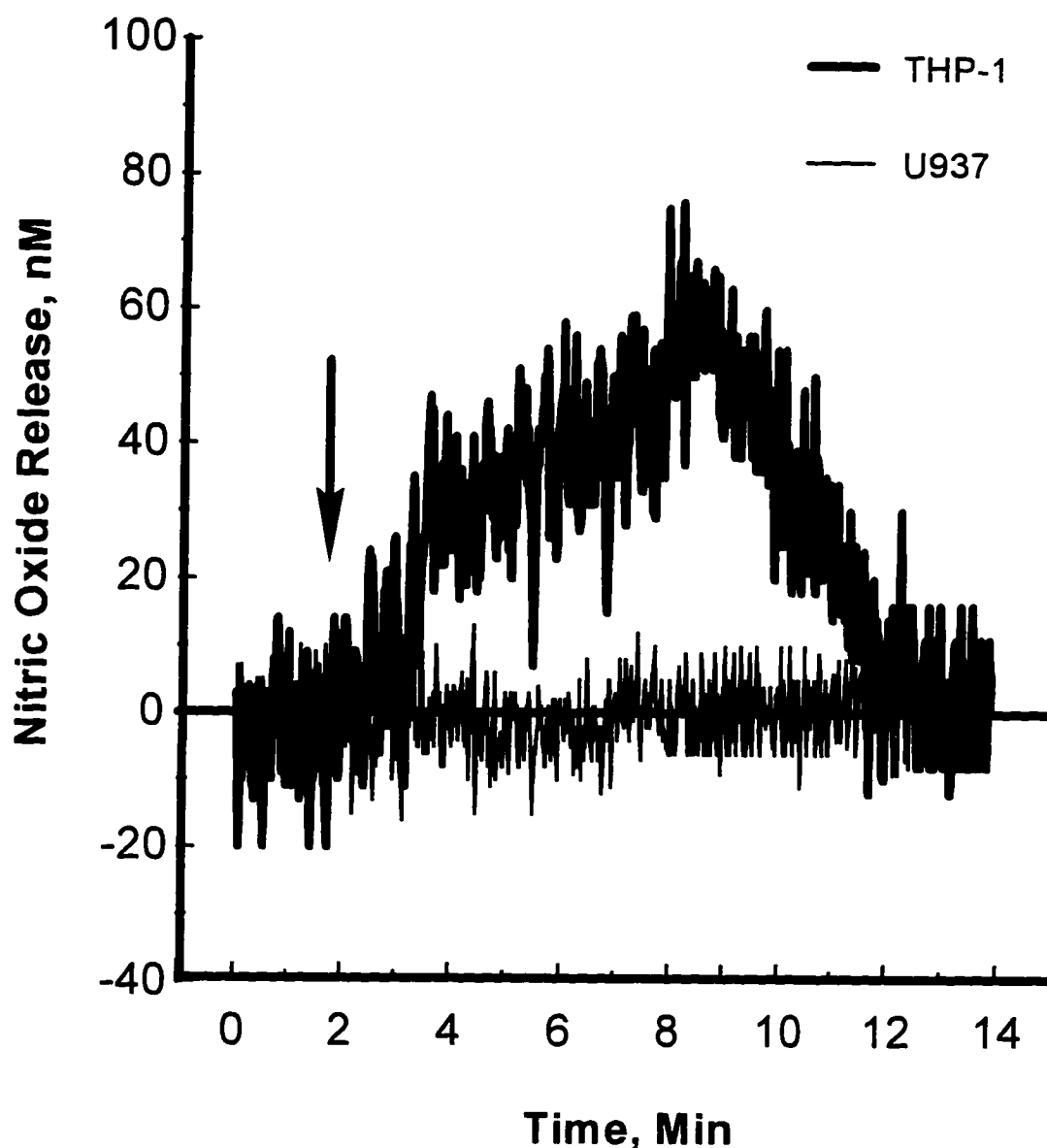


Figure 9: Induction of NO release in monocytic cell lines.

To evaluate coupling of ET receptors to NO release, 1×10^6 THP-1 or U937 cells were stimulated with 10 nM ET-1 and NO concentration in solution was measured in real time using an NO-selective amperometric probe (World Precision Instruments, FL). Data shown is a representative tracing of $n = 6$ experiments.

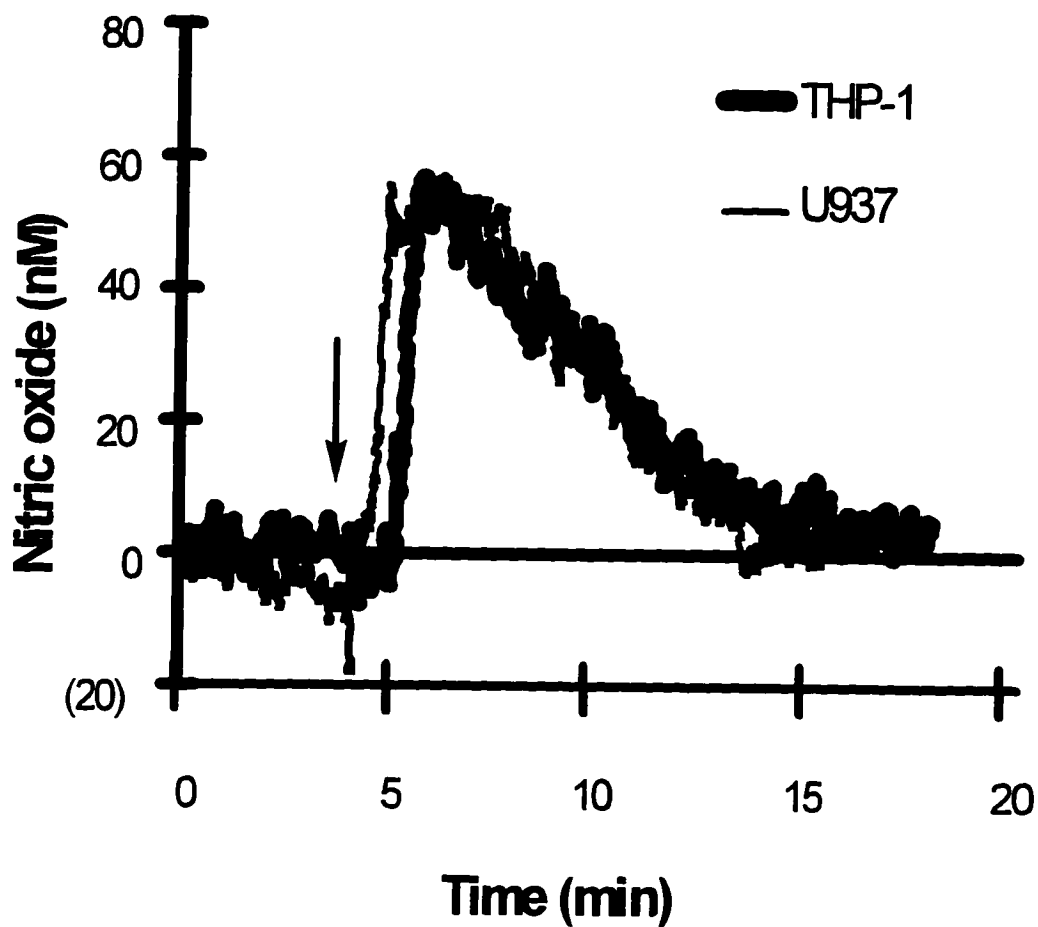


Figure 10: Induction of NO release in monocytic cells in response to ionomycin.

To evaluate calcium coupling to NO release, 1×10^6 THP-1 or U937 cells were stimulated with $1 \mu\text{M}$ ionomycin and NO concentration in solution was measured in real time using an NO-selective amperometric probe (World Precision Instruments, FL). Data shown is a representative tracing of $n = 4$ experiments.

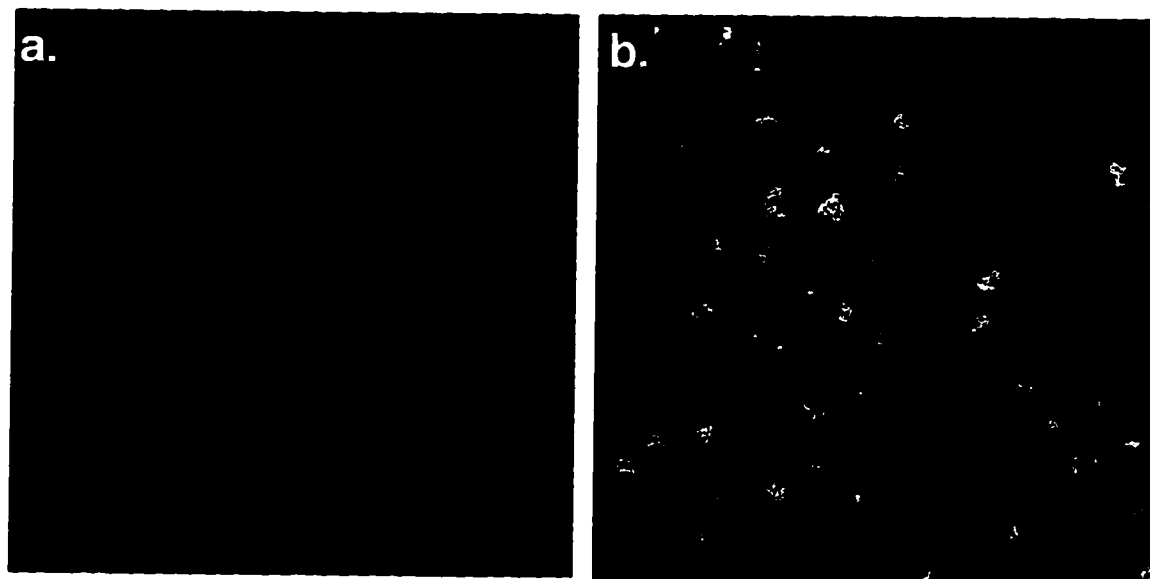


Figure 11: Immunocytochemistry of THP-1 ecNOS expression.

THP-1 cells were formaldehyde fixed to glass slides and permeabilized with 0.1% saponin. Panel A was treated with mouse serum IgG and Panel B was treated with mouse Anti human ecNOS. Subsequently, slides were treated with rhodamine conjugated Anti mouse IgG. Panel A represents background fluorescence, Panel B represents ecNOS expression in unstimulated THP-1 cells. Experiments examining both ecNOS and iNOS expression in THP-1 cells were performed by myself and Kamal D. Srivastava. Data presented in Figures 11 and 24 were provided by Kamal D. Srivastava. Immunocytochemical data is consistent with previous data examining ecNOS and iNOS expression in THP-1 cells (Reiling, N. et al. 1994).

CHAPTER THREE:

EVALUATE THE BIOLOGICAL IMPACT OF ET-1 INDUCED NO IN HUMAN MONOCYTES.

OBJECTIVE:	73
METHODS:	74
RESULTS.....	79
<i>a. ET_B receptor-coupled NO release induces conformational changes in THP-1 cells.</i>	79
<i>b. ET-1 modulation of PBM adhesion to human saphenous vein.</i>	80
<i>c. Evaluation of potential consequences of adherent monocytes on vascular contraction to ET-1.</i>	80
CONCLUSIONS:	82

Chapter Three:

Evaluate the biological impact of ET-1 induced NO in human monocytes.

OBJECTIVE:

Functional coupling of ET_B receptor-coupled NO is involved in regulation of monocyte activation at sites of vascular injury. Analysis of monocyte conformation permits the real-time analysis of cell behavior in response to hormonal stimulation. Examination of monocyte conformation upon ET-1 stimulation resulted in marked cell rounding and inactivation which was attributable to ET_B receptor-coupled NO release. Furthermore adhesion of PBM to human saphenous vein was attenuated by preincubation with ET-1 moreover adhesion was restored to level similar to that of control in the presence of BQ-788. In order to gain insight on the potential benefits of ET-1 induced cell rounding and inactivation, we measured the vascular contractile potency of ET-1 in rat aorta in the presence and absence of adherent monocytes. There was a significant increase in ET-1 potency in the presence of monocytes or monocytic membranes. ET-1 induced phosphatidylinositol hydrolysis in rat aorta was markedly elevated in the presence of monocytes. Cell-cell contact was required and the increase in ET-1 signaling was independent of released mediators, suggesting juxtacrine intercellular signaling. Thus, accumulation of monocytes as sites of vascular injury may augment vascular tone and contribute disease progression.

METHODS:

- a. Blood Monocytes and Cell culture: as described in Chapter One.
- b. Evaluation of Cell Conformation: Cells were washed extensively with RPMI 1640, diluted to a concentration of 10^6 cells/ml and maintained at 4°C prior to use. Cells suspensions were added to slides coated with 5 µg/ml fibronectin whereupon changes in cell conformation were evaluated for approximately 30 min. at 24°C as described (Magazine, H.I. et al. 1995b; Stefano, G.B. 1995a; Stefano, G.B. et al. 1995b). Briefly, the degree of conformational change was based on measurements of cellular area and perimeter using image analysis software (Image Analytics, Hauppauge, NY) (Stefano, G.B. 1995a). Changes in cellular conformation which ranged from inactive-rounded to active-ameboid were determined by measurements of cellular area and perimeter and were mathematically expressed by use of the form-factor (FF) formula $(4 \times \pi A)/P^2$, where A is the area of the cell and P is the perimeter of that given cell (Stefano, G.B. et al. 1989). The lower the FF value, the higher the cellular perimeter and the more ameboid the cellular shape (Stefano, G.B. et al. 1995b). The proportion of activated (ameboid) cells was determined by noting the proportion of ameboid verses round cells under phase contrast microscopy. ET-1-induced changes in cell conformation were evaluated by addition of 100 nM ET-1 or vehicle alone (PSS) to cells that had been allowed to adhere to fibronectin coated slides for 15 and changes in cell conformation were evaluated for an additional 15 min. For experiments in the presence of ET receptor antagonists or NOS inhibitors, these

agents were added to the cell suspensions just prior to addition of the cells to the slides for evaluation of cell conformation. Prior exposure of cells to L-NNA or ET antagonists alone, did not significantly alter their conformation or ability to adhere to the slide.

c. Monocyte Adhesion Assay: PBM were labeled with 1 μ M fluorescent dye Green CMFDA (5-chloromethylfluorescein diacetate) CellTracker™ (Molecular Probes, Or) at 37°C for 45 minutes. PBM were washed extensively with PSS by centrifugation. Prior to addition of PBM to strips of human saphenous vein, PBM were either treated with 10 nM ET-1 in the presence or absence of 1 μ M BQ-788 for 10 minutes at 37°C or untreated and then washed with PSS. Saphenous vein strips (10mm) were placed luminal side up in petri dishes containing PSS. Fluorescently labeled PBM (5×10^5) were added to the saphenous vein for 30 minutes. The saphenous vein was washed 3 times in PSS and then fixed in 4% paraformaldehyde overnight at 4°C. Monocyte adhesion was evaluated by confocal imaging (Meridian Instruments, MI) of the luminal surface.

d. Animal preparation: Male Sprague-Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) (250-400g) are anesthetized using 0.5ml euthanizing solution (Henry Schein Inc. Melville, NY). SD rats are fed standard rat chow and have free access to water. Thoracic aorta is immediately removed and placed in physiological saline solution PSS buffer. Connective and adipose tissue is dissected from the aorta. In preparing denuded rings a 1mm knife was

inserted into the lumen and was used to remove the endothelium (Magazine, H.I. et al. 1996a).

e. Organ Chamber Experiments: Rat aorta is cut into 3mm rings and mounted on metal tissue holders placed in a 5ml tissue bath (Kent Scientific Corp., CT). Bath temperature are maintained at 37°C and aerated with (95%O₂/5%CO₂). Rings are stretched stepwise to a passive isometric tension of 3.0g and allowed 90 min to equilibrate. Maximal contraction to 100 mM potassium chloride (KCl) is used to normalize each rings capacity to contract, vessels are allowed 45 minutes to equilibrate, 15 minutes prior to start of experiment, monocytes, monocyte membranes or inhibitors L-NNA (100uM) were directly added to appropriate baths. Concentration response curve to ET-1 (0.1 nM to 30 nM) are obtained by cumulative addition in quarter log increments. Time course experiments are performed by the addition of a single dose of 3 nM ET-1, 10 nM arginine vasopressin (AVP) or 10 nM Angiotensin II (BACHEM, King of Prussia, PA). The functional status of the endothelium was assessed by examining the relaxation response to acetylcholine (0.1 μM) after precontraction with norepinephrine (3 μM) (Bian, K. et al. 1995).

f. Peptide Synthesis: Arginine Vasopressin (AVP) (H-C-Y-F-Q-N-C-P-R-G-NH₂) and (G-R-G-D-S) were prepared in this laboratory using rink resin on the Advanced ChemTech peptide synthesizer Model 90 using standard solid phase peptide synthesis protocol (Magazine, H.I. et al. 1996a; Magazine, H.I. et al. 1996b). Peptides were cleaved with a cocktail of 90% Trifluoroacetic acid, 5% 1,2

ethanedithiol, 4% water and 1% thioanisol. Peptides were further purified using column chromatography. 40% acetonitril was used to extract the peptide from the column.

g. Preparation of Monocyte Membranes: Cells were homogenized in buffer containing 25mM HEPES, 50mM sodium fluoride, 5mM EDTA (pH 7.4), supplemented with 1 μ g/ml aprotinin, 5 μ g/ml pepstatin A, 5 μ g/ml Soybean Trypsin Inhibitor, 5 μ g/ml PMSF, (Sigma, MO) on ice with 50 strokes in a Dounce homogenizer. The homogenate centrifuged at 4°C 1,200 g 10 min and the supernatant centrifuged at 100,000 g for 60 min at 4°C. The pellet was resuspended in PSS with 0.1% Triton x-100 and stored at -70°C. Protein concentration was determined using the micro BCA assay kit (Pierce, IL).

h. Protocol for Inositol Phosphate Hydrolysis in Rat Aorta: Animal prepared exactly as described in section (d). The aorta was sectioned into twelve rings followed by incubation for 3 hr at 37°C in 0.5 ml PSS supplemented with 0.5% BSA + 300 μ l, 40 μ Ci/ml of [^{3} H]myoinositol (Amersham, Arlington Heights, IL). purged with 95%/5% O₂/CO₂. Subsequently aortic rings were washed three times in PSS supplemented with 0.5% BSA and 10 mM lithium chloride (LiCl) the monophosphate phosphatase inhibitor then transferred to individual polypropylene tubes. THP-1 cells were allowed 15 min to adhere aortic rings prior to ET-1 stimulation. Aortic rings were stimulated at 37°C for 60 min before stopping the reaction by addition of methanol and stored at 4°C overnight. Extraction of inositol phosphate from phosphatidylinositols is performed by

addition of chloroform, methanol, water mixture (2:1:1) v/v to a final concentration. Samples then vortexed and centrifuged for 10 min at 1000 rpm. To extract inositol phosphates Dowex AG1-X9 resin columns were used. Briefly, setup columns for each sample with 800 μ l Dowex AG1-X8 resin (Bio-Rad Laboratories, Hercules, CA) washed three times with dH₂O then load 500 μ l of aqueous sample to each column. Wash columns three times with 5mM inositol followed by elution of glycerophosphates with 60 mM ammonium formate, discard elutant. Then elute inositol phosphates with 1 M ammonium formate/ 0.1 M formic acid into scintillation vials. Add 10 ml scintillation cocktail to each vial and count on Beckman counter.

i. Statistics: Data were evaluated using the Student's *t*-test and a *P* value of < 0.05 was considered to be statistically significant. Multiple comparisons were adjusted using the Bonferroni multi-range test with alpha set at 0.005. Isometric tension was sampled every msec. and averaged at 5 sec intervals. All data are given as $\pm$ SE.

RESULTS

a. ET_B Receptor-Coupled NO Release Induces Conformational Changes in THP-1 Cells.

In the THP-1 cell preparations examined, the level of spontaneously activated and ameoboid cells was in the range of 9 to 14 % of the total cell population when placed on fibronectin coated slides at 24°C. This level of activation is consistent with that obtained using human PBM where approximately 9% of cells were found to be spontaneously activated (Magazine, H.I. et al. 1995b). The FF value for THP-1 cells evaluated immediately upon introduction to the slide was 0.64 ± 0.01 , indicating that the cells exhibit a rounded conformation (Figure 12). However, THP-1 cells when allowed to adhere to the slide for 30 min became ameoboid as evidenced by a significant decrease in the FF to 0.42 ± 0.01 (Figure 12)(Figure 13a). Stimulation of the THP-1 cells with 100 nM ET-1 following a 15 min. period of adhesion to the slide resulted in a rapid rounding of THP cells $FF = 0.58 \pm 0.01$ (Figure 12)(Figure 13b) to a level comparable to that of initial conditions. Pretreatment with 10 μ M L-NNA, a NOS inhibitor, prevented the rounding of ET-1 stimulated THP-1 cells $FF = 0.43 \pm 0.01$ (Figure 12)(Figure 13c), suggesting that the changes in cell conformation may be mediated by NO. In addition pretreatment of THP-1 cells with BQ-788 but not BQ-123 abrogated the rounding effect of ET-1 on THP-1 cells (Figure 13d) suggesting that ET-1 induced changes in THP-1 conformation appear to be the result of ET_B receptor coupled NO production.

b. ET-1 Modulation of PBM adhesion to human saphenous vein.

Modulation of THP-1 conformation by ET-1 suggested that induction of NO by ET-1 stimulated monocytic cells may impair cellular adhesion. Significant levels of PBM adhered to segments of human saphenous vein (Figure 14a) whereas adhesion was markedly reduced by preincubation of PBM with 10 nM ET-1 (Figure 14b). Consistent with ET_B receptor-dependent modulation of PBM adhesion, preincubation of PBM with 1 μ M BQ-788 prior to ET-1 restored PBM adhesion to control levels (Figure 14c). Examination of THP-1 cell adhesion to 3 mm rings of rat aorta was consistent with PBM adhesion to saphenous veins. ET-1 stimulation markedly impairs THP-1 adhesion to rat aorta as did treatment with the integrin inhibitor GRGDS (Table 6).

c. Evaluation of Potential Consequences of Adherent Monocytes on Vascular Contraction to ET-1.

We have demonstrated that adherent monocytes markedly alter VSM contractility to ET-1. Measuring vascular contraction in rat aorta in the absence and presence of the human monocytic cell line THP-1 we have observed a significant increase in ET-1 potency. The EC₅₀ decreased from 8.25 nM $\pm$ 1.1 nM to 3.61 nM $\pm$ 0.6 nM (n = 9, P < 0.01, ANOVA) (Figure 15). In addition, bolus injection of 3 nM ET-1 to rat aorta in the presence THP-1 or THP-1 cell membranes produced a significant increase in vascular tone (Figure 16)(Figure 19). The accumulation of THP-1 cells markedly altered ET-1 induced vascular contraction as demonstrated by (Figure 17) in which increasing number of THP-1 cell were added to rat aortic rings followed by bolus addition of 3 nM ET-1. To

determine the specificity of monocyte augmentation in vascular contraction we measured the effect of arginine vasopressin (AVP) and angiotensin II (ANG II) induced contraction in the absence and presence of THP-1. The THP-1 cells were without effect on AVP or ANG II induced contraction (Figure 18) as well as 100 mM potassium chloride (KCl) or epinephrine induced contraction (not shown). To further examine the mechanism by which monocytes augment vascular contraction we measured ET-1 induced phosphatidylinositol hydrolysis in rat aorta in the presence and absence of THP-1. ET-1 stimulation resulted in elevation of IP₃ levels in rat aorta. Figure 20 The presence of THP-1 alone was without effect on stimulation of IP₃ accumulation. IP₃ accumulation induced by ET-1 in the presence of THP-1 was increase markedly compared to aorta stimulated with ET-1 alone (Figure 20).

Conditions	%, of maximal unstimulated THP-1 adhesion
Control	100
ET-1 (1 nM)	65
GRGDS (333 μ M)	49
PMA (10 nM)	240

Table 6: Modulation of THP-1 adhesion to rat aortic rings.

CONCLUSIONS:

Vascular injury triggers intense accumulation of inflammatory cells, particularly PBM by adhesion to injured endothelium and exposed matrix (Cavender, D.E. et al. 1991; Van der Wal, A.C. et al. 1992). Activation of the serine protease α -thrombin is an important signal for monocyte chemotaxis (Bar-Shavit, R. et al. 1983; Bar-Shavit, R. et al. 1983) and upregulation of cellular adhesion proteins (Beretz, A. et al. 1990) resulting in increased interaction of PBM with vascular endothelium following episodes of injury. Unregulated adhesion of PBM would have damaging effects on thrombosis and hemostasis consequently, the recruitment and adhesion of PBM to sites of injury must be tightly regulated. In this regard, the constitutive release of NO by vascular endothelium has been suggested to be quite important. Damage to the endothelium following injury markedly impairs endothelium-dependent NO release (Morita, K. et al. 1996; Cohen, R.A. 1995) and thus, it is likely that the combined effects of thrombin mediated inflammatory cell recruitment and reduced endothelial cell derived NO release will result in increased cellular interactions with the vascular wall. We have demonstrated that release of NO by PBM may modulate interactions with the vascular wall by preventing changes in PBM conformation required for cell adhesion and spreading (Magazine, H.I. et al. 1995b; Bilfinger, T.V. et al. 1996) and inducing the down regulation and internalization of cellular adhesion proteins (Bath, P.M.W. et al. 1991; De Caterina, R. et al. 1995).

Exposure of monocytes to thrombin has been demonstrated to markedly alter a variety of cellular functions including motility, chemotaxis and adhesion (Altieri, D.C. 1995). THP-1 and U937 cells were demonstrated by RT-PCR to express mRNA for thrombin receptors consistent with previous reports of thrombin receptor expression by PBM (Fisker, S. et al. 1992; Goodnough, L.T. et al. 1982). Activation of ET_B receptors on PBM or THP-1 cells triggered rapid release of NO that resulted in significant cell rounding. Importantly cellular conformational changes that result in cell rounding have been associated with impaired cellular adhesion (Warren, A.P. et al. 1994) and recent reports have suggested that inhibition of actin polymerization may contribute to the impairment of cellular adhesion observed following NO exposure (Clancy, R. et al. 1995). Since activation of thrombin receptors with thrombin or a thrombin receptor agonist peptide triggers release of ET-1 from aortic rings within seconds of thrombin receptor activation (Magazine, H.I. et al. 1996c; Magazine, H.I. et al. 1996a) adhesion of monocytes at sites of acute injury may be attenuated by ET_B receptor-coupled NO released from vascular endothelium.

In the presence of an endothelium that is functionally compromised, autologous production of NO by PBM is likely to play preeminent role in attenuation of cellular adhesion. We hypothesized that exposure to PBM to ET-1 would reduce cellular interaction with the vascular wall and reduce cellular adhesion. Our results using PBM adhesion to segments of human saphenous vein confirm that a significant reduction in PBM adhesion is observed following exposure of PBM to ET-1. Activation of ecNOS induces the production of NO for

only a few min although this short burst of NO release appears to be sufficient to alter cellular spreading for several hours (Magazine, H.I. et al. 1995b; Bilfinger, T.V. et al. 1996). Indeed adhesion of PBM to human saphenous vein was markedly reduced by preincubation of cells with ET-1 prior to interaction with the vein. Under these conditions, NO release by the ET-1 stimulated PBM had already reached maximum and returned to baseline levels consistent with an inhibitory effect of NO on PBM adhesion that endures well beyond that of NO production.

To investigate the potentially deleterious role of monocyte adhesion at sites of acute vascular injury we measured ET-1 induced vascular contraction in rat aorta in the absence and presence of adherent monocytes. The presence of adherent monocytes markedly augmented the vascular contractile potency of ET-1. We conducted a series of experiments designed to test if ET-1 stimulated monocytes were releasing a stable transferable vasoactive mediator responsible for augmented the potency of ET-1. Experiments performed using supernatant transfer from ET-1 stimulated monocytes, or in the presence of inhibitors of arachidonic acid metabolites or oxygen radical production suggested that the release of a stable, transferable vasoactive mediator in response to ET-1 stimulation does not explain the augmentation of contraction to ET-1 observed in the presence of monocytes. Additionally, paraformaldehyde fixed monocytes and monocytic membrane preparations are capable of augmenting contraction in response to ET-1. These data suggest that a physical interaction between monocyte and vessel wall may be required for augmentation of ET-1 potency.

Monocytes may interact with VSM via juxtacrine interactions, where a stimulator cell induces functional changes in a target cell. (Zimmerman G.A. et al. 1993). Juxtacrine intercellular signaling is unlike that of paracrine and endocrine stimulation in that the molecule that induced functional changes in the target cell remains associated with the plasma membrane of the stimulator cell. The molecule does not act in the fluid phase and may provide a mechanism that allows strict spatial control of cellular activation. ET-1 stimulation of rat aorta results in inositol phosphate turnover followed by calcium transience which causes smooth muscle contraction. Examination of inositol phosphate accumulation in rat aorta in the presence and absence of adherent monocytes suggested that monocytes augment ET-1 signal transduction pathways promoting increased contractile responses in the presence of adherent monocytes. Inappropriate juxtacrine interactions or juxtacrine interactions that fail to terminate may contribute to the pathogenesis of vascular diseases such as atherosclerosis.

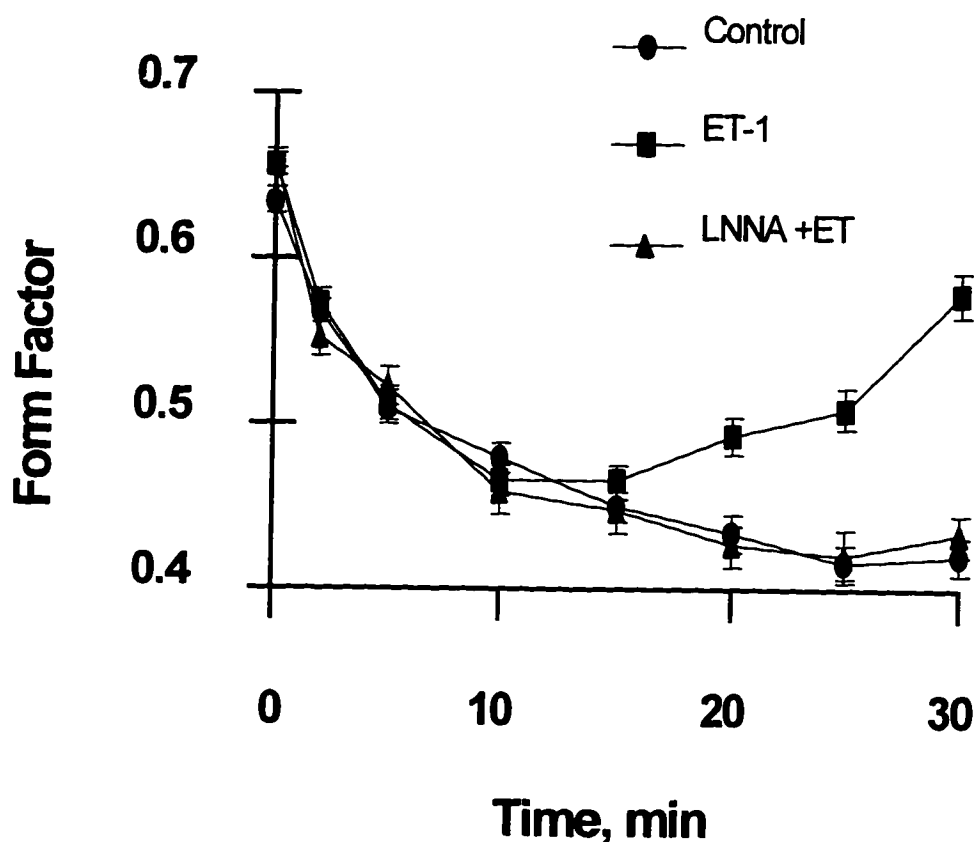


Figure 12: Adhesion of monoblastic cell line to fibronectin.

THP-1 cells were placed on fibronectin-coated slides and cell conformation was evaluated with image analysis software as described in *Materials and Methods*. Untreated THP-1 cells (●) changed from a rounded conformation to an ameboid conformation within 15 min following addition to the slide. Addition of 100 nM ET-1 to cells that were allowed to bind to the slide for 15 min resulted in a rapid rounding (■) of the cells. Pretreatment of THP-1 cells with 10 μ M L-NNA abrogated the rounding effect of 100 nM ET-1 (▲) and cell conformation was similar to that of untreated controls.

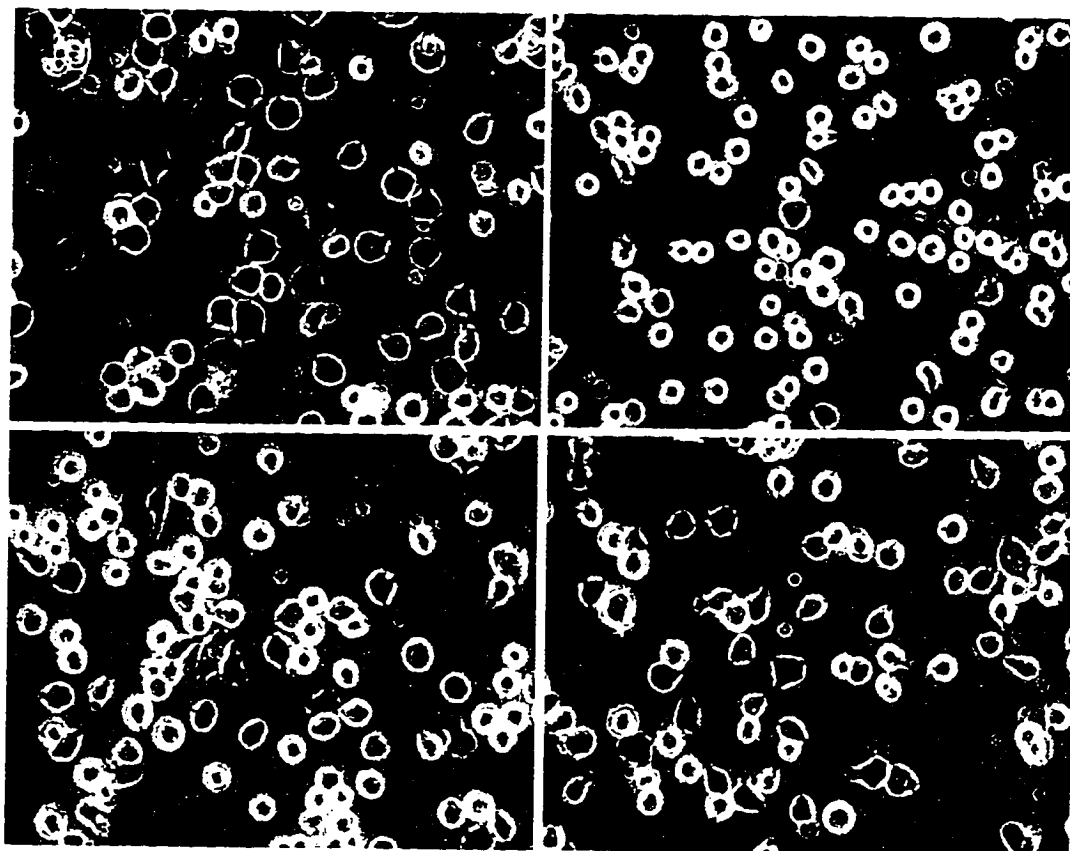


Figure 13: Adhesion of monoblastic cell line to fibronectin.

Phase Contrast images (400X) of THP-1 cells following 30 min. adhesion to fibronectin coated slides. Vehicle or 100 nM ET-1 was added after 15 min, in Panel C and Panel D inhibitors were added at the onset of the experiment. Panel A. Vehicle, Panel B. 100 nM ET-1, Panel C. 100 nM ET-1 in the presence of 10 μ M L-NNA, and Panel D. 100 nM ET-1 in the presence of 100 nM BQ-788.

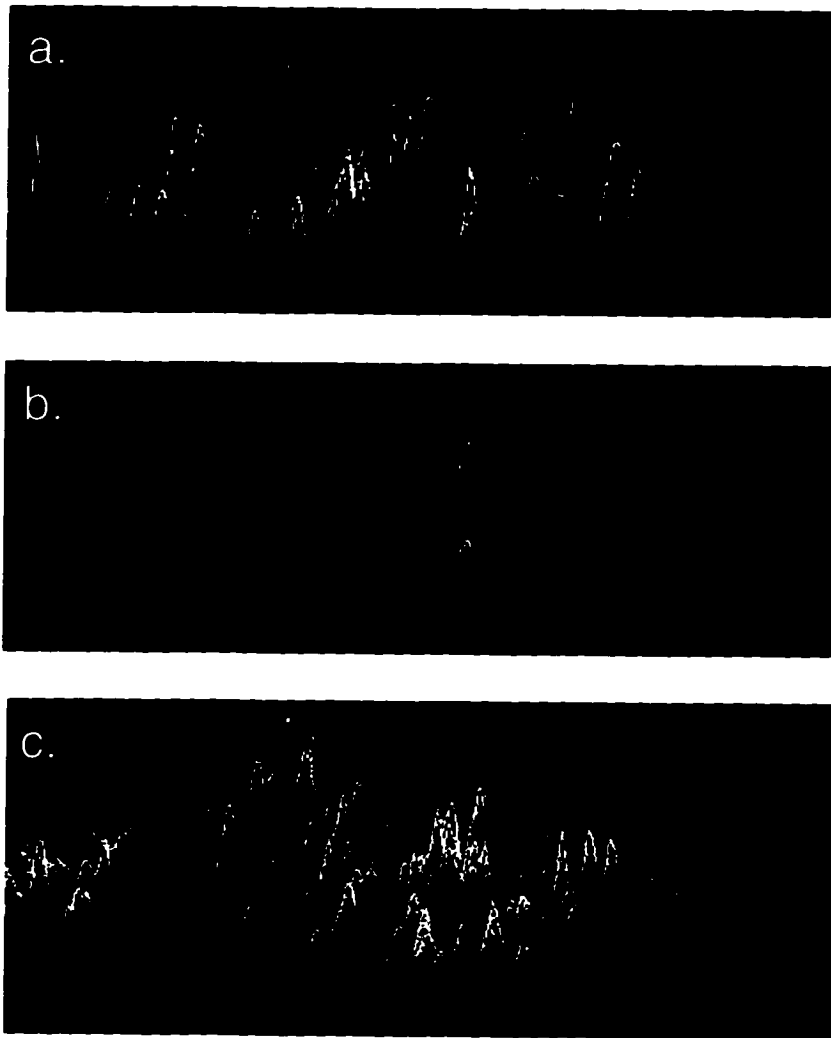


Figure 14: PBM adhesion to human saphenous vein is modulated by ET-1.

Topographic plots of PBM fluorescence on the luminal surface of human saphenous vein are shown; a $500\ \mu\text{m}^2$ area was scanned by confocal microscopy. Peaks of fluorescence represent sites of PBM adhesion. The luminal surface was visualized by exploiting tissue autofluorescence. The panels represent: A, Untreated; B, 10 min pretreatment of PBM with 10 nM ET-1; C, 10 min pretreatment of PBM with 10 nM ET-1 in the presence of $1\ \mu\text{M}$ BQ-788.

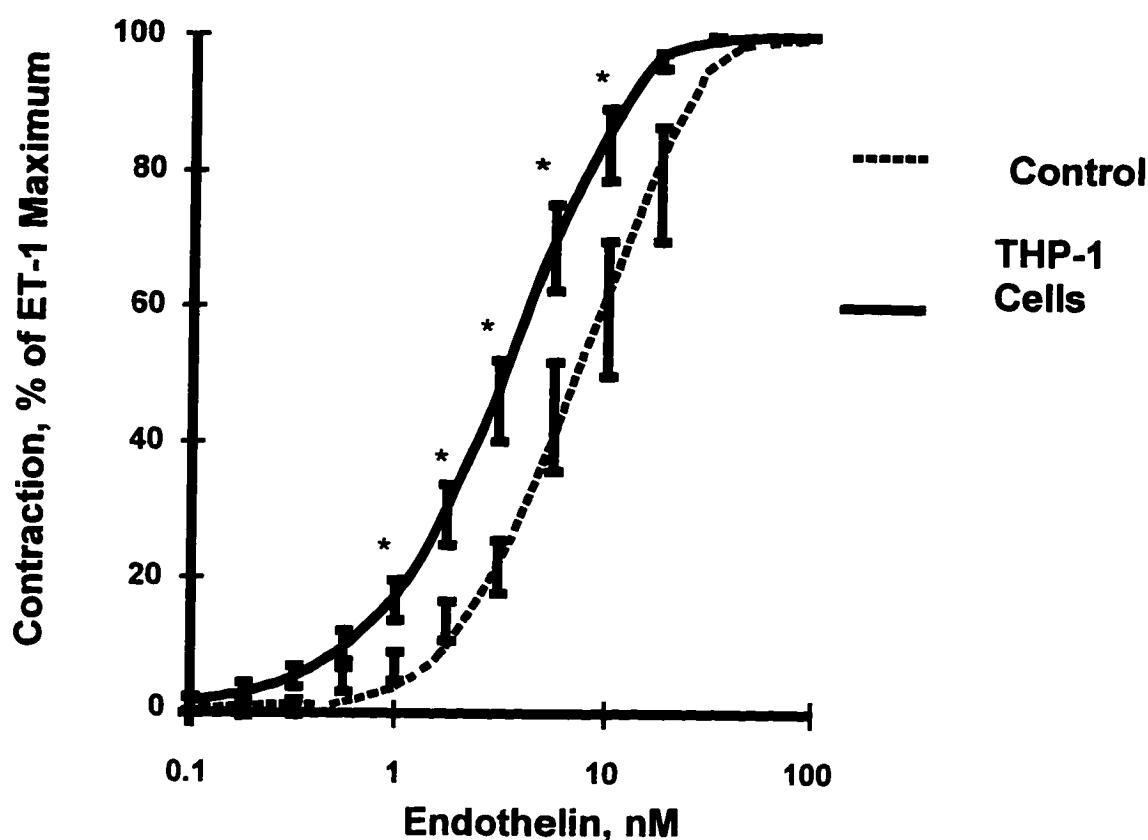


Figure 15: The Vascular Contractile Potency of Endothelin-1 is Increased in the Presence of Monocytic Cells.

Maximal developed isometric tension was recorded at each dose of ET-1. Aortic rings (3mm) were loaded with 3.5 g passive tension placed in organ baths maintained in aerated 37°C PSS. Individual rings were normalized according to their own maximal contraction to ET-1. The EC_{50} value for control was 6.0 nM and in the presence of THP-1 cells the EC_{50} was 2.0 nM and was significant with a p value < 0.001 as determined by ANOVA analysis.

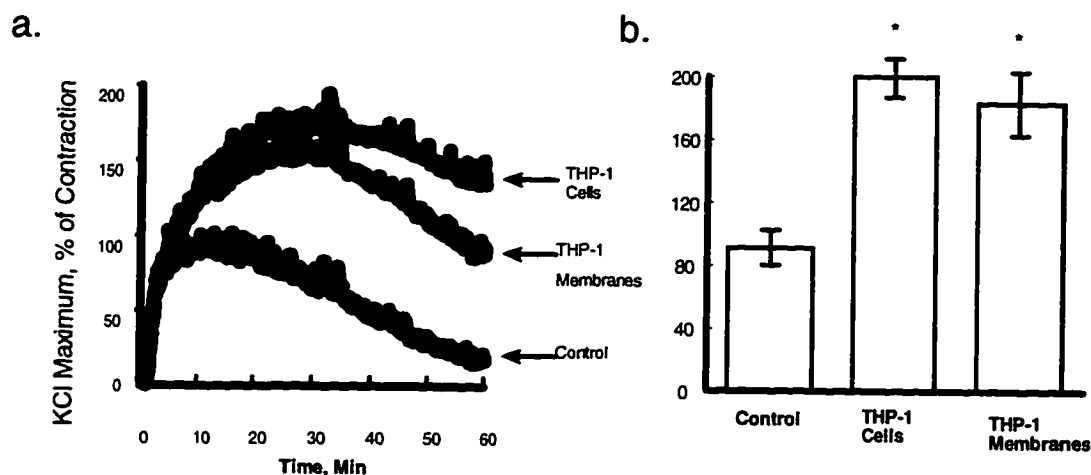


Figure 16: Effect of Monocytic Cells on Endothelin-1 Induced Contraction of Rat Aorta.

Panel A is a representative tracing of developed isometric tension to a 3 nM dose of ET-1. Aortic rings were normalized to their own maximal response to 100mM KCl and contraction was recorded for 60 min. Panel B is average maximal developed isometric tension to 3 nM ET-1. The present of intact THP-1 cells or THP-1 plasma membranes resulted in a twofold increase in contraction which is consistent with the THP-1 cells increase in ET-1 potency. The intact THP-1 cells and the THP-1 membranes were significantly different from the control with a p value < 0.001.

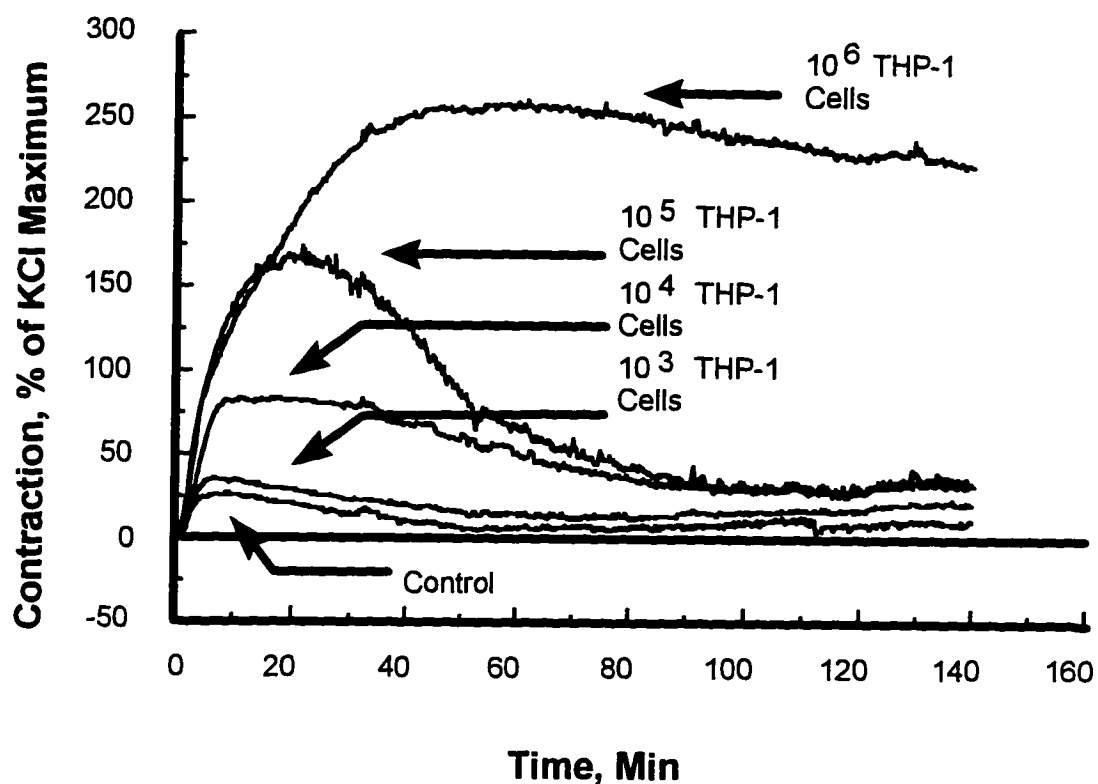


Figure 17: Effect of Monocytic Cell Number on Endothelin-1 Induced Contraction of Rat Aorta.

This representative tracing demonstrates the effect of THP-1 cells on augmenting vascular contraction to 3 nM ET-1. Aortic rings were normalized to their own 100 mM KCl maximum and developed isometric tension was recorded for 140 minutes.

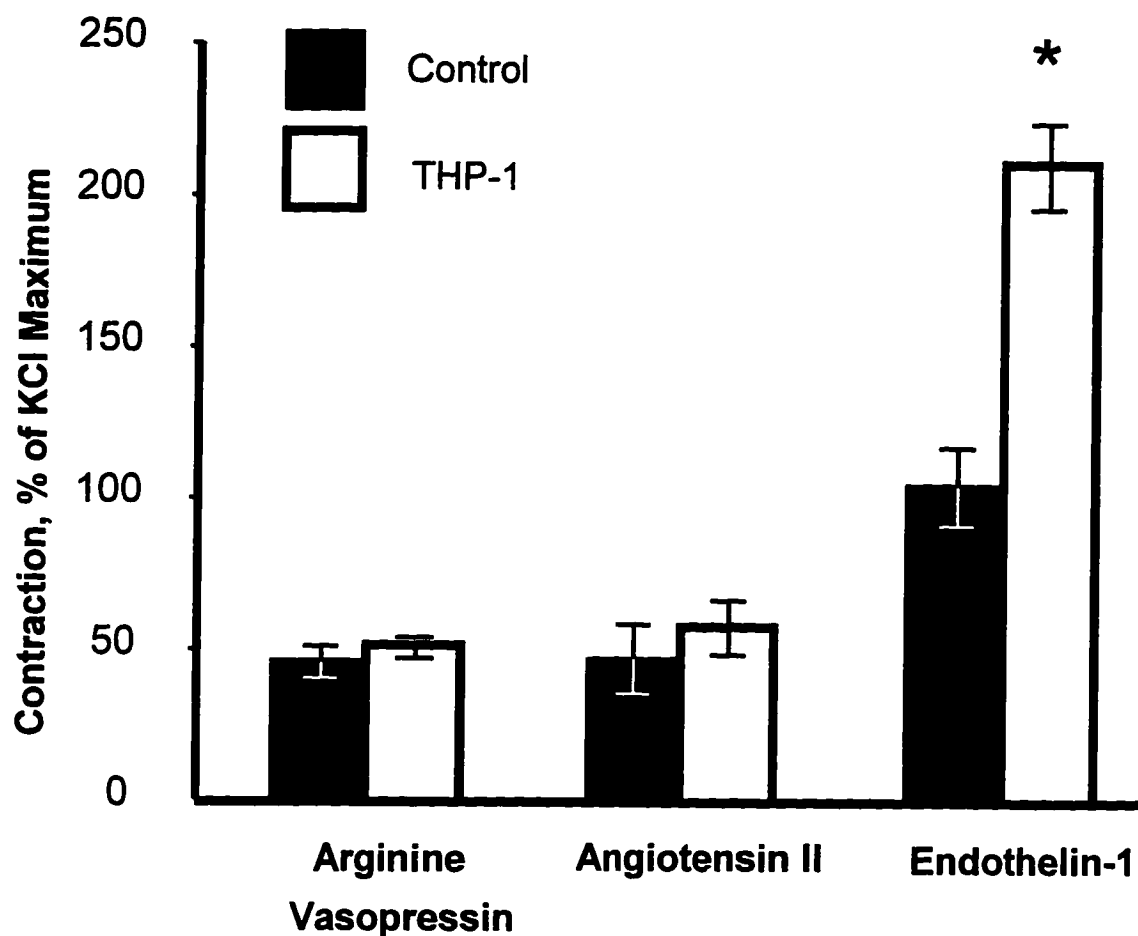


Figure 18: Effect of Monocytic Cells on Agonist Induced Contraction of Rat Aorta.

Average isometric tension was measured using known vasoconstrictors in the presence and absence of THP-1 cells. For each vasoconstrictor a EC_{50} was administered to aortic rings and maximal contraction was recorded. The data suggests that monocytes augment contraction specifically to ET-1.

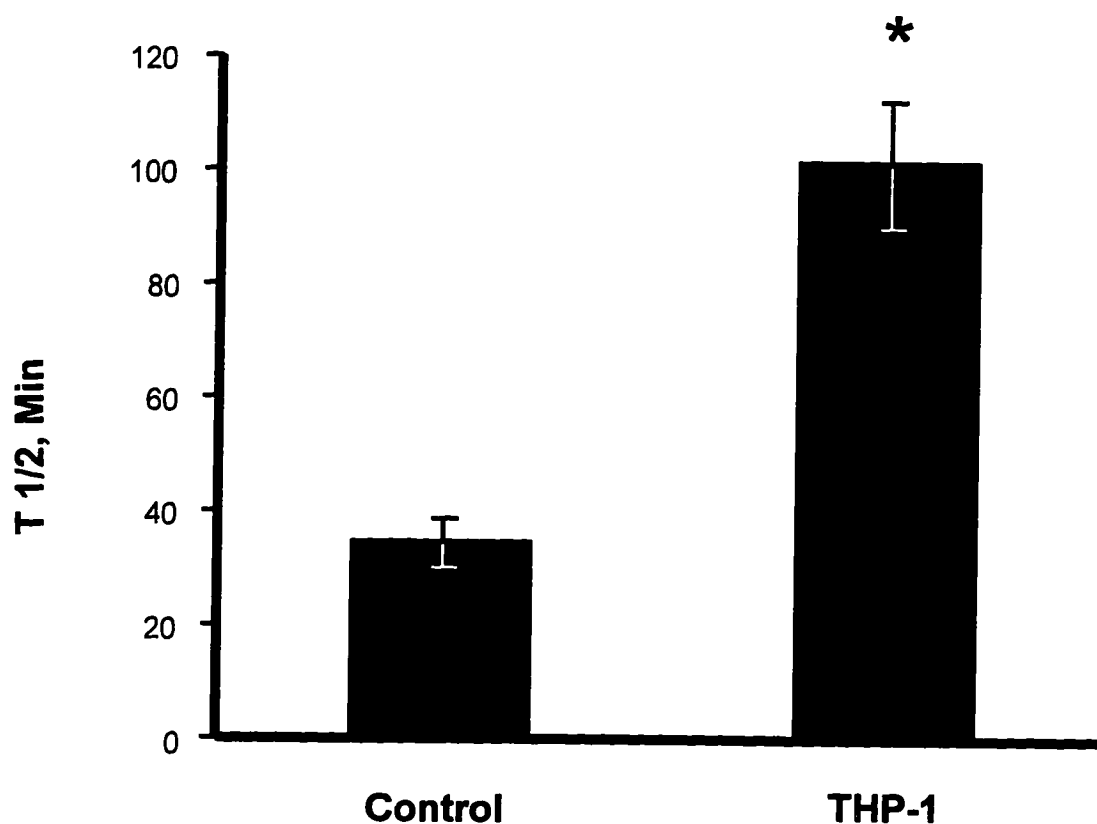


Figure 19: Relaxation of Rat Aorta following Contraction to Endothelin-1.

$T_{1/2}$ is a measure of the time required for 50% relaxation following maximal developed tension. THP-1 increased the $T_{1/2}$ more than twofold suggesting that monocytes are altering vascular tone. The monocytes don't appear to be inhibiting relaxation mechanisms rather increasing the maximal developed tension. The data was significant with a p value < 0.05

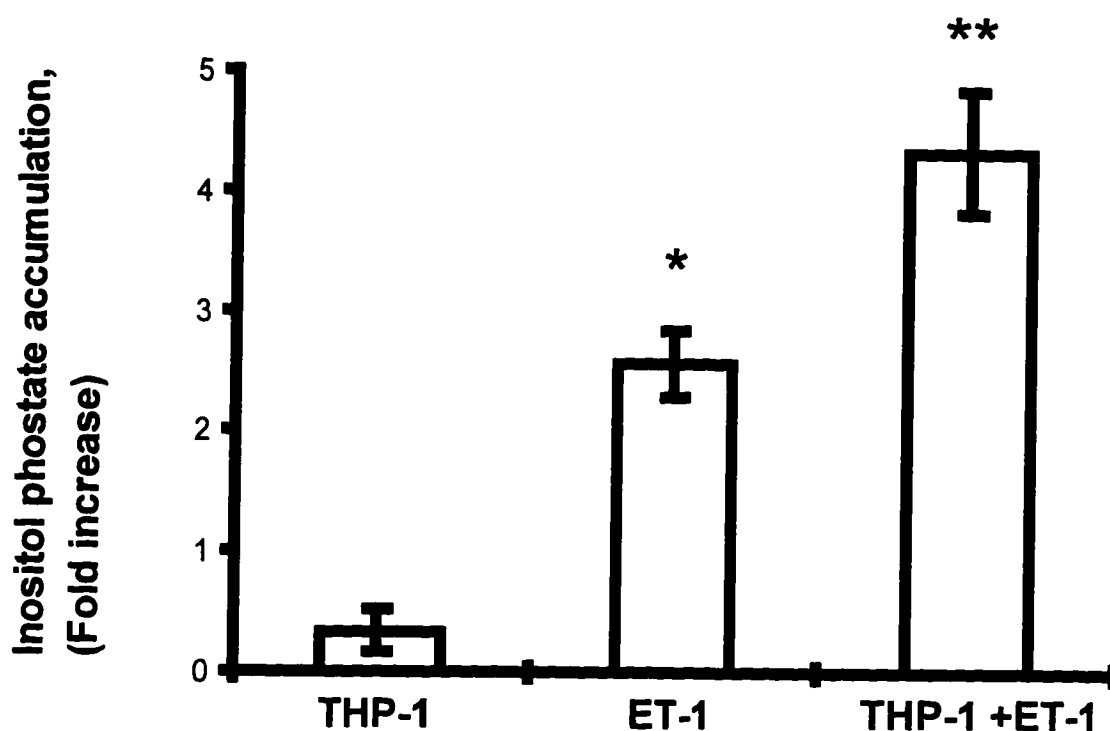


Figure 20: Stimulation of aortic smooth muscle by THP-1 cells.

Examination of IP₃ accumulation rat aorta to 10 nM ET-1 stimulation for 60 min in the absence and presence of 1×10^6 THP-1 cells. Data is reported as fold increase in IP₃ levels over unstimulated rat aorta, THP-1 cells alone did not stimulate IP₃ turnover. ET-1 stimulation resulted in a significant 2.5 fold increase ($P < 0.05$) in IP₃ levels and the addition of THP-1 cells significantly augmented IP₃ accumulation resulting in a 4 fold increase ($P < 0.05$). Samples were measured in quadruplicate $\pm$ SE.

CHAPTER FOUR:

EXAMINE MONOCYTE ACTIVATION STATE ON ET-1 COUPLED NO RELEASE.

OBJECTIVE: 96

METHODS: 98

RESULTS:..... 100

a. Examine the role of activation state on monocyte NO production..... 100

*b. Evaluate the effect of ET-1 stimulation on activated monocytes cellular
conformation..... 100*

*c. Examine the role of activation state on modulation of THP-1 cell adhesion
in response to ET-1. 101*

*d. Evaluation of ET-1 stimulation on immunocytes from the invertebrate
Mytilus edulis. 102*

CONCLUSIONS: 103

Chapter Four:

Examine Monocyte Activation State on ET-1 Coupled NO Release.

OBJECTIVE:

Monocyte recruitment to sites of vascular injury and subsequent activation may contribute to the pathogenesis of vascular disease. We have demonstrated that PBM adhesion is modulated by ET_B receptor coupled NO release. However, activation of monocytes with inflammatory cytokines or microbial products suppress ET_B receptor coupled NO release and subsequent monocyte inactivation. We examined iNOS and ecNOS expression in activated THP-1 cells using immunocytochemistry. We observed reciprocal regulation of the NOS isoforms upon monocyte activation; a marked increase in iNOS and a corresponding decrease in ecNOS expression was observed following activation. Monocyte activation with IFN + LPS enhanced THP-1 cell adhesion to human saphenous vein. In contrast to that observed in PBM and THP-1 cells, ET-1 stimulation failed to reduce adhesion of IFN γ activated THP-1 cells.

To further establish ET-1 as a immunomodulator we examined the effect of ET-1 stimulation on the immunocytes from an *invertebrate* *Mytilus edulis*. Using confocal microscopy we identified specific ET binding sites on the immunocytes and demonstrated a modulation of immunocyte behavior following ET-1 stimulation. The immunocytes adhered and spread rapidly when placed on a glass slide. However in the presence of ET-1 cell spreading was more rapid

and was followed by a retraction into a stellate conformation. The ability of both invertebrate and vertebrate immune cells to respond to ET-1 suggests that ET-1 may be part of a general regulatory mechanism of immune cell function.

METHODS:

- a. Blood Monocytes and Cell culture: as described in Chapter One.
- b. Monocyte activation: Monocytes were activated in RPMI-1640 supplemented with 10% FBS, 10 or 100 U/ml INF_γ , and 1 $\mu\text{g/ml}$ LPS at 37°C. Subsequently, monocytes were washed three times in PSS prior to use.
- c. Direct Measurement of NO Release: as described in Chapter Two.
- d. Immunohistochemical analysis of ecNOS and iNOS: Performed exactly as described in Chapter Two. Additionally human Anti-iNOS antibody was purchased from (Transduction Laboratories, KY).
- e. Evaluation of THP-1 cell conformation: as described in Chapter Three.
- f. Monocyte Adhesion Assay: THP-1 cells were labeled with 1 μM fluorescent dye Green CMFDA (5-chloromethylfluorescein diacetate) CellTracker™ (Molecular Probes, Or) at 37°C for 45 minutes. THP-1 cells were washed extensively with PSS by centrifugation. Prior to addition of THP-1 cells to strips of human saphenous vein, THP-1 cells were either activated for 1 hour with 10 U/ml INF_γ and 1 $\mu\text{g/ml}$ LPS at 37°C or untreated. THP-1 cells were subsequently treated in the absence and presence of 10 nM ET-1 for ten minutes and then washed with PSS. Saphenous vein strips (10mm) were placed luminal side up in petri dishes containing PSS. Fluorescently labeled THP-1 cells (5×10^5) were added to the saphenous vein for 30 minutes. The saphenous vein was washed 3 times in PSS and then fixed in 4% paraformaldehyde overnight at 4°C. Monocyte adhesion was evaluated by confocal imaging (Meridian Instruments, MI) of the luminal surface.

g. Invertebrate immunocyte preparation: The invertebrate species selected for use in these studies was a marine bivalve mollusk *Mytilus edulis* which were harvested from either Wading River or Queens College/Caumsett State Park shore area of Long Island Sound. Hemolymph was withdrawn from the extracellular space in the area of the posterior adductor muscle and diluted 1:1 (v/v) in Instant Ocean. Immunocytes present in hemolymph were placed on glass slides in the absence or presence of 100 nM ET-1. The receptor antagonist 1 μ M BQ-123 was added 10 min prior to ET-1 stimulation. Immunocytes cell conformation was analyzed as described in Chapter Three.

h. Confocal Imaging of invertebrate ET receptor: Immunocytes were fixed to glass slides using 1:1 methanol and acetone (v/v) for ten min at -20°C. Biotinylated ET-1 receptor labeling was performed as described in Chapter One.

RESULTS:

a. Examine the role of activation state on monocyte NO production.

Inflammatory cytokines and microbial products are capable of altering monocyte behavior. These molecular signals promote increases in adhesion receptors, enzymatic activity, and phagocytosis. We examined PBM NO release in response to 100 U/ml IFN γ and 1 μ g/ml LPS over a 24 hour time frame. There was an increase over baseline NO production within 3 hours and maximal NO production was obtained by 6 hours (Figure 21). The PBM produced NO for 24 hours without decrement indicating that NO release was mediated by iNOS rather than ecNOS (Figure 21). In addition THP-1 cells were stimulated with 10 U/ml IFN γ and 1 μ g/ml LPS for 24 hours and fixed to glass slides for immunocytochemical analysis of iNOS expression. Panel (A) depicts iNOS immunofluorescence of unstimulated THP-1 cells whereas Panel (B) represents iNOS immunofluorescence of activated THP-1 cells (Figure 24). Importantly, examination of ecNOS expression in activated THP-1 cells indicates that there is a marked decrease in ecNOS levels upon monocyte activation (Figure 24; panels C and D).

b. Evaluate the effect of ET-1 stimulation on activated monocytes cellular conformation.

We have demonstrated that PBM or THP-1 cells produce NO and rapidly alter their cellular conformation in response to ET-1 stimulation. To evaluate the role of ET-1 stimulation on activated THP-1 cells we examined cell conformation using phase contrast microscopy. In the untreated THP-1 cell preparations

examined, the level of spontaneously activated and ameoboid cells was in the range of 9 to 14 % of the total cell population when placed on fibronectin coated slides at 24°C whereas when treated with IFN γ and LPS there was a marked increase in THP-1 cell activation. THP-1 cells when allowed to adhere to the slide for 30 min became ameoboid as evidenced by a significant decrease in the (Figure 13a). Stimulation of the THP-1 cells with 100 nM ET-1 following a 15 min. period of adhesion to the slide resulted in a rapid rounding of THP cells (Figure 13b). THP-1 cells activated with 10 U/ml IFN γ and 1 μ g/ml LPS for 18 hours exhibited an ameoboid conformation (Figure 22a) and stimulation of activated THP-1 cells with 100 nM ET-1 was without effect as the cells retained their ameoboid conformation (Figure 22b).

c. Examine the role of activation state on modulation of THP-1 cell adhesion in response to ET-1.

THP-1 cells adhere to strips of human saphenous vein in a similar fashion as PBM. The presence of ameoboid cells following ET-1 stimulation of activated THP-1 cells suggested that monocyte activation alters ET $_B$ receptor signal transduction. We evaluated the role of IFN γ activation on THP-1 cell adhesion to strips of human saphenous vein in the absence and presence of 10 nM ET-1 prestimulation. THP-1 cells (5×10^5) were activated with 10 U/ml IFN γ and 1 μ g/ml LPS for one hour, then THP-1 cells were stimulated with 10 nM ET-1 for ten minutes followed by three washes in PSS before addition to the blood vessel. Significant levels of untreated (Figure 22a) and activated (Figure 22c) THP-1 cells adhered to segments of human saphenous vein. Stimulation of untreated

THP-1 cells modulated their adhesion (Figure 22b) in contrast to activated THP-1 cells in which ET-1 was without effect (Figure 22d). Consistent with $\text{IFN}\gamma$ activation, stimulation of THP-1 with 10 nM PMA for 10 min markedly enhanced adhesion to rings of at aorta (Table 6).

d. Evaluation of ET-1 stimulation on immunocytes from the invertebrate *Mytilus edulis*.

Studies have demonstrated that several bioactive neuropeptides have similar functions in both vertebrates and invertebrates. We examined the effect of ET-1 stimulation on immunocyte cell conformation. Analysis of biotinylated ET-1 binding suggested that *Mytilus edulis* immunocytes bind ET-1 in a specific manner (Figure 25). ET-1 Immunocyte cell behavior was markedly altered in the presence of 100 nM ET-1. Immunocyte cell conformation when initially placed on a glass slide was $\text{FF} = 0.87 \pm 0.03$ and after 40 min the immunocytes spread $\text{FF} = 0.58 \pm 0.04$ (Figure 26)(Figure 27a). In contrast, 1 nM ET-1 stimulation resulted in a decrease in form factor from 0.87 ± 0.02 to 0.28 ± 0.04 in 40 min. ET-1 induced immunocytes to adopt a stellate conformation with moderately ramified pseudopodia (Figure 26)(Figure 27b). In order to evaluate the marked ET-1 induced conformational changes we measured changes in immunocyte area over a 40 min period to 1 nM ET-1. Immunocytes rapidly spread with 1 nM ET-1 stimulation to $240 \pm 20 \text{ mm}^2$ at 20 min and had retracted to $134 \pm 17 \text{ mm}^2$ by 40 min (Figure 28). In contrast 1 nM ET-1 stimulation was inhibited by BQ-123, cellular area increased from $85 \pm 13 \text{ mm}^2$ at 20 min to 236

$\pm 18 \text{ mm}^2$ at 40 min (Figure 28) which is consistent with unstimulated immunocytes.

CONCLUSIONS:

An initial event in the development of an inflammatory response is monocyte activation. The molecular signals which regulate monocyte activation promote an increase in adhesion receptors, elevation of enzyme levels producing a respiratory burst and increase production of cytokines and growth factors which control the inflammatory response and promote tissue repair. We have demonstrated that ET-1 coupled NO produces conformational changes in cell behavior which modulates PBM adhesion to vascular tissue. Monocyte activation results in expression of iNOS and abrogation of ecNOS levels. Our data indicates that an increase in activation state interferes with the ability of ET-1 to modulate monocyte adhesion.

Monocytes are an important component of an individual's innate immunity. The ability of PBM to respond to both blood borne and intracellular parasites in a timely fashion is critical for host survival. After the preliminary identification of the insult monocytes are rapidly induced to express high levels of iNOS. Analysis of PBM NO production following cytokine activation demonstrated significant elevation in NO within three hours and maximal continual production for twenty four hours. The NO generated by iNOS is used for killing through the production of the free radical peroxynitrite. The profile of NO release coupled to iNOS is markedly different than ecNOS. The activation of iNOS results in tonic activation of the enzyme due to the relative calcium insensitivity of the iNOS isoform whereas ecNOS is dependent on extracellular calcium transience for activation.

Examination of activation state on monocyte conformation and adhesion indicated that an increased percentage of cells become ameboid increasing their ability to adhere to exposed extracellular matrix on blood vessels. Although the activated monocytes are continually producing NO through the induction of iNOS, ET-1 stimulation did not alter monocyte conformation or adhesion. These data together with the absolute requirement for extracellular calcium indicate that ET-1 coupled NO release is functionally coupled to ecNOS activation and increases activation state result in insensitivity to ET-1 stimulation.

Examination of ET-1 binding to immunocytes of the invertebrate *Mytilus edulis* has provided an interesting opportunity to evaluate ET-1 as a molecule with neuroimmunological ramifications. The existence of specific ET-1 binding sites on this invertebrate and its ability to markedly alter immunocyte conformation distinguishes ET-1 as an essential effector molecules which regulates immune cell function. The immunocyte response to ET-1 was extremely pronounced causing rapid spreading followed by retraction into a stellate conformation. We have also demonstrated that the pedal ganglia release ET-1 as demonstrated by enzyme immunoassay (not shown) suggesting that ET-1 may activate microglia contributing to their ramified conformation.

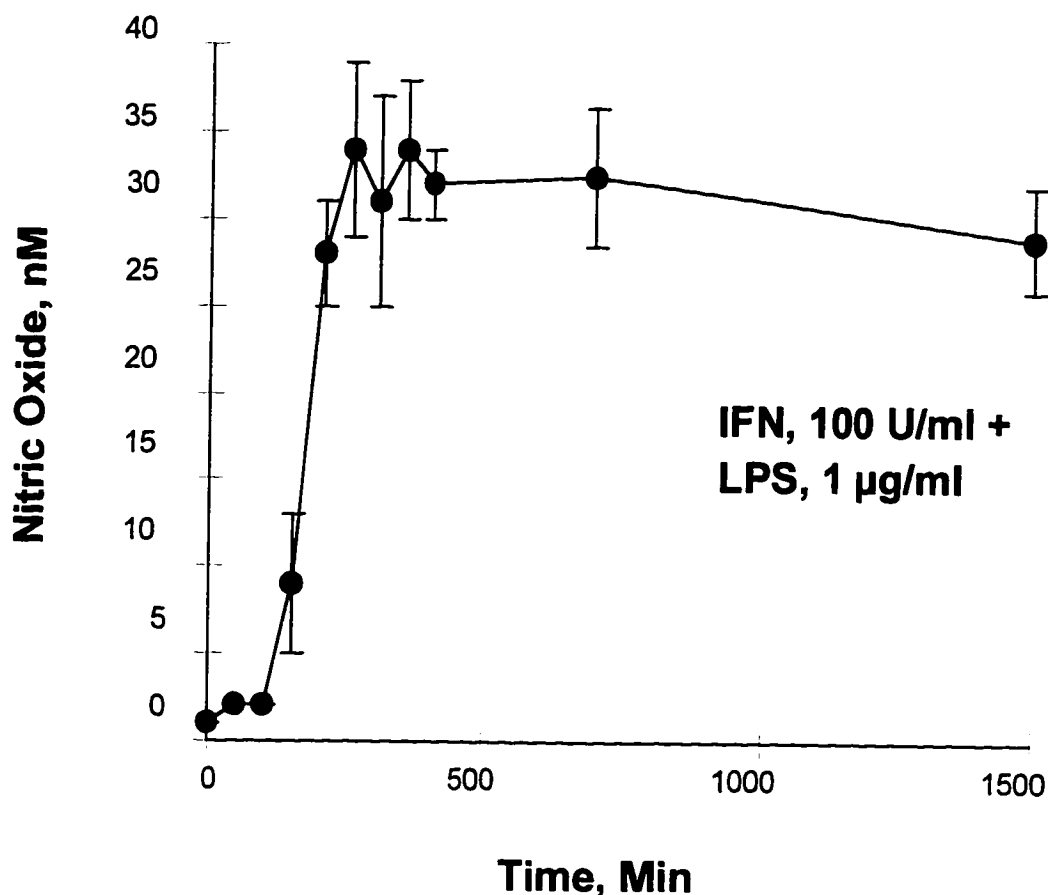


Figure 21: iNOS-Mediated Nitric Oxide Release from human monocytes.

Examination of NO release in human PBM (5×10^5) following cytokine activation. There was a marked increase in NO release within 4 hours after activation and NO release remained elevated after induction. Independence of extracellular calcium results in a tonic state of activation and is characteristic of iNOS activation. Samples were measured in triplicate $\pm$ SE.

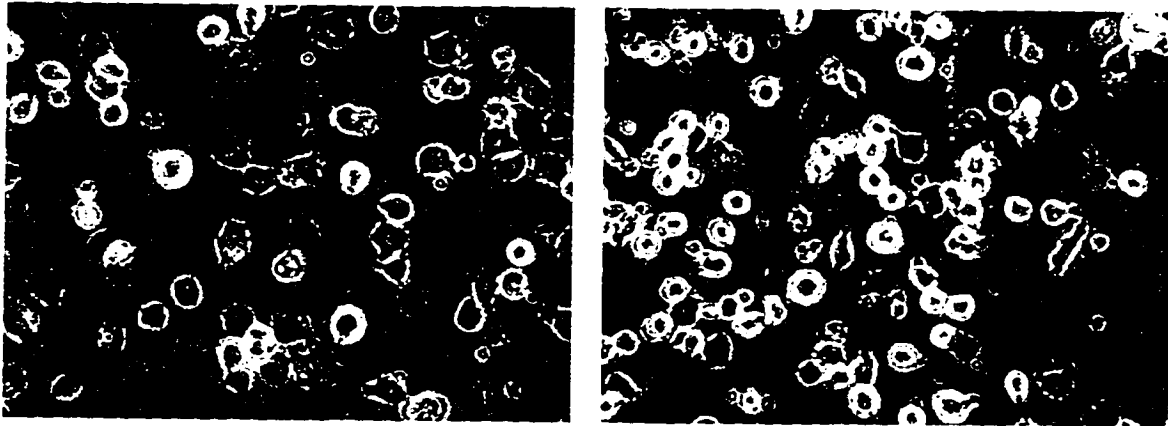


Figure 22: Monocyte cell conformation in the presence and absence of $\text{IFN}\gamma$.

Phase Contrast images (400X) of THP-1 cells following 30 min. adhesion to fibronectin coated slides. THP-1 cells have been activated with 10 U/ml $\text{IFN}\gamma$ and 1 $\mu\text{g/ml}$ LPS for 1 hour at 37°C. Vehicle or 100 nM ET-1 was added after 15 min, Panel A. Vehicle, Panel B. 100 nM ET-1.

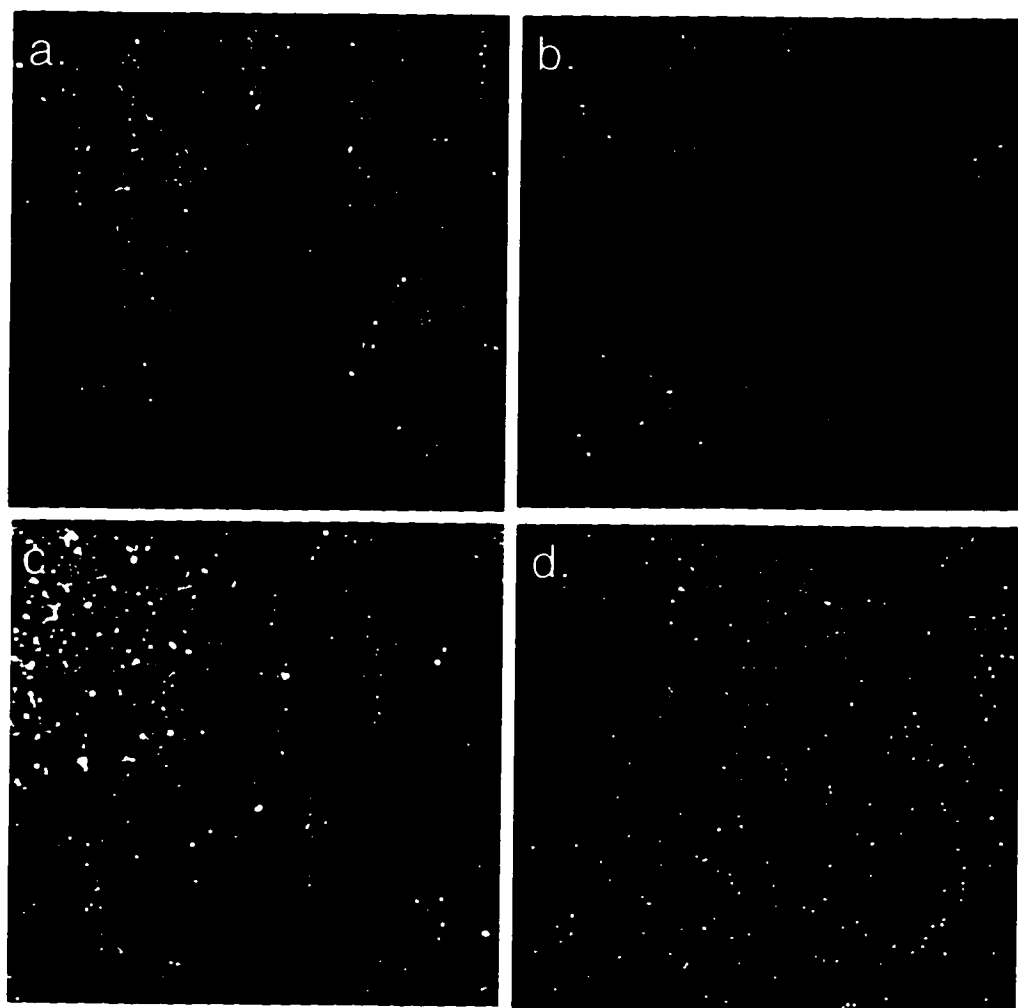


Figure 23: The effect of cytokine activation on monocyte adhesion to human saphenous vein.

THP-1 cell fluorescence on the luminal surface of human saphenous vein are shown; a $2000\ \mu\text{m}^2$ area was scanned by confocal microscopy. Fluorescence represents sites of THP-1 cell adhesion. The panels A and B are resting THP-1 cells whereas panels C and D the THP-1 cells were activated with 10 U/ml IFN γ and 1 $\mu\text{g}/\text{ml}$ LPS for 1 hour prior to adhesion assay. The panels represent: A and C, Untreated; B and D, 10 min pretreatment of THP-1 cells with 10 nM ET-1.

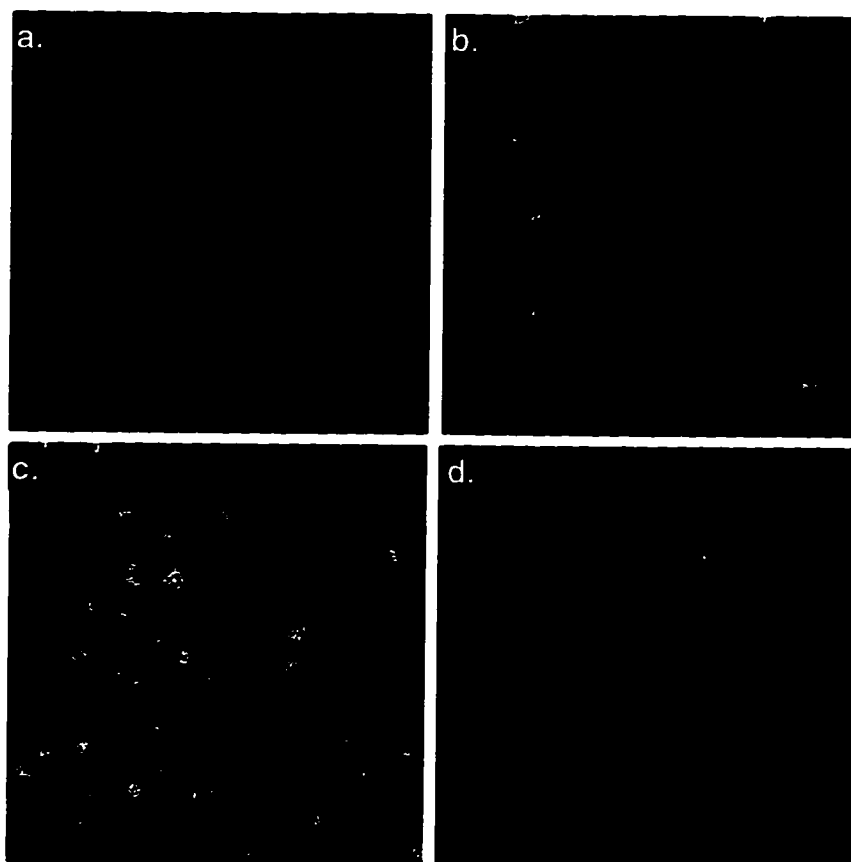


Figure 24: Immunocytochemistry of cytokine stimulated THP-1 NOS expression

THP-1 cells either activated or resting were stained for the presence of either ecNOS or iNOS. Panel A and B. was stained for iNOS expression whereas panels C and D were stained for ecNOS expression. Panels A and C are resting THP-1 cells whereas panels B and D have been activated with 10 U/ml IFN γ and 1 μ g/ml LPS for 1 hour prior to fixation. Subsequently, slides were treated with rhodamine conjugated Anti mouse IgG and fluorescence was detected by confocal microscopy.

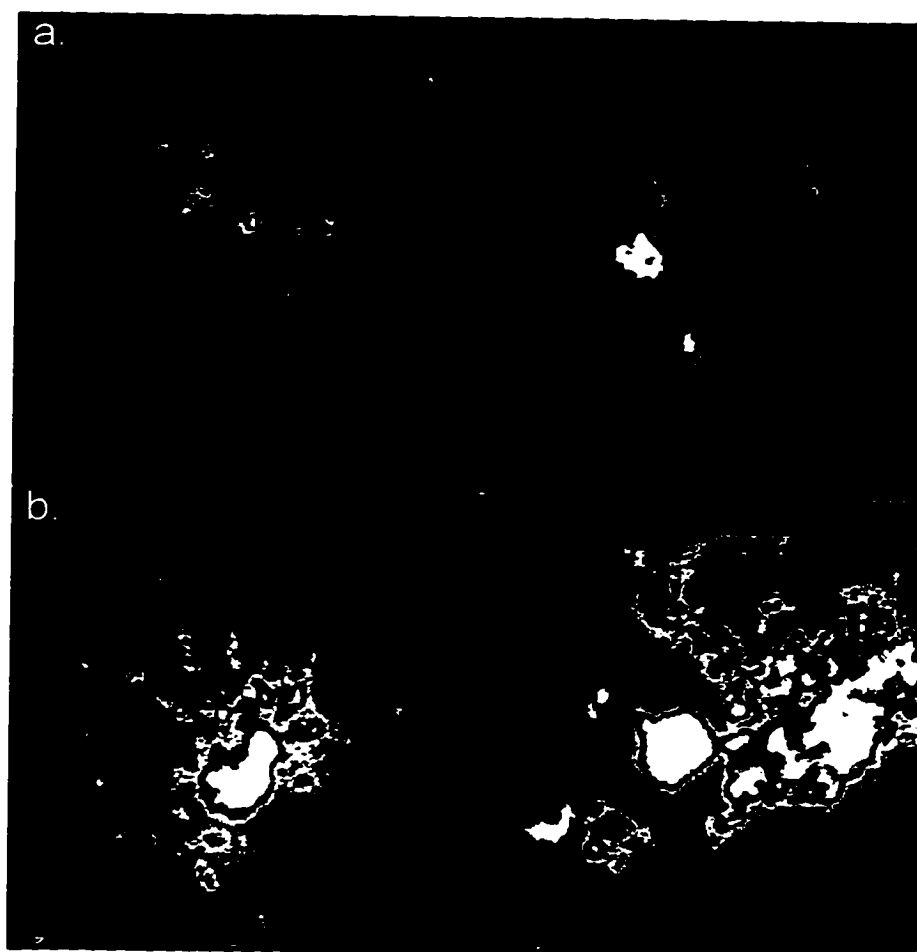


Figure 25: Confocal image of invertebrate immunocyte labeled with biotinylated ET-1.

Fluorescent confocal images (1000X) of invertebrate immunocytes fixed to glass slides. Immunocytes were treated with 300 pM biotinylated ET-1 in the absence (Panel B) and presence of 30 nM ET-1 (Panel A). Binding of biotinylated ET-1 was markedly abrogated by the addition of 100 fold excess unlabeled ET-1 suggesting the existence of specific ET-1 binding sites on immunocytes.

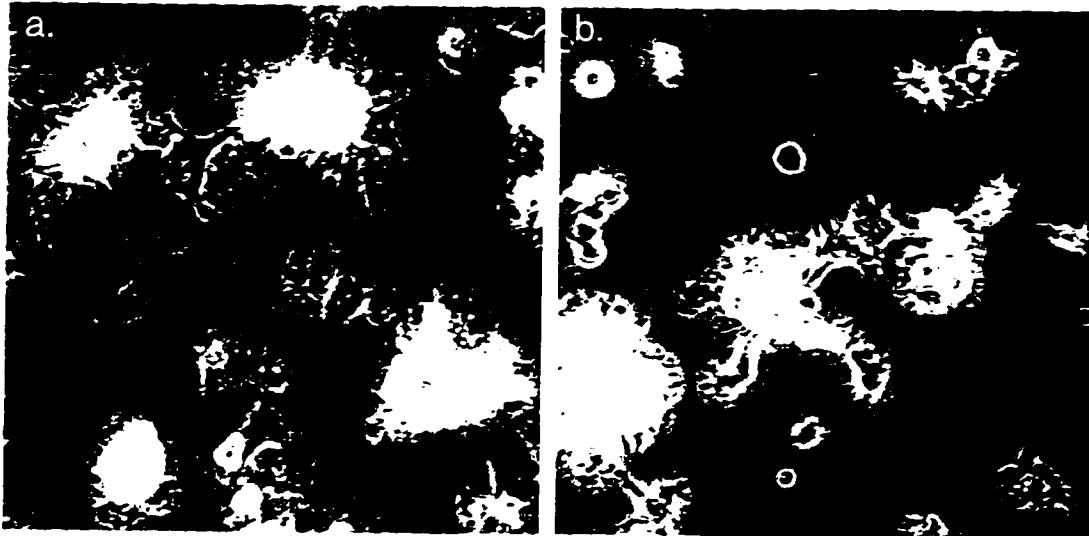


Figure 26: Invertebrate immunocyte response to ET-1 stimulation.

Phase contrast images (400X) of invertebrate immunocytes. Immunocytes when placed on a glass slide rapidly spread and obtain an ameboid conformation as demonstrated (Panel A). Stimulation with 1 nM ET-1 enhances immunocyte spreading and markedly alters their conformation (Panel B).

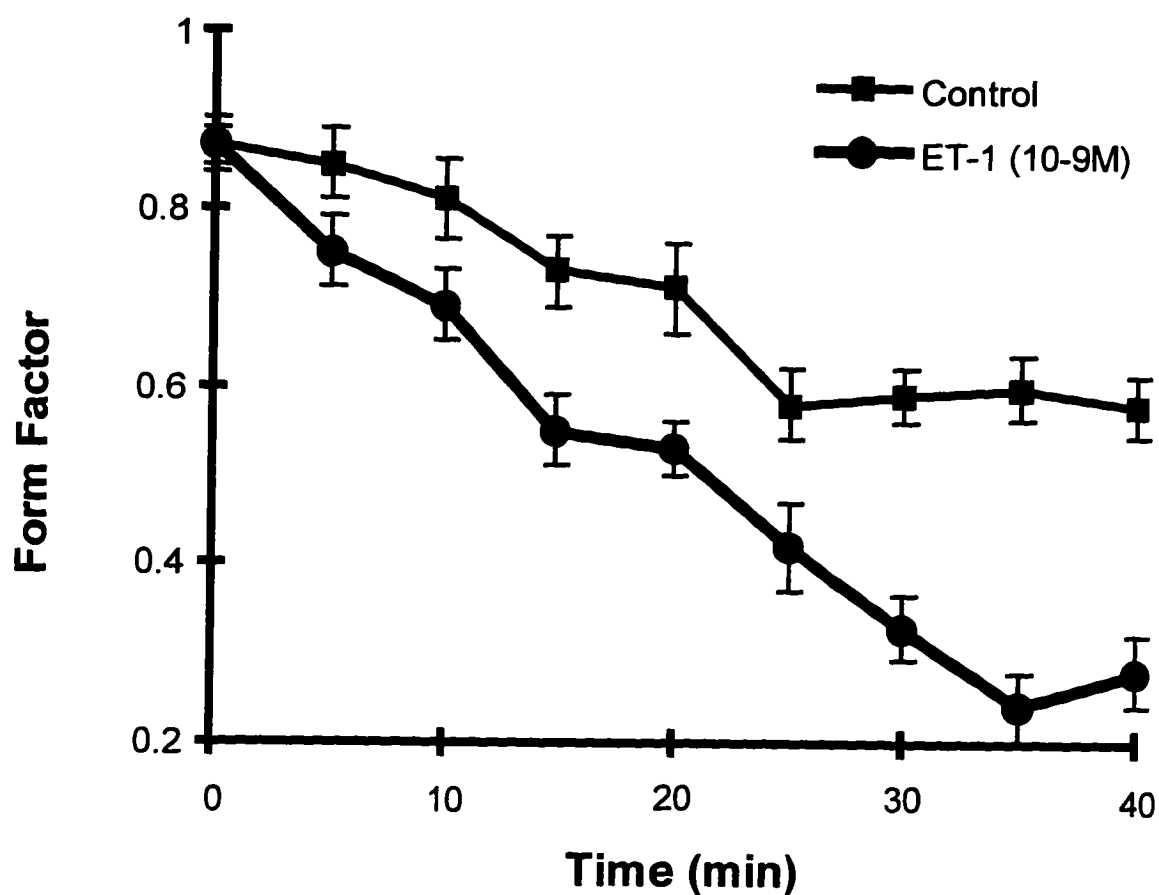


Figure 27: Image analysis of immunocyte response to ET-1.

Invertebrate immunocytes when placed on a glass slide rapidly spread and obtain an ameboid conformation as indicated by a decrease in FF. Stimulation with 1 nM ET-1 enhances immunocyte spreading and markedly alters their conformation resulting in a stellate immunocyte conformation.

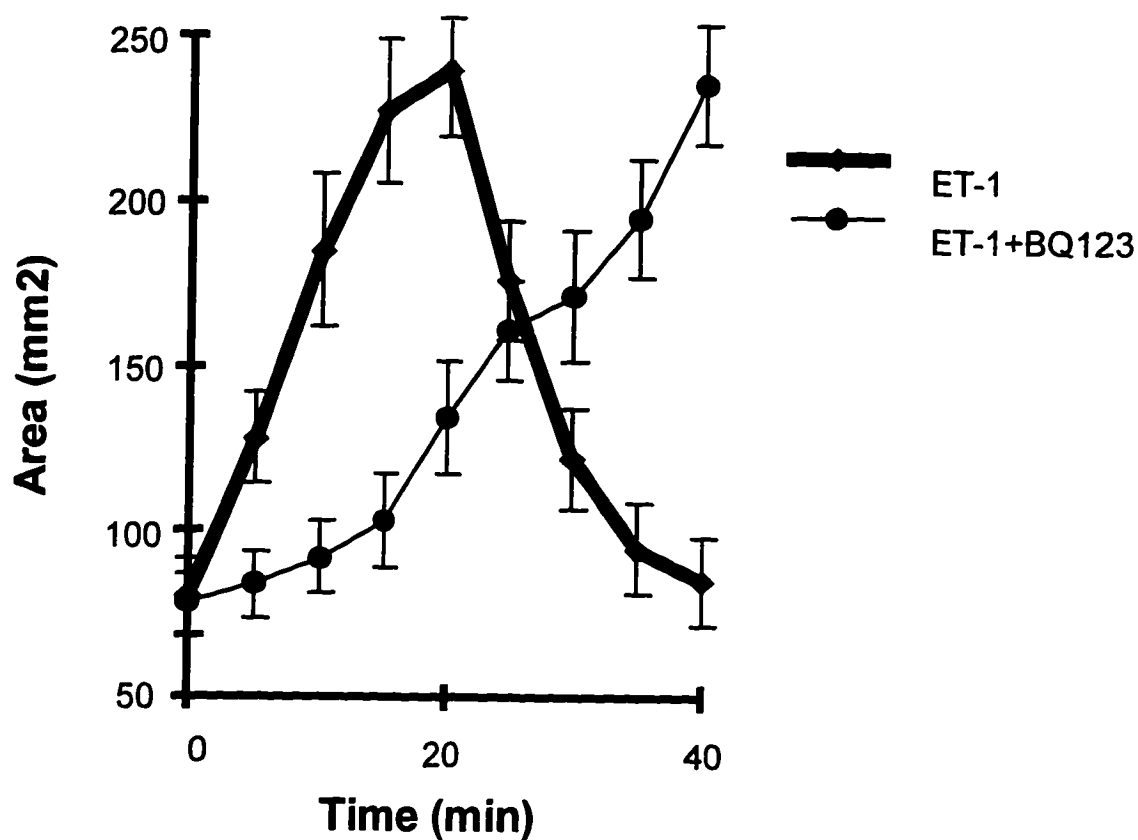


Figure 28: Image analysis of immunocyte response to ET-1.

Examination of immunocyte spreading on glass slides was performed by measuring immunocyte area for 40 min. ET-1 stimulation results in rapid spreading followed by retraction into a stellate conformation. ET-1 stimulation was inhibited by pretreatment with BQ-123 restoring immunocyte spreading to levels consistent with controls.

Reiteration:

A marked increase in circulating ET-1 is observed following injury (Luscher, T.F. 1992; Cernacek, P. et al. 1989; Lerman, A. et al. 1991) or during progression of diseases such as metastatic cancer (Ishibashi, M. et al. 1994) that compromise the integrity of vascular wall. We have demonstrated that PBM produce NO through an ET_B receptor-dependent mechanism. This suggests that ET_B receptor-coupled NO release by PBM may provide a mechanism for autoregulation of adhesion that may attenuate cellular interactions in the absence of functional endothelium. These data when taken together with our recent demonstration of rapid release of ET-1 within seconds of thrombin receptor activation (Magazine, H.I. et al. 1996a; Magazine, H.I. et al. 1996c) suggests that functional interactions between the thrombin and ET_B receptor may provide important mechanisms for regulation of cellular responses at sites of vascular injury (Figure 29).

Pathological conditions which activate PBM in conjunction with elevated plasma ET-1 levels may desensitize PBM ET_B receptors resulting in an augmented monocyte adhesiveness. In addition, NO release has been reported to be impaired in patients with a variety of vascular disorders. Therefore, at sites of vascular injury which promotes increase expression of PBM adhesion molecules the absence of ET-1 coupled NO will result in elevated PBM adhesion. We have demonstrated that monocyte adhesion markedly alters vascular reactivity to ET-1 and may contribute to the pathogenesis of vascular disease by autocrine production of ET-1, release of vasoactive factors

influencing local hemodynamics and stimulation of mitogenesis contributing to plaque formation and vasospasm (Figure 30).

ET-1 stimulation produces rapid and profound changes in inflammatory cell behavior. We have demonstrated that immunocytes from the invertebrate *Mytilus edulis* bind and respond to ET-1 stimulation. This observation suggests that ET-1 may be an essential effector molecules which regulates inflammatory cell behavior.

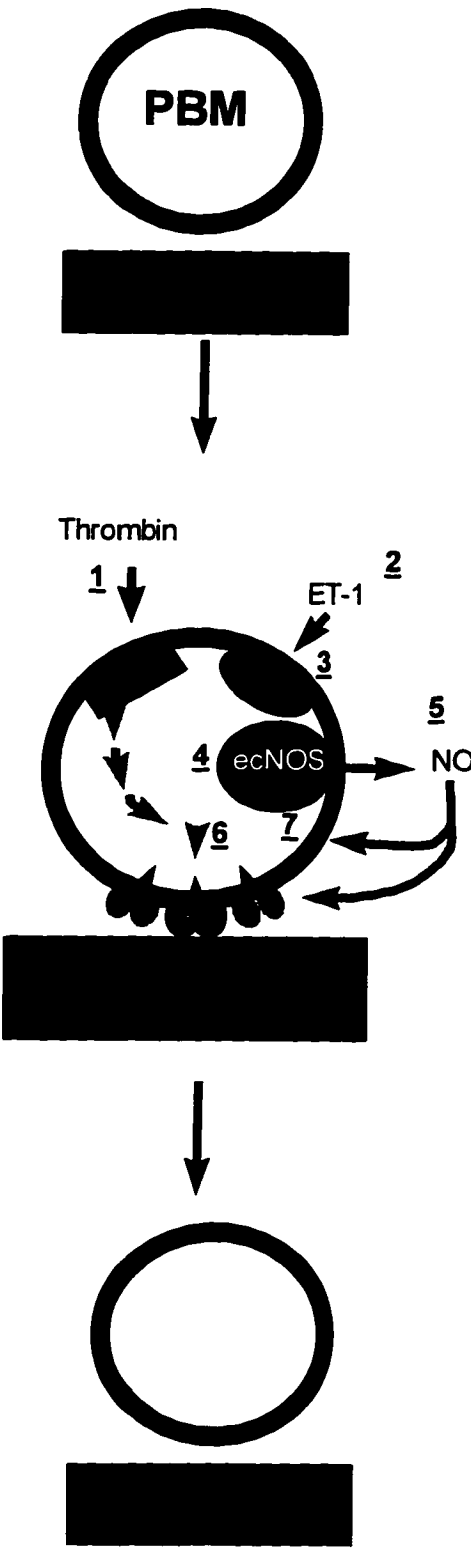


Figure 29: Proposed Mechanism of Modulation of Adhesion in Monocytes by ET-1 coupled NO release.

Representative scenario involving vascular injury and subsequent PBM accumulation. At sites of vascular injury thrombin (1) promotes PBM chemotaxis and adhesion. Typically ET-1 (2) concentration increase resulting in ET_B receptor (3) activation coupled to ecNOS (4), which produces a short burst of NO (5). PBM adhesion is modulated by NO (5) induced decreased expression of adhesion receptors (6) and conformational changes (7) in PBM. The resulting conformational changes (7) inhibit PBM adhesion to the vessel wall.

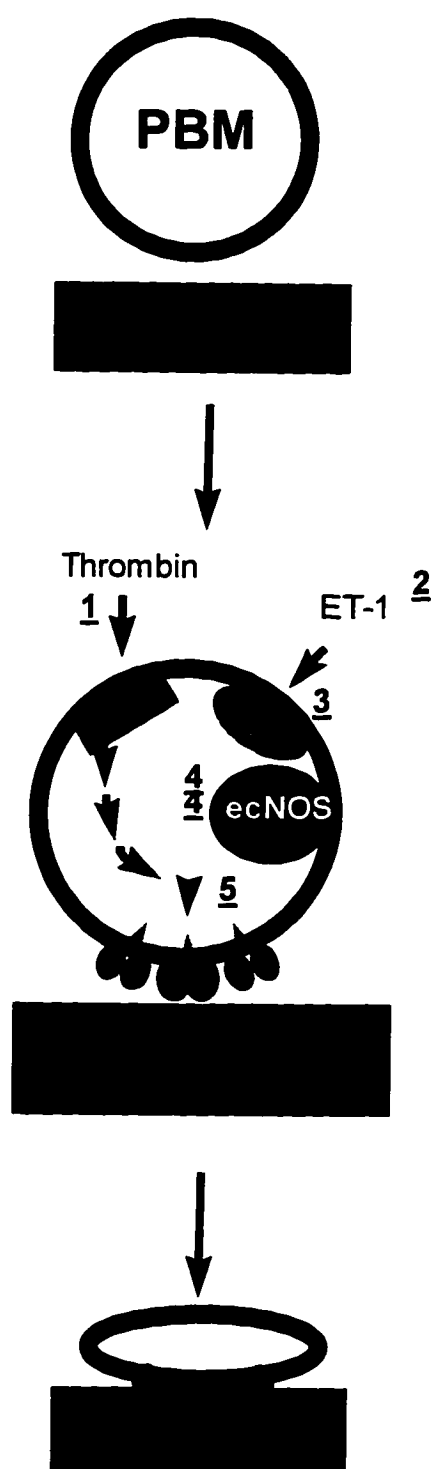


Figure 30: Proposed mechanism of monocyte accumulation during episodes of disease.

Many vascular disease states are connected to impaired NO production or elevated plasma ET-1 concentration. Unregulated PBM adhesion may elicit detrimental effects to the vascular wall. Injury produces thrombin (1) which results in PBM accumulation and adhesion. Elevated ET-1 (2) concentration has caused the PBM ET_B receptor to become tachyphlactic (3) and unresponsive to further ET-1 stimulation, importantly ecNOS (4) is impaired due to the functional coupling of ET_B receptor to ecNOS activation. PBM become adherent (5) and contribute to damaging the vascular wall.

Bibliography:

Abu-Soud, H.M., & Stuehr, D.J. (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc Natl Acad Sci U S A*, 90, 10769-10772.

Alberts, G.F., Peifley, K.A., Johns, A., Kleha, J.F., & Winkles, J.A. (1994). Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. *Journal of Biological Chemistry*, 269, 10112-10118.

Altieri, D.C. (1995). Proteases and protease receptors in modulation of leukocyte effector functions. *J Leukoc Biol*, 58, 120-127.

Anderson, C.L. (1982). Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. *J Exp Med*, 156, 1794-1806.

Anggard, E.E. (1990). The endothelium—the body's largest endocrine gland? *J Endocrinol*, 127, 371-375.

Aquilla, E., Whelchel, A., Knot, H.J., Nelson, M., & Posada, J. (1996). Activation of multiple mitogen-activated protein kinase signal transduction pathways by the endothelin B receptor requires the cytoplasmic tail. *Journal of Biological Chemistry*, 271, 31572-31579.

Aramori, I., & Nakanishi, S. (1992). Coupling of two endothelin receptor subtypes to different signal transduction in transfected Chinese hamster ovary cells. *Journal of Biological Chemistry*, 267, 12468-12474.

Arendt, R.M., Lampen, U.W., Heucke, L., Suhler, K., Ritter, M., & Richter, W.O. (1990). Ir-ET circulates in human plasma: Elevated concentrations in patients with hyperlipoproteinemia. *Am J Hypertens*, 3, 93A

Auger, M.J., & Ross, J.A. (1992). The biology of the macrophage. In C.E. Lewis & J.O. McGee (Eds.), *The macrophage*. (pp. 1-57). New York: Oxford University Press.

Bang, A., Speck, E.R., Blanchette, V.S., Freedman, J., & Semple, J.W. (1996). Recipient humoral immunity against leukoreduced allogeneic platelets is suppressed by aminoguanidine, a selective inhibitor of inducible nitric oxide synthase. *Blood*, 88, 2959-2966.

Bar-Shavit, R., Hruska, K.A., Kahn, A.J., & Wilner, G.D. (1986). Hormone-like activity of human thrombin. *Ann N Y Acad Sci*, 485, 335-348.

- Bar-Shavit, R., Kahn, A., Fenton, J.W., & Wilner, G.D. (1983). Chemotactic response of monocytes to thrombin. *J Cell Biol*, 96, 282-285.
- Bar-Shavit, R., Kahn, A., Wilner, G.D., & Fenton, J.W. (1983). Monocyte chemotaxis: stimulation by specific exosite region in thrombin. *Science*, 220, 728-731.
- Bastian, N.R., & Hibbs, J.B.J. (1994). Assembly and regulation of NADPH oxidase and nitric oxide synthase. *Curr Opin Immunol*, 6, 131-139.
- Bath, P.M.W., Hassal, D.J., Galdwin, A.M., Palmer, R.M.J., & Martin, J.F. (1991). Nitric oxide and prostacyclin: Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler Thromb*, 11, 254-260.
- Battistini, B., Chailier, P., D'Orleans-Juste, P., Breie, N., & Sirois, P. (1993). Growth Regulatory Properties of Endothelins. *Peptides*, 14, 385-399.
- Bauvois, B., Rouillard, D., Sanceau, J., & Wietzerbin, J. (1992). IFN-gamma and transforming growth factor-beta 1 differently regulate fibronectin and laminin receptors of human differentiating monocytic cells. *J Immunol*, 148, 3912-3919.
- Beretz, A., Klein-Soyer, C., Archipoff, G., Camilla, C., Brisson, C., & Freyssinet, J.M. (1990). Modulation by cytokines of leukocyte-endothelial cell interactions. Implications for thrombosis. *Biorheology*, 27, 455-60.
- Berridge, M.J. (1993). Inositol triphosphate and calcium signalling. *Nature*, 361, 315-325.
- Bian, K., & Bukoski, R.D. (1995). Modulation of resistance artery force generation by extracellular calcium. *Am J Physiol*, 269, H230-238.
- Bilfinger, T.V., Hartman, A.R., Liu, Y., Magazine, H.I., & Stefano, G.B. (1996). Myocardial revascularization with cryopreserved veins: A 5-year experience and a possible mechanism for their failure. *Ann Thoracic Surg*, (In Press).
- Billiar, T.R., Curran, R.D., Harbrecht, B.G., Stadler, J., Williams, D.L., Ochoa, J.B., Di Silvio, M., Simmons, R.L., & Murray, S.A. (1992). Association between synthesis and release of cGMP and nitric oxide biosynthesis by hepatocytes. *Am J Physiol*, 262, C1077-C1082.
- Billiar, T.R., Curran, R.D., Stuehr, D.J., West, M.A., & Bentz, B.G. (1989). An L-arginine dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis. *J Exp Med*, 169, 1467-1471.

Bluhm, R.E., Frazer, M.G., Vore, M., Pinson, C.W., & Badr, K.F. (1993). Endothelins 1 and 3: potent cholestatic agents secreted and excreted by the liver that interact with cyclosporine. *Hepatology*, 18, 961-968.

Bounancin, F., Audigier, Y., & Chabre, M. (1993). ADP-ribosylation of Gs by cholera toxin is potentiated by agonist activation of beta-adrenergic receptors in the absence of GTP. *Journal of Biological Chemistry*, 268, 17026-17029.

Cambrey, A.D., Harrison, N.K., Dawes, K.E., Southcott, A.M., Black, C.M., du Bois, R.M., Laurent, G.J., & McAnulty, R.J. (1994). Increased levels of endothelin-1 in bronchoalveolar lavage fluid from patients with systemic sclerosis contribute to fibroblast mitogenic activity in vitro. *Am J Respir Cell Mol Biol*, 11, 439-445.

Carlos, T.M., & Harlan, J.M. (1990). Membrane proteins involved in phagocyte adherence to endothelium. *Immunol.Rev.* 114, 5-27.

Cavender, D.E., Edelbaum, D., & Welkovich, L. (1991). Effects of inflammatory cytokines and phorbol esters on the adhesion of U937 cells, a human monocyte line. *Journal of Leukocyte Biology*, 49, 566-578.

Cernacek, P., & Stewart, D.J. (1989). Immunoreactive endothelin in human plasma: marked elevations in patients in cardiogenic shock. *Biochemical and Biophysical Research Communications*, 161, 562-567.

Chanez, P., Vignola, A.M., Albat, B., Springall, D.R., Polak, J.M., & Godard, P. (1996). Involvement of endothelin in mononuclear phagocyte inflammation in asthma. *J Allergy Clin Immunol*, 98, 412-420.

Cho, H.J., Xie, Q., & Calaycay, J. (1992). Calmodulin is a subunit of nitric oxide synthetase from macrophages. *J Exp Med*, 176, 599-604.

Chun, M., Liyanage, U.K., Lisanti, M.P., & Lodish, H.F. (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A*, 91, 11728-11732.

Clancy, R., Leszczynska, J., Amin, A., Levartovsky, D., & Abramson, S.B. (1995). Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *J Leukoc Biol*, 58, 196-202.

Clark, E.A., & Brugge, J.S. (1995). Integrins and signal transduction pathways: The road taken. *Science*, 268, 233-239.

- Cleland, J.G., & Oakley C.M. (1991). Vascular tone in heart failure: the neuroendocrine-therapeutic interface. *Br Heart J*, 66(4), 264-7.
- Clozel, M., Loffler, B.M., Breu, V., Hilfiger, L., Maire, J.P., & Butscha, B. (1993). Downregulation of endothelin receptors by autocrine production of endothelin-1. *Am J Physiol*, 265, C188-C192.
- Cody, W.L., Doherty, A.M., He, X.Q., Rapundalo, S.T., Hingorani, G.P., Panek, R.L., & Major, T.C. (1991). Monocyclic endothelins: examination of the importance of the individual disulfide rings. *J Cardiovasc Pharmacol*, 17 Suppl 7, S62-S64.
- Cohen, R.A. (1995). The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease. *Prog Cardiovasc Dis*, 38, 105-128.
- Conkling, P.R., Greenberg, C.S., & Weinberg, J.B. (1988). Tumor necrosis factor induces tissue factor-like activity in human leukemia cell line U937 and peripheral blood monocytes. *Blood*, 72, 128-133.
- Corbett, J.A., Lancaster, J.R.J., Sweetland, M.A., & McDaniel, M.L. (1991). Interleukin-1- β -induced formation of EPR-detectable iron-nitrosyl complexes in islets of Langerhans: role of nitric oxide in interleukin-1- β -induced inhibition of insulin secretion. *Journal of Biological Chemistry*, 266, 21351-21354.
- Cyr, C., & Kris, R.M. (1993). Desensitization of the human endothelin A receptor. *Journal of Cardiovascular Pharmacology*, 22, S11-14.
- D'Angelo, G., & Meininger, G.A. (1994). Transduction mechanisms involved in the regulation of myogenic activity. *Hypertension*, 23, 1096-1105.
- Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., & Stewart, T.A. (1993). Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science*, 259, 1739-1742.
- Davies, M.G., & Hagen, P. (1993). The Vascular Endothelium: A New Horizon. *Ann Surg*, 218, 593-609.
- De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A., Jr., Shin, W.S., & Liao, J.K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest*, 96, 60-68.
- Denis, M. (1994). Human monocytes/macrophages: NO or no NO? *Journal of Leukocyte Biology*, 55, 682-684.

- Eguchi, S., Hirata, Y., Imai, T., & Marumo, F. (1993). Endothelin receptor subtypes are coupled to adenylate cyclase via different guanyl nucleotide-binding proteins in vasculature. *Endocrinology*, 132, 524-529.
- Ehrenreich, H., Anderson, R.W., Fox, C.H., Rieckmann, P., Hoffman, G.S., Travis, W.D., Coligan, J.E., Kehrl, J.H., & Fauci, A.S. (1990). Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. *J Exp Med*, 172, 1741-1748.
- Ehrenreich, H., Kehrl, J.H., Anderson, R.W., Rieckmann, P., Vitkovic, L., Coligan, J.E., & Fauci, A.S. (1991). A vasoactive peptide, endothelin-3, is produced by and specifically binds to primary astrocytes. *Brain Res*, 538, 54-58.
- Eid, H., de Bold, D.L., Chen, J.H., & de Bold, A.J. (1994). Epicardial mesothelial cells synthesize and release endothelin. *J Cardiovasc Pharmacol*, 24, 715-720.
- Elshourbagy, N.A., Korman, D.R., Wu, H., Sylvester, D.R., Lee, J.A., Nuthalaganti, P., Bergsma, D.J., Kumar, C.S., & Nambi, P. (1993). Molecular characterization and regulation of the human endothelin receptors. *Journal of Biological Chemistry*, 268, 3873-3879.
- Feletou, M., & Vanhoutte, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol*, 93, 515-524.
- Feng, Q., & Hedner, T. (1990). Endothelium-derived relaxing factor (EDRF) and nitric oxide(NO), II. Physiology, pharmacology and pathophysiological implications. *Clinical Physiology*, 10, 503-526.
- Finder, J., Stark, W.W.J., Nakayama, D.K., Geller, D., Wasserloos, K., Pitt, B.R., & Davies, P. (1995). TGF- β regulates production of NO in pulmonary artery smooth muscle cells by inhibiting expression of NOS. *Am J Physiol*, 268, L862-L867.
- Fisker, S., Kudahl, K., & Sonne, O. (1992). In vivo inflammatory stimulation induces a transient change in the binding of thrombin to rat peritoneal macrophages. *Exp Cell Res*, 201, 145-153.
- Fujitani, Y., Ninomiya, H., Okada, T., Urade, Y., & Masaki, T. (1995). Suppression of endothelin-1 induced mitogenic responses of human aortic smooth muscle cells by interleukin-1 beta. *J Clin Invest*, 95, 2474-2482.
- Fukuroda, T., Fujikawa, T., Ozaki, S., Ishikawa, K., Yano, M., & Nishikibe, M. (1994). Clearance of Circulating Endothelin-1 by ETB Receptors in Rats. *Biochemical and Biophysical Research Communications*, 199, 1461-1465.

Garside, P., Hutton, A.K., Severn, A., Liew, F.Y., & Mowat, A.M. (1992). Nitric oxide mediates intestinal pathology in graft-vs-host disease. *Eur J Immunol*, 22, 2141-2145.

Gifford, R.W.J. (1993). The role of multiple risk factors in cardiovascular morbidity and mortality. *Cleve Clin J Med*, 60, 211-218.

Glassberg, M.K., Ergul, A., Wanner, A., & Puett, D. (1994). Endothelin-1 promotes mitogenesis in airway smooth muscle cells. *Am J Respir Cell Mol Biol*, 10, 316-321.

Goodnough, L.T., & Saito, H. (1982). Specific binding of thrombin by human peripheral blood monocytes: possible role in the clearance of activated clotting factors from the circulation. *J Lab Clin Med*, 99, 873-884.

Gordon, S., Clarke, S., Greaves, D., & Doyle, A. (1995). Molecular immunobiology of macrophages: recent progress. *Curr Opin Immunol*, 7, 24-33.

Gross, S.S., & Levi, R. (1992). Tetrahydrobiopterin synthesis: An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *Journal of Biological Chemistry*, 267, 25722-25729.

Haak, T., Jungmann, E., Hillmann, U., Felber, A., & Usadel, K.H. (1993). [Sie of increased endothelin level for development of sequelae of diabetes mellitus.]. *Med Klin*, 88, 291-296.

Hajjar, D.P., & Pomerantz, K.B. (1992). Signal transduction in atherosclerosis: integration of cytokines and the eicosanoid network. *The FASEB Journal*, 6, 2933-2941.

Harbrecht, B.G., Billiar, T.R., Stadler, J., Demetris, A.J., Ochoa, J., Curran, R.D., & Simmons, R.L. (1992). Inhibition of nitric oxide sythesis during endotoxemia promotes intrahepatic thrombosis and an oxygen radical-mediated hepatic injury. *Journal of Leukocyte Biology*, 52, 390-394.

Harrison, V.J., Barnes, K., Turner, A.J., Wood, E., Corder, R., & Vane, J.R. (1995). Identification of endothelin 1 and big endothelin 1 in secretory vesicles isolated from bovine aortic endothelial cells. *Proceedings of the National Academy of Science*, 92, 6344-6348.

Hasegawa, K., & Kobayashi, H. (1991). Immunohistochemical localization of endothelin-1 in the nervus system of the earthworm *Eisenia foetida*. *General and Comparative Endocrinology*, 81, 433-441.

- Hathaway, D.R., March, K.L., Lash, J.A., Adam, L.P., & Wilensky, R.L. (1992). Vascular smooth muscle: A review of the molecular basis of contractility. *Circulation*, 83, 382-389.
- Hay, D.W. (1992). Pharmacological evidence for distinct endothelin receptors in guinea-pig bronchus and aorta. *Br J Pharmacol*, 106, 759-761.
- Hecker, M., Sessa, W.C., & Harris, H.J. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci U S A*, 87, 8612-8616.
- Hemsen, A., & Lundberg, J.M. (1991). Presence of endothelin-1 and endothelin-3 in peripheral tissues and central nervous system of the pig. *Regul Pept*, 36, 71-83.
- Hepler, J.R., & Gilman, A.G. (1992). G proteins. *Trends in Biochemical Science*, 17, 383-387.
- Hexum, T.D., Hoeger, C., Rivier, J.E., Baird, A., & Brown, M.R. (1990). Characterization of Endothelin Secretion by Vascular Endothelial Cells. *Biochemical and Biophysical Research Communications*, 167, 294-300.
- Hirata, Y., Emori, T., Eguchi, S., Kanno, K., Imai, T., Ohta, K., & Marumo, F. (1993). Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *Journal of Clinical Investigation*, 91, 1367-1370.
- Hoffman, R.A., Langrhr, J.M., Billiar, T.R., Curran, R.D., & Simmons, R.L. (1990). Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *The Journal of Immunology*, 145, 2220-2226.
- Horie, K., Morita, A., & Yokogoshi, H. (1995). Endothelin-1 and endothelin-3 modulate dopaminergic neurons through different mechanisms. *Life Sciences*, 57, 735-741.
- Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilek, J., Zinkernagel, R.M., & Aguet, M. (1993). Immune response in mice that lack the interferon-gamma receptor. *Science*, 259, 1742-1745.
- Imokawa, G., Yada, Y., & Miyagishi, M. (1992). Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. *J Biol Chem*, 267, 24675-24680.
- Inagaki, H., Bishop, A.E., Yura, J., & Polak, J.M. (1991). Localization of endothelin-1 and its binding sites to the nervous system of the human colon. *J Cardiovasc Pharmacol*, 17, S455-457.

- Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K., & Masaki, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*, 86, 2863-2867.
- Irons, C.E., Flynn, M.A., Mok, L.M., & Reynolds, E.E. (1996). Endothelin and PDGF enhance arachidonic acid release and DNA synthesis in vascular smooth muscle cells. *Am J Physiol*, 270, C1642-C1646.
- Ishibashi, M., Ito, N., Fujita, M., Furue, H., & Yamaji, T. (1994). Endothelin-1 as an aggravating factor of disseminated intravascular coagulation associated with malignant neoplasms. *Cancer*, 73, 191-195.
- Ishikawa, K., Ihara, M., Noguchi, K., Mase, T., Mino, N., Saeki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., Nishikibe, M., & Yano, M. (1994). Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proceedings of the National Academy of Science*, 91, 4892-4896.
- Jiang, H., & Stephens, N.L. (1994). Calcium and smooth muscle contraction. *Mol. Cell Biochem.* 135, 1-9.
- Joossens, J.V. (1980). Trends in cardiovascular mortality. *Int Congr Ser*, 530, 12-36.
- Jouneaux, C., Mallat, A., Serradeil-Le, G.C., Goldsmith, P., Hanoune, J., & Lotersztajn, S. (1994). Coupling of endothelin B receptors to the calcium pump and phospholipase C via Gs and Gq in rat liver. *Journal of Biological Chemistry*, 269, 1845-1851.
- Karaki, H., Sudjarwo, S.A., Hori, M., Takai, M., Urade, Y., & Okada, T. (1993). Induction of endothelium-dependent relaxation in the rat aorta by IRL 1620, a novel and selective agonist at the endothelin ETB receptor. *Br J Pharmacol*, 109, 486-490.
- Kasinath, B.S., Fried, T.A., Davalath, S., & Marsden, P.A. (1992). Glomerular epithelial cells synthesize endothelin peptides. *Am J Pathol*, 141, 279-283.
- Kilborn, R.G., Jubran, A., & Gross, S.S. (1991). Reversal of endotoxin mediated shock by NG-methyl-L-arginine, and inhibitor of nitric oxide synthetase. *Biochemical and Biophysical Research Communications*, 172, 1132-1138.
- King, J.M., Srivastava, K.D., Bahou, W.F., Stefano, G.B., & Magazine, H.I. (1997). Human monocyte adhesion is modulated by endothelin B receptor coupled nitric oxide release. *The Journal of Immunology*, 158, 880-886.

- Kishino, J., Hanasaki, K., Kato, T., & Arita, H. (1991). Endothelin-induced intracellular Ca^{2+} mobilization through its specific receptors in murine peritoneal macrophages. *FEBS Lett*, 280, 103-106.
- Kohno, M., Yasunari, K., Murakawa, K., Yokokawa, K., Horio, T., Fukui, T., & Takeda, T. (1990). Release of immunoreactive endothelin from porcine aortic strips. *Hypertension*, 15, 718-723.
- Kohno, M., Horio, T., Yokokawa, K., Yasunari, K., Kurihara, N., & Takeda, T. (1994). Endothelin modulates the mitogenic effect of PDGF on glomerular mesangial cells. *Am J Physiol*, 266, F894-F900.
- Koller, A., Sun, D., Messina, E.J., & Kaley, G. (1993). L-Arginine analogues blunt prostaglandin-related dilation of arterioles. *Am J Physiol*, 264, 1194-1199.
- Krystek, S.R.J., Bassolino, D.A., Novotny, J., Chen, C., Marschner, T.M., & Andersen, N.H. (1991). Conformation of endothelin in aqueous ethylene glycol determined by ^1H -NMR and molecular dynamics simulations. *FEBS*, 281, 212-218.
- Kubrina, L.N., Mikoyan, V.D., Mordvintcev, P.I., & Vanin, A.F. (1993). Iron potentiates bacterial lipopolysaccharide-induced nitric oxide formation in animal organs. *Biochim Biophys Acta*, 1176, 240-244.
- Kuchan, M.J., & Frangos, J.A. (1993). Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *American Journal of Physiology*, 264, H150-H156.
- Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., & Karlsson, S. (1993). Transforming growth factor β 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A*, 90, 770-774.
- Ladenheim, R.G., Lacroix, I., Foignant-Chaverot, N., Strosberg, A.D., & Couraud, P.O. (1993). Endothelins stimulate c-fos and nerve growth factor expression in astrocytes and astrocytoma. *J Neurochem*, 60, 260-266.
- LaDouceur, D.M., Flynn, M.A., Keiser, J.A., Reynolds, E., & Haleen, S.J. (1993). ETA and ETB receptors coexist on rabbit pulmonary vascular smooth muscle mediating contraction. *Biochemical and Biophysical Research Communications*, 196, 209-215.
- Langrehr, J.M., White, D.A., Hoffman, R.A., & Simmons, R.L. (1993). Macrophages produce nitric oxide at allograft sites. *Ann Surg*, 218, 159-166.

- Laporte, L., D'Orleans-Juste, P., & Sirois, P. (1996). Guinea pig Clara cells secrete endothelin-1 through a phosphoramidon-sensitive pathway. *Am J Respir Cell Mol Biol*, 14, 356-362.
- Larsen, G.R., Sako, D., Ahern, T.J., Shaffer, M., Erban, J., Sajer, S.A., Gibson, R.M., Wagner, D.D., Furie, B.C., & Furie, B. (1992). P-selectin and E-selectin. *Journal of Biological Chemistry*, 267, 11104-11110.
- Lerman, A., Edwards, B.S., Hallett, J.W., Heublein, D.M., Sandberg, S.M., & Burnett, J.C., Jr. (1991). Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med*, 325, 997-1001.
- Lin, H.Y., Kaji, E.H., Winkel, G.K., Ives, H.E., & Lodish, H.F. (1991). Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proceedings of the National Academy of Science*, 88, 3185-3189.
- Lin, L., & Nasjletti, A. (1991). Role of endothelium-derived prostanoid in angiotensin-induced vasoconstriction. *Hypertension*, 18, 158-164.
- Lisanti, M.P., Scherer, P.E., Tang, Z., & Sargiacomo, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends in Cell Biology*, 4, 231-235.
- Liu, Y., Magazine, H.I., King, J.M., & Stefano, G.B. (1996). Endothelins as neuroregulatory signal molecules. *Animal Biology*, 1, 25-28.
- Locksley, R.M., & Fearon, D.T. (1996). The instructive role of innate immunity in the acquired immune response. *Science*, 272, 50-53.
- Lopez-Farre, A., Caramelo, C., Esteban, A., Alberola, M.L., Millas, I., Monton, M., & Casado, S. (1995). Effects of aspirin on platelet-neutrophil interactions. Role of nitric oxide and endothelin-1. *Circulation*, 91, 2080-2088.
- Lukic, M.L., Stosic-Grujicic, S., Ostojic, N., Chan, W.L., & Liew, F.Y. (1991). Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. *Biochemical and Biophysical Research Communications*, 178, 913-920.
- Luscher, T.F. (1992). Endothelin: systemic arterial and pulmonary effects of a new peptide with potent biologic properties. *Am Rev Respir Dis*, 146, S56-S60.
- Luscher, T.F. (1993). The endothelium as a target and mediator of cardiovascular disease. *Eur J Clin Invest*, 23, 670-685.
- Lyons, C.R. (1995). The role of nitric oxide in inflammation. *Advances in Immunology*, 60, 323-371.

Lyons, C.R., Orloff, G.J., & Cunningham, J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem*, 267(9), 6370-6374.

Maemura, K., Kurihara, H., Morita, T., Oh-hashii, Y., & Yazaki, Y. (1992). Production of endothelin-1 in vascular endothelial cells is regulated by factors associated with vascular injury. *Gerontology*, 38 Suppl 1, 29-35.

Magazine, H.I. (1995a). Detection of endothelial cell-derived nitric oxide: Current trends and future directions. *Adv Neuroimmunol*, 5, 479-490.

Magazine, H.I., Andersen, T.T., Goligorsky, M.S., & Malik, A.B. (1991). Evaluation of endothelin receptor populations using endothelin-1 biotinylated at lysine-9 sidechain. *Biochemical and Biophysical Research Communications*, 181, 1245-1250.

Magazine, H.I., Bruner, C.A., Andersen, T.T., & Malik, A.B. (1994). Vascular contractile potency of endothelin-1 is increased in the presence of monocytes or macrophages. *American Journal of Physiology*, 266, H1620-H1625.

Magazine, H.I., Butt, O., & Yaghoutiel, H.R. (1996a). Endothelin-1 and nitric oxide release modulates contraction of rat aorta to selected thrombin receptor agonists. *American Journal of Physiology*, 270, C1814-C1818.

Magazine, H.I., King, J.M., & Srivastava, K.D. (1996b). Protease activated receptors modulate aortic vascular tone. *International Journal of Cardiology*, 53, S75-S80.

Magazine, H.I., Liu, Y., Bilfinger, T.V., & Stefano, G.B. (1995b). Morphine-induced conformational changes in immunocytes, endothelial cells and microglia are mediated by nitric oxide. *The Journal of Immunology*, 56, 4845-4850.

Magazine, H.I., & Srivastava, K.D. (1996c). Thrombin-induced vascular reactivity is modulated by ETB receptor-coupled nitric oxide release in rat aorta. *American Journal of Physiology*, 271, C923-C928.

Makman, M.H., Bilfinger, T.V., & Stefano, G.B. (1995). Human granulocytes contain an opiate alkaloid-selective receptor mediating inhibition of cytokine-induced activation and chemotaxis. *J Immunol*, 154, 1323-1330.

Malinski, T., & Taha, Z. (1992). Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature*, 358, 676-678.

Mantovani, A., Bussolino, F., & Dejana, E. (1992). Cytokine regulation of endothelial cell function. *The FASEB Journal*, 6, 2591-2599.

- Masaki, T., Kimura, S., & Yanagisawa, M. (1991). Molecular and cellular mechanism of endothelin regulation. Implications for vascular function. *Circulation*, 84, 1457-1468.
- McMillen, M.A., Huribal, M., Cunningham, M.E., Kumar, R., & Sumpio, B.E. (1995). Endothelin-1 increases intracellular calcium in human monocytes and causes production of interleukin-6. *Crit Care Med*, 23(1), 34-40.
- Miles, A.M., Owens, M.W., Milligan, S., Johnson, G.G., Fields, J.Z., Ing, T.S., Kottapalli, V., Keshavarzian, A., & Grisham, M.B. (1995). Nitric oxide synthase in circulating vs. extravasated polymorphonuclear leukocytes. *J Leukoc Biol*, 58, 616-622.
- Millul, V., Lagente, V., Gillaudeaux, O., Boichot, E., Dugas, B., Mencia-Huerta, J.M., Bereziat, G., Braquet, P., & Masliah, J. (1991). Activation of guinea pig alveolar macrophages by endothelin-1. *J Cardiovasc Pharmacol*, 17 Suppl 7, S233-S235.
- Mirza, H., Yatsula, V., & Bahou, W.F. (1996). The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells: molecular characterization and evidence for functional coupling to the thrombin receptor. *Journal of Clinical Investigation*, 97, 1705-1714.
- Moncada, S., & Higgs, A. (1993). The L-arginine nitric oxide pathway. *N Engl J Med*, 329, 2002-2012.
- Moncada, S., Palmer, R.M.J., & Higgs, E.A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, 43, 109-141.
- Moncada, S., & Vane, J.R. (1979). Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A₂ and prostacyclin. *Pharmacological Reviews*, 30, 293-331.
- Morano, I.L. (1992). Molecular biology of smooth muscle. *Hypertension*, 10, 411-416.
- Morita, K., Ihnken, K., Buckberg, G.D., Sherman, M.P., & Ignarro, L.J. (1996). Pulmonary vasoconstriction due to impaired nitric oxide production after cardiopulmonary bypass. *Ann Thoracic Surg*, 61, 1775-1780.
- Muller, W.A., Weigl, S.A., Deng, X., & Phillips, D.M. (1993). PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med*, 178, 449-460.
- Murphy, R.A. (1994). What is special about smooth muscle? The significance of covalent crossbridge regulation. *FASEB J*, 8, 311-318.

Nakajima, K., Kubo, S., Kumagaye, S., Nishio, H., Tsunemi, M., Inui, T., Kuroda, H., Chino, N., Watanabe, T.X., Kimura, T., & et al. (1989). Structure-activity relationship of endothelin: importance of charged groups. *Biochemical and Biophysical Research Communications*, 163, 424-429.

Parums, D.V. (1992). Macrophages in cardiovascular disease. In C.E. Lewis & J.O. McGee (Eds.), *The macrophage*. (pp. 359-385). New York: Oxford University Press.

Patel, K.V., & Schrey, M.P. (1995). Human breast cancer cells contain a phosphoramidon-sensitive metalloproteinase which can process exogenous big endothelin-1 to endothelin-1: a proposed mitogen for human breast fibroblasts. *Br J Cancer*, 71, 442-447.

Pribnow, D., Muldoon, L.L., Fajardo, M., Theodor, L., Chen, L.Y., & Magun, B.E. (1992). Endothelin induces transcription of fos/jun family genes: a prominent role for calcium ion. *Mol Endocrinol*, 6, 1003-1012.

Provost, P., Lam, J.Y., Lacoste, L., Merhi, Y., & Waters, D. (1994). Endothelium-derived nitric oxide attenuates neutrophil adhesion to endothelium under arterial flow conditions. *Arterioscler Thromb*, 14, 331-335.

Rapoport, R.M., Stauderman, K.A., & Highsmith, R.F. (1990). Effects of EDCF and endothelin on phosphatidylinositol hydrolysis and contraction in rat aorta. *Am J Physiol*, 258, C122-C131.

Reiling, N., Ulmer, A.J., Duchrow, M., Ernst, M., Flad, H.D., & Hauschildt, S. (1994). Nitric oxide synthase: mRNA expression of different isoforms in human monocytes/macrophages. *Eur J Immunol*, 24(8), 1941-1944.

Resink, T.J., Scott-Burden, T., Weber, E., & Buhler, F.R. (1990). Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular responses to endothelin in human vascular smooth muscle cells. *Biochemical and Biophysical Research Communications*, 166, 1213-1219.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, 362, 801-808.

Rubanyi, G.M., & Parker-Botelho, L.H. (1991). Endothelins. *The FASEB Journal*, 5, 2713-2720.

Sakal, N., & Milstien, S. (1993). Availability of tetrahydrobiopterin is not a factor in the inability to detect nitric oxide production by human macrophages. *Biochemical and Biophysical Research Communications*, 193, 378-383.

Sakamoto, A., Yanagisawa, M., Sawamura, T., Enoki, T., Ohtani, T., Sakurai, T., Nakao, K., Toyooka, T., & Masaki, T. (1993). Distinct Subdomains of Human Endothelin Receptors Determine Their Selectivity to Endothelin A-selective Antagonist and Endothelin B-selective Agonists. *J Biol Chem*, 268, 8547-8553.

Sakurai, T., Yanagisawa, M., Inoue, A., Ryan, U.S., Kimura, S., Mitsui, Y., Goto, K., & Masaki, T. (1991). cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. *Biochemical and Biophysical Research Communications*, 175, 44-47.

Schiffrin, E.L. (1995). Endothelin in hypertension. *Current Opinion in Cardiology*, 10, 485-494.

Schneemann, M., Schoedon, G., Hoefer, S., Blau, N., Guerrero, L., & Schaffner, A. (1993). Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. *Journal of Infectious Disease*, 167, 1358-1363.

Schramek, H., Sorokin, A., Watson, R.D., & Dunn, M.J. (1995). ET-1 and PDGF BB induce MEK mRNA and protein expression in mesangial cells. *J Cardiovasc Pharmacol*, 26, S95-S99.

Schramek, H., Wang, Y., Konieczkowski, M., Rose, P.M., Sedor, J.R., & Dunn, M.J. (1994). Endothelin-1 stimulates cytosolic phospholipase A2 in chinese hamster ovary cells stably expressing the human ETA or ETB receptor subtype. *Biochemical and Biophysical Research Communications*, 199, 992-997.

Schrey, M.P., Patel, K.V., & Tezapsidis, N. (1992). Bombesin and glucocorticoids stimulate human breast cancer cells to produce endothelin, a paracrine mitogen for breast stromal cells. *Cancer Res*, 52, 1786-1790.

Schwartz, I., Ittoop, O., Davidai, G., & Hazum, E. (1992). Endothelin rapidly stimulates tyrosine phosphorylation in osteoblast-like cells. *Peptides*, 13, 159-163.

Sessa, W.C. (1994). The nitric oxide synthetase family of proteins. *J Vasc Res*, 31, 131-143.

Sessa, W.C., Kaw, S., Hecker, M., & Vane, J.R. (1991). The biosynthesis of endothelin-1 by human polymorphonuclear leukocytes. *Biochemical and Biophysical Research Communications*, 174, 613-618.

Severn, A., Wakelam, M.J., & Liew, F.Y. (1992). The role of protein kinase C in the induction of nitric oxide synthesis by murine macrophages. *Biochemical and Biophysical Research Communications*, 188, 997-1002.

- Sheffler, L.A., Wink, D.A., Melillo, G., & Cox, G.W. (1995). Characterization of nitric oxide-stimulated ADP-ribosylation of various proteins from the mouse macrophage cell line ANA-1 using sodium nitroprusside and the novel nitric oxide-donating compound diethylamine dinitric oxide. *Journal of Leukocyte Biology*, 57, 152-159.
- Shepherd J.T. (1995). Interactions of neurotransmitters and endothelial cells in determining vascular tone. *Adv Exp Med Biol*, 381, 1-13.
- Shetty, S.S., Okada, T., Webb, R.L., DelGrande, D., & Lappe, R.W. (1993). Functional Distinct Endothelin B Receptors in Vascular Endothelium and Smooth Muscle. *Biochemical and Biophysical Research Communications*, 191, 459-464.
- Simonson, M.S., & Dunn, M.J. (1990). Cellular signaling by peptides of the endothelin gene family. *FASEB*, 4, 2989-2999.
- Simonson, M.S., & Dunn, M.J. (1991). Endothelins: A Family of Regulatory Peptides. *Hypertension*, 17, 856-863.
- Simonson, M.S., & Herman, W.H. (1993). Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1. Cross-talk between G protein-coupled receptors and pp60c-src. *J Biol Chem*, 268, 9347-9357.
- Simonson, M.S., Jones, J.M., & Dunn, M.J. (1992). Differential regulation of fos and jun gene expression and AP-1 cis-element activity by endothelin isopeptides. Possible implications for mitogenic signaling by endothelin. *J Biol Chem*, 267, 8643-8649.
- Spinella, M.J., Kottke, R., Magazine, H.I., Healy, M.S., Catena, J.A., Wilkin, P., & Andersen, T.T. (1993). Endothelin-receptor interactions: Role of a putative sulfhydryl on the endothelin receptor. *FEBS Letters*, 328, 82-88.
- Springer, T.A. (1990). Adhesion receptors of the immune system. *Nature*, 346, 425-434.
- Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*, 76, 301-314.
- Stadler, J., Curran, R.D., & Ochoa, J.B. (1991). Effect of endogenous nitric oxide on mitochondrial respiration of rat hepatocytes in vitro and in vivo. *Arch. Surg.* 126, 186-191.
- Stamler, J., Simon, D., Osborne, J., Mullins, M., Jaraki, O., Michel, T., Singel, D., & Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: Synthesis and

characterization of biologically active compounds. *Proc Natl Acad Sci U S A*, 89, 444-448.

Stefano, G.B. (1995a). *Computer-assisted microscopic image analysis in neuroimmunology*. Orlando, FL: Academic Press, Inc.

Stefano, G.B., Hartman, A., Bilfinger, T.V., Magazine, H.I., Liu, Y., Casares, F., & Goligorsky, M.S. (1995b). Presence of the μ_3 opiate receptor in endothelial cells. Coupling to nitric oxide production and vasodilation. *Journal of Biological Chemistry*, 270, 30290-30293.

Stefano, G.B., Leung, M.K., Zhao, X., & Scharrer, B. (1989). Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes. *Proc Natl Acad Sci U S A*, 86, 626-630.

Stefano, G.B., Rodriguez, M., Glass, R., Cesares, F., Hughes, T.K., & Bilfinger, T.V. (1995c). Hyperstimulation of leukocytes by plasma from cardiopulmonary by-pass patients is diminished by morphine and IL-10 pretreatment. *J Cardiovasc Surg (Torino)*, 36, 25-30.

Stewart, D.J., Levy, R.D., Cernacek, P., & Langleben, D. (1991). Increased plasma ET-1 in pulmonary hypertension: Marker or mediator of disease. *Ann Intern Med*, 114, 464-469.

Stull, J.T., Tansey, M.G., Tang, D., Word, A., & Kamm, K.E. (1993). Phosphorylation of myosin light chain kinase: a cellular mechanism for calcium desensitization. *Mol. Cell Biochem.* 127/128, 229-237.

Sumner, M.J., Cannon, T.R., Mundin, J.W., White, D.G., & Watts, I.S. (1992). Endothelin ETA and ETB receptors mediate vascular smooth muscle contraction. *Br J Pharmacol*, 107, 858-860.

Suzuki, T., Kumazaki, T., & Mitsui, Y. (1993). Endothelin-1 is produced and secreted by neonatal rat cardiac myocytes in vitro. *Biochemical and Biophysical Research Communications*, 191, 823-830.

Swope, V.B., Medrano, E.E., Smalara, D., & Abdel-Malek, Z.A. (1995). Long-term proliferation of human melanocytes is supported by the physiologic mitogens alpha-melanotropin, endothelin-1, and basic fibroblast growth factor. *Exp Cell Res*, 217, 453-459.

Takagi, Y., Ninomiya, H., Sakamoto, A., Miwa, S., & Masaki, T. (1995). Structural basis of G protein specificity of the human endothelin receptors. A study with endothelin A/B chimeras. *Journal of Biological Chemistry*, 270, 10072-10078.

- Takasuka, T., Sakurai, T., Goto, K., Furuichi, Y., & Watanabe, T. (1994). Human Endothelin Receptor ETB. *J Biol Chem*, 269, 7509-7513.
- Takemoto, F., Uchida, S., Katagiri, H., Oka, Y., Nakao, A., & Kurokawa, K. (1995). Densitization of endothelin-1 binding by vasopressin via cAMP-mediated pathway in rat CCD. *Am J Physiol*, 268, F385-390.
- Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K., & Masaki, T. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem*, 264, 7856-7861.
- Taylor, C.W., & Marshall, C.B. (1992). Calcium and inositol 1,4,5-triphosphate receptors: a complex relationship. *Trends in Biochemical Science*, 17, 403-407.
- Tsukahara, H., Ende, H., Magazine, H.I., Bahou, W.F., & Goligorsky, M.S. (1994). Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells: receptor coupling to nitric oxide synthetase. *Journal of Biological Chemistry*, 269, 21778-21785.
- Tsukahara, H., Gordienko, D.V., & Goligorsky, M.S. (1993). Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochemical and Biophysical Research Communications*, 193, 722-729.
- Van der Wal, A.C., Das, P.K., Tigges, A.J., & Becker, A.E. (1992). Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *American Journal of Pathology*, 141, 1427-1433.
- Vane, J.R., & Botting, R.M. (1992a). The role of chemical mediators released by the endothelium in the control of the cardiovascular system. *Int J Tissue React*, 14, 55-64.
- Vane, J.R., & Botting, R.M. (1992b). Secretory functions of the vascular endothelium. *J Physiol Pharmacol*, 43, 195-207.
- Walsh, M.P. (1994). Calmodulin and the regulation of smooth muscle contraction. *Mol. Cell Biochem*. 135, 21-41.
- Warren, A.P., Owens, C.N., Borysiewicz, L.K., & Patel, K. (1994). Down-regulation of integrin alpha 1/beta 1 expression and association with cell rounding in human cytomegalovirus-infected fibroblasts. *J Gen Virol*, 75, 3319-3325.
- Watanabe, T.X., Itahara, Y., Nakajima, K., Kumagaye, S., Kimura, T., & Sakakibara, S. (1991). The biological activity of endothelin-1 analogues in three different assay systems. *J Cardiovasc Pharmacol*, 17 Suppl 7, S5-S9.

- Weinberg, J.B., Misukonis, M.A., Shami, P.J., Mason, S.N., Sauls, D.L., Dittman, W.A., Wood, E.R., Smith, G.K., McDonald, B., Haney, A.F., & Granger, D.L. (1995). Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood*, 86, 1184-1195.
- Weiss, G., Werner-Felmayer, G., Werner, E.R., Grunewald, K., Wachter, H., & Hentze, M.W. (1994). Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med*, 180, 969-946.
- Weissberg, P.L., Witchell, C., Davenport, A.P., Hesketh, T.R., & Metcalfe, J.C. (1990). The endothelin peptides ET-1, ET-2, ET-3 and sarafotoxin S6b are comitogenic with platelet-derived growth factor for vascular smooth muscle cells. *Arteriosclerosis*, 85, 257-262.
- Wilkins, F.C., Alberola, A., Mizelle, H.L., Opgenorth, T.J., & Granger, J.P. (1993). Chronic pathophysiologic circulating endothelin levels produce hypertension in conscious dogs. *Journal of Cardiovascular Pharmacology*, 8, S325-327.
- Wolff, D.J., & Lubeskie, A. (1995). Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch Biochem Biophys*, 316, 290-301.
- Wu-Yong, J.R., Chiou, W., & Opgenorth, T.J. (1993). Phosphoramidon modulates the number of endothelin receptors in cultured Swiss 3T3 fibroblasts. *Mol Pharmacol*, 44, 422-429.
- Xuan, Y.T., Watkins, W.D., & Whorton, A.R. (1991). Regulation of endothelin-mediated calcium mobilization in vascular smooth muscle cells by isoproterenol. *Am J Physiol*, 260, C492-C502.
- Yamashita, K., Niwa, M., Kataoka, Y., Shigematsu, K., Himeno, A., Tsutsumi, K., Nakano-Nakashima, M., Sakurai-Yamashita, Y., Shibata, S., & Taniyama, K. (1994). Microglia with an endothelin ETB receptor aggregate in rat hippocampus CA1 subfields following transient forebrain ischemia. *J Neurochem*, 63, 1042-1051.
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., & Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, 332, 411-415.
- Yohn, J.J., Moreli, J.G., Walchak, S.J., Rundell, K.B., Norris, D.A., & Zamora, M.R. (1993). Cultured human keratinocytes synthesize and secrete endothelin-1. *J Invest Dermatol*, 100, 23-26.

Yoshizawa, T., Shinmi, O., Giaid, A., Yanagisawa, M., Gibson, S.J., Kimura, S., Uchiyama, Y., Polak, J.M., Masaki, T., & Kanazawa, I. (1990). Endothelin: a novel peptide in the posterior pituitary system. *Science*, 247, 462-464.

Zimmerman, G.A., Lorant, D.E., McIntyre, T.M., & Prescott, S.M. (1993). Juxtacrine intercellular signaling: Another way of doing it. *Am J Respir Cell Mol Biol*, 9, 573-577.