THE ROLE OF $P2Y_2$ NUCLEOTIDE RECEPTORS IN VASCULAR INFLAMMATION

A Dissertation presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

NINGPU YU

Drs. Cheikh I. Seye and Gary A. Weisman, Dissertation Supervisors

DECEMBER 2007

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

THE ROLE OF $P2Y_2$ NUCLEOTIDE RECEPTORS

IN VASCULAR INFLAMMATION

presented by Ningpu Yu,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Gary A. Weisman

Professor Cheikh I. Seye

Professor Peter A. Wilden

Professor Grace Y. Sun

Professor Charlotte L. Phillips

Professor Laurie Erb

ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisors, Dr. Gary Weisman and Dr. Cheikh Seye, for providing me this opportunity to study in their laboratory, for their guidance and support in exploring science, and for their invaluable encouragement when I encountered difficulties in my research or life. I would never have finished the doctoral education and training without their knowledge, perceptiveness and enthusiasm.

I also want to thank my doctoral committee members, Dr. Grace Sun, Dr. Charlotte Phillips, Dr. Peter Wilden, and Dr. Laurie Erb. They have spent a great amount of their precious time on giving me critiques and inspiring suggestions on my research. My special thanks go to Dr. Michael Sturek and Dr. Dennis Lubahn for their tremendous help in accomplishing my research goals. I would also like to acknowledge my friends and colleagues in our laboratory and the Department of Biochemistry, particularly Qiongman Kong, Zhongji Liao, Shivaji Rikka, Nishant Jain, Min Wang, Jun Liu, Jennifer Hamilton, Jean Camden, and Olga Baker, who have helped me in various ways.

I would like to thank the American Heart Association-Heartland Affiliate for the award of the Predoctoral Fellowship, which supported my research for two years at the University of Missouri-Columbia.

I cannot end without thanking my parents, Liqun and Huizhen Yu, for whose constant encouragement and love I have relied on throughout my life. Their support in my pursuit of scientific research will continue to be most treasured.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……..…….…….……..…ii

LIST OF FIGURES……..…….…….……vi

LIST OF ABBREVIATIONS……...viii

ABSTRACT……..xii

CHAPTER

I. Introduction…….…….....1

A. Inflammation in vascular disease……1

B. P2 receptors in cardiovascular system…….3

B1. P2 receptors in vascular cells……..4

B2. P2 receptors in blood cells……..6

C. Modulation of P2 receptors in vascular injury and related pathophysiology……7

D. Regulation of smooth muscle cells cytoskeletal reorganization by nucleotides…….12

E. Endothelial cell-to-cell junctions and leukocyte permeability……...……15

E1. Endothelial cell-to-cell junctions seal endothelium and regulate permeability……..…..15

E2. The role of actin cytoskeleton in the regulation of tight junctions…….17

II. The $P2Y_2$ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells …..……..…..……26

Abstract……..……..,……27

Introduction…….……..…...…….28

Experimental procedures……..……31

Results……..…….……35

Discussion……..……...……39

III. The $P2Y_2$ nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF Receptor-2 (KDR/Flk-1)…..……59

Abstract……...……..…….60

Introduction……..……..…...…….61

Experimental procedures……...……63

Results…….……...……68

Discussion……...……...……73

IV. Binding of the $P2Y_2$ nucleotide receptor to filamin A regulates migration of vascular smooth muscle cells……..……...98

Abstract…….……...……98

Introduction…….……100

Experimental procedures……..……102

Results…….……109

Discussion……...……..……113

V. Ongoing project: The $P2Y_2$ nucleotide receptor regulates tight junction complex assembly and endothelial cell permeability properties……129

Introduction……..……129

Experimental procedures……..……132

Results……..……133

VI. Summary and general discussion……...……...138

References……143

Vita……...…165

List of Figures

Figure I-1. Signaling mechanism of $P2Y_2$ receptor-induced VEGFR-2 activation and VCAM-1 expression in endothelial cells……..……...20

Figure I-2. $P2Y_2$ receptor is linked to actin cytoskeleton via filamin A.……...22

Figure I-3. Structure of tight junctions and adherens junctions……...24

Figure II-1. UTP or ATP induces VCAM-1 expression in HCAEC……...43

Figure II-2. ICAM-1 expression in HCAEC is unaffected by UTP or ATP……45

Figure II-3. Detection of VCAM-1 expression in endothelial cells by immunofluorescence…….47

Figure II-4. Calcium release from internal stores mediates UTP-induced VCAM-1 expression…….49

Figure II-5. UTP-induced VCAM-1 expression is mediated by $P2Y_2$ receptors…….51

Figure II-6. $P2Y_2$ receptors mediate VCAM-1 expression in transfected 1321N1 astrocytoma cells……...53

Figure II-7. Inhibitors of p38 and Rho kinase, but not MEK1/2, attenuate UTP-induced VCAM-1 expression…….55

Figure II-8. U937 monocyte adherence to UTP-stimulated HCAEC is inhibited by anti-VCAM-1 antibody……....57

Figure III-1. UTP induces tyrosine phosphorylation of VEGFR-2……...…77

Figure III-2. VEGFR-2 siRNA duplex efficiently silences VEGFR-2 gene and protein expression and inhibits $P2Y_2$ receptor-mediated VCAM-1 expression……79

Figure III-3. Inhibition of VEGFR-2 tyrosine phosphorylation prevents UTP-induced VCAM-1 expression…….82

Figure III-4. The $P2Y_2$ receptor and VEGFR-2 co-localize and interact upon UTP stimulation……84

Figure III-5. Src kinase activity and SH3-binding domains in the C-terminal tail of the $P2Y_2$ receptor are required for VEGFR-2 transactivation……86

Figure III-6. Deletion or mutation of the SH3-binding sites in the $P2Y_2$ receptor prevents UTP-induced VCAM-1 expression in HCAEC……90

Figure III-7. UTP-induced transactivation of VEGFR-2 causes tyrosine phosphorylation of Vav2 in HCAEC……...92

Figure III-8. UTP stimulates the activity of RhoA and Rac1……....94

Figure III-9. Dominant negative RhoA inhibits VCAM-1 expression……..96

Figure IV-1. FLNa binds to the $P2Y_2$ receptor C-terminal domain (CTD) and co-immunoprecipitates with the HA-$P2Y_2$ receptor……117

Figure IV-2. Identification of the FLNa binding domain of the $P2Y_2$ receptor……..119

Figure IV-3. UTP induces FLNa phosphorylation in aortic SMCs……121

Figure IV-4. Spreading of SMCs on collagen-coated coverslips……....123

Figure IV-5. Loss of FLNa/$P2Y_2$ receptor interaction affects UTP-induced SMC migration……125

Figure IV-6. Migration of SMCs out of mouse aortic explants……...127

Figure V-1. UTP stimulation causes a decrease in tight junction protein levels in HMVEC……..134

Figure V-2. The $P2Y_2$ receptor interacts with ZO-1/ZO-2 upon UTP stimulation…….136

List of Abbreviations:

$[Ca^{2+}]_i$	intracellular calcium concentration
2-MeS-ATP	2-methyl-thio-adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
AJ	adherens junction
ATCC	American Type Cell Collection
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CASMC	coronary artery smooth muscle cells
CTD	carboxyl terminal domain
EBM	endothelium basal medium
ECM	extracellular matrix
EDRF	endothelial-dependent relaxing factor
EGF	epithelial growth factor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FCS	fetal calf serum
FLN	filamin
GMP	guanine 5'-monophosphate
GST	glutathione S-transferase
GUK	guanine kinase
HA	hemagglutinin
HCAEC	human coronary artery endothelial cells

HMVEC	human microvascular endothelial cells
IB	immunoblotting
ICAM	intercellular cell adhesion molecule
IgG	immunoglobulin G
IP	immunoprecipitation
IP_3	inositol 1,4,5-triphosphate
JAM	junction adhesion protein
KO	knockout
LDL	low density lipoprotein
M-CSF	macrophage-colony stimulating factor
MAGUK	membrane associated guanine kinase
MCP	monocyte chemotactic protein
MAPK	mitogen-activated protein kinase
NFκB	nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase
oxLDL	oxidized low density lipoprotein
PAK	p21-activated kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
pERK	phosphorylated extracellular signal-regulated kinase
PGI2	prostaglandin I_2

PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
PY	phosphotyrosine
RBD	Rhotekin binding domain
RIPA	radio-immunoprecipitation
S.E.	standard error
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
siRNA	short interfering RNA
SMC	smooth muscle cells
TBST	tris-buffered saline Tween-20
TJ	tight junction
TNF	tumor necrosis factor
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
WT	wild type
ZAK	ZO-1-associated kinase

ZO	zona occludens

The Role of $P2Y_2$ Nucleotide Receptors in Vascular Inflammation

Abstract

Extracellular nucleotides and nucleosides can regulate vascular tone. Extracellular nucleotides are released at the site of tissue injury and may play an important role in the atherogenesis process by promoting monocyte/macrophage adherence and infiltration, impairing endothelial barrier, and stimulating migration and proliferation of vascular smooth muscle cells, thereby contributing to the development of intimal thickening. In vascular injury models, including collared rabbit carotid arteries and rat aorta balloon angioplasty, $P2Y_2$ mRNA was significantly up-regulated while $P2Y_4$ mRNA was unaffected and $P2Y_6$ mRNA was undetectable. This dissertation concerns the specific roles played by the $P2Y_2$ receptor in pathophysiological vascular development.

Promotion of monocyte adherence to vascular endothelium by nucleotides was first suggested by the observation that activation of up-regulated $P2Y_2$ receptors in the collared rabbit carotid artery increased monocyte/macrophage infiltration and intimal hyperplasia. We addressed the hypothesis that activation of the $P2Y_2$ receptor modulates the expression of VCAM-1 in vascular endothelial cells that is important for monocyte recruitment. Suppression of $P2Y_2$ receptor expression by $P2Y_2$ anti-sense oligonucleotide inhibited UTP-induced VCAM-1 expression. UTP-induced VCAM-1 expression in human coronary artery endothelial cells (HCAEC) is dependent on intracellular calcium stores, p38 mitogen-activated protein kinase (MAPK) and Rho kinase, but independent of MEK1/2. Expression of VCAM-1 in HCAEC increased the adherence of monocytic

U937 cells to HCAEC monolayers. We report here that $P2Y_2$ receptor-induced VCAM-1 expression is mediated by rapid tyrosine phosphorylation of VEGFR-2 in HCAEC. RNA interference (RNAi) targeting of VEGFR-2 expression or inhibition of VEGFR-2 tyrosine kinase activity abolished $P2Y_2$ receptor-mediated VCAM-1 expression. Furthermore, VEGFR-2 association with the $P2Y_2$ receptor upon UTP stimulation is dependent on the integrity of SH3 binding sites in the $P2Y_2$ receptor C-terminal tail and Src kinase activity, and is required for VCAM-1 expression.

We also discovered that the $P2Y_2$ receptor is linked to the cytoskeleton through direct interaction with the actin-binding protein filamin A (FLNa), which is a large protein of 280 kD and serves as a cross-linker of actin polymers and as a scaffolding protein for various signaling molecules. This interaction was mapped to the C-terminal tail of the $P2Y_2$ receptor (amino acids 322 to 333) and is required for FLNa phosphorylation induced by extracellular nucleotides. $P2Y_2$ receptor activation causes spreading and migration of vascular smooth muscle cells (SMC) *in vitro* and *ex vivo*. Disruption of this interaction impairs the ability of the receptor to induce the spreading and migration of SMC.

Taken together, these results demonstrated that extracellular nucleotides and the $P2Y_2$ receptor have several important functions in vascular endothelial cells and smooth muscle cells and may be a key contributor to the progression of cardiovascular diseases, such as atherosclerosis and hypertension. These results encourage drug design targeting the $P2Y_2$ receptor as a means to prevent and/or treat arterial disease.

Chapter I

Introduction

A. Inflammation in vascular disease

The connection between atherosclerosis and lipid storage was observed a long time ago. Although hypercholesterolemia was theorized as pathogenic and is still under intensive study, until the 1990's this phenomenon was poorly understood. In the last fifteen years, substantial advances in basic and experimental sciences have elucidated the role of vascular inflammation and associated molecular and cellular mechanisms that contribute to atherogenesis (reviewed by Libby, 2002). Understanding the connection between inflammation and atherosclerosis could be of considerable prognostic and therapeutic value.

In the progression of atherosclerosis, inflammation participates in each step (Libby, 2002). Normal vascular endothelium is not adhesive to leukocytes (Poole and Florey, 1958). However, at atherosclerotic lesion sites, the endothelium is inflamed and expresses certain adhesion molecules that can bind their cognate ligands on leukocytes in the circulation (Cybulsky and Gimbrone, 1991). Initially, selectins expressed on the endothelial cell luminal surface cause leukocytes to slow down and roll on the

endothelium (Dong *et al.*, 1998). Then, cytokines up-regulate the integrins α_4 and β_2 in the leukocytes, which promote binding to vascular cell adhesion molecule (VCAM)-1 and intercellular cell adhesion molecule (ICAM)-1 on endothelium, respectively (Springer, 1994). Interestingly, VCAM-1 can bind monocytes and T lymphocytes, two types of leukocytes found in atheromas, making it an important player and potential target for therapeutic strategies (Elices *et al.*, 1990; Walpola *et al.*, 1995). In addition, pro-inflammatory factors expressed in the atheroma are chemoattractants for leukocytes and direct the leukocytes to migrate into the vascular intima. Such chemoattractants include monocyte chemotactic protein (MCP)-1, which can guide monocytes into the intima (Boring *et al.*, 1998; Gu *et al.*, 1998) and T lymphocyte-activating CXC chemokines, which guide T cells into the intima (Mach *et al.*, 1999). Other pro-inflammatory factors in the intima, such as macrophage-colony stimulating factor (M-CSF), can up-regulate the leukocytic expression of macrophage scavenger receptors that promote the uptake of modified lipoprotein particles (*i.e.* oxLDL) and the differentiation of monocytes into macrophages (de Villiers *et al.*, 1994). M-CSF can also stimulate the replication of macrophages in the intima (Sherr, 1991; Stanley *et al.*, 1997). Pro-inflammatory factors released by T cells (*e.g.* interferon-γ and lymphotoxin) stimulate the migration and proliferation of macrophages and smooth muscle cells (SMC) (Libby and Hansson, 1991), resulting in intimal hyperplasia and a decrease in vessel inner diameter. Leukocytes and resident arterial cells also release fibrogenic mediators that cause the formation of a fibrous cap, mainly composed of collagen, to protect the atheroma from further exposure to invading leukocytes (Schneider *et al.*, 1999). As inflammation progresses, the leukocytes release collagenase to prevent the formation of new collagen

and thin the fibrous cap of the atherosclerotic plaque, rendering it susceptible to rupture (Galis *et al.*, 1994; Sukhova *et al.*, 1999; Herman *et al.*, 2001). Activated T lymphocytes release interferon-γ, that suppresses production of collagen by SMC (Libby *et al.*, 1995; Libby, 2000). Inflammatory mediators cause the macrophages in the atherosclerotic plaque to produce tissue factor, which is a pro-coagulator (Wilcox *et al.*, 1989). Once vascular rupture occurs, tissue factors are released that can trigger thrombosis and result in fatal acute myocardial infarction (Libby and Simon, 2001).

B. P2 receptors in the cardiovascular system

Extracellular nucleotides bind to cell-surface receptors known as P2 receptors, that are present in many tissues. To date, these receptors have been classified into two main families: the P2X receptors that are ligand-gated ion channels comprised of homo- or hetero-oligomers (North and Surprenant, 2000), and P2Y receptors that are seven-membrane-spanning receptors coupled via G proteins ($G_{q/11}$ or $G_{i/o}$) to phospholipase C (PLC) and/or adenylate cyclase (Cooper and Rodbell, 1979; North and Surprenant, 2000; Communi *et al.*, 2001). In turn, PLC activation generates inositol 1,4,5-triphosphate (IP_3), a mediator of Ca^{2+} release from intracellular stores, and diacylglycerol, an activator of protein kinase C (PKC) whereas adenylate cyclase generates cyclic AMP, an activator of protein kinase A (PKA). The identification of seven P2X ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$) (Valera *et al.*, 1994; Le *et al.*, 1997; Garcia-Guzman *et al.*, 1997a, 1997b; Rassendren *et al.*, 1997; Urano *et al.*, 1997; Lynch *et al.*, 1999) and eight P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Parr *et al.*, 1994; Nguyen *et al.*, 1995; Ayyanathan *et al.*, 1996; Chambers *et al.*, 2000; Communi *et al.*, 1995, 1996, 1997, 2001; Hollopeter *et al.*, 2001) receptor subtypes has made it possible to use molecular and

pharmacological approaches to study the distribution and functional properties of specific P2 receptor subtypes at the tissue and cellular level.

B1. P2 receptors in vascular cells The normal arterial wall consists of three layers: intima, media, and adventitia. The single layer of endothelial cells facing the vessel lumen is a very important component of the vascular wall in terms of releasing both vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors such as thromboxane A2 and endothelin (Weksler *et al.*, 1978; Nawroth *et al.*, 1985; Salvemini *et al.*, 1990; McClellan *et al.,* 1994). The principal P2Y receptor subtypes that have been functionally characterized in endothelial cells are $P2Y_1$ and $P2Y_2$, but mRNAs for $P2Y_4$ and $P2Y_6$ receptors have also been detected (Di Virgilio and Solini, 2002). Endothelium-dependent vasorelaxation has been attributed to the release of NO and PGI_2 after binding of nucleotides to $P2Y_1$ and $P2Y_2$ receptors in endothelial cells (Ralevic and Burnstock, 1991) whereas vasoconstrictor effects in SMC result from the action of nucleotides on $P2Y_2$ and P2X receptors (von Kugelgen and Starke, 1990; Saiag *et al*., 1992). In most blood vessels, $P2Y_1$ receptors, which preferentially bind ADP, are present on the endothelium and regulate vasodilatation by Ca^{2+}-dependent (PLC-mediated) activation of NO synthase (NOS) and generation of endothelial-dependent relaxing factor (EDRF) (Di Virgilio and Solini, 2002). Endothelial PGI_2 production is also stimulated by $P2Y_1$ and $P2Y_2$ receptors, but this seems to play a minimal role in vasodilatation, at least under physiological conditions (Ralevic and Burnstock, 1998). Recent studies have indicated that in the aortas of $P2Y_2$-null mice, endothelium-dependent relaxation induced by ATP and ATPγS was inhibited, demonstrating the role of $P2Y_2$ receptors, but the response to0 UTP and UDP was maintained, suggesting the additional involvement of

$P2Y_6$ receptors (Guns *et al*., 2006). The majority of cells in intact blood vessels are SMC, which occupy most of the media and are involved in vasoconstriction and vasorelaxation. $P2Y_2$ receptors in SMC mediate the induction of immediate-early and delayed-early cell-cycle-dependent genes, consistent with a role for $P2Y_2$ receptors in vascular proliferation of SMC (Malam-Souley *et al*., 1995; Seye *et al*., 1997). A recent study demonstrated that the $P2Y_2$ receptor is the predominant functional receptor that responds to ATP and UTP in rat aortic SMC (Kumari *et al*., 2003). In human cerebral arteries, the $P2Y_6$ receptor seems to be the predominant subtype and induces vasoconstriction when activated by UDP/UTP (Malmsjo *et al*., 2003a, b). This is consistent with findings from rat pulmonary and mesenteric arteries (Hartley *et al*., 1998; Malmsjo *et al*., 2000). In addition, a recent study with $P2X_1$ receptor knockout mice further supports the prominent contractile effect of the $P2Y_6$ receptor subtype in mesenteric arterial trees (Vial and Evans, 2002). Taken together, it seems that the principal receptor mediating UTP/UDP-induced contractile responses in blood vessels might be the $P2Y_6$ receptor subtype. Other studies have reported the presence of both $P2Y_4$ and $P2Y_6$ receptors in rat aortic SMC (Chang *et al*., 1995; Malam-Souley *et al*., 1995; Erlinge *et al*., 1998a). $P2Y_1$ receptors are expressed in SMC of a number of blood vessel types and, like their endothelial cell counterparts, mediate vasodilatation most likely through the activation of K^+ channels (Ralevic and Burnstock, 1998). The presence of several P2X receptor subtypes also has been reported in human saphenous vein SMC, including $P2X_1$, $P2X_2$, $P2X_4$, and $P2X_7$ receptors (Cario-Toumaniantz *et al*., 1998). The outermost layer of the blood vessel consists of connective tissue and fibroblasts, which have not been appreciated in the regulation of vascular tone. A recent study showed that fibroblasts can migrate into the neointima, suggesting their

possible involvement in the development of vascular diseases such as atherosclerosis and restenosis after angioplasty (Zalewski *et al*., 2002). Since human and rat fibroblasts are known to express $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2X_3$, $P2X_4$, and $P2X_7$ receptors (von Kugelgen and Wetter, 2000), further investigation is needed to establish the role of fibroblast P2 receptors under physiological and pathophysiological conditions.

B2. P2 receptors in blood cells G-protein-coupled P2Y receptors, the activation of which leads to intracellular calcium mobilization, have been observed in neutrophils (Kuhns *et al*., 1988; Cowen *et al*., 1989; Di Virgilio and Solini, 2002). Turkey erythrocytes express both $P2Y_1$ and $P2X_7$ receptors (Parker and Snow, 1972; Boyer *et al*., 1989). ATP and UTP act as secretagogues by binding to $P2Y_2$ receptors to enhance exocytosis of primary granules in neutrophils (Cockcroft and Stutchfield, 1989). In macrophages, ATP activates a $P2X_7$ receptor that differs from other ligand-gated ion-channel P2X receptors by its ability to generate plasma membrane pores when activated (Steinberg *et al*., 1987). Human T lymphocytes also have been shown to express $P2X_7$ receptors (Baricordi *et al*., 1996). Human monocytes and macrophages co-express $P2X_1$, $P2X_4$, and $P2X_7$ receptors whereas granulocytes only express $P2X_7$ receptors (Suh *et al*., 2001). $P2Y_1$, $P2Y_2$, and $P2Y_6$ receptors also are expressed in monocytes, B lymphocytes, and polymorphonuclear granulocytes (Di Virgilio *et al*., 2001). Human platelets express $P2Y_1$, $P2Y_{12}$, and $P2X_1$ receptors (Leon *et al*., 1997; Sun *et al*., 1998; Hollopeter *et al*., 2001). Thus, the diversity of P2 receptor expression in blood cells, as well as endothelial cells, SMC and fibroblasts, suggests that this receptor superfamily plays a significant role in the regulation of cardiovascular functions.

C. Modulation of P2 receptors in vascular injury and related pathophysiology

Experimental arterial intimal hyperplasia can be evoked by balloon angioplasty, or by the perivascular placement of a silicone collar around an artery. An influx of leukocytes precedes the migration and proliferation of vascular SMC into the intima in both of these models (De Meyer *et al*., 1997). In normal adult aorta, $P2Y_2$ receptor mRNA was found in the endothelial cell lining, while a sustained expression of this receptor was detected in few SMC disseminated in all the media (Seye *et al*., 1997). In contrast, $P2Y_2$ mRNA was detected in all medial SMC of fetal aortas and in most of the cells of intimal lesions obtained after aortic ballooning, with high expression in cells lining the lumen, both 1 and 3 weeks after injury (Seye *et al*., 1997).

In the collar model, neointimal formation appears to be triphasic (De Meyer *et al*., 1997). The first phase characterized by vascular infiltration of leukocytes begins 2 h after collar placement around a rabbit carotid artery. The second phase begins within 12 h of collar placement and is characterized by medial replication of SMC. The third phase is characterized by the appearance, beginning at day 3 after collar placement, of sub-endothelial SMC. *In situ* hybridization with sham-operated arteries indicated that $P2Y_2$ receptor mRNA was localized to CD31-positive endothelial cells and not medial SMC (Seye *et al*., 2002). High levels of $P2Y_2$ mRNA were detected in medial SMC 3 days after collar placement, before appearance of neo-intima. At day 14, all intimal and medial cells were $P2Y_2$ positive. Fura-2 digital imaging of single SMC that was used to measure changes in myoplasmic calcium concentration in response to P2Y receptor agonists

showed an increase in $P2Y_2$ receptor activity. However, the same study showed that $P2Y_4$ receptor mRNA was equivalently expressed in sham-operated, collared arteries, or cultured SMC, whereas $P2Y_6$ receptor mRNA was not detected in carotid arteries or in cultured carotid SMC. In a more recent study, it was shown that $P2Y_2$ receptor up-regulation occurs in a porcine model of coronary artery stent, a clinically relevant model of arterial injury (Shen *et al*., 2004). $P2Y_2$ receptor mRNA levels were significantly increased in coronary SMC dispersed from stented segments of coronary arteries 3 weeks after stent angioplasty compared with SMC from unstented segments. There was no significant difference observed in levels of $P2Y_6$ receptor mRNA in the stented and unstented artery segments, whereas $P2Y_4$ receptor mRNA was undetectable.

Up-regulation of functional P2Y receptors also occurs in the basilar artery of the rat double-hemorrhage model (Carpenter *et al*., 2001), in the coronary artery of diabetic dyslipidemic pigs (Hill *et al*., 2001), and in human atherosclerotic lesions (Seye and Desgranges, personal communication). It has been proposed that up-regulation of P2Y receptors could be a potential diagnostic indicator for the early stages of atherosclerosis (Elmaleh *et al*., 1998). Interestingly, a more recent study showed that high shear stress associated with vascular diseases can selectively up-regulate $P2Y_2$ and $P2Y_6$ receptors in perfused arterial SMC (Wang *et al*., 2003). $P2X_1$ and $P2X_4$ receptors have been shown to be up-regulated in rabbit intimal thickenings (Pulvirenti *et al*., 2000). Taken together, these findings strongly suggest that at least some P2 receptor subtypes (most notably $P2Y_2$) are implicated in the development of vascular disease.

A high level of $P2Y_2$ receptor expression could be related to the altered phenotype of SMC in intimal thickenings. Migrating and proliferating SMC in the arterial media,

together with infiltrating macrophages and T lymphocytes, are the main cell types that comprise atherosclerotic and restenotic lesions (Ross, 1993). In the early stages of intimal hyperplasia, SMC are modified from a differentiated, contractile phenotype to an immature, synthetic phenotype, which enables them to migrate into the intima, proliferate, and secrete extracellular matrix components (Bochaton-Piallat *et al.*, 1996). In many aspects, this shift in phenotype is a reversal of the normal differentiation pattern of vascular SMC during fetal and early postnatal life (Owens, 1995; Katoh and Periasamy, 1996). However, there is still a lack of knowledge concerning the phenotypic regulation of SMC during vasculogenesis and vascular disease. Partially dedifferentiated SMC are found in rat arterial intimal lesions after balloon angioplasty (Gabbiani *et al*., 1984; Kocher *et al*., 1991), and their phenotype has been compared with that of newborn rat aortic SMC (Schwartz *et al*., 1995). Interestingly, it has been reported that medial SMC of rat embryonic aorta also exhibit high $P2Y_2$ receptor expression similar to intimal thickenings (Seye *et al*., 1997). Other studies have shown that $P2Y_1$ and $P2Y_2$ receptor transcripts are strongly up-regulated with phenotypic changes in rat SMC whereas $P2X_1$ mRNA is completely down-regulated and $P2Y_4$ and $P2Y_6$ mRNA levels are unchanged (Erlinge *et al*., 1998a; Pillois *et al*., 2002). Taken together, these results suggest that $P2Y_2$ receptor expression is up-regulated in the entire cell population of intimal thickenings and is closely associated with a poorly differentiated phenotype of SMC. The dramatic increase in $P2Y_2$ mRNA expression observed in balloon angioplasty-induced intimal lesions would suggest increased activity of extracellular nucleotides with consequent enhancement of cell proliferation and vasoreactivity. Indeed, extracellular nucleotides, particularly ATP and UTP, have been shown to induce cell-cycle progression and

proliferation of cultured arterial SMC (Erlinge *et al*., 1993, 1995; Malam-Souley *et al*., 1993, 1995), and vasoconstriction in the absence of endothelial cells (Saiag *et al*., 1990; von Kugelgen and Starke, 1990; Ralevic and Burnstock, 1991). Since both neointimal hyperplasia and vasoconstrictive remodeling have been found to be involved in postangioplasty restenosis (Ross, 1993; Lafont *et al*., 1995; Schwartz *et al*., 1995; Andersen *et al*., 1996), these findings suggest that extracellular nucleotides may play a significant role in these processes, at least as long as functional endothelial cells, which regulate intimal thickening (Haudenschild and Schwartz, 1979; Clowes *et al*., 1986) and nucleotide-induced vasorelaxation (Shimokawa *et al*., 1989; Weidinger *et al*., 1990), are not regenerated.

Increased $P2Y_2$ receptor expression in the neointima may by itself be sufficient to enhance local effects of extracellular nucleotides on proliferation of SMC. Although expression of other P2 receptors has been described in arterial SMC (Valera *et al*., 1994; Chang *et al*., 1995; Malam-Souley *et al*., 1995), the $P2Y_2$ receptor seems to be more specifically involved in the response of SMC to ATP and UTP (Nicholas *et al*., 1996), particularly in potentiation of proliferation (Erlinge *et al*., 1995; Malam-Souley *et al*., 1995). Effects of extracellular nucleotides are not only dependent on the nature and number of P2 receptors present on target cells but also on local concentrations of nucleotide agonists. Although *in vivo* concentrations of extracellular nucleotides are difficult to measure, *in vitro* experiments suggest that extracellular nucleotides are released from blood and vascular cells exposed to various physicochemical conditions, *e.g.*, stress, hypoxia (Gordon, 1986; Bergfeld and Forrester, 1992; Bodin and Burnstock, 1995) associated with the angioplasty process.

$P2Y_2$ receptors in SMC are involved in nucleotide-induced constriction of normal arteries (Saiag *et al*., 1990; von Kugelgen and Starke, 1990; Ralevic and Burnstock, 1991). Long-lasting alterations in vasomotricity after endothelial cell denudation, resulting in increased sensitivity to vasoconstrictive substances, have previously been demonstrated (Shimokawa *et al*., 1989; Weidinger *et al*., 1990). It appears that, like other receptors for vasoconstrictive factors such as angiotensin II (Viswanathan *et al*., 1992), endothelin (Winkles *et al*., 1993), and platelet-derived growth factor (PDGF) (Majesky *et al*., 1990), which are overexpressed in neointima, $P2Y_2$ receptors may play an important role in controlling vasoactive properties of pathological arteries, particularly in chronic constriction at the lesion site that is postulated to be one of the processes leading to postangioplasty restenosis (Lafont *et al*., 1995; Andersen *et al*., 1996)

In the collared artery, up-regulation of the $P2Y_2$ receptor ($P2Y_2R$) and *in vivo* activation of the $P2Y_2$ receptor by continuous delivery of UTP with an osmotic mini-pump significantly increases macrophage infiltration (Seye *et al.*, 2002). The macrophages in the intima originate from monocytes in the blood recruited to the endothelium via binding to adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular cell adhesion molecule (ICAM)-1, and selectins. Importantly, VCAM-1 is expressed in atherosclerosis-prone areas, such as aorta, one week after feeding the animal with a atherogenic diet, which is before the appearance of macrophages in the intima (Li *et al.*, 1993). VCAM-1 binds to monocytes and T lymphocytes, which are found in the atheromas (Elices *et al.*, 1990; Walpola *et al.*, 1995). Chapter II clarifies the role of the $P2Y_2R$ in VCAM-1 induction in endothelial cells and monocyte recruitment to endothelium. Furthermore, we explored downstream signaling

effectors that mediate UTP-induced VCAM-1 expression. Especially, the role of VEGFR-2, a key player in vascular inflammation and associated signaling events, is discussed in Chapter III. A schematic representation of the mechanism of $P2Y_2R$-induced activation of VEGFR-2 and induction of VCAM-1 is shown in Figure I-1.

D. Regulation of smooth muscle cell motility and cytoskeletal reorganization by nucleotides

Recent studies indicate that the extracellular nucleotides ATP, ADP, UTP, and UDP serve as directional cues for migration of rat aortic SMC (Chaulet *et al*., 2001). At identical concentrations, the most powerful migratory response induced by these nucleotides was elicited by UTP. Nucleotide-induced migration of SMC is the consequence of both chemotaxis and chemokinesis and may result from activation of one or several P2 nucleotide receptor subtype(s) (Ikeda *et al.*, 1997; Pillois *et al.*, 2002)). The ability of UTP at submicromolar levels to stimulate migration of SMC supports the hypothesis that this response could have physiological consequences and is essentially mediated by $P2Y_2$ receptor activation without excluding participation of other P2Y receptor subtypes. The difference in the capacity of UTP and ATP to elicit migration of SMC could be due to inhibition of nucleotide-induced cell migration by adenosine generated from ATP catabolism by cell-surface ectonucleotidases (Pillois *et al.*, 2002). Indeed, ATP and UTP were equally effective in causing migration of SMC when ATP was prevented from degradation by addition of the ectonucleotidase inhibitor α,β-methylene-ATP (Pillois *et al*., 2002). It has been shown in rat aortic SMC that the $P2Y_2$ receptor is the predominant P2Y receptor subtype (Erlinge *et al*., 1998a; Pillois *et al*.,

2002) whereas lower levels of $P2Y_6$ and $P2Y_4$ receptor mRNA and very low level of $P2Y_1$ receptor mRNA were detected (Pillois *et al*., 2002). The very low level of $P2Y_1$ receptor mRNA expression was consistent with the absence of ADP-induced migration of cultured rat aortic SMC, demonstrating that $P2Y_1$ is not involved in this process (Pillois *et al*., 2002). In addition, the same study showed that a commercially available solution of hexokinase-treated UDP (UTP-free) induced cell migration equally as well as untreated UDP, thereby demonstrating that UDP is chemotactic for aortic SMC by activation of the $P2Y_6$ receptor (Pillois *et al*., 2002). Conversely, migration of rat aortic SMC induced by UTP occurred even when UTP degradation by nucleoside diphosphate kinase was inhibited, demonstrating the involvement of $P2Y_2$ and/or $P2Y_4$ receptor(s) (Pillois *et al*., 2002).

The increased migration of SMC in response to extracellular nucleotides could be related to increases in extracellular matrix (ECM) protein expression. Indeed, previous studies have shown that UTP induces osteopontin expression in rat and rabbit aortic SMC (Malam-Souley *et al*., 1993; Seye *et al*., 2002). Increased expression of osteopontin, an integrin-binding RGD motif-containing ECM protein, is associated with the activation of rat arterial SMC *in vitro* and *in vivo* (Chaulet *et al*., 2001). The increase in osteopontin expression plays a key role in UTP-induced migration of rat aortic SMC since a monoclonal antibody against osteopontin fully abolished UTP-induced migration (Chaulet *et al*., 2001) whereas an antibody against vitronectin, another ECM protein also involved in migration of human SMC (Dufourcq *et al*., 1998), had no effect on the migration of rat aortic SMC (Chaulet *et al*., 2001). UTP can increase osteopontin mRNA expression by increasing both osteopontin mRNA stabilization and osteopontin promoter

activity (Renault *et al*., 2003). Recent studies have shown that activation of an AP-1 binding site located 76 bp upstream of the transcription initiation site in the rat osteopontin promoter is involved in UTP-induced osteopontin expression (Renault *et al.*, 2003). Using a luciferase promoter deletion assay, Renault *et al*. (2005) identified a new region of the rat osteopontin promoter (−1837 to −1757) that is responsive to UTP. This region contains an NFκB site located at −1800 and an Ebox located at −1768. Electrophoretic mobility supershift and chromatin immunoprecipitation assays identified NFκB and USF-1/USF-2 as DNA-binding proteins induced by UTP (Renault *et al.*, 2005). Using dominant negative mutants of IκB kinase and USF transcription factors, it was confirmed that NFκB and USF-1/USF-2 are involved in the UTP-induced expression of osteopontin.

The ability of nucleotides to act as chemoattractants for rat arterial SMC in a concentration range potentially found in pathological vessels (Communi *et al*., 2000) and the findings of previous studies demonstrating the mitogenic activity of extracellular nucleotides for these cells suggest that nucleotides released from mechanically stretched vascular or damaged cells during the angioplasty process may participate in arterial wall remodeling. Activation of the $P2Y_2$ receptor in SMC can cause the reorganization of actin filaments (Sauzeau *et al.*, 2000). Filamins (also named FLN, actin-binding protein, or ABP) form a family of molecules that are large actin-binding proteins that can crosslink and stabilize three-dimensional networks of actin filaments (Cunningham *et al*., 1992; Stossel *et al*., 2001). Three isoforms of FLN have been reported in humans and named FLNa, FLNb, and FLNc (Gorlin *et al.*, 1990; Xie *et al.*, 1998; Xu *et al.*, 1998). Tissue-specific gene splicing further contributes to FLN diversity. The overall homology

between different isoforms of FLN is about 60-80%, where the C-terminal region is the least conserved (Stossel *et al*., 2001). FLNa isoform-specific functions have been suggested, since membrane instability and defects in cell migration occurred in FLNa-deficient, but not FLNb-deficient, M2 melanoma cells (Cunningham, 1995). FLNa is an actin-binding protein that promotes the orthogonal cross-linking of actin filaments, raising the possibility that it may regulate cytoskeletal rearrangement and other related cell functions such as cell motility (Cunningham *et al*., 1992; Li *et al*., 1999). FLNa can be phosphorylated by a wide variety of kinases, such as Src, p90RSK, PKA, and PAK-1. FLNa also has been found to be targeted by small GTPases of the Rho- and Rac-families, suggesting a role in cell motility and cell shape change. However, it is not well understood how phosphorylation regulates FLNa function (Ridley and Hall, 1992; Kozma *et al*., 1996; Hall, 1998; Ohta *et al*., 1999; Vadlamudi *et al*., 2002). In Chapter IV, we report that $P2Y_2$ receptor is linked to actin cytoskeleton via interaction with filamin A and regulate SMC migration. The model of the role of filamin A is shown in Fig I-2.

E. Endothelial cell-to-cell junctions and leukocyte permeability

E1. Endothelial cell-to-cell junctions seal endothelium and regulate leukocyte permeability Vascular endothelium is a monolayer of endothelial cells that separates the intima from the circulating blood. Endothelial cells adhere to neighboring cells by homodimerization of transmembrane adhesion proteins (Breier *et al.*, 1996; Bazzoni *et al.*, 2000b). Similar to epithelium, in the endothelium, mainly three types of junctions have been reported, which are tight junctions (TJ), adherens junctions (AJ) and gap junctions (reviewed by Bazzoni and Dejana, 2004). Both TJ and AJ are composed of transmembrane proteins and soluble proteins. In TJ, the transmembrane proteins occludin,

claudins, junction adhesion molecule (JAM), and the intracellular proteins zona occludens, ZO-1, ZO-2, and ZO-3 have been reported (Fig. I-1) (Stevenson *et al.*, 1986; Gumbiner *et al*., 1991; Furuse *et al*., 1993; 1998; Haskins *et al.*, 1998; Martin-Padura *et al*., 1998). In AJ, E-cadherin and VE-cadherin are transmembrane components and they bind to intracellular proteins α- and β-catenin (Fig. I-1) (Takeichi, 1993; Dejana *et al.*, 1995; Aberle *et al.*, 1996; Gumbiner, 1996). In each junctional complex, the transmembrane proteins can associate via their extracellular domain to the same protein on adjoining cells, thus form an endothelium that is impermeable to leukocytes. Intracellular junctional proteins are important in the regulation of tight and adherens junctional functions, such as anchorage of transmembrane proteins on the cell surface through linkage to actin cytoskeleton (Schneeberger and Lynch, 2004; Perez-Moreno and Fuchs, 2006). Although the TJ are usually located at the apical side of adherens junction in epithelium, such ordered structure is less defined in endothelium and TJ and AJ are intermingled (Simionescu, 2000). Gap junctions formed by connexin proteins are also found in the junctional complex, which mediate intercellular communication by forming channels between cells that allow the passage of electrical signals and small secondary messenger molecules (Simon and Goodenough, 1998). Desmosomes, which are seen in epithelium (Geisenheimer and Han, 1971), are not present in endothelium (Rubin, 1992; Lampugnani and Dejana, 1997; Bazzoni *et al*., 1999; Dejana *et al*., 1999; Simionescu, 2000).

The main functions of these junctions include restriction of membrane molecules from moving between apical and basalateral domains to maintain cell polarity (*i.e.* fence function), and fine-regulation of the extravasation and infiltration of leukocytes at sites of

vascular inflammation (*i.e.* barrier function) (Johnson-Leger *et al*., 2000; Muller, 2003). Endothelium of different blood vessels have different organization of junctions (Simionescu, 2000). Brain vessels, where maintaining the blood-brain barrier is required, display a well-developed junctional complex system (Rubin and Staddon, 1999; Wolburg and Lippoldt, 2002). In contrast, postcapillary endothelial cells, where frequent trafficking of cells and proteins is required, express low levels of junctional proteins (Jiang *et al.*, 1999). During embryogenesis in mice, development of junctions occurs in the late stages (Rubin and Staddon, 1999). It was found that formation of a fully functional blood-brain barrier is reached only after birth (Rubin and Staddon, 1999; Stewart, 2000).

E2. The role of actin cytoskeleton in the regulation of tight junctions TJ appear like fibrillar strands within the plasma membrane of endothelial cells at sites of cell-cell connection in electron micrographs. Cohesion of tight junctional strands on adjoining cell plasma membranes occludes the paracellular space and provides the mechanical basis for the barrier and fence functions (Tsukita *et al*., 2001). Actin filaments terminate at these highly ordered membrane contact sites, suggesting a physical linkage and functional/structural regulation of TJ by actin cytoskeleton.

Transmembrane proteins in TJ have been found to contribute to paracellular seals *in vitro*. Mutation of such proteins causes disruption of both fence and barrier functions (Balda *et al*., 1996b; Furuse *et al*., 1998). In freeze fracture electron micrographs, both membrane proteins (occludin and claudins) and cytosolic plaque proteins (ZO-1, ZO-2, and ZO-3) are localized at the fibrillar strand structures (Stevenson *et al*., 1986; Balda *et al*., 1993; Jesaitis and Goodenough, 1994; Fannning *et al*., 1998). Occludin, the first

adhesion protein discovered in TJ, has four transmembrane domains, two extracellular loops, a short N-terminal tail and a long C-terminal tail (Amieva *et al*., 2003). Occludin phosphorylation occurs when TJ assemble. The C-terminal tail of occludin has been reported to bind to the guanine kinase (GUK) domain of ZO-1 (Schmidt *et al*., 2001). ZO-1 belongs to the Membrane Associated Guanine Kinase (MAGUK) family, which is a family of diverse proteins that mediates the linkage between membrane proteins and cytoskeleton (Fanning *et al.*, 1998). All proteins in the MAGUK family have three PDZ (PSD-95, Dlg, ZO-1) domains, one SH3 (Src Homology 3) domain, and one guanine kinase (GUK) domain in their N-terminal halves, although kinase activity is not present in these proteins (Bazzoni and Dejana, 2004). The GUK domain of ZO-1 is predicted to be unable to bind guanine monophosphate (GMP), but it is reported to bind to the C-terminal tail of occludin (Schmidt *et al*., 2001). Multiple serine/threonine and tyrosine residues in ZO-1 are predicted to be phosphorylation targets (Beatch *et al*., 1996; Bazzoni *et al*., 1999, 2000a, b; Bazzoni and Dejana, 2001; Bazzoni, 2003). A serine kinase has been identified that can specifically phosphorylate the C-terminal tail of ZO-1, and was named ZAK-1 (ZO-1-associated kinase) (Behrens *et al*., 1985). The actin-binding activity is predicted to reside in the C-terminal domain of ZO-1. Unlike full-length ZO-1 or the N-terminal domain of ZO-1, expression of a construct encoding the C-terminal domain of ZO-1 does not localize to the cell membrane. Instead, it often targets to actin filaments that are connected to focal adhesions, intercellular junctions, microvilli, and lamellae in subconfluent cells (Fanning *et al*., 1998). ZO-1 also has been found to localize with actin filaments at intercellular junctions and in lamellae of astrocytes and transformed fibroblasts in cell culture (Howarth *et al*., 1992; Howarth and Stevenson, 1995). The N-

teriminal domain of ZO-1, which is unable to bind actin, can be as efficiently incorporated into cell-cell contacts as full length ZO-1. However, modulation of the structure of perijunctional actin cytoskeleton affects junction assembly and maintenance of the paracellular seal in both endothelium and epithelium (Madara *et al*., 1992; Anderson and Van Itallie, 1995). Expression of constitutive or dominant negative forms of RhoA or Rac1 in epithelial cells caused a marked change in perijunctional actin organization and disrupted the paracellular seal in epithelium (Jou *et al*., 1998).

Leukocyte extravasation is controlled by finely regulated opening and closing of the tight junction, which requires the coordination of actin cytoskeleton (Lampugnani *et al.*, 1993). The endothelial permeability of leukocytes is an important factor in vascular inflammation (de Boer *et al.*, 1999). Inflammation represents an attempt by the organism to combat on the infection, but could be detrimental if it persists in vascular tissue (Cotran *et al.*, 1999). Clarification of the molecular and signaling mechanisms that regulate assembly of the components that seal interendothelial junctions is key to understanding and controlling chronic vascular inflammation.

Figure I-1 Signaling mechanism of $P2Y_2$ receptor-induced VEGFR-2 activation and VCAM-1 expression in endothelial cells.

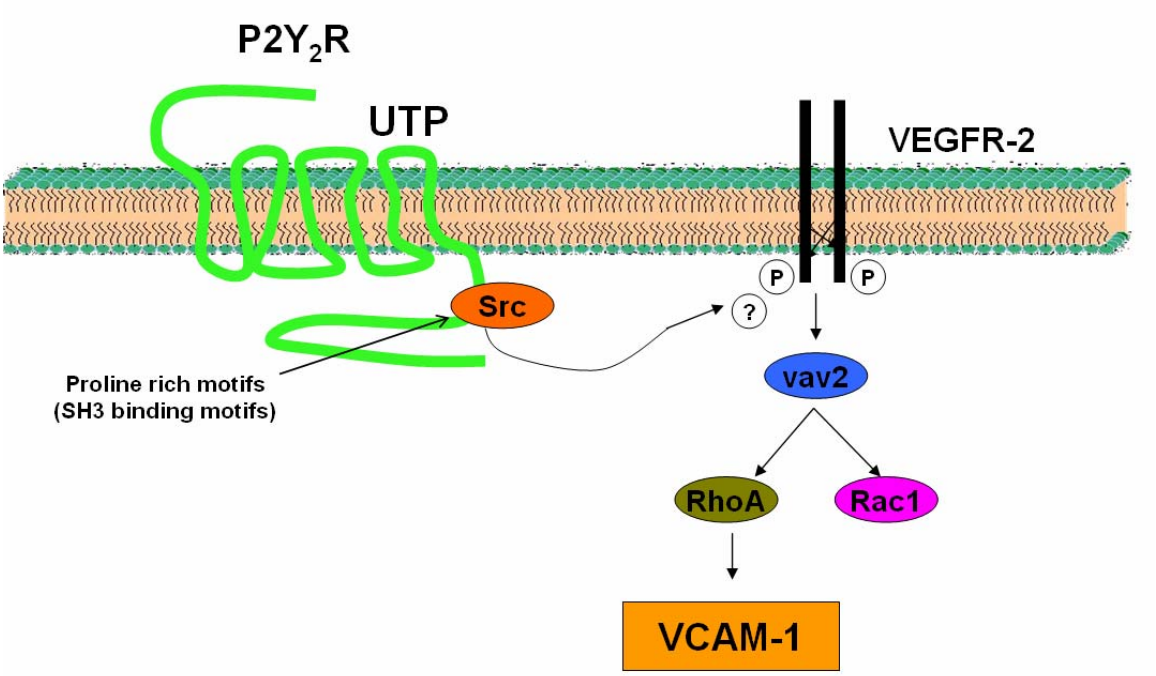

P2Y2R
UTP
VEGFR-2
P
P
Src
?
Proline rich motifs
(SH3 binding motifs)
vav2
RhoA
Rac1
VCAM-1

Figure I-2 The $P2Y_2$ receptor is linked to actin cytoskeleton via filamin A.

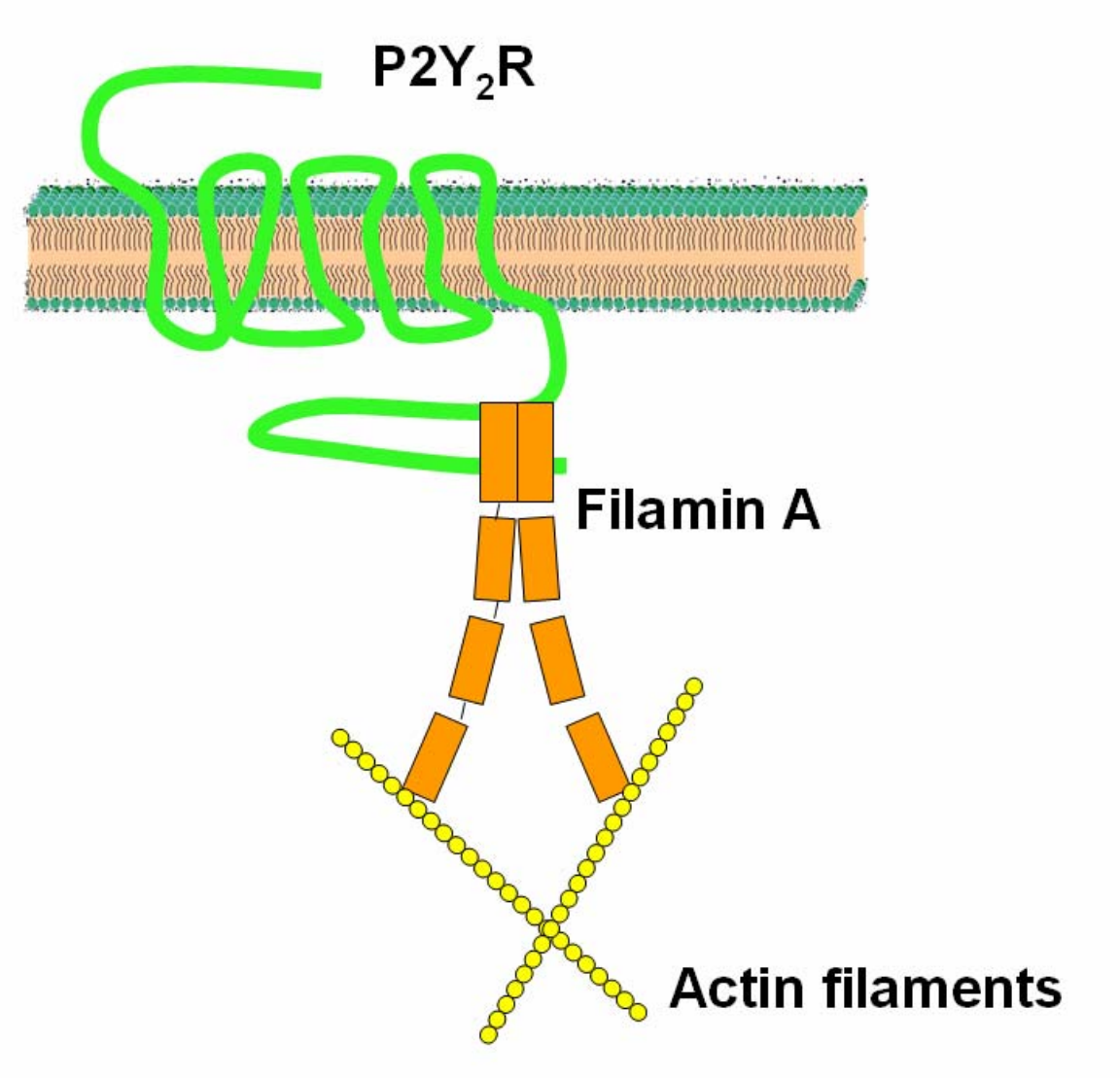
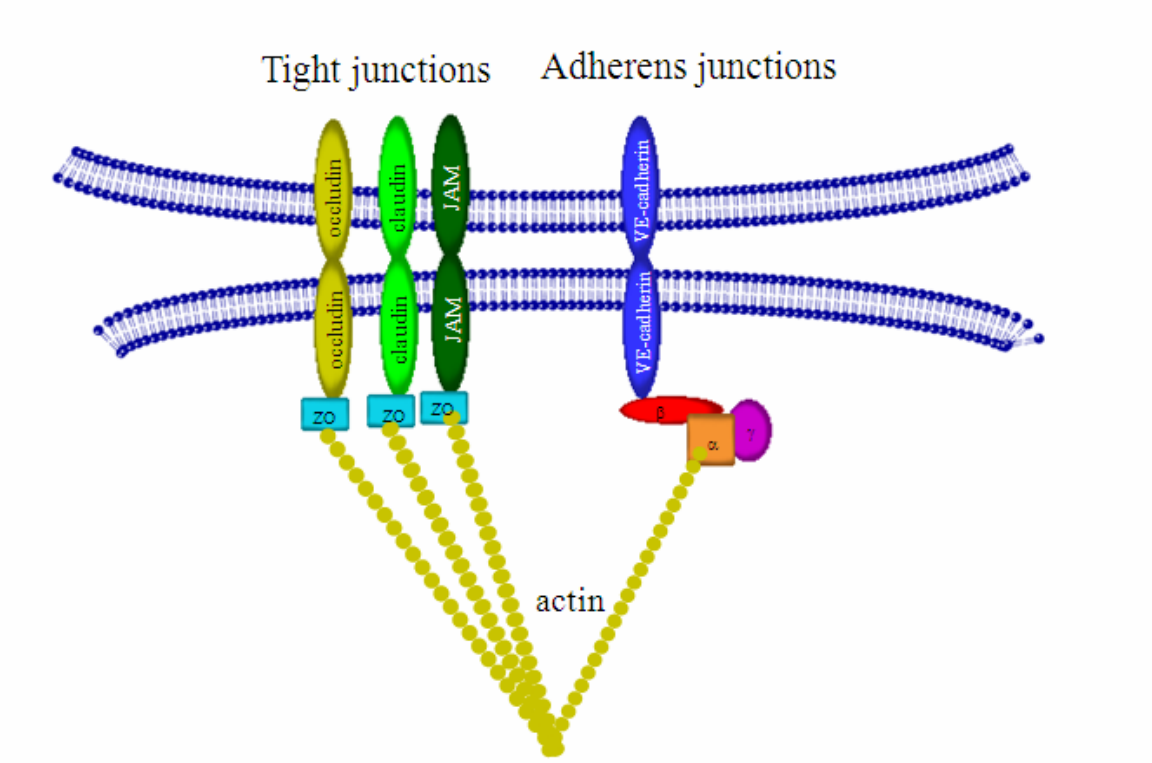

P2Y2R
Filamin A
Actin filaments

Figure I-3 Structure of tight junctions and adherens junctions.


Tight junctions
Adherens junctions
occludin
claudin
JAM
VE-cadherin
occludin
claudin
JAM
VE-cadherin
ZO
ZO
ZO
β
α
γ
actin

Chapter II

The $P2Y_2$ Nucleotide Receptor Mediates UTP-induced Vascular Cell Adhesion Molecule-1 Expression in Coronary Artery Endothelial Cells

This work is published in:

Cheikh I. Seye, Ningpu Yu, Renu Jain, Qiongman Kong, Tess Minor, Jessica Newton, Laurie Erb, Fernando A. González, and Gary A. Weisman. The $P2Y_2$ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *Journal of Biological Chemistry* 2003 Jul 4; 278(27): 24960-5

Abstract

$P2Y_2$ receptor up-regulation and activation induce intimal hyperplasia and monocyte/macrophage infiltration in the collared rabbit carotid artery model of vascular injury, suggesting a potential role for $P2Y_2$ receptors in monocyte recruitment by vascular endothelium. In this study, we addressed the hypothesis that activation of $P2Y_2$ receptors by extracellular nucleotides modulates the expression of adhesion molecules on vascular endothelial cells that are important for monocyte recruitment. Results indicated that the equipotent $P2Y_2$ receptor agonists UTP or ATP (1–100 μM) stimulated the expression of vascular cell adhesion molecule-1 (VCAM-1) in human coronary artery endothelial cells (HCAEC) in a time- and dose-dependent manner. $P2Y_2$ antisense oligonucleotides inhibited VCAM-1 expression induced by UTP but not by tumor necrosis factor-α. Furthermore, UTP induced VCAM-1 expression in human 1321N1 astrocytoma cell transfectants expressing the recombinant $P2Y_2$ receptor, whereas vector-transfected control cells did not respond to UTP. The effect of UTP on VCAM-1 expression in HCAEC was prevented by depletion of intracellular calcium stores with thapsigargin or by inhibition of p38 mitogen-activated protein kinase or Rho kinase, but was not affected by inhibitors of the mitogen-activated protein/extracellular signal-regulated kinase pathway (*i.e.* MEK1/2). Consistent with a role for VCAM-1 in the recruitment of monocytes, UTP or ATP increased the adherence of monocytic U937 cells to HCAEC, an effect that was inhibited by anti-VCAM-1 antibodies. These findings suggest a novel role for the $P2Y_2$ receptor in the p38- and Rho kinase-dependent expression of VCAM-1 that mediates the recruitment of monocytes by vascular endothelium associated with the development of atherosclerosis.

Introduction

Extracellular nucleotides cause a wide range of cellular responses and appear to play a role in the regulation of many vascular functions (Dubyak and el-Moatassim, 1993; Erlinge, 1998a). Vascular cells release nucleotides when exposed to stimuli such as ischemia, hypoxia, and chemical or mechanical stress (Pearson and Gordon, 1979; Grierson and Meldolesi, 1995). It also is becoming apparent that extracellular nucleotides can promote the development of a variety of pathologies including disorders of the immune system, and neurodegenerative and vascular diseases (Dubyak and el-Moatassim, 1993). Indeed, ATP or UTP induces proliferation and migration of vascular smooth muscle cells, two processes involved in the development of intimal lesions found in atherosclerosis and post-angioplasty restenosis (Malam-Souley *et al.*, 1993; Pillois *et al.*, 2002). The biological effects of extracellular nucleotides are mediated through activation of P1 and P2 purinergic receptors (Burnstock, 1998; Di Virgilio *et al*., 2001). P1 receptors are responsive to adenosine, whereas P2 receptors are activated by a variety of nucleotides including ATP and UTP (Burnstock, 1998; Di Virgilio *et al*., 2001). The P2 receptors are subdivided into two distinct categories, the metabotropic G protein-coupled (P2Y) receptors and the ionotropic ligand gated channel (P2X) receptors (Burnstock, 1998; Zhang *et al*., 2002). Vascular cells have been shown to express metabotropic P2Y and ionotropic P2X receptors (Radisavljevic *et al*., 2000; Di Virgilio *et al*., 2001). It has been reported that $P2Y_2$ receptors are up-regulated in cells of rat intimal lesions following balloon angioplasty (Seye *et al*., 1997). Recent studies in our group showed that placement of a silicone collar around the rabbit carotid artery promoted up-regulation of $P2Y_2$ receptors in vascular smooth muscle cells and endothelium (Seye *et*

al., 2002). Subsequently, local infusion of UTP was shown to stimulate intimal hyperplasia and increase intimal monocyte infiltration (Seye *et al*., 2002), suggesting a role for $P2Y_2$ receptors in the recruitment of blood monocytes leading to inflammation in atherosclerosis.

Monocyte recruitment into the vessel wall is a complex process that includes cell rolling, firm attachment, and directed migration (Reviewed by Libby, 2002). It is now becoming evident that adhesion molecules such as VCAM-1 play an important role in leukocyte adherence to vascular endothelial cells (Faruqi and DiCorleto, 1993; Cybulsky *et al*., 2001). VCAM-1 expression is induced or up-regulated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α (Dubyak and El-Moatassim 1993) and interleukin 1-β on cellular components of the arterial wall including endothelial cells, smooth muscle cells, and fibroblasts (Rice *et al*., 1990; Braun *et al*., 1995; Iademarco *et al*., 1995). ATP and UTP have been shown to induce cell-cell adhesion in a monocyte/macrophage lineage and neutrophil adherence to an endothelial cell monolayer (Ventura and Thomopoulos, 1995; Parker *et al*., 1996), raising the possibility that released ATP and UTP could induce endothelial cell activation by an autocrine/paracrine mechanism to regulate leukocyte adherence.

The purpose of this study was to test the hypothesis that extracellular nucleotides stimulate the expression of adhesion molecules on vascular endothelial cells and promote monocyte adherence. Results showed that the $P2Y_2$ receptor agonists UTP or ATP induced the expression of VCAM-1 in human coronary artery endothelial cells (HCAEC). The ability of $P2Y_2$ antisense oligonucleotides to inhibit UTP-induced VCAM-1 expression strongly suggests that this response is mediated via $P2Y_2$ receptors.

The effects of UTP were dependent upon release of calcium from intracellular stores and activation of the p38 kinase pathway. Finally, results indicated that nucleotide-induced VCAM-1 expression increased monocyte binding to endothelial cells, supporting the hypothesis that nucleotides released during vascular injury contribute to leukocyte/endothelial cell interactions and promote inflammatory responses involved in the development of atherosclerosis.

Experimental Procedures

Materials

HCAEC and endothelial cell basal medium-2 (EBM-2) were purchased from Clonetics (Walkerville, MD). U937 cells were obtained from American Type Cell Collection (ATCC), and RPMI 1640 medium was purchased from Invitrogen. Anti-VCAM-1 (P3C4), anti-ICAM-1 (P2A4), and anti-E-selectin (P2H3) antibodies used in blocking studies were purchased from Chemicon International (Temecula, CA). Anti-ICAM-1 (clone G-5), anti-β-actin (rabbit polyclonal), anti-VCAM-1 (c-19), and anti-VCAM-1 (HAE-2z) antibodies used for Western analysis and immunofluorescence, respectively, were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Oregon green-labeled goat anti-rabbit IgG antibody and Hoechst 33258 were purchased from Molecular Probes (Eugene, OR). The polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody was obtained from Cell Signaling (Beverly, MA). The p38 inhibitor SB203580, MEK1/2 inhibitor U0126, and Rho kinase 1/2 inhibitor Y27632 were purchased from Calbiochem (Indianapolis, IN). DOTAP liposomal reagent was purchased from Roche Diagnostics. Phosphorothioate-modified oligonucleotides to human *$P2Y_2$* were synthesized and purified by Integrated DNA Technologies. All other reagents including nucleotides and the PKH2 green fluorescent cell linker were obtained from Sigma, unless otherwise specified.

Cell Cultures

HCAEC were cultured in EBM-2 supplemented with SingleQuot® Kit (Lonza Group Ltd, Switzerland) at 37 °C in a humidified atmosphere of 5% CO_2. HCAEC were used between the third and tenth passages. Monocytic U937 cells were grown in RPMI

1640 medium supplemented with 5% (v/v) fetal bovine serum. The retroviral vector pLXSN was used for stable expression of the human $P2Y_2$ receptor in human 1321N1 astrocytoma cells, as described previously (Erb *et al*., 1995). Briefly, the recombinant $P2Y_2$-pLXSN construct or pLXSN (control) was used to transfect PA317 amphotropic packaging cells for production of the viral vectors. Then, 1321N1 cells were infected with the viral vectors and cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 1 mg/ml G418 (Invitrogen) for neomycin resistance selection.

$P2Y_2$ Antisense S-oligonucleotides

Sequences including translation initiation site were as follows: sense, 5'-GGCGATGGCAGCAGACCTGGCCCCTGGA-3'; antisense, 5'-TCCAGGGGCCAGG TCTGCTGCCATCGCC-3'. The sequences were checked for uniqueness using the National Center for Biotechnology Information's Local Alignment Search Tool (BLAST) (Erlinge, 1998b). Briefly, HCAEC were incubated with 2 μM $P2Y_2$ sense or antisense S-oligonucleotides for 6 h at 37 °C in EBM-2 medium containing 1.4% (v/v) DOTAP liposomal reagent. Then, fresh EBM-2 medium was added and cells were cultured for an additional 20 h, as previously reported (Warny *et al*., 2001). Fluorescein isothionate-conjugated $P2Y_2$ antisense oligonucleotides were used to monitor cellular oligonucleotide uptake by HCAEC.

Immunofluorescence Staining

HCAEC were made quiescent by incubation in EBM-2 for 24 h at 37 °C before exposure to UTP. After treatment with UTP (100 μM) or TNF-α (10 ng/ml) for 8 h at 37 °C, the cells were fixed in acetone for 15 min and treated with 0.2% (v/v) Triton X-100 in

phosphate-buffered saline (PBS) at room temperature. After washing in PBS, VCAM-1 expression was detected in cells by incubation for 2 h at room temperature with a rabbit polyclonal anti-VCAM-1 (HAE-2z) antibody (1:200 dilution in PBS containing 5% (v/v) normal goat serum), followed by Oregon green-labeled goat anti-rabbit IgG antibody (1:200 dilution in PBS). Then, the cells were incubated in PBS containing 2 μM Hoechst 33258 for nuclear counterstaining.

Binding of U937 Cells to Human Endothelial Cells

HCAEC were grown to confluence in 24-well plates in EBM-2 supplemented with 5% (v/v) fetal bovine serum, and then the cells were incubated for 24 h at 37 °C in EBM-2 followed by stimulation with 100 μM ADP, ATP, UTP, adenosine, or 2MeS-ATP for an additional 8 h at 37 °C. In some experiments, HCAEC were preincubated with BAPTA/AM (10 μM) for 30 min before the addition of nucleotides. U937 cells were labeled with the green fluorescent dye PKH2, as described previously (Allen *et al*., 1998). Labeled U937 cells (106 cells/well) were added to confluent cultures of HCAEC for 1 h at 37 °C. The cells were washed several times in PBS, and adherent U937 cell numbers were determined by microscopy using fluorescein isothiocyanate illumination. In experiments designed to investigate the effect of blocking antibodies on binding of U937 cells, HCAEC were treated with UTP for 8 h at 37 °C and then 10 μg/ml anti-VCAM-1, anti-ICAM-1, or anti-E-selectin antibody was added to HCAEC for 45 min before addition of U937 cells. For each condition, cell numbers were determined in seven different fields of view in three wells.

Western Blot Analysis

Equivalent amounts of protein (100 μg) in extracts of HCAEC were subjected to

7.5% (w/v) SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. Detection of VCAM-1 and ICAM-1 was performed using goat anti-mouse VCAM-1 or goat anti-mouse ICAM-1 monoclonal antibodies (1:1000 dilution), followed by horseradish peroxidase-conjugated anti-mouse IgG (1:2000 dilution). For signal normalization, membranes were probed with polyclonal anti-β-actin antibody (1:2000 dilution). A mouse monoclonal pERK (E-4) antibody and a rabbit polyclonal anti-p38 MAP kinase antibody were used to analyze ERK1/2 and p38 phosphorylation.

Statistical Analysis

Results are represented as mean ± S.E. Statistical analyses were performed by analysis of variance and unpaired Student's t test (significance, $p < 0.05$).

Results

ATP and UTP Induce VCAM-1 Expression in Vascular Endothelial Cells

Incubation of serum-starved HCAEC with UTP or ATP for 8 h caused a dose-dependent increase in the expression of VCAM-1 (Fig. II-1A and B). In contrast, UDP, ADP, 2MeS-ATP, or adenosine had no significant effect even at concentrations as high as 100 μM (not shown). VCAM-1 expression was induced within 2 h of UTP or ATP addition, reached a maximum level within 6 h, and remained elevated through 16 h (Fig. II-1C and D). A significant increase in VCAM-1 expression occurred even when HCAEC were exposed to 100 μM UTP for as little as 30–60 min before the nucleotide agonist was removed (Fig. II-1E). Incubation of HCAEC with the enzyme potato apyrase grade III (2 units/ml) that degrades nucleoside di- and triphosphates inhibited UTP or ATP-induced VCAM-1 expression (not shown). In contrast to VCAM-1 expression, ATP or UTP did not significantly increase ICAM-1 expression in quiescent HCAEC (Fig. II-2). These data suggest a selective effect of UTP and ATP on VCAM-1 expression in human endothelial cells. Immunofluorescence staining of quiescent untreated HCAEC detected a low basal level of VCAM-1 expression with a punctate staining (Fig. II-3A). In cells treated with UTP (100 μM), there was diffuse VCAM-1 staining over the entire cell surface (Fig. II-3B). A similar VCAM-1 staining pattern was observed when cells were treated with 10 ng/ml TNF-α (Fig. II-3C).

Calcium Release from Internal Stores Is Required For UTP-induced VCAM-1 Expression

UTP caused an increase in the intracellular free calcium concentration, $[Ca^{2+}]_i$, in HCAEC, a response that was inhibited by the intracellular calcium chelator BAPTA (not

shown). VCAM-1 expression induced by UTP also was inhibited in HCEAC that were preincubated with 10 μM BAPTA/AM for 30 min at 37 °C (Fig. II-4). Furthermore, thapsigargin (2 μg/ml), an agent that depletes intracellular calcium stores, also inhibited UTP-induced VCAM-1 expression (Fig. II-4), further demonstrating a role for UTP-induced increases in $[Ca^{2+}]_i$ in the expression of VCAM-1. Thapsigargin treatment had no apparent toxic effect on HCEAC as measured by trypan blue exclusion (not shown).

UTP-induced VCAM-1 Expression Is Mediated by $P2Y_2$ receptors

Pertussis toxin (100 ng/ml), an inhibitor of $G_{\alpha i/o}$ proteins, attenuated the effects of UTP on VCAM-1 expression in HCAEC (Fig. II-5A). Because the $P2Y_2$ receptor subtype has been shown to be partially sensitive to pertussis toxin (Erb *et al*., 2001), we evaluated the effect on UTP-stimulated VCAM-1 expression in HCAEC of inhibition of $P2Y_2$ receptor activity with $P2Y_2$ antisense S-oligonucleotides. Treatment of HCAEC with $P2Y_2$ antisense S-oligonucleotides (2 μM), complementary to the $P2Y_2$ translation initiation region, inhibited UTP-induced VCAM-1 expression by ~50% (Fig. II-5B and C; $p < 0.05$) compared with cells treated with $P2Y_2$ sense oligonucleotides. In contrast, $P2Y_2$ antisense S-oligonucleotides had no effect on TNF-α-induced VCAM-1 expression in HCAEC (Fig. II-5B and C). To confirm the involvement of $P2Y_2$ receptors in UTP-induced VCAM-1 expression in HCAEC, we stably expressed $P2Y_2$ receptor cDNA in 1321N1 cells that lack endogenous P2 receptors (Parr *et al*., 1994). Cells transfected with $P2Y_2$ receptor cDNA and treated with UTP for 8 h at 37 °C expressed VCAM-1, whereas vector-transfected control cells failed to respond to UTP (Fig. II-6). Taken together, these results demonstrate that $P2Y_2$ receptors mediate UTP-induced VCAM-1 expression.

Rho Kinase Activation and p38 Phosphorylation Mediate UTP-induced VCAM-1 Expression

The UTP-induced increase in $[Ca^{2+}]_i$ mediates mitogen-activated protein kinase phosphorylation in vascular cell types including endothelial cells (Boarder and Hourani, 1998; Chaulet *et al*., 2001). In HCAEC, UTP (100 μM) caused the rapid phosphorylation of ERK1/2 and p38 that was inhibited by the MEK1/2 inhibitor U0126 (5 μM) or the p38 inhibitor SB203580 (10 μM), respectively (Fig. II-7A). Preincubation of HCAEC with 10 μM SB203530 prevented UTP-induced VCAM-1 expression in HCAEC, whereas the MEK1/2 inhibitor U0126 (5 μM) had no effect (Fig. II-7B). Because P2Y receptors have been shown to couple to activation of the small GTPase RhoA (Sauzeau *et al*., 2000), we examined the effect of the Rho kinase inhibitor Y27632 on UTP-induced VCAM-1 expression. Pretreatment of HCAEC with 10 μM Y27632 inhibited the expression of VCAM-1 induced by UTP (Fig. II-7B). Thus, phosphorylation of Rho kinase and p38, but not ERK1/2, is involved in the intracellular signaling pathways leading to VCAM-1 expression induced by UTP.

VCAM-1 Expression Correlates with Increased Monocytic Cell Adherence to Endothelial Cells

Stimulation of HCAEC with UTP or ATP (100 μM) for 8 h increased the binding of U937 monocytes to HCAEC by ~4- and ~3.6-fold, respectively, compared with HCAEC incubated in the absence of nucleotides (Fig. II-8). In contrast, treatment of HCAEC with UDP, adenosine, or 2MeS-ATP (Fig. II-8) or ADP (not shown) did not

significantly increase U937 cell adherence to HCAEC. Introduction of BAPTA/AM (10 μM) into HCAEC prior to treatment with UTP attenuated U937 cell binding to endothelial cells (Fig. II-8). Incubation of HCAEC with anti-VCAM-1 antibody, but not anti-ICAM-1 or anti-E-selectin antibody, significantly decreased U937 cell binding to UTP-treated HCAEC (Fig. II-8). These antibodies did not affect U937 cell adherence to HCAEC in the absence of UTP (not shown).

Discussion

The present study demonstrates that exogenous UTP or ATP stimulate VCAM-1 expression via $P2Y_2$ receptors in HCAEC. It also indicates that the $P2Y_2$ receptor-mediated increase in VCAM-1 expression promotes the adherence of U937 monocytes to HCAEC. These results, for the first time, link P2Y purinergic receptor signaling to the mechanisms controlling the expression of an endothelial cell adhesion molecule involved in monocyte recruitment in early atherosclerosis.

Vascular diseases such as atherosclerosis and post-angioplasty restenosis are initiated by vascular injury. Previous reports demonstrated that large amounts of nucleotides are released into the extracellular milieu in response to vascular stress conditions including ischemia/oxidative stress, flow, and mechanical stretch (Pearson and Gordon, 1979; Di Virgilio *et al*., 2001). In addition, high concentrations of extracellular ATP and UTP generated *in vivo* by platelet aggregation or cell damage that occurs during transluminal angioplasty should increase the concentration of nucleotides in the vessel wall. Although the metabolism of extracellular nucleotides by ecto-nucleotidases plays a role in the regulation of purinergic signaling, recent studies indicate that chronic hypoxic exposure of pulmonary artery endothelial cells can decrease the rate of ecto-nucleotidase activity (Gerasimovskaya *et al*., 2002). In addition, it also has been reported that certain pathological conditions, especially those associated with endothelial cell activation, can decrease ATP-diphosphorylase activity (Robson *et al*., 1997), suggesting that pathological conditions affecting blood vessels contribute to local elevations in nucleotide concentrations in the vessel wall.

The $P2Y_2$ receptor is up-regulated in activated thymocytes (Koshiba *et al*., 1997) and in epithelial cells in response to ligation of the salivary gland duct (Turner *et al*., 1998). It is now becoming apparent that $P2Y_2$ receptor up-regulation occurs in various models of vascular injury including balloon denudation of the rat aorta (Seye *et al*., 1997) and placement of a silicone collar around the rabbit carotid artery (Seye *et al*., 2002). In the latter model, we showed that activation of the $P2Y_2$ receptor by UTP stimulated intimal hyperplasia and induced monocyte/macrophage infiltration into the vessel wall, suggesting a potential role for this receptor in monocyte recruitment by vascular endothelium. The relative equipotency and equiefficacy of UTP and ATP for induction of VCAM-1 expression in HCAEC (Fig. II-1) strongly suggests the involvement of $P2Y_2$ receptors. Nonetheless, the ability of antisense, but not sense, $P2Y_2$ oligonucleotides to inhibit UTP-induced VCAM-1 expression (Fig. II-5) unambiguously demonstrates the role of $P2Y_2$ receptors. Furthermore, UTP stimulated the expression of VCAM-1 in human 1321N1 astrocytoma cells expressing recombinant $P2Y_2$ receptors but not in control 1321N1 cells (Fig. II-6) that lack endogenous P2 receptors (Parr *et al*., 1994). Moreover, the inability of 2MeS-ATP and UDP to induce VCAM-1 expression in HCAEC suggests that activation of $P2Y_1$, $P2Y_4$, and $P2Y_6$ receptors is not involved.

Stimulation of the $P2Y_2$ receptor in endothelial cells is known to activate a variety of signaling molecules including the mitogen- and stress-activated protein kinases ERK1/2, p38, and c-Jun NH2-terminal kinase, and the small GTPase RhoA, and the release of the second messenger Ca^{2+} into the cytoplasm (Boarder and Hourani, 1998; Sauzeau *et al*., 2000; Chaulet *et al*., 2001). In this study, UTP-induced VCAM-1

expression in HCAEC was prevented by the calcium chelator BAPTA or by depletion of intracellular calcium stores with thapsigargin (Fig. II-4), indicating a role for $P2Y_2$ receptor-mediated increases in $[Ca^{2+}]_i$ in VCAM-1 expression. UTP-induced VCAM-1 expression also was suppressed by inhibitors of p38 and Rho kinase but not by inhibition of ERK1/2 (Fig. II-7), suggesting that p38 and RhoA are involved in the increased expression of VCAM-1 mediated by $P2Y_2$ receptors.

Vascular endothelial growth factor induces VCAM-1, ICAM-1, and E-selectin expression in endothelial cells through a pathway involving the MEK/ERK-independent activation of NF-κB (Kim *et al*., 2001). Activation of the $P2Y_2$ receptor has been reported to increase prostacyclin and nitric oxide release from endothelial cells (Lustig *et al*., 1992; Shen *et al*., 1999). Studies have shown that nitric oxide modulates the level of VCAM-1 protein expression in endothelial cells (De Caterina *et al*., 1995). Although nitric oxide did not play a role in vascular endothelial growth factor-induced adhesion molecule expression (Kim *et al*., 2001), it could potentially modulate the effects of UTP on VCAM-1 expression in endothelial cells. Further studies are needed to identify the factors involved in nucleotide-induced VCAM-1 expression both at the transcriptional and translational levels.

Consistent with earlier reports showing that ATP and UTP stimulate cell-cell adhesion in a monocyte/macrophage lineage and enhance neutrophil adherence to endothelial cell monolayers (Ventura and Thomopoulos, 1995), our data demonstrate that ATP and UTP stimulate U937 monocyte adherence to HCAEC (Fig. II-8). The $P2Y_2$ receptor contains the integrin binding domain arginine-glycine-aspartic acid (RGD) in its

first extracellular loop (Erb *et al*., 2001). The RGD motif has been shown to be the core recognition sequence for many integrins including $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Hynes, 1992). Recent studies have indicated that the RGD sequence in the $P2Y_2$ receptor mediates its interaction with $\alpha_v\beta_3/\beta_5$ integrins, and is required for G_o-mediated signal transduction (Erb *et al*., 2001). It has been reported previously that the $\alpha_v\beta_3$ integrin on monocytes mediates their adherence to endothelial and epithelial cells, an early event in the acute inflammatory response (Beekhuizen and van Furth, 1993). Thus, protein complex formation between $\alpha_v\beta_3/\beta_5$ integrins and $P2Y_2$ receptors also may be involved in the regulation of monocyte binding to endothelial cells mediated by $P2Y_2$ receptors.

In summary, this study is the first to demonstrate that extracellular nucleotides and $P2Y_2$ receptor activation can induce VCAM-1 expression in endothelial cells and promote the adherence of monocytes. In combination with our previous studies, these results indicate that $P2Y_2$ receptor up-regulation in vascular cells mediates the nucleotide-stimulated proliferation of smooth muscle cells and the adherence of monocytes to endothelium, responses associated with intimal hyperplasia and inflammation seen in atherosclerosis. These results encourage the development of therapeutic strategies to limit $P2Y_2$ receptor up-regulation and activation as a means to prevent arterial disease.

Figure II-1. UTP or ATP induces VCAM-1 expression in HCAEC.

A and *B*, quiescent HCAEC (serum-free-medium for 24 h at 37 °C) were stimulated with the indicated concentrations of UTP or ATP for 8 h at 37 °C. *C* and *D*, quiescent HCAEC were stimulated with 100 μM UTP or ATP for the indicated times at 37 °C. *E*, quiescent HCAEC were stimulated with 100 μM UTP for 5, 15, 30, or 60 min at 37 °C (*lanes 2–5*), the medium was removed and replaced with serum-free medium, and the cells were incubated for 8 h at 37 °C. Also, HCAEC were continuously incubated in the absence (*lane 1*) or presence (*lane 6*) of 100 μM UTP for 8 h at 37 °C. Protein extracts of HCAEC were prepared and 100 μg of extract protein from each sample were subjected to electrophoresis and transferred to nitrocellulose, as described under "Experimental Procedures." The nitrocellulose membrane was divided into two pieces and probed with either anti-VCAM-1 antibody or anti-β-actin antibody to verify the equivalence of protein loading. Results shown are representative of four independent experiments.

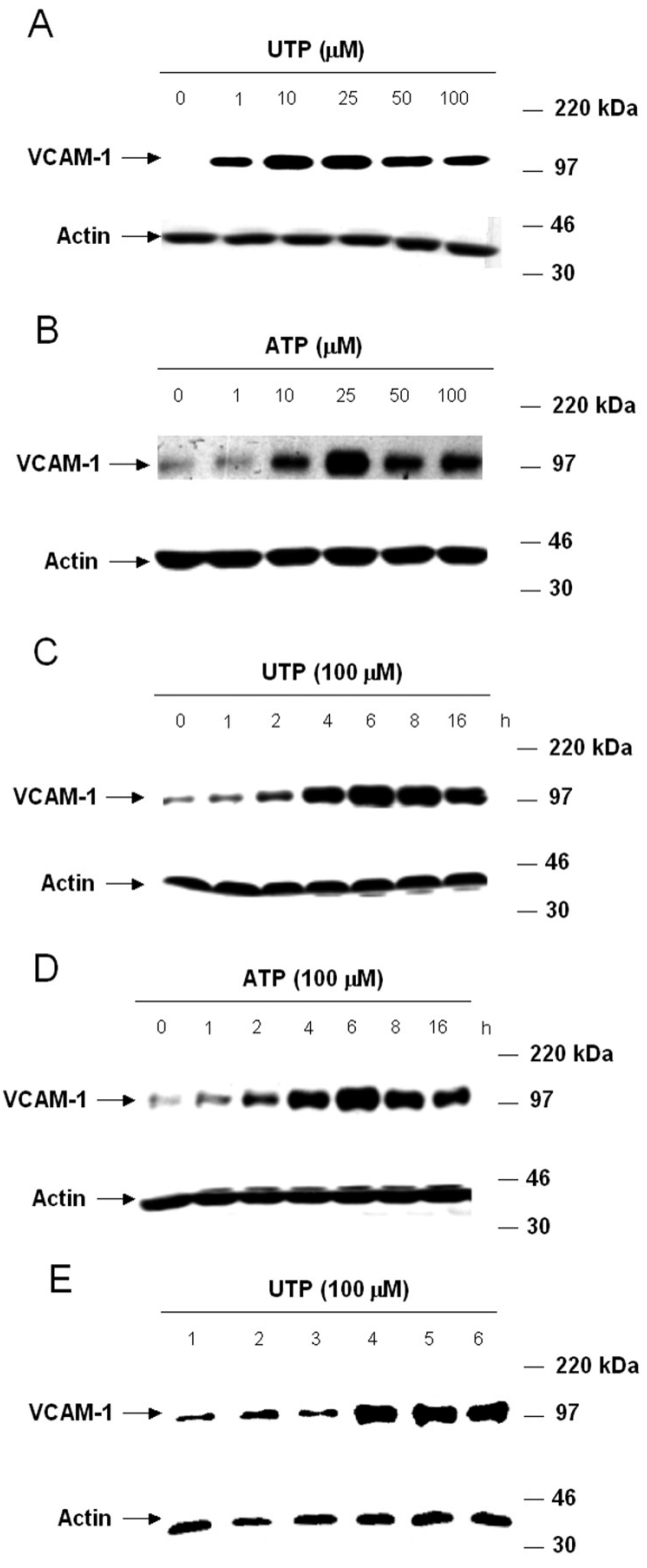

A
UTP (μM)
0 1 10 25 50 100
220 kDa
VCAM-1
97
Actin
46
30
B
ATP (μM)
0 1 10 25 50 100
220 kDa
VCAM-1
97
Actin
46
30
C
UTP (100 μM)
0 1 2 4 6 8 16 h
220 kDa
VCAM-1
97
Actin
46
30
D
ATP (100 μM)
0 1 2 4 6 8 16 h
220 kDa
VCAM-1
97
Actin
46
30
E
UTP (100 μM)
1 2 3 4 5 6
220 kDa
VCAM-1
97
Actin
46
30

Figure II-2. ICAM-1 expression in HCAEC is unaffected by UTP or ATP.

Quiescent endothelial cells were stimulated with the indicated concentrations of UTP or ATP for 8 h at 37 °C, and then ICAM-1 protein levels were determined by Western analysis with anti-ICAM-1 antibody, as described in the legend to Figure II-1. Results shown are representative of five independent experiments.

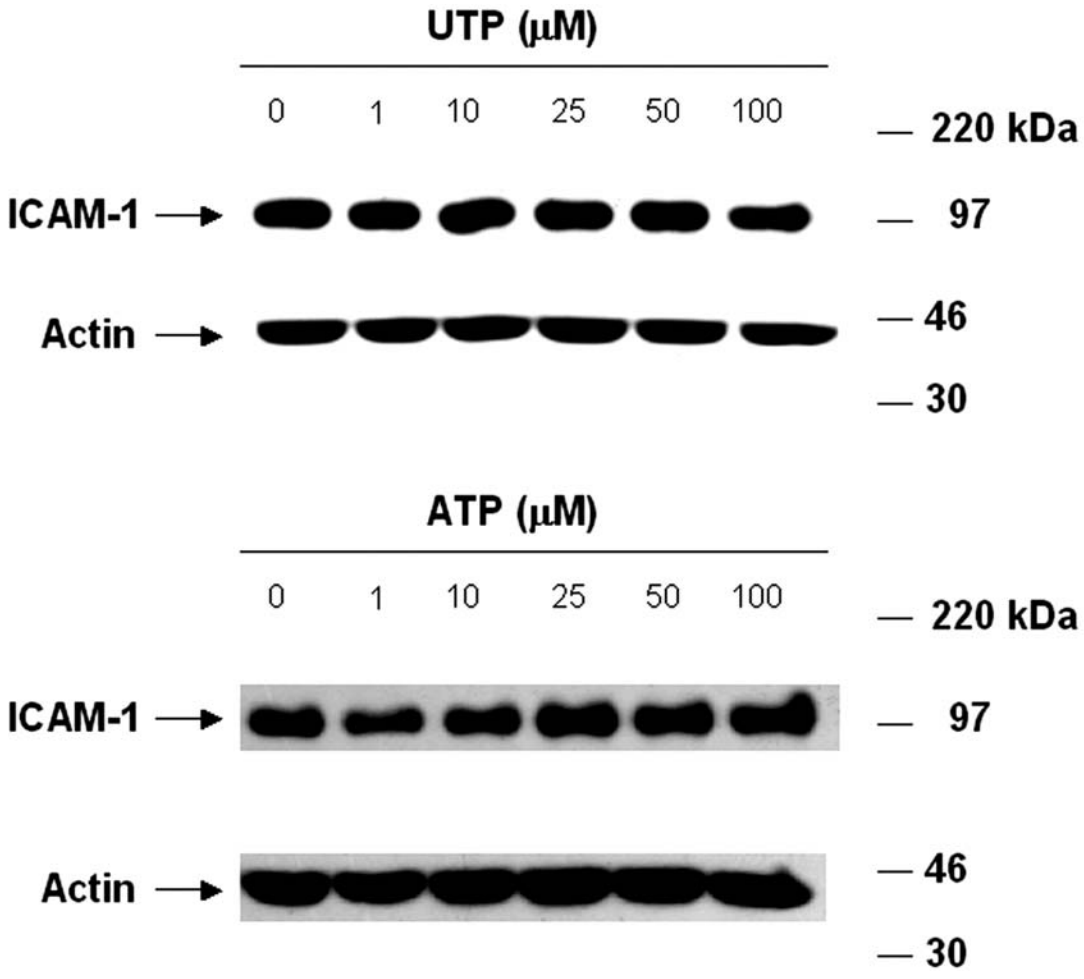
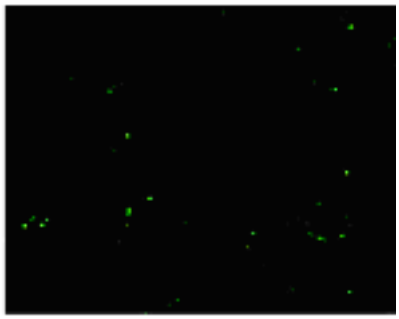

UTP (μM)
0
1
10
25
50
100
220 kDa
ICAM-1
97
Actin
46
30
ATP (μM)
0
1
10
25
50
100
220 kDa
ICAM-1
97
Actin
46
30

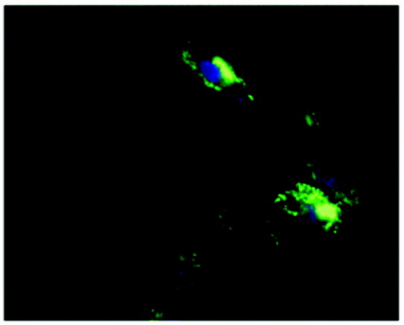
Figure II-3. Detection of VCAM-1 expression in endothelial cells by immunofluorescence.

Cultured HCAEC were serum-starved for 24 h at 37 °C and then incubated in the absence (*A*) or presence (*B*) of 100 μM UTP for 8 h at 37 °C. As a positive control (*C*), HCAEC were stimulated with TNF-α (10 ng/ml) for 8 h at 37 °C and analyzed for VCAM-1 expression. VCAM-1 staining (*green*) was obtained by indirect immunofluorescence, as described under "Experimental Procedures". Nuclei were counterstained with Hoechst (*blue*), and the images were merged. Fluorescence micrographs representative of three individual experiments are shown.

A



B



C

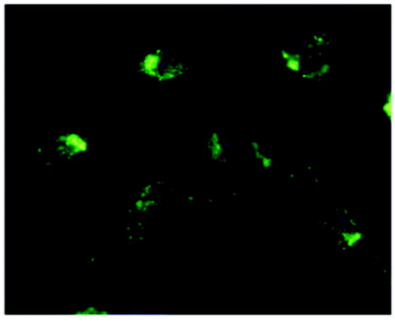


Figure II-4. Calcium release from internal stores mediates UTP-induced VCAM-1 expression.

HCAEC incubated in serum-free medium for 24 h at 37 °C were then incubated with BAPTA-AM (10 μM) or thapsigargin (2 μg/ml) for 30 min, and stimulated with 100 μM UTP (when indicated) for 8 h at 37 °C. VCAM-1 protein expression was determined in cell lysates by Western analysis, as described in the legend to Figure II-1. Results shown are representative of three independent experiments.

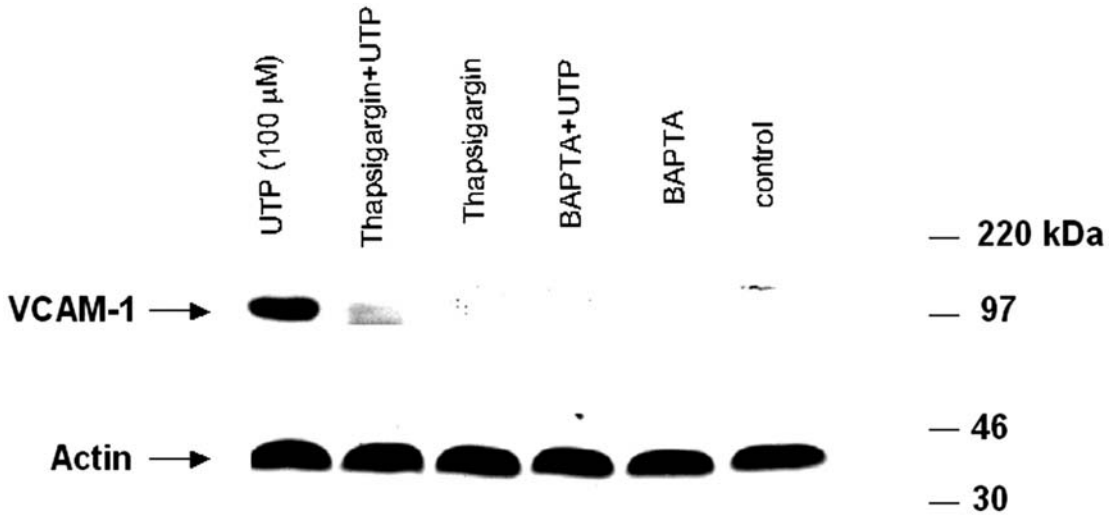

UTP (100 μM)
Thapsigargin+UTP
Thapsigargin
BAPTA+UTP
BAPTA
control
220 kDa
VCAM-1
97
Actin
46
30

Figure II-5. UTP-induced VCAM-1 expression is mediated by $P2Y_2$ receptors.

A, serum-starved HCAEC were incubated in the presence or absence of pertussis toxin (*PTX*; 100 ng/ml) for 1 h at 37 °C, and then incubated with or without UTP (100 μM) for 8 h at 37 °C. *B*, cells were transfected with 2 μM $P2Y_2$ antisense (*AS*) or sense (*S*) S-oligonucleotides for 4 h at 37 °C in EBM-2 containing 1.4% (v/v) DOTAP liposomal reagent. Then, cells were cultured for an additional 20 h at 37 °C in serum-free EBM-2 followed by addition of UTP (100 μM) or TNF-α for 8 h at 37 °C, and VCAM-1 expression was determined by Western analysis, as described in the legend to Figure II-1. *C,* bar graphs indicate VCAM-1 expression normalized to β-actin staining and represent the mean ± S.E. of results from three independent experiments.

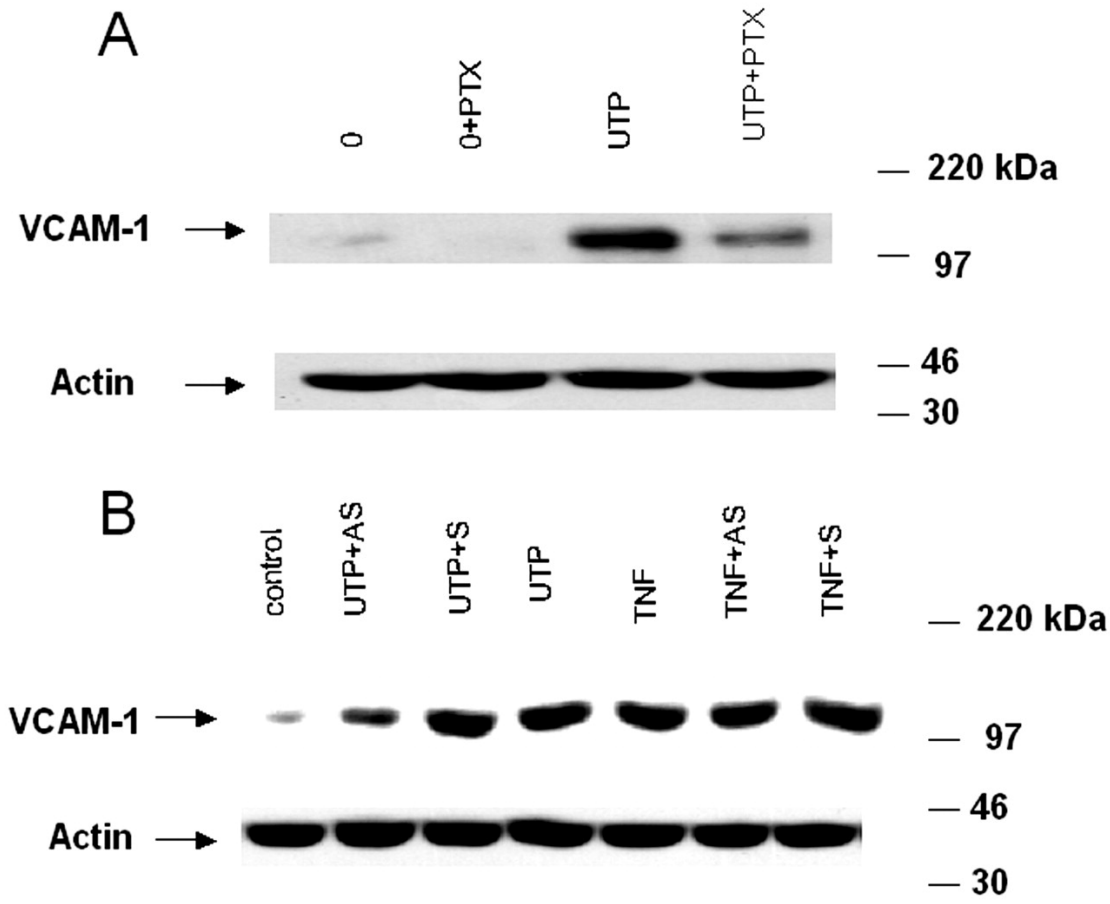

A
0
0+PTX
UTP
UTP+PTX
220 kDa
VCAM-1
97
Actin
46
30
B
control
UTP+AS
UTP+S
UTP
TNF
TNF+AS
TNF+S
220 kDa
VCAM-1
97
Actin
46
30
C
VCAM-1
(arbitrary units)
10.0
7.5
5.0
2.5
0.0
control
UTP
antisense
sense
TNF-α
antisense
sense

Figure II-6. $P2Y_2$ receptors mediate VCAM-1 expression in transfected 1321N1 astrocytoma cells.

Human 1321N1 cells expressing the $P2Y_2$ receptor (*$P2Y_2$-1321N1 cells*) or vector transfected controls (*1321N1 cells*) were incubated for the indicated times at 37 °C with 100 μM UTP and VCAM-1 expression was determined by Western analysis, as described in the legend to Figure II-1. Results shown are representative of three independent experiments.

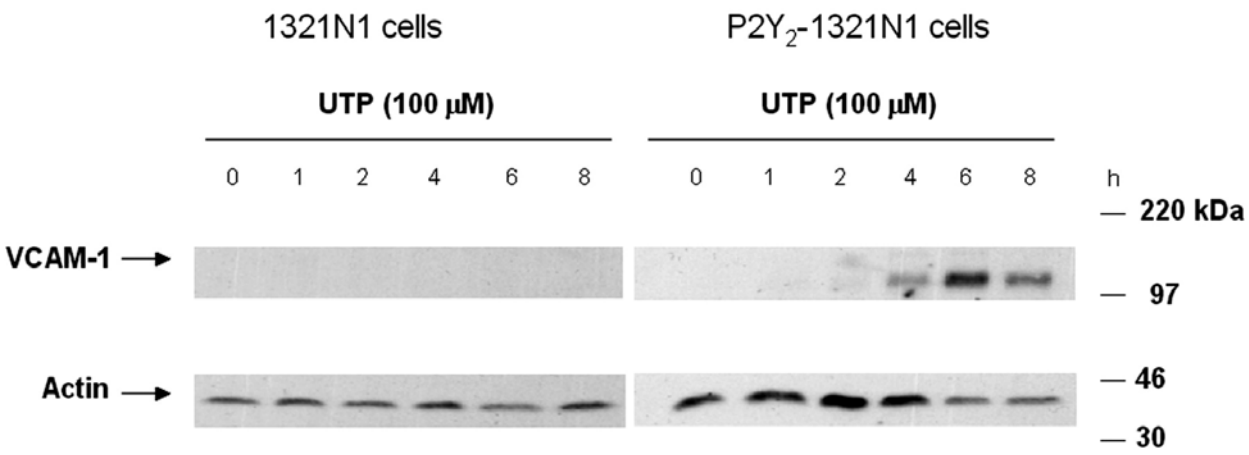

1321N1 cells
P2Y2-1321N1 cells
UTP (100 µM)
UTP (100 µM)
0 1 2 4 6 8
0 1 2 4 6 8
h
220 kDa
VCAM-1
97
Actin
46
30

Figure II-7. Inhibitors of p38 and Rho kinase, but not MEK1/2, attenuate UTP-induced VCAM-1 expression.

A, serum-starved HCAEC were incubated for 2 h at 37 °C in the presence or absence of the MEK1/2 inhibitor U0126 (5 μM) or the p38 inhibitor SB203580 (10 μM), followed by stimulation with 100 μM UTP for 10 min at 37 °C. Phosphorylated ERK1/2 and p38 were detected in cell extracts by Western analysis. *B*, serum-starved HCAEC were incubated in the presence or absence of the MEK1/2 inhibitor U0126 (5 μM), the p38 inhibitor SB203580 (10 μM), or the Rho kinase 1/2 inhibitor Y27632 for 2 h at 37 °C followed by stimulation with 10 μM UTP for 8 h at 37 °C. VCAM-1 expression was determined by Western analysis, as described in the legend to Figure II-1. Results shown are representative of three independent experiments.

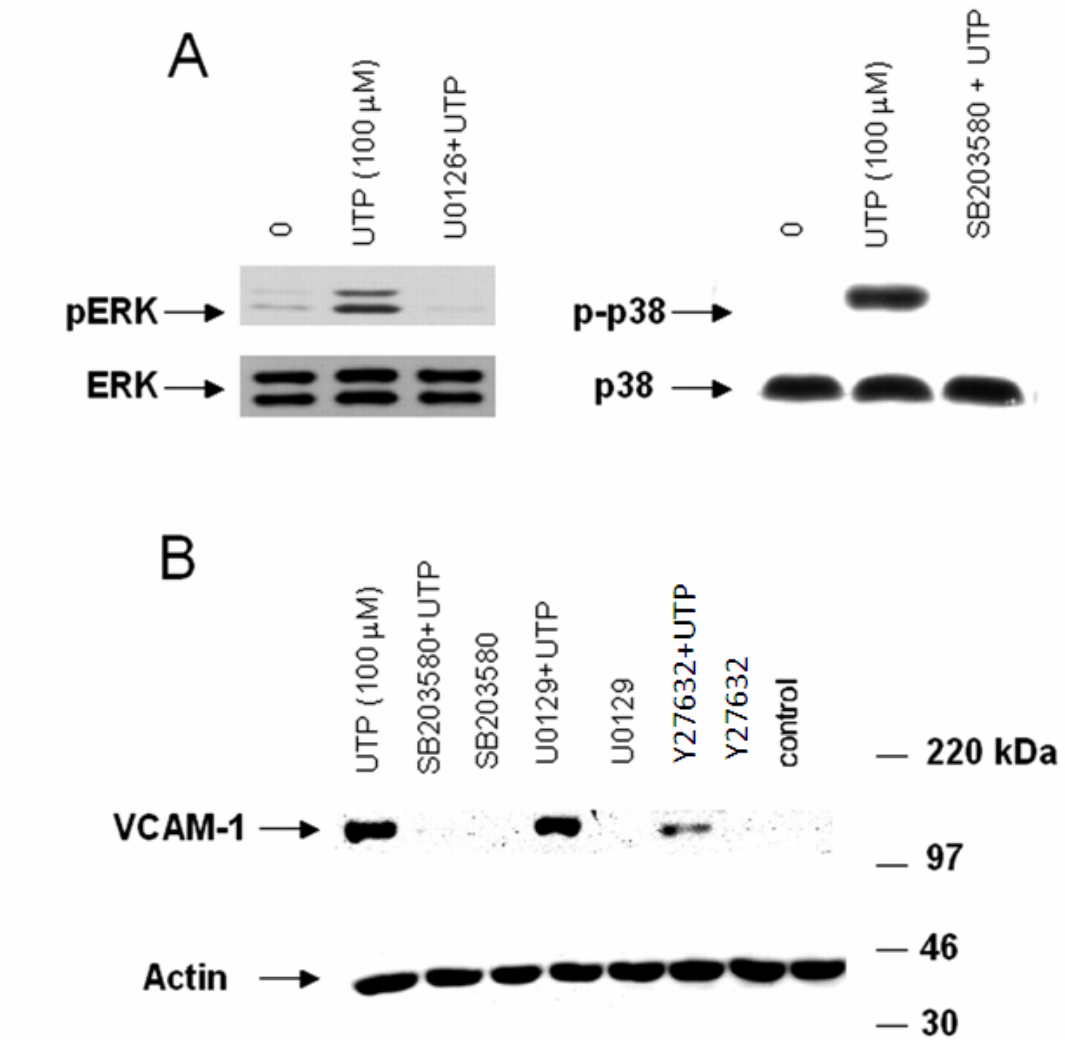
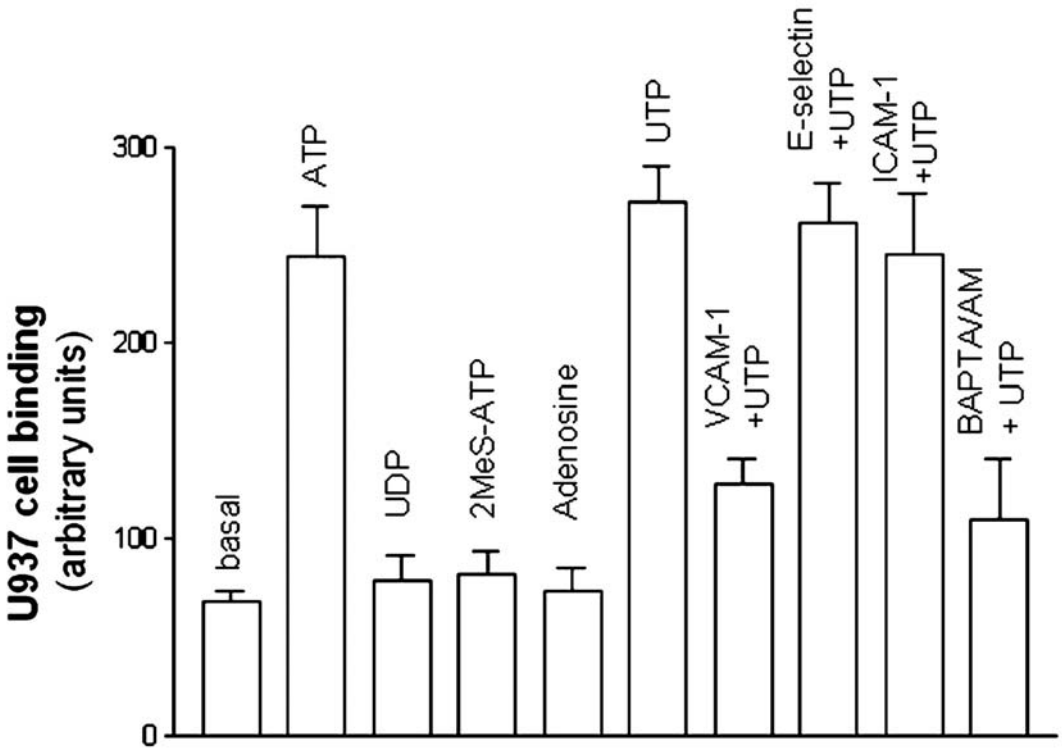

A
0
UTP (100 µM)
U0126+UTP
pERK
ERK
0
UTP (100 µM)
SB203580 + UTP
p-p38
p38
B
UTP (100 µM)
SB203580+UTP
SB203580
U0129+UTP
U0129
Y27632+UTP
Y27632
control
220 kDa
VCAM-1
97
Actin
46
30

Figure II-8. U937 monocyte adherence to UTP-stimulated HCAEC is inhibited by anti-VCAM-1 antibody.

Confluent serum-starved HCAEC in 24-well plates were stimulated with the indicated nucleotide or adenosine (100 μM) for 8 h at 37 °C then incubated for 1 h at 37 °C with U937 cells (10^6 cells/well) pre-labeled with the PKH2 green fluorescent cell linker. In some experiments, HCAEC were preincubated with BAPTA/AM (10 μM) for 30 min prior to the addition of UTP. In other experiments, 10 μg/ml anti-VCAM-1, anti-ICAM-1, or anti-E-selectin antibody was added to UTP-stimulated HCAEC for 45 min prior to the addition of U937 cells. The results shown represent the number of U937 cells (mean ± S.E.) bound to confluent HCAEC in seven wells from three independent experiments.


U937 cell binding
(arbitrary units)
300
200
100
0
basal
ATP
UDP
2MeS-ATP
Adenosine
UTP
VCAM-1
+UTP
E-selectin
+UTP
ICAM-1
+UTP
BAPTA/AM
+ UTP

Chapter III

The $P2Y_2$ Nucleotide Receptor Mediates Vascular Cell Adhesion Molecule-1 Expression through Interaction with VEGF Receptor-2 (KDR/Flk-1)

This work is published in:

Cheikh I. Seye, Ningpu Yu, Fernando A. González, Laurie Erb, Gary A. Weisman. The $P2Y_2$ nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). *Journal of Biological Chemistry*. 2004 Aug 20;279(34):35679–86.

Abstract

UTP stimulates the expression of pro-inflammatory vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells through activation of the $P2Y_2$ nucleotide receptor. Here, we demonstrated that activation of the $P2Y_2$ receptor induced rapid tyrosine phosphorylation of vascular endothelial growth factor receptor (VEGFR)-2 in human coronary artery endothelial cells (HCAEC). RNA interference targeting VEGFR-2 or inhibition of VEGFR-2 tyrosine kinase activity abolishes $P2Y_2$ receptor-mediated VCAM-1 expression. Furthermore, VEGFR-2 and the $P2Y_2$ receptor co-localize upon UTP stimulation. Deletion or mutation of two Src homology-3-binding sites in the C-terminal tail of the $P2Y_2$ receptor or inhibition of Src kinase activity abolished the $P2Y_2$ receptor-mediated transactivation of VEGFR-2 and subsequently inhibited UTP-induced VCAM-1 expression. Moreover, activation of VEGFR-2 by UTP leads to the phosphorylation of Vav2, a guanine nucleotide exchange factor for Rho family GTPases. Using a binding assay to measure the activity of the small GTPase Rho, we found that stimulation of HCAEC by UTP increased the activity of RhoA and Rac1 (but not Cdc42). Significantly, a dominant negative form of RhoA inhibited $P2Y_2$ receptor-mediated VCAM-1 expression, whereas expression of dominant negative forms of Cdc42 and Rac1 had no effect. These data indicate a novel mechanism whereby a nucleotide receptor transactivates a receptor tyrosine kinase to generate an inflammatory response associated with atherosclerosis.

Introduction

Extracellular nucleotides released by vascular cells act via P2 nucleotide receptors to regulate the vascular tone (Dubyak and el-Moatassim, 1993). There is increasing evidence to support a role for nucleotides in the development of vascular diseases such as atherosclerosis and restenosis after angioplasty. Indeed, UTP and ATP, equipotent agonists of the $P2Y_2$ receptor (Dubyak and el-Moatassim, 1993), induce proliferation and migration of vascular smooth muscle cells(Erlinge *et al*., 1995; Malam-Souley *et al*., 1995; Chaulet *et al*., 2001), two key processes involved in atherogenesis. Furthermore, previous studies by our group have shown that the development of intimal lesions in experimental models of atherosclerosis is closely associated with up-regulation of a functional $P2Y_2$ receptor (Seye *et al*., 1997; Seye *et al*., 2002). The first direct evidence supporting a role for nucleotides in atherogenesis is the demonstration that local application of UTP to collared rabbit carotid arteries enhanced intimal hyperplasia and promoted infiltration of monocytes into the neointima (Seye *et al*., 2002). In Chapter II, we showed that activation of the $P2Y_2$ receptor in vascular endothelial cells induces VCAM-1 expression and promotes the adherence of monocytes (Figs. II-1A and B, II-8). Similarly, VEGF, a pleiotropic factor that regulates multiple biological phenomena, including endothelial cell survival, proliferation, and migration, also stimulates endothelial cell expression of adhesion molecules, including VCAM-1, ICAM-1 (intracellular adhesion molecule-1), and selectins (Kim *et al*., 2001). These multiple responses are mediated through the interaction of VEGF with two receptor tyrosine kinases, Flt-1 or VEGF receptor-1 and Flk-1/KDR or VEGF receptor-2 (Kim *et al*., 2001). It is widely accepted that VEGFR-2 mediates the growth and migration of

endothelial cells, the permeability of blood cells, and the expression of VCAM-1 in human umbilical vascular endothelial cells (Zeng *et al*., 2001). It is increasingly apparent that VEGFR-2 serves as a point of convergence of multiple signals arising from diverse stimuli. VEGFR-2 transactivation follows activation of G protein-coupled receptors, including the endothelial differentiation gene product and the bradykinin receptor (Tanimoto *et al*., 2002; Thuringer *et al*., 2002). We hypothesized that transactivation of VEGFR-2 may be involved in $P2Y_2$ receptor-mediated VCAM-1 expression in endothelial cells. Here, we show that $P2Y_2$ receptor activation by UTP induced tyrosine phosphorylation of VEGFR-2. Importantly, we demonstrated that inhibition of the tyrosine kinase activity of VEGFR-2 prevented UTP-induced VCAM-1 expression. Furthermore, we elucidated the structural determinants within the $P2Y_2$ receptor that are responsible for activating the signaling pathways involved in nucleotide-induced VCAM-1 expression.

Experimental Procedures

Materials

VEGF-165, rabbit anti-human Flk-1/VEGFR-2 polyclonal antibody, A/G PLUS-agarose, monoclonal anti-phosphotyrosine (4G10) antibody, and transfection grade eukaryotic expression vector pUSEamp (+) containing human dominant negative RhoA, Rac1, or Cdc42 were purchased from Upstate Biotechnology (Lake Placid, NY). A rabbit anti-VCAM-1 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The antibody against Vav2 (rabbit polyclonal) was generously provided by Dr. Keith Burridge (University of North Carolina at Chapel Hill). Anti-VEGF neutralization monoclonal antibody was from Chemicon (Temecula, CA). Specific inhibitors for VEGFR-2 tyrosine phosphorylation (SU1498) and Src (PP2) were obtained from Calbiochem (San Diego, CA). LipofectAMINE 2000 reagent was purchased from Invitrogen. The rat monoclonal anti-HA 3F10 affinity matrix was obtained from Roche Diagnostics. Dominant negative Src (K296R/Y528F) and its control vector (pUSE) were purchased from Upstate Biotechnology. All other reagents were from Sigma unless otherwise specified.

Cell Culture and Transfection

HCAEC were cultured in endothelial basal medium-2 (Clonetics, Walkerville, MD) at 37 °C in a humidified atmosphere of 5% CO_2. HCAEC were used between the third and sixth passages. The retroviral vector pLXSN was used for stable expression of the human $P2Y_2$ receptor in human 1321N1 astrocytoma cells, as described previously (Erb *et al*., 2001). For transient transfections, plasmid constructs were delivered using LipofectAMINE 2000 reagent according to the manufacturer's instructions.

$P2Y_2$ Receptor cDNA Constructs

The open reading frame of the wild type human $P2Y_2$ receptor cDNA was modified using PCR to incorporate the HA epitope (YPYDVPDYA) from influenza virus at the N-terminus of the expressed protein, as described previously (Garrad *et al*., 1998). Three primers synthesized in the DNA Core Facility of the University of Missouri-Columbia (primer 1, 5'-AGGCTCGTACGCTTTGCCCGAGATGCCAAGGCTCGCCG CAGGCTGGGCCTGCGCAGATC-3'; primer 2, 5'-ATCATGGATCCTTACTTGGCA TCTCGGGC-3'; and primer 3, 5'-CACACCCTAACTGACAC-3') were used in PCR to generate cDNA encoding HA-tagged deleted (del) (using primer 1 and 3) or 4A (using primer 1 and 2) mutant $P2Y_2$ receptors. After verification of the size of the PCR products by agarose gel electrophoresis, the products were purified using the PCR Wizard kit (Amersham Biosciences), digested with BsiWI and BamHI, and inserted into pLXSN. The mutant cDNAs were sequenced to verify that the mutations were incorporated correctly.

siRNAs Treatment

The sense and antisense strands of siRNAs were: VEGFR-2, beginning at nucleotide 3172, 5'-GTGGATCTGAAACGGCGCTTGT-3' (sense) and 5'-CCGGCGACTCAATCTACTAGCT-3' (antisense). The control siRNAs were: scrambler, beginning at nucleotide 3172, 5'-GCTAGTAGATTGAGTCGCCGGT-3' (sense) and 5'-CCGGCGACTCAATCTACTAGCT-3' (antisense). Double-stranded siRNAs were generated in the pRNAT-U6.1 Neo vector (GenScript) that carries a green fluorescent protein marker used to track transfection efficiency. HCAEC were transfected with VEGFR-2 siRNA or control scrambler siRNA for 48 h in normal culture medium.

Activity Assay for Rho, Rac1, and Cdc42

A Rho activation assay kit (Upstate Biotechnology) was used to assess Rho activity in HCAEC. Briefly, HCAEC were cultured in 150-mm tissue culture dishes and stimulated with UTP for the times indicated in the figure legends. The cells were then washed three times with ice-cold Tris-buffered saline and suspended in lysis buffer A containing 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% (v/v) Igepal CA-630, 50 mM $MgCl_2$, 5 mM EDTA, and 10% (v/v) glycerol, and the lysates were transferred to 1.5-ml tubes. Fifty microliters of Rhotekin Rho-binding domain (RBD)-agarose were added to each tube for 45 min at 4 °C. The beads were pelleted by centrifugation (10s, 14,000 x g, 4 °C) and washed three times with lysis buffer A. Finally, the beads were resuspended in 40 μl of 2x Laemmli buffer, and Western blot analysis was performed with anti-Rho antibody (Upstate Biotechnology). The assay for Rac1 and Cdc42 was done using an immobilized GST fusion construct of the Rac1-binding domain of p65 Pak (p21 Rac-binding domain that binds to Rac1-GTP but not to Rac1-GDP). Because Cdc42 also binds to the p21 Rac-binding domain construct, the same assay was used to measure Cdc42 activity, except that the membranes were probed with an anti-Cdc42 antibody (Upstate Biotechnology).

Immunoprecipitation and Immunoblotting

HCAEC or 1321N1 cell transfectants were washed with PBS and suspended in lysis buffer B containing 0.5% (v/v) Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 0.1% (w/v) protease inhibitor mixture. Protein concentrations were measured using the Coomassie protein assay reagent following the manufacturer's instructions (Pierce), and then 500 mg of the protein lysate

were incubated overnight at 4 °C with anti-HA affinity matrix, anti-Flk1/VEGFR-2, anti-Vav2, or anti-phosphotyrosine antibodies. The immunoprecipitates were collected by incubation with protein A/G PLUS-agarose (Upstate Biotechnology) for 2 h when applicable and washed three times with lysis buffer B. Bound proteins were eluted by boiling in SDS-polyacrylamide gel electrophoresis sample buffer, and then immunoblotting was performed.

Immunofluorescence

Human 1321 astrocytoma cells stably expressing an N-terminal HA epitope-tagged human $P2Y_2$ receptor were grown to 60% confluence on coverslips in 35-mm dishes. The cells were serum-starved overnight followed by incubation for 1 h at 37 °C with rabbit anti-HA (1:100) (Santa Cruz Biotechnologies) and mouse anti-VEGFR-2 (1:100) (Upstate Biotechnology) antibodies that recognize cell surface epitopes. The cells were washed three times with PBS and then incubated with 1:200 dilution of goat anti-rabbit Alexa Fluor™ 594- and goat anti-mouse Oregon Green™ 488-conjugated IgG (Molecular Probes, Eugene, OR) in serum-free medium for 1 h at 37 °C. The cells were washed in PBS and re-equilibrated in serum-free medium for 1 h and then exposed to 100 μM UTP for 5 min at 37 °C, promptly washed in PBS, and immersed in PBS containing 4% (v/v) paraformaldehyde for 10 min. Coverslips were washed three times in PBS, rinsed in ddH_2O, and mounted on glass slides in ProLong® antifade reagent (Molecular Probes). Microscopy was performed on a Bio-Rad Radiance 2000 system coupled with an Olympus IX70 microscope using a 60x water immersion lens (numerical aperture 1.3). Data were processed with Laser Sharp 2000 software. Co-localization experiments with Alexa Fluor™ 594-conjugated IgG to detect VEGFR-2 and Oregon Green™ 488-

conjugated IgG to detect the HA-tagged $P2Y_2$ receptor were performed at 590 nm excitation/617 nm emission and 496 nm excitation/524 nm emission wavelengths for the respective antibodies.

Results

UTP Induces Tyrosine Phosphorylation of VEGFR-2

We have shown in Chapter II that UTP induces VCAM-1 expression via the $P2Y_2$ receptor in vascular endothelial cells (Figs. II-1A and B, II-5B and C). VEGF also stimulates the expression of endothelial cell adhesion molecules including VCAM-1 via activation of VEGFR-2 (Zeng *et al*., 2001). Because ligand-independent activation of receptor tyrosine kinases is involved in some biological responses initiated by G protein-coupled receptors (Thuringer *et al*., 2002; Seta and Sadoshima, 2003), we hypothesized that activation of the $P2Y_2$ receptor would increase VEGFR-2 phosphorylation in HCAEC. Indeed, we detected a rapid and intense tyrosine phosphorylation of VEGFR-2 in HCAEC as early as 1 min after UTP stimulation, which reached a peak within 5 min (Fig. III-1A). Importantly, treatment of HCAEC with anti-VEGF antibody did not suppress UTP-induced VEGFR-2 phosphorylation, indicating that activation of VEGFR-2 did not occur secondarily to VEGF release (not shown). To determine whether the $P2Y_2$ receptor could mediate VEGFR-2 phosphorylation, we utilized G protein-coupled P2 receptor-deficient 1321N1 cells transfected with the human $P2Y_2$ receptor. We first verified that VEGF could induce tyrosine phosphorylation of VEGFR-2 in 1321N1 cells prior to and after expression of the human $P2Y_2$ receptor (not shown). We then determined that UTP caused a time-dependent increase in VEGFR-2 tyrosine phosphorylation in 1321N1 cells expressing the $P2Y_2$ receptor (Fig. III-1B) but not in untransfected 1321N1 cells (not shown). Thus, our data clearly indicate that the $P2Y_2$ receptor can mediate transactivation of the VEGFR-2.

RNA Interference Targeting of VEGFR-2 Inhibits $P2Y_2$ Receptor-mediated VCAM-1 Expression

The rapid activation of VEGFR-2 by UTP prompted us to consider whether the VEGFR-2 signaling pathway was involved in UTP-induced $P2Y_2$ receptor-mediated VCAM-1 expression. To assess the role of VEGFR-2 in this process, siRNA was used to suppress the expression of the VEGFR-2 gene. Synthetic siRNAs of 21–23 nucleotides in length have been shown to silence cellular and viral gene expression in mammalian cells both *in vitro* and *in vivo* (Caplen *et al*., 2001; Song *et al*., 2003). Results indicated that VEGFR-2 siRNA significantly decreased VEGFR-2 mRNA and protein levels 2 days after transfection (Fig. III-2A and B) without affecting UTP-induced mobilization of intracellular calcium (not shown). Treatment of HCAEC with VEGFR-2 siRNA completely inhibited UTP-induced VCAM-1 expression (Fig. III-2C). Furthermore, SU1498, a specific inhibitor of VEGFR-2 tyrosine kinase activity, inhibited both UTP-induced phosphorylation of VEGFR-2 and VCAM-1 expression (Fig. III-3), indicating that activation of VEGFR-2 is necessary for $P2Y_2$ receptor-mediated VCAM-1 expression in HCAEC.

UTP-dependent Co-localization and Interaction of VEGFR-2 with the $P2Y_2$ Receptor

To determine whether the VEGFR-2 and the $P2Y_2$ receptor co-localize upon UTP stimulation, 1321N1 cells were transfected with the wild type $P2Y_2$ receptor in which a HA epitope tag was incorporated at the N-terminus to facilitate immunodetection of the receptor. In the absence of UTP, confocal microscopy of dual labeled cells showed no co-localization between the VEGFR-2 (red) and the $P2Y_2$ receptor (green) in 1321N1 cell transfectants (Fig. III-4A). Upon UTP stimulation (100 μM, for 5 min), the $P2Y_2$ receptor

and VEGFR-2 co-localized in the plasma membrane as indicated by the appearance of yellow staining due to the merged immunofluorescence from the $P2Y_2$ receptor and VEGFR-2 (Fig. III-4A). These data indicate that activation of the $P2Y_2$ receptor facilitates its association with VEGFR-2. We next examined whether the $P2Y_2$ receptor and VEGFR-2 physically interact with each other. An anti-HA affinity matrix was used to immunoprecipitate the HA-tagged $P2Y_2$ receptor expressed in 1321N1 cells, enabling us to demonstrate that the $P2Y_2$ receptor and VEGFR-2 could be co-immunoprecipitated as early as 1 min after UTP stimulation (Fig. III-4B). However, this interaction was transient because it was not observed 10 min after UTP stimulation. Interestingly, phosphorylation of VEGFR-2 in 1321N1 cells persists for 10 min after UTP stimulation (Fig. III-1B) even though the physical interaction between VEGFR-2 and the $P2Y_2$ receptor was lost (Fig. III-4B), suggesting that once phosphorylation of VEGFR-2 is initiated the activity of VEGFR-2 may be maintained through autophosphorylation.

SH3-binding Sites in the C-terminal Domain of the $P2Y_2$ Receptor and Src Kinase Activity Are Required for VEGFR-2 Tyrosine Phosphorylation

It is unclear how the $P2Y_2$ receptor, which lacks intrinsic tyrosine kinase activity, activates VEGFR-2. It has been reported previously that the angiotensin II type I receptor, which also lacks intrinsic tyrosine kinase activity, can activate the EGF receptor through binding with the SH2 domain containing tyrosine phosphatase 2 (Seta and Sadoshima, 2003). There is also evidence that Src kinase activity is necessary for transactivation of the EGF receptor by the $P2Y_2$ receptor (Soltoff, 1998). Recently, it has been shown that $P2Y_2$ receptor-mediated transactivation of the EGF receptor requires Src activity and two PXXP motifs in the C-terminus of the $P2Y_2$ receptor that represent

minimal consensus motifs for SH3 domain-binding proteins, including Src (Liu *et al*., 2004). We found that $P2Y_2$ receptor-mediated tyrosine phosphorylation of VEGFR-2 was dependent on Src kinase activity, as demonstrated by concentration-dependent sensitivity to PP2, the Src tyrosine kinase inhibitor (Fig. III-5A). Furthermore, expression of dominant negative Src abolished the physical interaction between VEGFR-2 and the $P2Y_2$ receptor (Fig. III-5B). These results indicate that Src plays a critical role in mediating UTP-induced activation of VEGFR-2. To test the role of the $P2Y_2$ receptor SH3-binding domains in UTP-induced transactivation of VEGFR-2, we stably expressed in 1321N1 cells $P2Y_2$ receptor cDNA encoding a mutant receptor (Fig. III-5C) in which the PXXP sequences were deleted (del) or mutated to replace the prolines with alanines (4A). VEGF induced tyrosine phosphorylation of VEGFR-2 in 1321N1 cells expressing wild type or mutant $P2Y_2$ receptors (Fig. III-5D). In contrast to cells expressing the wild type receptor, UTP failed to activate VEGFR-2 in cells expressing the del or 4A mutant $P2Y_2$ receptors (Fig. III-5D). These data clearly indicate that the SH3-binding domains in the C-terminus of the $P2Y_2$ receptor mediate UTP-induced transactivation of VEGFR-2. We also found that UTP did not induce VCAM-1 expression in cells expressing del or 4A mutant $P2Y_2$ receptors in contrast to cells expressing the wild type receptor (Fig. III-6).

Activation of RhoA Downstream of VEGFR-2 Mediates VCAM-1 Expression in HCAEC

We next sought to identify the signaling mechanisms downstream of VEGFR-2 activation that are responsible for UTP-induced VCAM-1 expression. Activation of the $P2Y_2$ receptor and VEGFR-2 are linked to activation of the small GTPase RhoA (Sauzeau *et al*., 2000; Zeng *et al*., 2002). Stimulation of the EGF receptor or the PDGF

receptor leads to phosphorylation and activation of Vav2, a specific Rho exchange factor that activates RhoA (Liu and Burridge, 2000). Therefore, we investigated whether Vav2 could be phosphorylated in response to $P2Y_2$ receptor-mediated VEGFR-2 transactivation. We found that UTP caused a strong time-dependent increase in the tyrosine phosphorylation of Vav2 in HCAEC (Fig. III-7A). UTP-induced Vav2 tyrosine phosphorylation was blocked by pretreatment of cells with SU1498, an inhibitor of VEGFR-2 tyrosine phosphorylation (Fig. III-7B). Because Vav2 can activate all three major Rho family GTPases, including RhoA, Rac, and Cdc42, we tested whether these G proteins were activated by UTP. Glutathione-Sepharose beads complexed to GST constructs were used to affinity-precipitate GTP-bound RhoA, Rac1, and Cdc42. As shown in Fig. III-8A, RhoA was activated as early as 2 min after stimulation of HCAEC with UTP and remained activated through 10 min. We found a transient but significant UTP-induced increase in the activity of Rac-1 in HCAEC, whereas Cdc42 was not activated (Fig. III-8B and C). To determine which of these G proteins was involved in UTP-induced VCAM-1 expression, we transiently expressed dominant negative forms of RhoA, Rac1, and Cdc42 in HCAEC. We found that expression of dominant negative forms of RhoA (but not Rac1 or Cdc42) blocked UTP-induced VCAM-1 expression (Fig. III-9), indicating that RhoA (but not Rac1 or Cdc42) mediates VCAM-1 expression downstream of Vav2 activation.

Discussion

The $P2Y_2$ receptor is increasingly recognized as an important contributor to the development of atherosclerosis or restenosis after angioplasty (Seye *et al*., 1997; Di Virgilio and Solini, 2002). We have reported previously that this G protein-coupled receptor mediates VCAM-1 expression and adhesion of leukocytes to vascular endothelial cells (Chapter II). This study focuses on the mechanisms whereby $P2Y_2$ receptor activation increases the expression of VCAM-1. We demonstrated for the first time that activation of the $P2Y_2$ receptor in human coronary artery endothelial cells induces the rapid tyrosine phosphorylation of VEGFR-2 and the activation of Vav2, a Rho guanine exchange factor, leading to the expression of VCAM-1. Two lines of evidence demonstrated the involvement of VEGFR-2 in this process. First, SU1498, a specific VEGFR-2 tyrosine kinase inhibitor, diminished UTP-induced VCAM-1 expression (Fig. III-3B). Second, selective inhibition of VEGFR-2 expression by siRNA inhibited UTP-induced VCAM-1 expression (Fig. III-2C). Ligand-independent activation of receptor tyrosine kinases is involved in biological responses initiated by G protein-coupled receptors. It has been reported that activation of the endothelial differentiation gene in response to sphingosine 1-phosphate stimulates endothelial nitric-oxide synthase through activation of Flk1/KDR (Tanimoto *et al*., 2002). EGF receptor transactivation by G protein-coupled receptor ligands may involve activation of matrix metalloproteinases, release of membrane heparin-bound EGF, and binding of heparin-bound EGF to the EGF receptor (Prenzel *et al*., 1999; Gschwind *et al*., 2001; Pierce *et al*., 2001). At the structural level, two SH3-binding sites (PXXP motifs) have been identified in the intracellular C-terminus of the $P2Y_2$ receptor and have been shown to interact with Src

and regulate the activity of the EGF and PDGF receptors (Liu *et al*., 2004). In this study, we demonstrated that PP2, the Src kinase inhibitor, decreased $P2Y_2$ receptor-mediated VEGFR-2 tyrosine phosphorylation (Fig. III-5A) consistent with the involvement of Src in EGF and PDGF receptor transactivation by $P2Y_2$ receptors (Liu *et al*., 2004). We also found that the $P2Y_2$ receptor and VEGFR-2 co-localized in cells upon UTP stimulation and demonstrated for the first time a direct interaction between these receptors in a UTP-dependent manner (Fig. III-4). Deletion or mutation of the SH3-binding domains of the $P2Y_2$ receptor prevented UTP-induced expression of VCAM-1 (Fig. III-6). Because transfection of dominant negative Src inhibited the $P2Y_2$ receptor-VEGFR-2 interaction (Fig. III-5B), we concluded that Src binding to the SH3-binding domains in the $P2Y_2$ receptor mediates both the UTP-induced $P2Y_2$ receptor and VEGFR-2 interaction and the transactivation of VEGFR-2 involved in VCAM-1 expression.

VEGF-stimulated expression of VCAM-1 is regulated mainly through nuclear factor κB (NFκB) activation with phosphatidylinositol 3-kinase-mediated suppression (Kim *et al*., 2001). $P2Y_2$ receptors in endothelial cells activate a variety of signaling pathways including but not restricted to the mitogen- and stress-activated protein kinases ERK1/2 and p38, and the small GTPase RhoA (Sauzeau *et al*., 2000; Chapter II). However, it is unclear whether activation of the small GTPase RhoA is involved in UTP-induced VCAM-1 expression. Previous work has demonstrated that Vav and Vav2, two members of the Dbl family of proteins, act as guanine nucleotide exchange factors for Rho family proteins (Cerione and Zheng, 1996; Whitehead *et al*., 1997). Rho family proteins function as GDP/GTP-regulated molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (Narumiya *et al*., 1997; Van Aelst and

D'Souza-Schorey, 1997; Zohn *et al*., 1998). Although Dbl family proteins stimulate GDP dissociation to promote the formation of the active GTP-bound protein, Rho family proteins are inactivated by GTPase-activating proteins that stimulate the intrinsic rate of GTP hydrolysis (Abe *et al*., 2000). Recent studies have demonstrated EGF- or PDGF-stimulated tyrosine phosphorylation of Vav2 in hematopoietic cells (Liu and Burridge, 2000). We have found that UTP induced a rapid but transient phosphorylation of Vav2 (Fig. III-7A), a Rho exchange factor that activates RhoA (Liu and Burridge, 2000), and UTP-induced Vav2 phosphorylation was inhibited by SU1498 (Fig. III-7B), a specific inhibitor of VEGFR-2 tyrosine kinase activity. In addition, UTP failed to activate Vav2 in HCAEC transfected with VEGFR-2 siRNA (data not shown). Therefore, we concluded that Vav2 is activated downstream of tyrosine phosphorylation of VEGFR-2.

Our results indicate that RhoA and Rac1 (but not Cdc42) activities were increased by UTP in HCAEC (Fig. III-8). The tyrosine phosphorylation of Vav2 in response to UTP suggests that Vav2 contributes to RhoA and Rac1 activity. Although we cannot conclude that elevation of RhoA and Rac1 activity was due to direct activation by Vav2, preliminary experiments (not shown) indicated that over-expression of a constitutively active Vav2 in HCAEC activates Rho and Rac1 (but not Cdc42). Others studies have indicated that Vav2 overexpression elevates the activities of Rac1, RhoA, and Cdc42 (Liu and Burridge, 2000). Although our data demonstrated that Rho and Rac1 were both activated downstream of VEGFR-2 tyrosine phosphorylation, only RhoA activation is required for VCAM-1 expression, because expression of dominant negative forms of RhoA (but not Rac1 or Cdc42) blocked UTP-induced VCAM-1 expression (Fig. III-9).

In summary, results from the present study indicate that activation of the G protein-coupled $P2Y_2$ receptor promotes Src binding to SH3-binding motifs in the $P2Y_2$ receptor and facilitates the interaction and transactivation of VEGFR-2, resulting in the sequential activation of Vav2 and RhoA leading to VCAM-1 expression. The present study elucidated for the first time a novel mechanism whereby a nucleotide receptor interacts with a growth factor receptor to generate a pro-inflammatory response in vascular endothelial cells. Further delineation of the signaling pathway involved in this process may help prevent inflammation associated with atherosclerosis by limiting $P2Y_2$ receptor up-regulation and/or the consequences of $P2Y_2$ receptor activation.

Figure III-1. UTP induces tyrosine phosphorylation of VEGFR-2.

A, HCAEC were treated with UTP (10 μM) for the indicated times. Cell lysates were subjected to immunoprecipitation (*IP*) with anti-VEGFR-2 antibody and were immunoblotted (*IB*) with anti-phosphotyrosine (*PY*) antibody. *B*, 1321N1 cells stably expressing the human $P2Y_2$ receptor were stimulated with UTP (100 μM) for the indicated times, and cell lysates were immunoprecipitated and immunoblotted as indicated in *A*. Immunoblots shown in *A* and *B* are representative of results from three independent experiments. The blots were stripped and reprobed with anti-VEGFR-2 antibody (*lower immunoblots* in *A* and *B*) to normalize for protein loading.

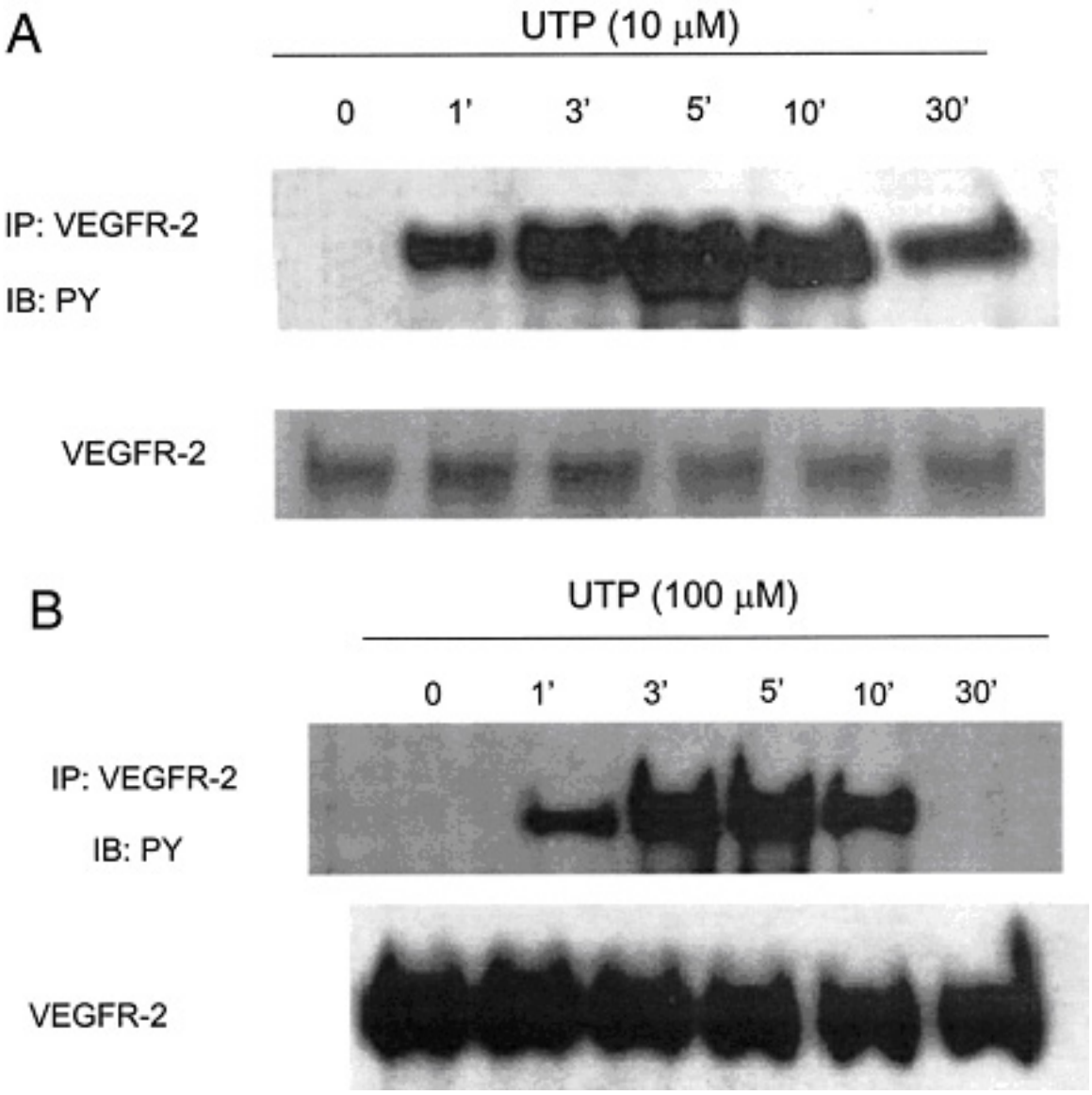
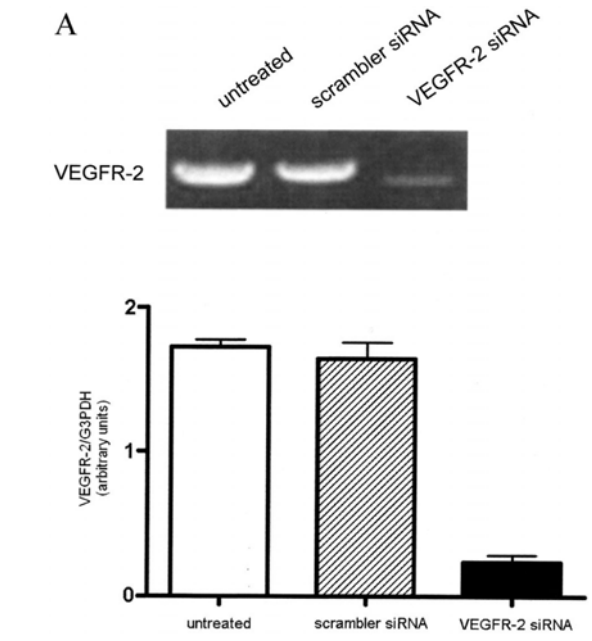
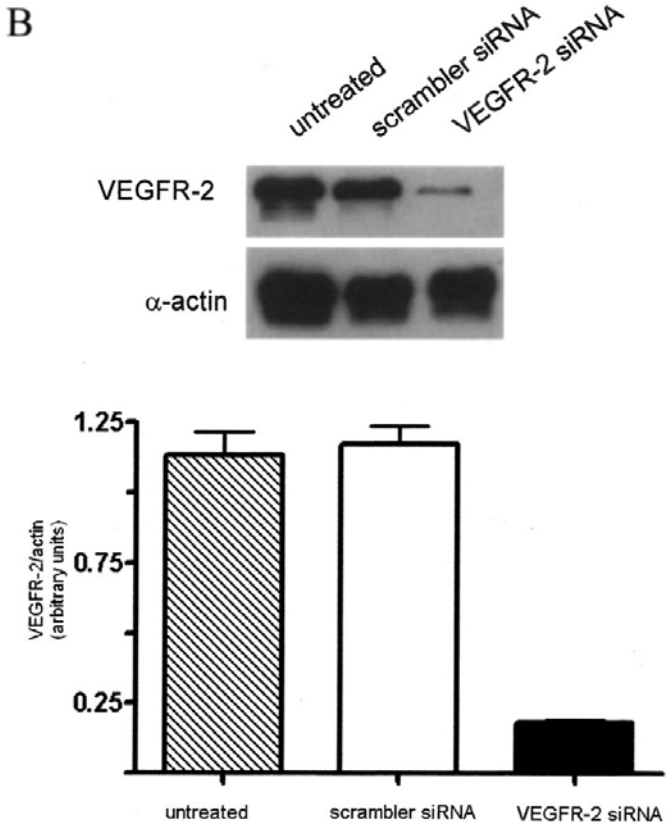

A
UTP (10 μM)
0
1'
3'
5'
10'
30'
IP: VEGFR-2
IB: PY
VEGFR-2
B
UTP (100 μM)
0
1'
3'
5'
10'
30'
IP: VEGFR-2
IB: PY
VEGFR-2

Figure III-2. VEGFR-2 siRNA duplex efficiently silences VEGFR-2 gene and protein expression and inhibits $P2Y_2$ receptor-mediated VCAM-1 expression.

HCEAC were transfected with VEGFR-2 siRNA or scrambler siRNA as described under "Experimental Procedures," and then VEGFR-2 mRNA (*A*) and protein (*B*) levels were analyzed by PCR and Western analysis, respectively. *A*, gel electrophoresis of PCR products and quantification of relative amounts of VEGFR-2 mRNA normalized to G3PDH mRNA (n = 3, mean ± S.E.). *B*, Western blot and quantification of VEGFR-2 protein levels normalized to α-actin (n = 3, mean ± S.E.). *C*, quantification of VCAM-1 expression in HCAEC treated with or without VEGFR-2 or control scrambler siRNA followed by 10 μM UTP for 6 h.


A
untreated
scrambler siRNA
VEGFR-2 siRNA
VEGFR-2
VEGFR-2/G3PDH (arbitrary units)
2
1
0
untreated
scrambler siRNA
VEGFR-2 siRNA


B
untreated
scrambler siRNA
VEGFR-2 siRNA
VEGFR-2
α-actin
VEGFR-2/actin (arbitrary units)
1.25
0.75
0.25
untreated
scrambler siRNA
VEGFR-2 siRNA

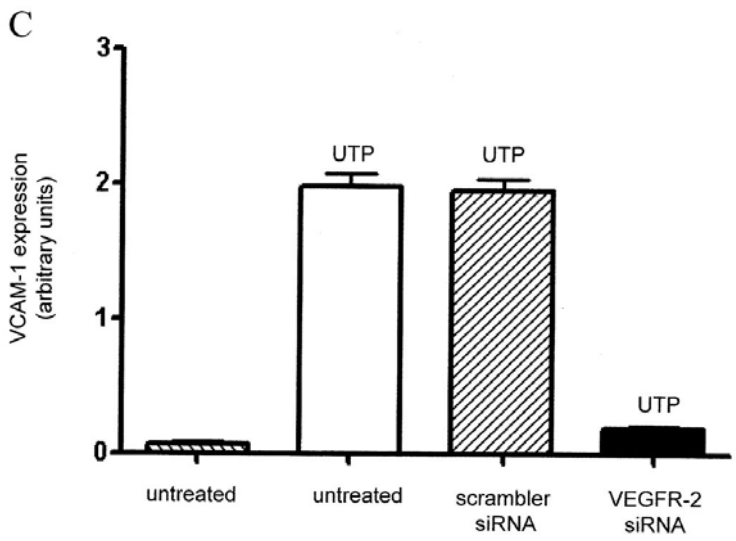

C
VCAM-1 expression (arbitrary units)
3
2
1
0
UTP
UTP
UTP
untreated
untreated
scrambler siRNA
VEGFR-2 siRNA

Figure III-3. Inhibition of VEGFR-2 tyrosine phosphorylation prevents UTP-induced VCAM-1 expression.

A, HCEAC were pretreated with the indicated concentrations of SU1498 for 30 min and then stimulated with 10 μM UTP for 5 min. VEGFR-2 was immunoprecipitated (*IP*) and tyrosine phosphorylation (*PY*) was detected by immunoblotting (*IB*), as described in the legend to Figure III-1. The blots were stripped and reprobed with anti-VEGFR-2 antibody. *B*, HCEAC were pretreated with the indicated concentrations of SU1498 for 30 min and stimulated with 10 μM UTP for 6 h, and VCAM-1 expression was determined by Western blot analysis. The blots were stripped and reprobed with anti-α-actin antibody. The data are representative of results from four independent experiments.

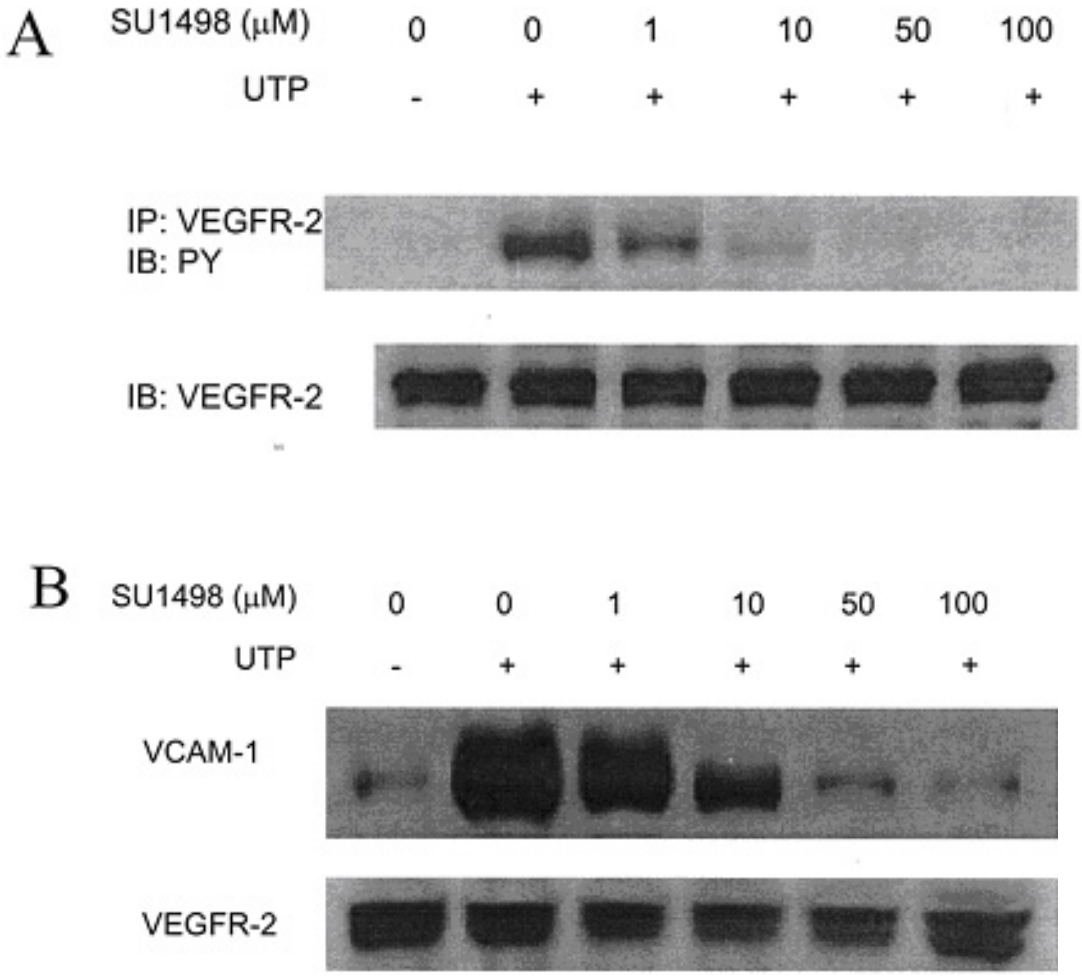

A
SU1498 (μM)
0
0
1
10
50
100
UTP
-
+
+
+
+
+
IP: VEGFR-2
IB: PY
IB: VEGFR-2
B
SU1498 (μM)
0
0
1
10
50
100
UTP
-
+
+
+
+
+
VCAM-1
VEGFR-2

Figure III-4. The $P2Y_2$ receptor and VEGFR-2 co-localize and interact upon UTP stimulation.

A, 1321N1 cells stably expressing the HA-tagged $P2Y_2$ receptor were incubated in the presence (+) or absence (–) of 100 μM UTP at 37 °C for 5 min. Immunofluorescence of IgG-conjugated Oregon Green™ (*green*) and IgG-conjugated Alexa Fluor™ (*red*) was used to detect anti-HA-tagged $P2Y_2$ receptor and anti-VEGFR-2 antibodies, respectively, in intact cells as described under "Experimental Procedures". Confocal micrographs are representative of results from four separate experiments. Magnification: 60x. *B*, 1321N1 cells stably expressing the HA-tagged $P2Y_2$ receptor were stimulated with 100 μM UTP for the indicated times, and cell lysates were subjected to immunoprecipitation (*IP*) with anti-HA antibody and immunoblotted (*IB*) with anti-VEGFR-2 antibody. Prior to immunoprecipitation, total VEGFR-2 was detected with anti-VEGFR-2 antibody using duplicate samples (*lower blot*). The results are representative of four independent experiments.

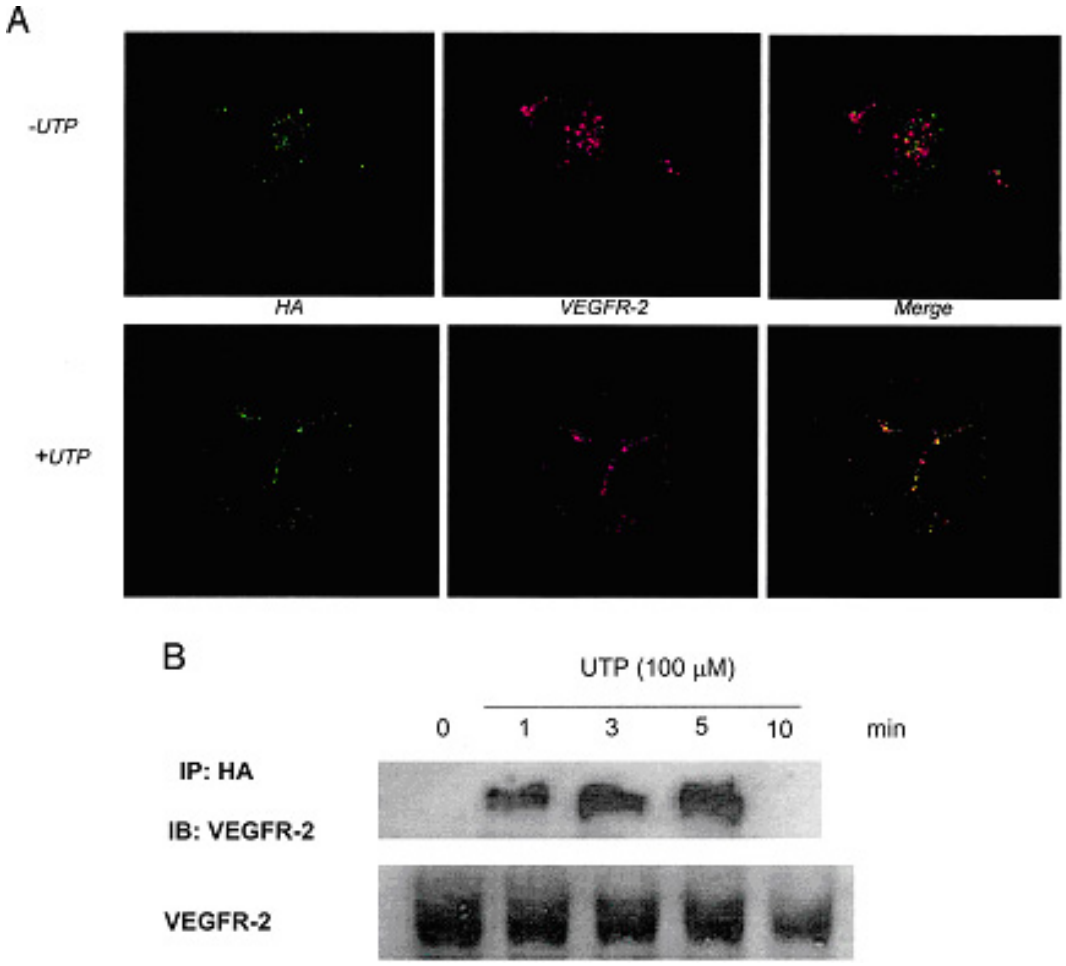
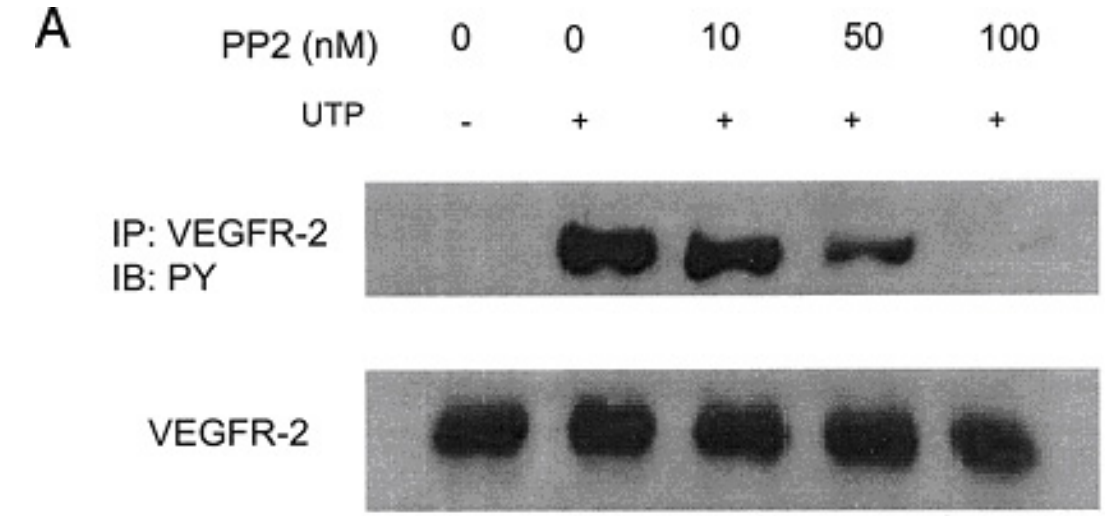
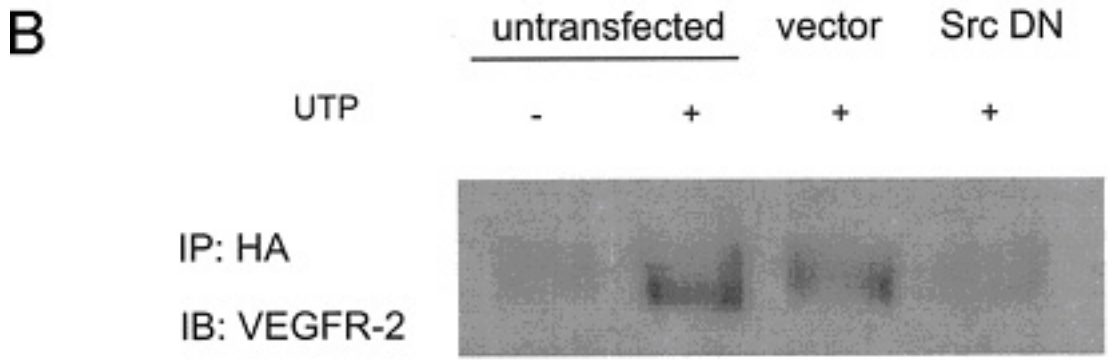

A
-UTP
+UTP
HA
VEGFR-2
Merge
B
UTP (100 μM)
0
1
3
5
10
min
IP: HA
IB: VEGFR-2
VEGFR-2

Figure III-5. Src kinase activity and SH3-binding domains in the C-terminal tail of the $P2Y_2$ receptor are required for VEGFR-2 transactivation.

A, PP2 inhibits UTP-induced VEGFR-2 phosphorylation. Human 1321N1 cells stably expressing the $P2Y_2$ receptor were incubated for 1 h with the indicated concentrations of the specific inhibitor of Src kinase PP2 and stimulated with 100 μM UTP for 5 min. VEGFR-2 phosphorylation was determined by immunoprecipitation (*IP*) with anti-VEGFR-2 antibody and immunoblotting (*IB*) with anti-phosphotyrosine (*PY*) antibody. The blots were stripped and reprobed with anti-VEGFR-2 antibody. *B*, dominant negative Src inhibits the interaction between VEGFR-2 and the $P2Y_2$ receptor. Human 1321N1 cells stably expressing the $P2Y_2$ receptor were transfected with dominant negative Src or control plasmid. 48 h after transfection, the cells were stimulated with 100 μM UTP for 5 min, and the cell lysates were subjected to immunoprecipitation with anti-HA antibody and immunoblotting with anti VEGFR-2 antibody. *Lane 1* (*left* to *right*), untransfected cells without UTP; *lane 2*, untransfected cells treated with UTP; *lane 3*, vector transfected cells with UTP; *lane 4*, dominant negative Src transfected cells with UTP. *C*, partial amino acid sequence of the C-terminal tail of the $P2Y_2$ receptor including the SH3-binding domains of the wild type receptor (*wt*) and mutant receptors in which prolines in the SH3-binding domains were mutated to alanines (*4A*) or deleted (*del*). *D*, VEGFR-2 phosphorylation in 1321N1 cells expressing the wt (*upper panel*), del (*middle panel*), or 4A mutant $P2Y_2$ receptor (*lower panel*). The cells were stimulated with 100 μM UTP for the indicated times or 10 ng/ml VEGF for 10 min, and the cell lysates were subjected to immunoprecipitation with anti-VEGFR-2 antibody and immunoblotting with anti-phosphotyrosine antibody. The blots were stripped and reprobed with anti-VEGFR-2

antibody. The results shown are representative of at least three independent experiments for each figure.





C

wt -----VRFARDAKPPTGPSPATPARRRLGL----------

4A -----VRFARDAKPATGASAATAARRRLGL----------

del -----VRFARDAK ARRRLGL----------

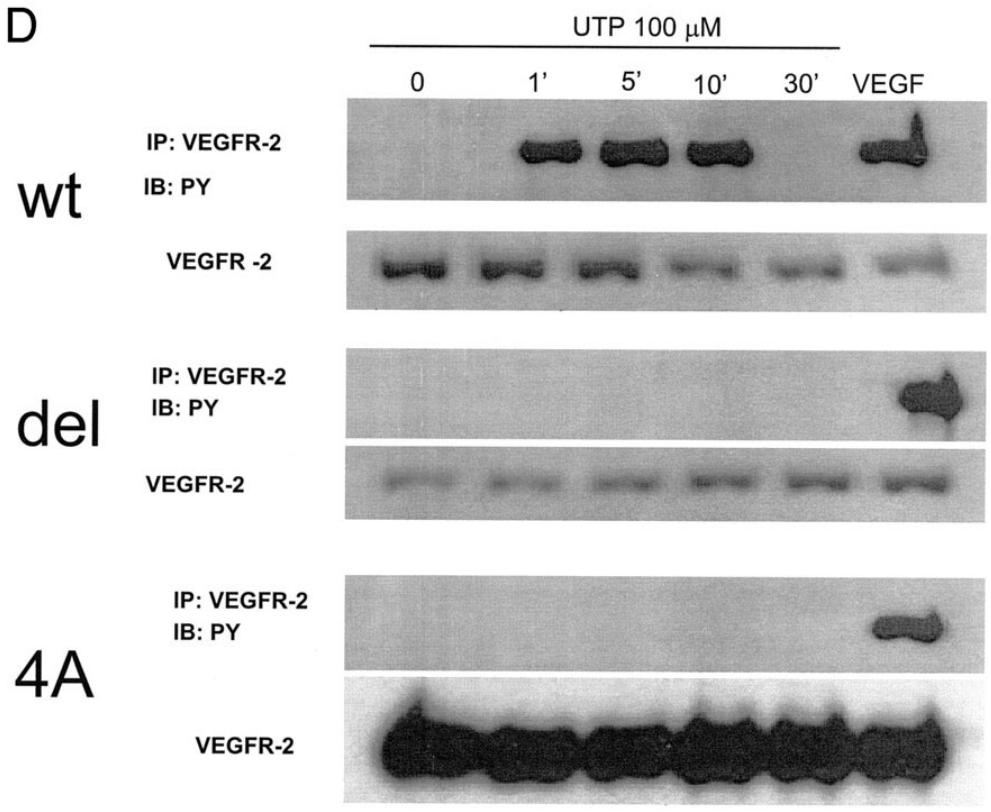

D
UTP 100 μM
0
1'
5'
10'
30'
VEGF
wt
IP: VEGFR-2
IB: PY
VEGFR -2
del
IP: VEGFR-2
IB: PY
VEGFR-2
4A
IP: VEGFR-2
IB: PY
VEGFR-2

Figure III-6. Deletion or mutation of the SH3-binding sites in the $P2Y_2$ receptor prevents UTP-induced VCAM-1 expression in HCAEC.

Wild type (*WT*) and mutant $P2Y_2$ receptors in which prolines in the SH3-binding domains were deleted (*del*) or mutated to alanines (*4A*) were stimulated with the indicated concentrations of UTP for 6 h or 10 ng/ml VEGF for 6 h, and then VCAM-1 expression was determined by Western analysis. The blots were stripped and reprobed with anti-VEGFR-2 antibody. The data are representative of results from three independent experiments.

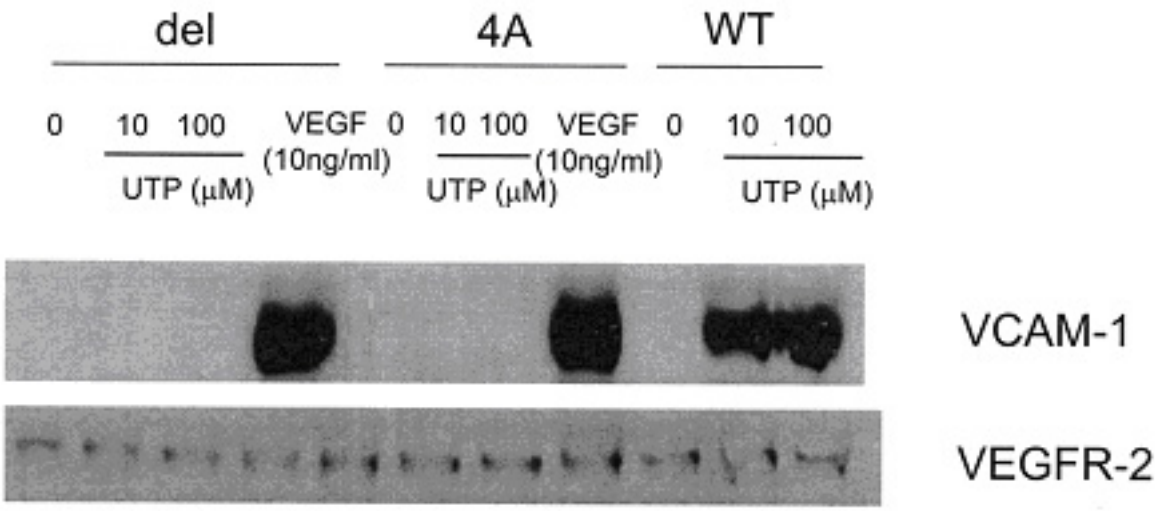
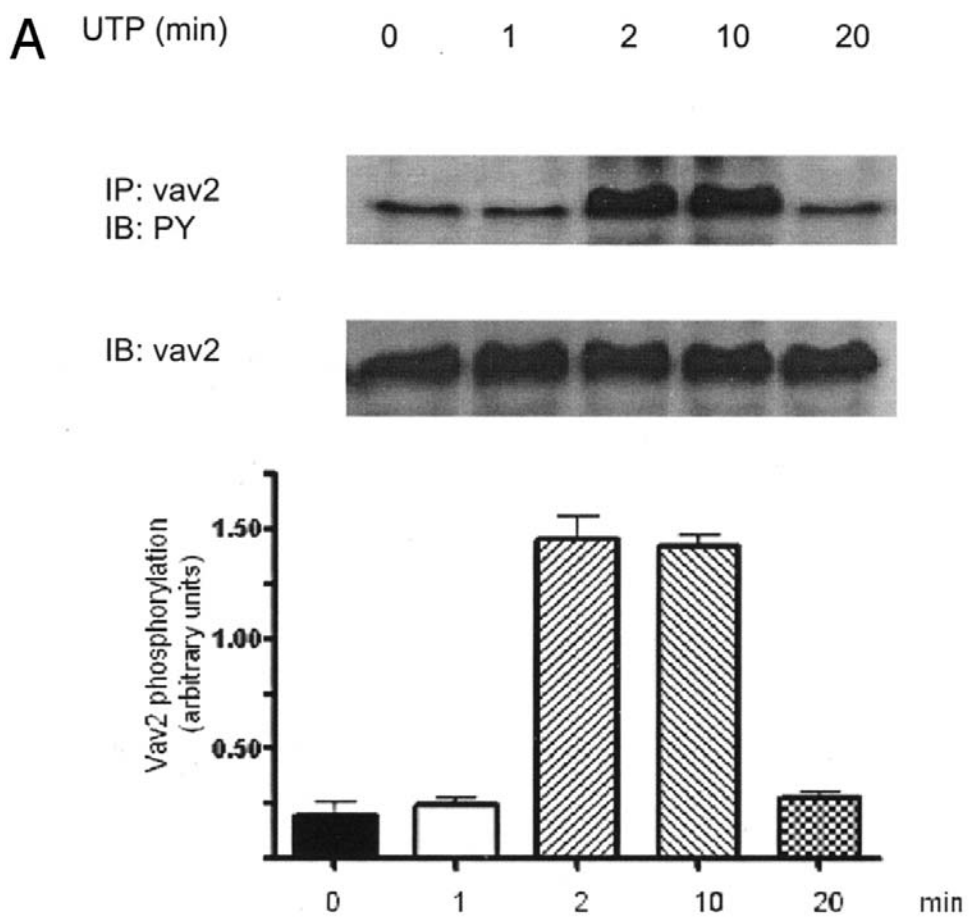

del
4A
WT
0
10
100
VEGF
(10ng/ml)
UTP (μM)
VCAM-1
VEGFR-2

Figure III-7. UTP-induced transactivation of VEGFR-2 causes tyrosine phosphorylation of Vav2 in HCAEC.

A, HCAEC were treated with 10 μM UTP for the indicated times, and cell lysates were immunoprecipitated (*IP*) with polyclonal anti-Vav2 antibody and immunoblotted (*IB*) with anti-phosphotyrosine (*PY*) antibody. The blots were stripped and reprobed with anti-Vav2 antibody. Shown are *bar graphs* of densitometric quantifications of three independent experiments normalized to total Vav2 protein expression (n = 3, mean ± S.E.). *B*, serum-starved HCAEC were pretreated with the indicated concentrations of SU1498 for 30 min and stimulated with 10 μM UTP for 2 min. Phosphorylation of Vav2 was detected as described in *A*.


A
UTP (min)
0
1
2
10
20
IP: vav2
IB: PY
IB: vav2
Vav2 phosphorylation
(arbitrary units)
1.50
1.00
0.50
0
1
2
10
20
min

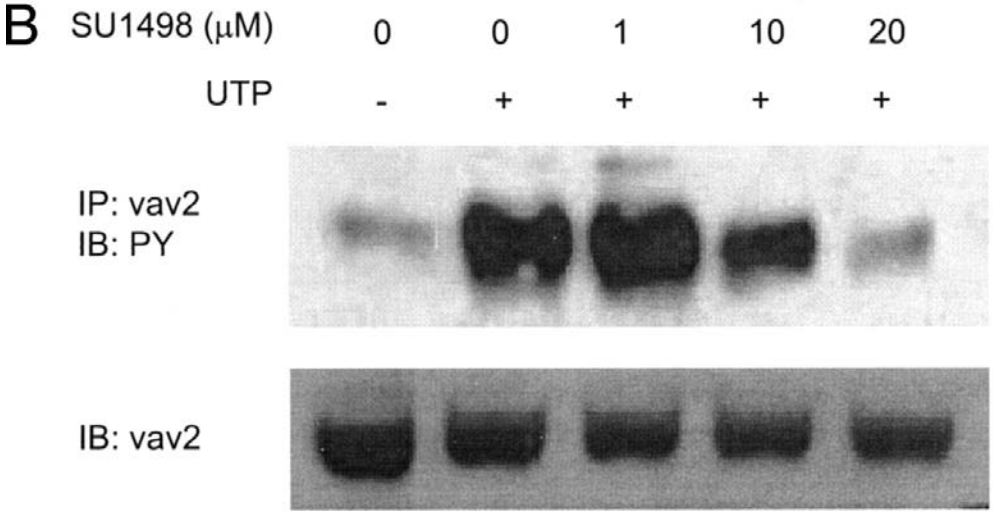

B
SU1498 (μM)
0
0
1
10
20
UTP
-
+
+
+
+
IP: vav2
IB: PY
IB: vav2

Figure III-8. UTP stimulates the activity of RhoA and Rac1.

HCAEC were treated with 10 μM UTP for the indicated times. Immunoblots of RhoA (*A*), Rac1 (*B*), and Cdc42 (*C*) are shown. The *top panels* show immunoblots of protein from cell lysates precipitated by either GST-RBD beads (for Rho-GTP) or GST-p21-binding domain beads (for Rac1-GTP or Cdc42-GTP). The signal from the stimulated cell lysates was compared with signals from lysates loaded with guanosine 5'-*O*-(3-thiotriphosphate) (*GTPγS*) before precipitation with GST-RBD or PAK-1 p21-binding domain beads. The *lower panels* show immunoblots of total RhoA, Rac1, or Cdc42 in total cell lysates.

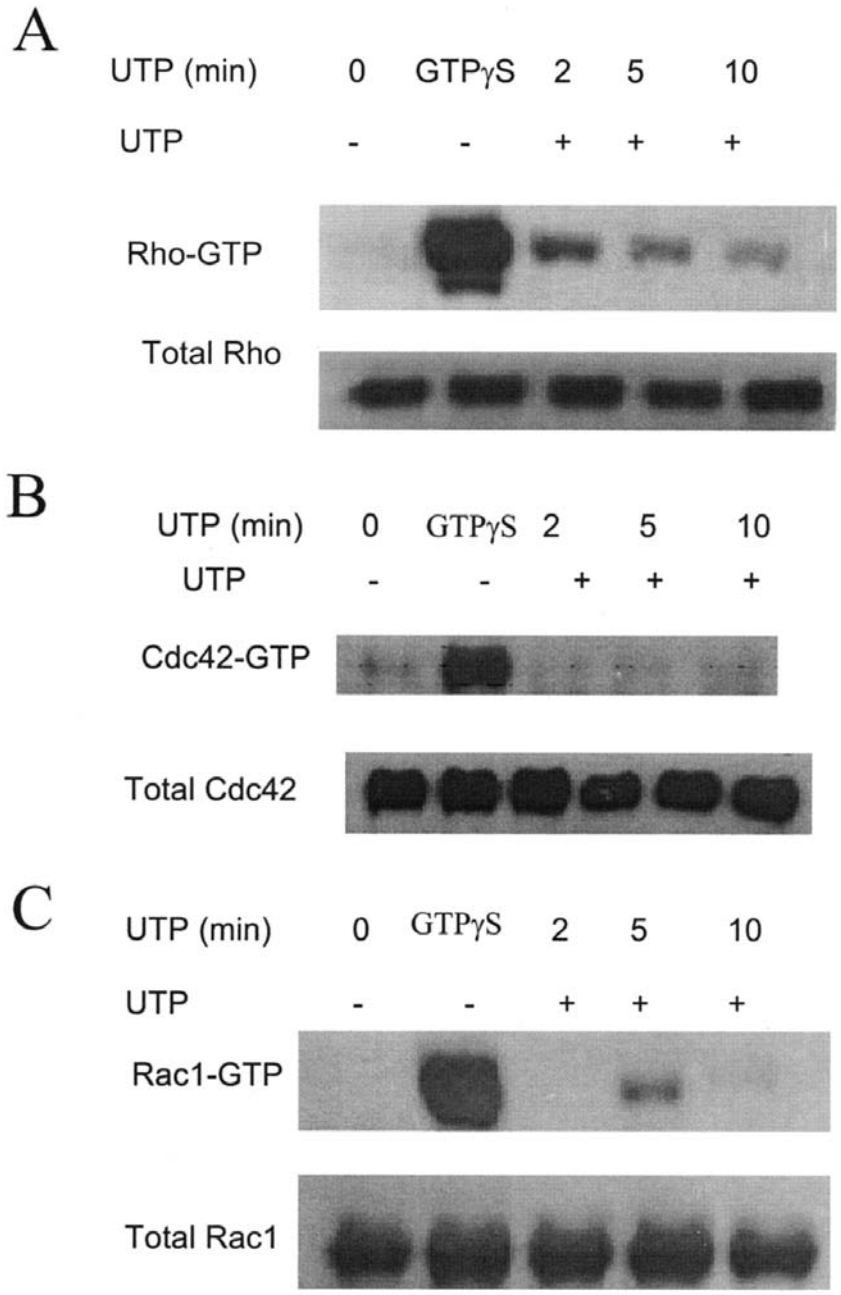
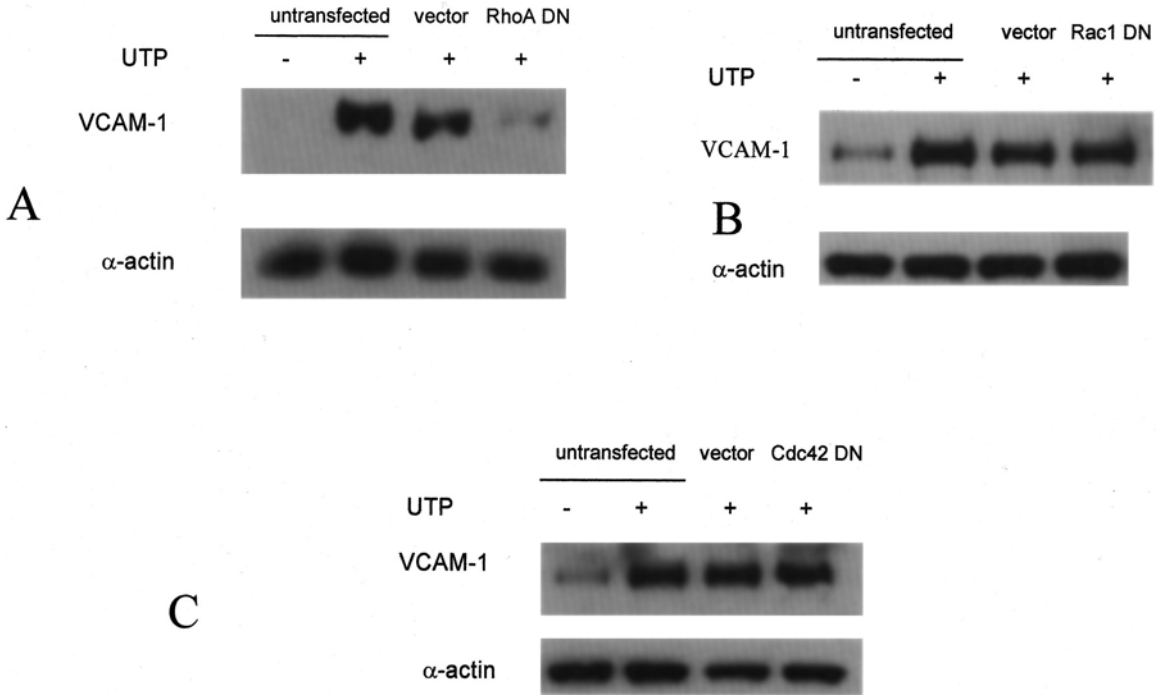

A
UTP (min) 0 GTPγS 2 5 10
UTP - - + + +
Rho-GTP
Total Rho
B
UTP (min) 0 GTPγS 2 5 10
UTP - - + + +
Cdc42-GTP
Total Cdc42
C
UTP (min) 0 GTPγS 2 5 10
UTP - - + + +
Rac1-GTP
Total Rac1

Figure III-9. Dominant negative RhoA inhibits VCAM-1 expression.

HCAEC were transiently transfected with vector or dominant negative (*DN*) RhoA (*A*), Rac1 (*B*), or Cdc42 (*C*). After stimulation with 10 μM UTP for 6 h, VCAM-1 was analyzed by Western analysis. The blots were stripped and reprobed with anti-actin antibody. The data are representative of results from three independent experiments.


untransfected
vector
RhoA DN
UTP
-
+
+
+
VCAM-1
α-actin
A
untransfected
vector
Rac1 DN
UTP
-
+
+
+
VCAM-1
α-actin
B
untransfected
vector
Cdc42 DN
UTP
-
+
+
+
VCAM-1
α-actin
C

Chapter IV

Binding of the $P2Y_2$ Nucleotide Receptor to Filamin A Regulates Migration of Vascular Smooth Muscle Cells

Abstract

The functional expression of the G protein-coupled $P2Y_2$ nucleotide receptor has been associated with proliferation and migration of vascular smooth muscle cells (SMC), two processes involved in atherosclerosis and restenosis. Activation of the $P2Y_2$ receptor causes dynamic reorganization of the actin cytoskeleton, which transmits biochemical signals and forces necessary for cell locomotion, suggesting that $P2Y_2$ receptors may be linked to the actin cytoskeleton. Here, we identified filamin A (FLNa) as a $P2Y_2$ receptor interacting protein using a yeast two-hybrid system screen with the amino terminus of the $P2Y_2$ receptor as bait. The FLNa binding site in the $P2Y_2$ receptor is localized between amino acids 322 and 333. Deletion of this region led to selective loss of FLNa binding to the $P2Y_2$ receptor and abolished tyrosine phosphorylation of FLNa induced by the $P2Y_2$ receptor agonist UTP. Using both time-lapse microscopy and the Transwell cell migration assay, we showed that UTP significantly increased smooth muscle cell spreading on collagen I (6.8 fold; $P \leq 0.01$) and migration (3.6 fold; $P \leq 0.01$) of aortic SMC isolated from wild type mice, as compared to unstimulated SMC. UTP-induced spreading and migration of aortic SMC did not occur with cells isolated from $P2Y_2$ receptor

knockout mice. Expression of the full length $P2Y_2$ receptor in SMC isolated from $P2Y_2$ receptor knockout mice restored both UTP-induced spreading and migration. In contrast, UTP-induced spreading and migration did not occur in SMC isolated from $P2Y_2$ receptor knockout mice and transfected with a mutant $P2Y_2$ receptor that does not bind FLNa. Furthermore, *ex vivo* studies showed that both ATP and UTP (10 μM) promoted migration of SMC out of aortic explants isolated from wild type but not $P2Y_2$ receptor knockout mice. Thus, this study demonstrates that $P2Y_2$ receptor/FLNa interaction selectively regulates spreading and migration of vascular SMC.

Introduction

Migration of vascular smooth muscle cells (SMC) plays a key role in the development of prominent vascular diseases, including atherosclerosis and restenosis after angioplasty (Ross, 1993; Schwartz, 1998). Extracellular nucleotides released in the vascular wall from perivascular nerves, activated platelets and mechanically stretched cells (Ellsworth *et al*., 1995; Lazarowski *et al*, 1997; Hamada *et al*., 1998) are the focus of ongoing studies aimed at determining the role of released nucleotides in the development of vascular diseases. Previous studies have indicated that extracellular nucleotides serve as directional cues for the migration of rat SMC *in vitro* through activation of G protein-coupled P2Y receptors (Chaulet *et al*., 2001). Up-regulation and activation of the $P2Y_2$ receptor subtype has been shown to stimulate neointimal growth in the collared rabbit carotid artery (Seye *et al*., 2002), suggesting an important role for this receptor in intimal hyperplasia and vascular remodeling.

As with many other cellular processes, the molecular components involved in smooth muscle cell migration are being identified at a rapid rate but like most cell functions, the manner in which these components work together as a dynamic, integrated system is less well understood. Although it has been shown that activation of P2Y receptors induces migration of SMC through increased production of osteopontin (Chaulet *et al*., 2001), an extracellular matrix protein that interacts with integrins, it is not known whether the $P2Y_2$ receptor interacts with intracellular components that modulate the migration process. Activation of P2Y receptors by nucleotides has been shown to induce reorganization of the actin cytoskeleton in vascular SMC (Sauzeau *et al*., 2000). One of the earliest responses to extracellular signals is a rapid reorganization of the actin

cytoskeleton, which leads to the formation of motile structures, alterations in cell shape, and changes in cell adhesiveness and locomotion (Thomas and Advani,, 2006). Actin-binding proteins play a pivotal role in reorganizing the actin cytoskeleton in response to signals exchanged between cells (Viola and Gupta, 2007). Accordingly, actin-binding proteins are increasingly a focus of investigations into effectors of cell signaling and the coordination of cellular functions that regulate tissue development. Using the yeast two-hybrid system, we report for the first time that an interaction occurs between the cytoplasmic domain of the $P2Y_2$ receptor and filamin A (FLNa), an actin binding protein that crosslinks filamentous actin into either orthogonal or parallel bundles (Cunningham *et al*., 1992). FLNa is required for cell migration (Cunningham *et al*., 1992) and mutations in human FLNa are responsible for impaired migration of cerebral neurons and give rise to periventricular heterotopia, leading to epilepsy and vascular disorders, as well as embryonic lethality (Fox *et al*., 1998).

FLNa anchors actin filaments to cell-extracellular matrix adhesion sites by binding to β-integrin subunits (Calderwood *et al*., 2000) and also provides a scaffold for small GTPases of the Ras and Rho families (Ohta *et al*, 1999). We characterized the interaction between FLNa and the $P2Y_2$ receptor in biochemical assays and used wild type and $P2Y_2$ receptor knockout mice to show that the loss of this interaction affects spreading and migration of aortic SMC.

Experimental Procedures

Materials

All reagents, unless specified otherwise, were purchased from Sigma Alderich (St. Louis, MO).

Plasmid Constructs

All cDNAs were generated by PCR. The sequence and insert orientation of all constructs were verified by DNA sequencing at the DNA Core Facility of the University of Missouri-Columbia. For the yeast two-hybrid assay, the EcoRI-BamHI fragment encoding a C-terminal domain (CTD) of the $P2Y_2$ receptor (amino acids 317-360) was subcloned into the yeast GAL4 DNA binding domain expression vector pGBKT7 (BD Biosciences, Palo Alto, CA) between EcoRI and BamHI restriction sites. Accordingly, the following $P2Y_2$ receptor primers: forward (5'-GTA GGA ATT CCG AGA TGC CAA GCC ACC CAC T-3') and reverse (5'-TAT GGG ATC CCC TGA AGT CCT CAC TGC TGC C-3') were used to produce the bait plasmid (pGBKT7-$P2Y_2$CTD). For the GST pull down assay, the BamHI-EcoRI fragment encoding the $P2Y_2$CTD was inserted between BamHI and EcoRI restriction sites of the bacterial expression vector pGEX-5X-1 (Amersham Biosciences, Piscataway, NJ) to produce pGEX-5X-1-$P2Y_2$CTD using the following primers: forward (5'-GTA GGG ATC CCC CGA GAT GCC AAG CCA CCC ACT-3') and reverse (5'-TAT GGA ATT CCC TGA AGT CCT CAC TGC TGC C-3').

Mutagenesis and Epitope Tagging of the $P2Y_2$ Receptor

The open reading frame of $P2Y_2$ receptor cDNA was modified to incorporate the hemagglutinin (HA) epitope (YPYDVPDYA) at the amino terminus of the recombinant protein, as described previously (Garrad *et al*., 1998). Six C-terminal truncation mutants

of the $P2Y_2$ receptor were generated by PCR. In each case, the forward primer was 5'-GAT CGT GAA TTC TGA TGT ATC CAT ATG ATG TTC CAG ATT ATG CTG CAG CAG ACC TGG AAC CCT GG-3', which maintains the HA epitope at the amino terminus and incorporates an EcoRI restriction site, and the reverse primer differed for each mutant: 5'-TGC TAG GGA TCC CGT CAT CAG CCC AAC ACA TCT TCT ATC CTC TGC AT-3' (truncation mutant 1; 1-354), 5'-TGC TAG GGA TCC CGT CAT CAC ATG TCA GTT CTG TCG GAT CTG CGC AG-3' (truncation mutant 2; 1-346), 5'-TGC TAG GGA TCC CGT CAT CAT CTG CGC AGG CCC AGC CTG CGG CGA GC-3' (truncation mutant 3; 1-340), 5'-TGC TAG GGA TCC CGT CAT CAG CGA GCC GGG GTG GCA GGG CTG GGG CC-3' (truncation mutant 4; 1-333), 5'-TGC TAG GGA TCC CGT CAT CAC TTG GCA TCT CGG GCA AAG CGT ACG AG-3' (truncation mutant 5; 1-321), and 5'-TGC TAG GGA TCC CGT CAT CAG GCA AAG CGT ACG AGC CTC TGC CCA GC-3' (truncation mutant 6; 1-317). The PCR was carried out using Advantage 2 Polymerase under conditions suggested by the manufacturer (BD Biosciences, Palo Alto, CA). The DNA amplification was verified by agarose gel electrophoresis, and PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ). Mutations were confirmed by DNA sequencing.

Yeast Two-Hybrid Library Screening

A cDNA library from human aorta constructed in the GAL4 activation domain vector pACT2 was purchased from BD Biosciences (Palo Alto, CA). The cDNA library was co-transformed with the bait plasmid pGBKT7-$P2Y_2$CTD into Saccharomyces cerevisiae strain AH109 following the manufacturer's instructions (BD Biosciences). The

interaction between bait and prey proteins was determined using the β-galactosidase assay, as described in the Yeast Protocol Handbook (Clontech Laboratories, Palo Alto, CA). Positive prey plasmids were extracted from yeast and transformed into *E. coli* TOP10 chemically competent cells (Invitrogen, Carlsbad, CA). The identity of the prey was determined by DNA sequencing with the 5'-pACT2 primer 5'-CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC-3' and the 3'-pACT2 primer 5'-GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG A-3' and the Basic Local Alignment Search Tool (BLAST) against the human protein database (tblastx; http://www.ncbi.nlm.nih.gov/BLAST). To confirm the interaction between bait and prey in the yeast two-hybrid system, purified prey plasmids were re-transformed with the bait plasmid or empty pGBKT7 and tested for growth on the same plates.

Glutathione S-Transferase (GST) Pull-Down Assay

The GST-fusion protein pull-down assay was carried out according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Briefly, pGEX-5X-1-$P2Y_2CTD$ was transformed into *E. coli* strain BL21-Gold (Stratagene, La Jolla, CA). Fusion protein expression was induced with 1 mM IPTG (Sigma, St. Louis, MO) for 2 h at 37 °C and the fusion protein was immobilized by glutathione covalently linked to Sepharose. Lysates from human coronary artery smooth muscle cells (CASMC) were incubated with fusion protein immobilized by glutathione-linked Sepharose, and bound proteins were eluted with soluble glutathione (100 mM). The eluted proteins were resolved by SDS-PAGE and analyzed by Western analysis using a mouse monoclonal anti-FLNa antibody (1:1000 dilution; Chemicon, Temecula, CA).

Adenovirus Production and Infection of Cultured Cells

The $P2Y_2$ receptor cDNA constructs encoding full length and truncated $P2Y_2$ receptors were inserted into the adenoviral vector Ad5mCMV. Virus production was performed at the Gene Transfer Vector Core at the University of Iowa (Iowa City, IA). Mouse aortic smooth muscle cells (SMC), at 70-80% confluence, were incubated for 4 h with adenovirus at 200 PFU/cell in growth medium comprised of DMEM, 2% (v/v) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Then, the cells were washed and cultured in fresh growth medium for 24-48 h to allow protein expression.

Immunoprecipitation and Western Blot Analysis

Mouse aortic SMC or 1321N1 astrocytoma cell transfectants expressing full length or truncated HA-tagged $P2Y_2$ receptors were washed with cold PBS and lysed in modified RIPA buffer at 4 ^{o}C for 15 min. The lysates were cleared by centrifugation at 10,000 X g at 4 ^{o}C for 15 min. The protein concentrations in the supernatant were determined according to the Lowry assay and equal amounts of supernatant protein (200 μg) were incubated with 50 μl of HA-affinity matrix (Roche Applied Sciences, Indianapolis, IN) with gentle rocking at 4 ^{o}C for 2 h. The immunoprecipitate was sedimented by centrifugation, washed with RIPA buffer, resuspended in 50 μl of 2X Laemmli's buffer and heated at 95 ^{o}C for 4 min. The solubilized proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) non-fat milk in TBST with gentle rocking at room temperature for 2 h. Then, the membrane was incubated at room temperature in TBST containing mouse monoclonal anti-FLNa antibody (1/1000 dilution; Chemicon), washed five times with TBST for 10 min each time, incubated with 1/2000 dilution of HRP-conjugated goat anti-

mouse IgG for 1 h, and washed four times with TBST for 10 min each time. Proteins were visualized with Luminol reagent (Bio-Rad Laboratories, Hercules, CA).

Cell Culture

Mouse aortic SMC were obtained from 4-6 week-old mice. Briefly, thoracic aorta was denuded of adventitia, cut into 1 mm cubes and digested with 0.1% (w/v) collagenase (Worthington Biochemical Corp, Lakewood, NJ) in DMEM containing 1% (w/v) BSA at 37 ^{o}C with gentle rocking. Cells dissociated within the first 30 min were discarded to improve smooth muscle cell purity. Subsequently, dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Cells were identified as vascular SMC through their characteristic hill-and-valley growth pattern and by detection of α–smooth muscle actin. Human 1321N1 astrocytoma cells were cultured in DMEM with 10% (v/v) fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Transfection of 1321N1 cells with wild type or mutant $P2Y_2$ receptor cDNA was performed using Lipofectamine 2000 following the manufacturer's (Invitrogen) instructions.

Smooth Muscle Cell Migration in Aortic Explants

$P2Y_2$ receptor knockout (KO) mice on a B6D2 genetic background and the corresponding wild type mice were generous gifts from Dr. B. H. Koller (University of North Carolina-Chapel Hill). Genotypes were verified by tail snip assay. Descending mouse aorta was thoroughly dissected free from connective tissue, cut open longitudinally, and the intima and a thin portion of the subjacent media were removed. The descending aorta was weighed and trimmed to normalize the weight to 5 mg. The

aorta was cut into 4 pieces and each piece was placed in a corner of a chambered slide with the endothelium facing down and cultured in DMEM containing appropriate concentrations of nucleotides or supplemented with 10% (v/v) FBS. For the creatine phosphokinase (CPK) regenerating system, cells were preincubated with agonist for 10 min, and then 2 U ml^{-1} CPK and 1 mM phosphocreatine were added. Medium was replaced every day for 5 days prior to analysis of the explants by confocal microscopy. To count cells that migrated out of the explant, the remaining tissue was removed and cells that had migrated away from the explant were detached by 0.05% (w/v) trypsin-EDTA (Invitrogen), and counted under a microscope.

Cell Migration Assay

Cell migration assays were performed on Transwells (Costar), as previously described (Chaulet *et al*., 2001). Briefly, cells were resuspended in serum-free DMEM ($5x10^5$ cells/ml) and added to the upper chamber of Transwells. The lower chamber was filled with 600 μl of DMEM with or without 10 μM UTP. After an 18 h incubation with nucleotides, filters were removed, and cells that had not migrated (*i.e.*, cells on the upper surface of the membrane) were removed with a cotton swab. Then, cells present beneath the membrane were fixed with cold methanol and stained with hematoxylin (Sigma, St. Louis, MO). Migrating cells were counted in 10 high-power microscope fields (20X magnification). Analysis was performed on three Transwells for each condition, and each experiment was repeated three times.

Cell Spreading Assay and Time-Lapse Microscopy

Cell spreading was determined by measuring the number of cells that spread after a defined time, as described previously (Yamamoto and Yamamoto, 1994). Coverslips

placed in welled plates were coated with type I collagen (20 μg/ml) overnight at 4 °C. To suppress nonspecific binding, coverslips were blocked with 1% (w/v) BSA in PBS at 37 °C for 1 h. Cells in DMEM containing 0.4% (v/v) FCS were added at a concentration of 2000 cells per 100 μl and maintained at 37 ºC for 1 h in a POC-R chamber inside a pre-heated microscope stage insert (LaCon, Staig, Germany). Then, cell spreading was followed by time-lapse microscopy to monitor movement of cells containing nuclei or with a recognizable noncircular shape using an Axiovert 200M inverted microscope equipped with a 40X/0.6 NA LD Plan-Neofluar objective (LSM 510 Meta System, Zeiss, Thornwood, NY). Four fields of view for each coverslip were chosen by visual inspection and the position of the stage in each field was marked by the microscope control software (Zeiss LSM 5 version 4.0). At different time points, the microscope stage was positioned at each marked location, the fields were focused manually, and cells were imaged by detecting transmitted light while scanning with a 488 nm laser.

Statistical Analysis

ANOVA and paired and unpaired *t* tests were performed for statistical analysis, as appropriate. Values of $P<0.05$ were considered to be statistically significant.

Results

FLNa Interacts Directly With Amino Acids 322-333 in the C-Terminus of the $P2Y_2$ Receptor

Extracellular nucleotides have been shown to induce reorganization of the actin cytoskeleton (Sauzeau *et al*., 2000), a key step in cell migration. Since $P2Y_2$ receptor activation causes migration of SMC, we hypothesized that the $P2Y_2$ receptor is directly linked to the actin cytoskeleton. Thus, we used a 45-amino acid fragment (aa 318-362) in the intracellular C-terminal tail of the $P2Y_2$ receptor (*i.e.*, $P2Y_2$CTD) as bait to screen for binding of proteins expressed from a human aorta cDNA library using the AH109 yeast two-hybrid system. Screening of 10^6 independent clones yielded $P2Y_2$CTD-binding proteins in several clones expressing an identical 1.2-kb cDNA insert that encoded the C-terminal region of FLNa, a dimeric actin-crosslinking phosphoprotein that promotes orthogonal branching of actin filaments. The specificity of the interaction between $P2Y_2$CTD and FLNa was verified by one-on-one transformation into yeast strain AH109 (data now shown). To further verify that a specific interaction occurs between the $P2Y_2$CTD and FLNa, we tested the ability of a Glutathione S-Transferase (GST)-fusion protein containing the C-terminus of the $P2Y_2$ receptor (GST-$P2Y_2$CTD) to associate with endogenously expressed full length FLNa. Lysates from human coronary artery SMC (CASMC) contained a 280 kDa protein that immunoreacted with anti-FLNa antibody (Fig. IV-1A). As shown in Fig. IV-1B, this protein also was detected in pull-down assays when lysates from CASMC were incubated with GST-$P2Y_2$CTD, but not GST, immobilized by glutathione-linked Sepharose. To verify that full length $P2Y_2$ receptor and FLNa interact in mammalian cells, we transfected P2 receptor-null 1321N1

astrocytoma cells with cDNA encoding the HA-tagged $P2Y_2$ receptor, then immunoprecipitated the HA-$P2Y_2$ receptor from cell lysates using anti-HA antibody and detected co-precipitation of FLNa using anti-FLNa antibody (Fig. IV-1C). To identify the domain in the C-terminal tail of the $P2Y_2$ receptor that interacts with FLNa, cDNA encoding $P2Y_2$ receptor with progressive deletion mutants of the CTD were generated by PCR using specific primers (Fig. IV-2A). All of the $P2Y_2$ receptor mutants expressed in 1321N1 cells showed the same ability as the wild type $P2Y_2$ receptor to mediate UTP-induced intracellular calcium mobilization (data not shown). HA-tagged $P2Y_2$ receptor or $P2Y_2$CTD deletion mutants expressed in 1321N1 cells were immunoprecipitated with anti-HA antibody, and co-precipitation of FLNa was assessed. As indicated in Fig. IV-2B, deletion of amino acids 322-333 results in the loss of FLNa binding to the HA-$P2Y_2$ receptor.

$P2Y_2$ Receptor Activation Mediates FLNa Phosphorylation

Phosphorylation and dephosphorylation events are thought to regulate FLNa activity (Gibbons and Dzau, 1994), and therefore we determined whether $P2Y_2$ receptor activation by UTP in mouse aortic SMC induces the phosphorylation of FLNa. For this purpose, the full length $P2Y_2$ receptor or the mutant $P2Y_2$ receptor lacking the FLNa binding amino acids 322-332 (*i.e.*, truncation mutant 5) were expressed in aortic SMC isolated from $P2Y_2$ receptor knockout (KO) mice. UTP (10 μM) stimulation of SMC expressing the full length $P2Y_2$ receptor, but not truncation mutant 5 $P2Y_2$ receptor, caused a rapid and sustained increase in tyrosine phosphorylation of FLNa (Fig. IV-3). These results indicate that binding of FLNa to the $P2Y_2$ receptor is necessary for UTP-induced activation of FLNa.

$P2Y_2$ Receptor and FLNa Interaction Promotes Spreading of SMC

To gain further insight into the role of $P2Y_2$ receptors in the function of vascular SMC, we studied the ability of UTP to induce the spreading of mouse aortic SMC on collagen I. Treatment of aortic SMC isolated from wild type mice with UTP for 2 h caused a 6.8 fold increase ($P \leq 0.01$) in the spreading of cells on collagen I, as compared to unstimulated cells (Fig. IV-4). In contrast, UTP did not increase spreading of aortic SMC isolated from $P2Y_2$ receptor KO mice. To test the role of $P2Y_2$ receptor/FLNa interaction in UTP-induced cell spreading, we transfected aortic SMC obtained from $P2Y_2$ receptor KO mice with either full length (WT) or truncation mutant 5 $P2Y_2$ receptor cDNA, and successful receptor expression was demonstrated by the appearance of UTP-induced calcium mobilization and $P2Y_2$ receptor mRNA in the cell transfectants (data not shown). Expression of the full length $P2Y_2$ receptor in aortic SMC from $P2Y_2$ receptor KO mice completely restored UTP-induced cell spreading whereas cells that expressed the truncation mutant 5 $P2Y_2$ receptor exhibited significantly less spreading (Fig. IV-4). These results indicate that the $P2Y_2$ receptor C-terminal tail is required for optimum UTP-induced spreading of SMC.

$P2Y_2$ Receptor and FLNa Interaction Mediates Migration of SMC

UTP increased by 3.4 fold ($P \leq 0.01$) the migration of aortic SMC isolated from wild type mice, as compared to unstimulated SMC or UTP-stimulated SMC from $P2Y_2$ receptor KO mice (Fig. IV-5A). Expression of the full length $P2Y_2$ receptor in aortic SMC from $P2Y_2$ receptor KO mice increased UTP-induced cell migration (Fig. IV-5B) to levels seen with SMC from wild type mice (Fig. IV-5A). In contrast, aortic SMC from $P2Y_2$ receptor KO mice expressing the truncation mutant 5 $P2Y_2$ receptor that is

defective in FLNa binding did not exhibit UTP-induced cell migration (Fig. IV-5B), indicating that loss of $P2Y_2$ receptor/FLNa interaction prevents UTP-induced migration of SMC.

$P2Y_2$ Receptors Mediate Migration of SMC Out of Mouse Aortic Explants

To validate our *in vitro* data, we investigated whether extracellular nucleotides serve as directional cues for SMC within a vessel fragment. Therefore, we examined the ability of SMCs to migrate out of aortic explants isolated from wild type and $P2Y_2$ receptor KO mice. The $P2Y_2$ receptor agonists ATP or UTP (10 μM) induced significant migration of SMC out of aortic explants isolated from wild type but not $P2Y_2$ receptor KO mice, as compared to untreated controls (Fig. IV-6, *A-B*). ATP and UTP also increased migration of SMC out of explants in the presence of 2U/ml CPK and 1 mM phosphocreatine (Fig. IV-6B), a system for regenerating nucleoside triphosphates from cell-hydrolyzed ATP or UTP, suggesting that ectonucleotidase-mediated nucleoside generation was not responsible for cell migration induced by ATP or UTP. In contrast to ATP and UTP, 5% serum was equally effective in stimulating migration of SMC out of aortic explants from both wild type and $P2Y_2$ receptor KO mice (Fig. IV-6B). UDP and 2MeS-ADP did not induce migration of SMC out of the explants (data not shown). These data indicate that ATP and UTP promote migration of SMC from aortic tissue via activation of $P2Y_2$ receptors.

Discussion

In the present study, we demonstrated that extracellular nucleotides serve as directional cues not only for cultured SMC but also for SMC within a vessel segment suggesting that nucleotides released *in vivo* at the site of vessel injury could participate in the remodeling process accompanying prominent vascular diseases. We showed that the nucleotides ATP and UTP act directly on $P2Y_2$ receptors, since nucleotides were not effective in promoting the motility of SMC derived from the $P2Y_2$ receptor knockout (KO) mouse. Furthermore, we showed that ATP and UTP significantly increased the spreading of mouse aortic SMC on collagen I, a process that involves rearrangement of the cytoskeleton and increased contractility of vascular SMC (Calderwood *et al*., 2000). Both cytoskeletal rearrangement and contraction of SMC are essential for the control of vascular remodeling and repair mechanisms that are induced by increased shear stress or vascular damage (Gibbons and Dzau, 1994). To date, this is the first report demonstrating that ATP and UTP promote cell spreading in intact tissue.

Nucleotides act on P2Y receptors expressed in vascular SMC to promote actin cytoskeletal reorganization in a Rho kinase-dependent manner (Sauzeau *et al*., 2000). Furthermore, the $P2Y_2$ receptor mediates UTP-induced cytoskeleton rearrangement and cell migration induced by Rho/Rac activation (Bagchi *et al*., 2005; Liao *et al*., 2007). This prompted us to investigate whether the $P2Y_2$ receptor is directly linked to the actin cytoskeleton. Using a yeast two-hybrid system screen and *in vitro* and *in vivo* binding assays, we demonstrated that the scaffold protein filamin A (FLNa) interacts directly with the $P2Y_2$ receptor. FLNa is an actin crosslinking protein and is involved in the maintenance of the cytoskeletal architecture (Cunningham *et al*., 1992). FLNa functions

as a dimer and is composed of 23 approximately 96 amino acid long repeats flanked by an N-terminal actin-binding domain and a C-terminal homodimerization domain that regulate organization of the cortical actin network (Cunningham *et al*., 1992). FLNa is essential for mammalian cell locomotion (Cunningham *et al*., 1992). Human melanoma cell lines that do not express detectable levels of FLNa do not migrate, and expression of FLNa in these cells restores their locomotion (Cunningham *et al*., 1992). FLNa is known to be a phosphoprotein (Ohta *et al*., 1996), but it is not understood how phosphorylation regulates FLNa function. In the present study, we found that stimulation of quiescent SMC with UTP results in a rapid increase in the phosphorylation of FLNa (Fig. IV-3). FLNa is targeted by a variety of signaling molecules, *e.g.*, Src, ribosomal S6 kinase, protein kinase C, and small GTPases of the Rho- and Rac-families, which thereby implicate FLNa in cell shape modulation and integrin-dependent adhesion and motility (Ohta *et al*., 1996; Nagano *et al*., 2002; Tigges *et al*., 2003; Woo *et al*., 2004; Pal Sharma and Goldmann, 2004). Previous results have shown that PAK-1 mediates FLNa phosphorylation in MCF-7 breast cancer cells (Vadlamudi *et al*., 2002). Rac1, an activator of PAK-1, has been found to be activated by the $P2Y_2$ receptor in vascular endothelial cells (Chapter III). We also have found that UTP induces PAK activity in mouse SMC (unpublished data), suggesting a possible role of PAK-1 in UTP-induced FLNa phosphorylation. Activation of the $P2Y_2$ receptor can cause tyrosine phosphorylation of Src, a kinase family member that is known to phosphorylate FLNa on tyrosine residues (Pal Sharma and Goldmann, 2004; Liu *et al*., 2004). Further studies are needed to define the roles of Src and PAK-1 in $P2Y_2$ receptor-mediated FLNa phosphorylation.

The biological significance of the loss of $P2Y_2$ receptor/FLNa interaction was a major focus of the present study. We have identified amino acids in the C-terminal domain of the $P2Y_2$ receptor that are required for $P2Y_2$ receptor/FLNa interaction and UTP-induced migration of SMC (Figs. IV-2 and IV-5). Our data indicate that UTP did not induce spreading and migration of aortic SMC isolated from $P2Y_2$ receptor KO mice, and expression of the $P2Y_2$ receptor in these cells restored nucleotide-induced spreading and migration of SMC (Figs. IV-4 and IV-5). In further support of a role for $P2Y_2$ receptor/FLNa interaction in nucleotide-induced spreading and migration of SMC, expression in SMC from $P2Y_2$ receptor KO mice of a mutant $P2Y_2$ receptor that does bind FLNa fails to restore cell spreading and migration in response to UTP, as compared to expression of the WT $P2Y_2$ receptor (Figs. IV-4 and IV-5). Since loss of the $P2Y_2$ receptor/FLNa interaction also prevented UTP-induced FLNa phosphorylation in mouse aortic SMC (Fig. IV-3), we conclude that activation of FLNa is a prerequisite for $P2Y_2$ receptor-mediated spreading and migration of SMC.

Rearrangement of actin filaments plays an essential role in cell growth, survival, locomotion, and shape. For example, cell-cycle progression depends on cell spreading and adhesion, whereas apoptosis is initiated after cells are detached (Assoian and Marcantonio, 1996; Meredith and Schwartz, 1997). In cardiovascular diseases, such as hypertension, atherosclerosis, and restenosis after angioplasty, vascular remodeling clearly requires rearrangement of the cytoskeleton of vascular SMC (Gibbons and Dzau, 1994; Schwartz *et al*., 1995). Nucleotides are thought to play important roles in cell remodeling by virtue of their effects on migration and growth of SMC (Erlinge, 2004).

$P2Y_2$ receptors are highly expressed in rat and rabbit intimal lesions (Seye *et al.*, 1997; 2002), and therefore, $P2Y_2$ receptor interactions with FLNa may play an important role in the regulation of vascular remodeling and tone by modulating migration of SMC in response to nucleotides released at the site of vascular injury.

Figure IV-1. FLNa binds to the $P2Y_2$ receptor C-terminal domain (CTD) and co-immunoprecipitates with the HA-$P2Y_2$ receptor.

A, Lysates from CASMC were analyzed by SDS-PAGE and immunoblotted by goat anti-FLNa antibody. M2 melanoma cells (kindly provided by Dr J. H. Hartwig, Harvard Medical School) that are deficient in FLNa were used in parallel assays as a negative control, and FLNa transfected cells (A7 cells) were used as a positive control. Results are representative of three independent experiments. *B,* GST-$P2Y_2$CTD or GST expression was induced in *E. coli*, as described in the "Experimental Procedures". GST-$P2Y_2$CTD or GST in bacterial lysates from 5 ml of IPTG-induced bacterial cultures were immobilized by incubation with glutathione-linked Sepharose for 4 h at 4 °C with gentle rocking. After washing 3X with PBS, glutathione-linked Sepharose-bound protein was incubated with lysates from 10^7 CASMC overnight at 4 °C with gentle rocking. Bound proteins were eluted with 100 mM glutathione, and Western analysis was performed with anti-FLNa antibody. Results are representative of three independent experiments. *C,* Human 1321N1 cells transfected with cDNA encoding the HA-tagged $P2Y_2$ receptor or with the pLXSN vector alone were lysed, and immunoprecipitated (IP) with anti-HA affinity matrix or normal IgG and immunoprecipitates were analyzed by immunoblotting (IB) with anti-FLNa antibody, as described in the "Experimental Procedures". FLNa levels in whole cell lysates are shown in the lower panel. Results are representative of three independent experiments.

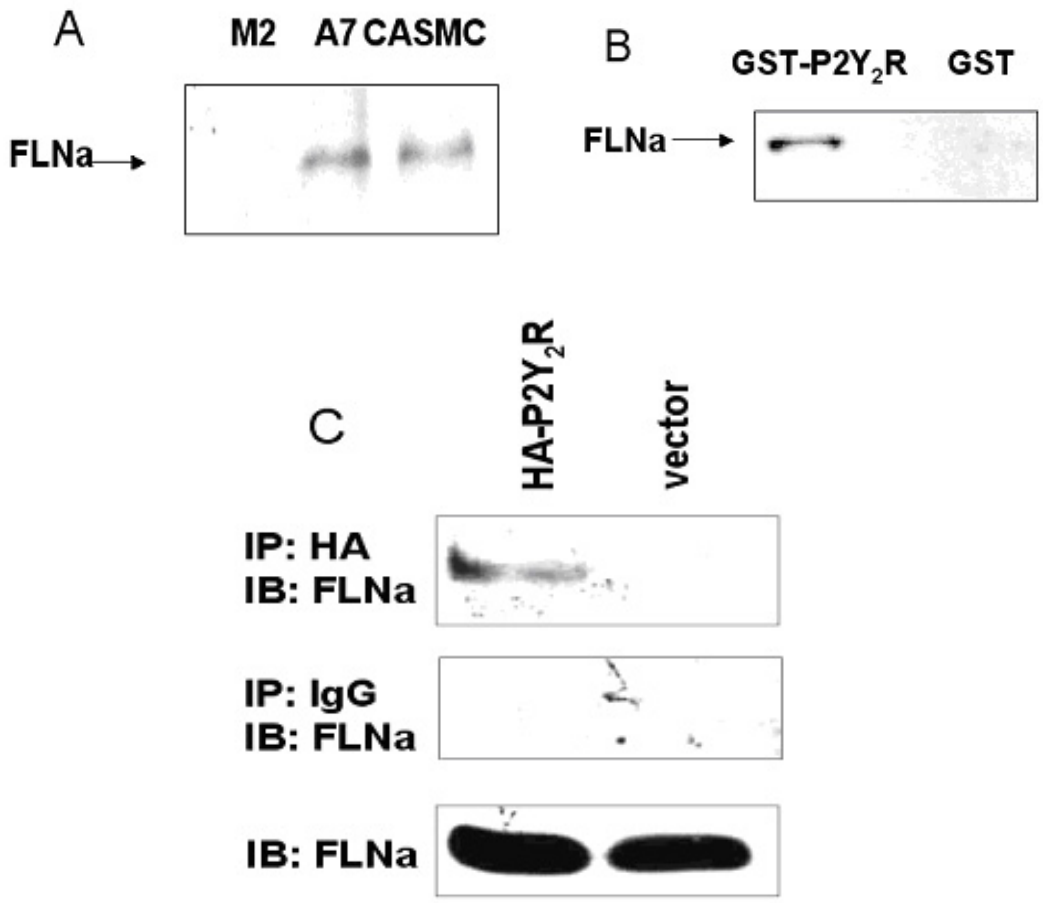

A
M2
A7
CASMC
FLNa
B
GST-P2Y2R
GST
FLNa
C
HA-P2Y2R
vector
IP: HA
IB: FLNa
IP: IgG
IB: FLNa
IB: FLNa

Figure IV-2. Identification of the FLNa binding domain of the $P2Y_2$ receptor.

Full length (wt) and progressive C-terminal truncation mutants of the HA-tagged $P2Y_2$ receptor (*A*) were expressed in human 1321N1 astrocytoma cells. *B,* Lysates from transfected cells were immunoprecipitated (IP) with anti-HA affinity matrix, and immunoprecipitates were analyzed by immunoblotting (IB) with anti-FLNa antibody, as described in the "Experimental Procedures". FLNa levels in whole cell lysates were used to normalize FLNa binding data (means ± S.E.) from three independent experiments (bar graphs) where ** indicates $P<0.01$ as compared to wild type control, with a representative blot shown above.

A

```
Wild type     ---QRLVRFARDAKPPTGPSPATPARRRLGLRRSDRTDMQRIEDVLGSSEDSRRTESTPAGSENTKDIRL     377

Truncation 1 ---QRLVRFARDAKPPTGPSPATPARRRLGLRRSDRTDMQRIEDVLG     354

Truncation 2 ---QRLVRFARDAKPPTGPSPATPARRRLGLRRSDRTDM     346

Truncation 3 ---QRLVRFARDAKPPTGPSPATPARRRLGLRR     340

Truncation 4 ---QRLVRFARDAKPPTGPSPATPAR     333

Truncation 5 ---QRLVRFARDAK     321

Truncation 6 ---QRLVRFA     317
```

B

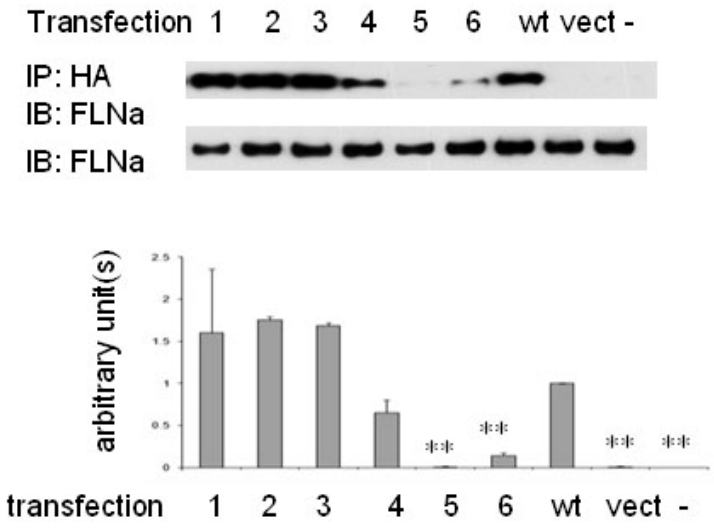


Figure IV-3. UTP induces FLNa phosphorylation in aortic SMC.

SMC from aortas of $P2Y_2$ receptor knockout (KO) mice were transfected with cDNA encoding the full length (wt) or truncation mutant 5 $P2Y_2$ receptor (Fig. IV-2A), as described in the "Experimental Procedures". Cell transfectants were serum-starved for 48 h and stimulated with UTP (10 μM) for the indicated time. Cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibody and immunoprecipitates were analyzed by immunoblotting (IB) with anti-FLNa antibody, as described in the "Experimental Procedures". FLNa levels in total cell lysates were used to normalize phosphotyrosine data (mean ± S.E.) from three independent experiments, where * indicates $P<0.05$ and ** indicates $P<0.01$, as compared to the wt $P2Y_2$ receptor at 0 min.

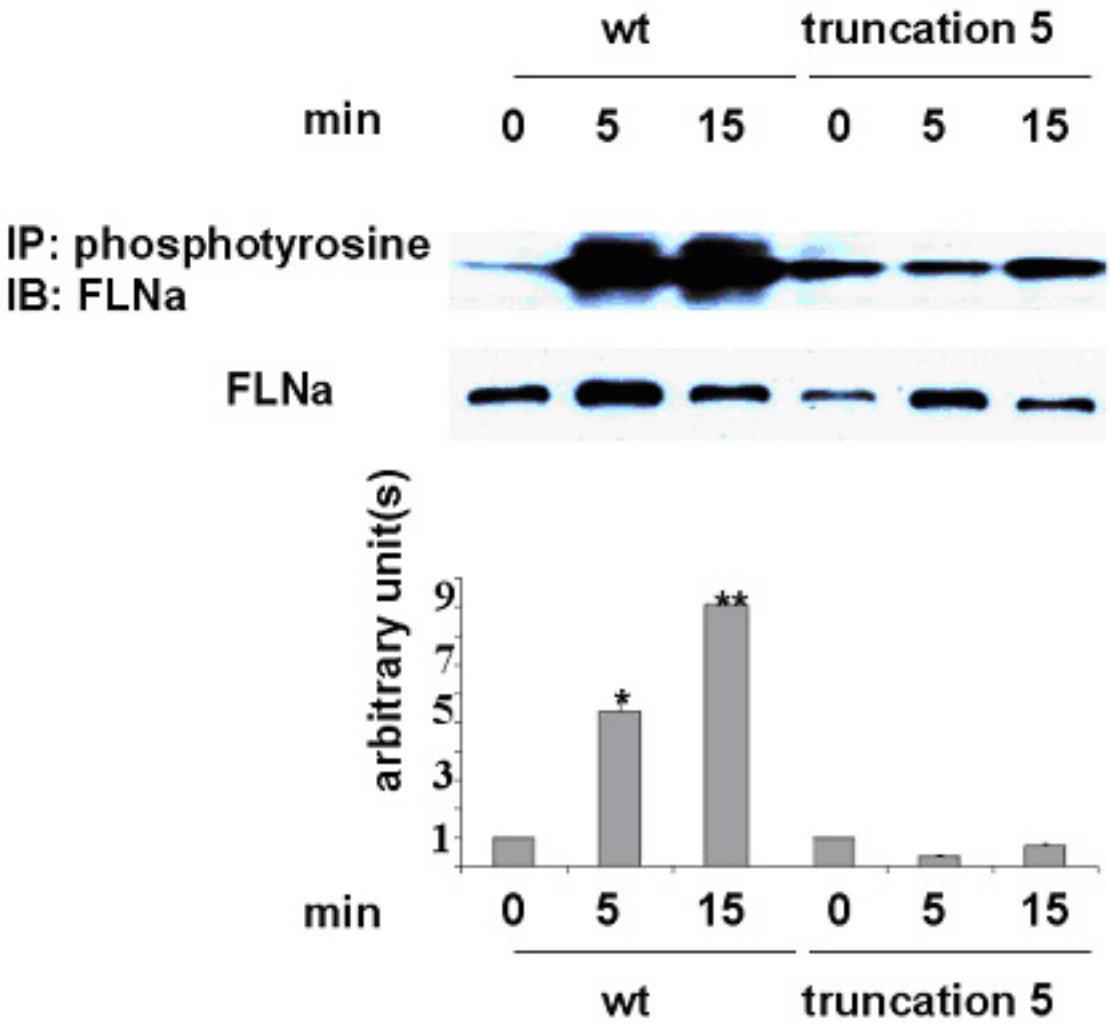

wt
truncation 5
min
0
5
15
0
5
15
IP: phosphotyrosine
IB: FLNa
FLNa
arbitrary unit(s)
9
7
5
3
1
*
**
min
0
5
15
0
5
15
wt
truncation 5

Figure IV-4. Spreading of SMCs on collagen-coated coverslips.

Aortic SMC from wild type (WT) or $P2Y_2$ receptor knockout (KO) mice were plated on collagen I-coated coverslips, as described in the "Experimental Procedures", and then were incubated for 1 h in serum-free medium with or without UTP (10 μM). *A,* Cell spreading was visualized with an Axiovert 200M inverted microscope and a representative micrograph is shown with arrows indicating spreading cells. *B,* Aortic SMC from wild type (WT) or $P2Y_2$ receptor KO mice were treated as above and cell spreading was visualized by time-lapse microscopy, as described in the "Experimental Procedures". Also, aortic SMC from $P2Y_2$ receptor KO mice were transfected with cDNA encoding full length (WT) or truncation mutant 5 $P2Y_2$ receptor. Then, cell transfectants were incubated with or without UTP (10 μM) for 1 h, and the percentage of cells exhibiting UTP-induced cell spreading was determined by time-lapse microscopy, as described in the "Experimental Procedures". Data represent means ± S.E., where ** indicates $P<0.01$, as compared to the indicated condition for three independent experiments performed in triplicate.

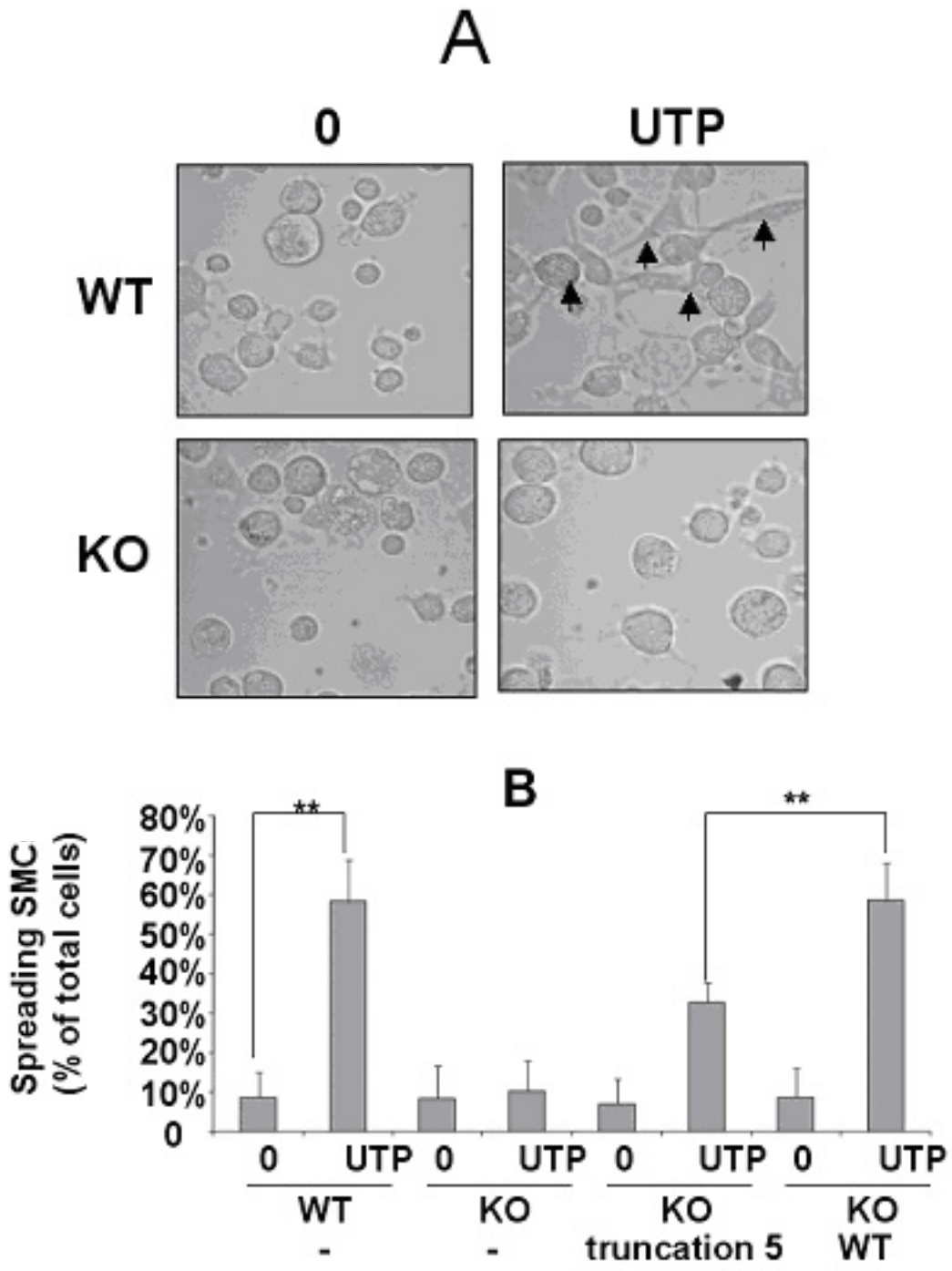

A
0
UTP
WT
KO
B
Spreading SMC
(% of total cells)
80%
70%
60%
50%
40%
30%
20%
10%
0
**
**
0
UTP
0
UTP
0
UTP
0
UTP
WT
-
KO
-
KO
truncation 5
KO
WT

Figure IV-5. Loss of FLNa/$P2Y_2$ receptor interaction affects UTP-induced migration of SMC.

A, Aortic SMC from wild type (WT) or $P2Y_2$ receptor KO mice were added to the upper chamber of Transwells. The lower chamber contained serum-free DMEM with or without UTP (10 μM). Cell migration expressed as the number of cells migrating across the membrane was evaluated after 18 h, as described in the "Experimental Procedures". *B,* Aortic SMC from $P2Y_2$ receptor KO mice expressing full length (WT) or truncation mutant 5 $P2Y_2$ receptor, or vector alone were assayed for UTP-induced cell migration, as described above. Incubation for 18 h with 10% FBS in the lower chamber was used as a positive control. Data with UTP and FBS are expressed as fold increase over untreated control and represent means ± S.E. of results from three independent experiments, where ** indicates $P<0.01$, as compared to untreated cells.

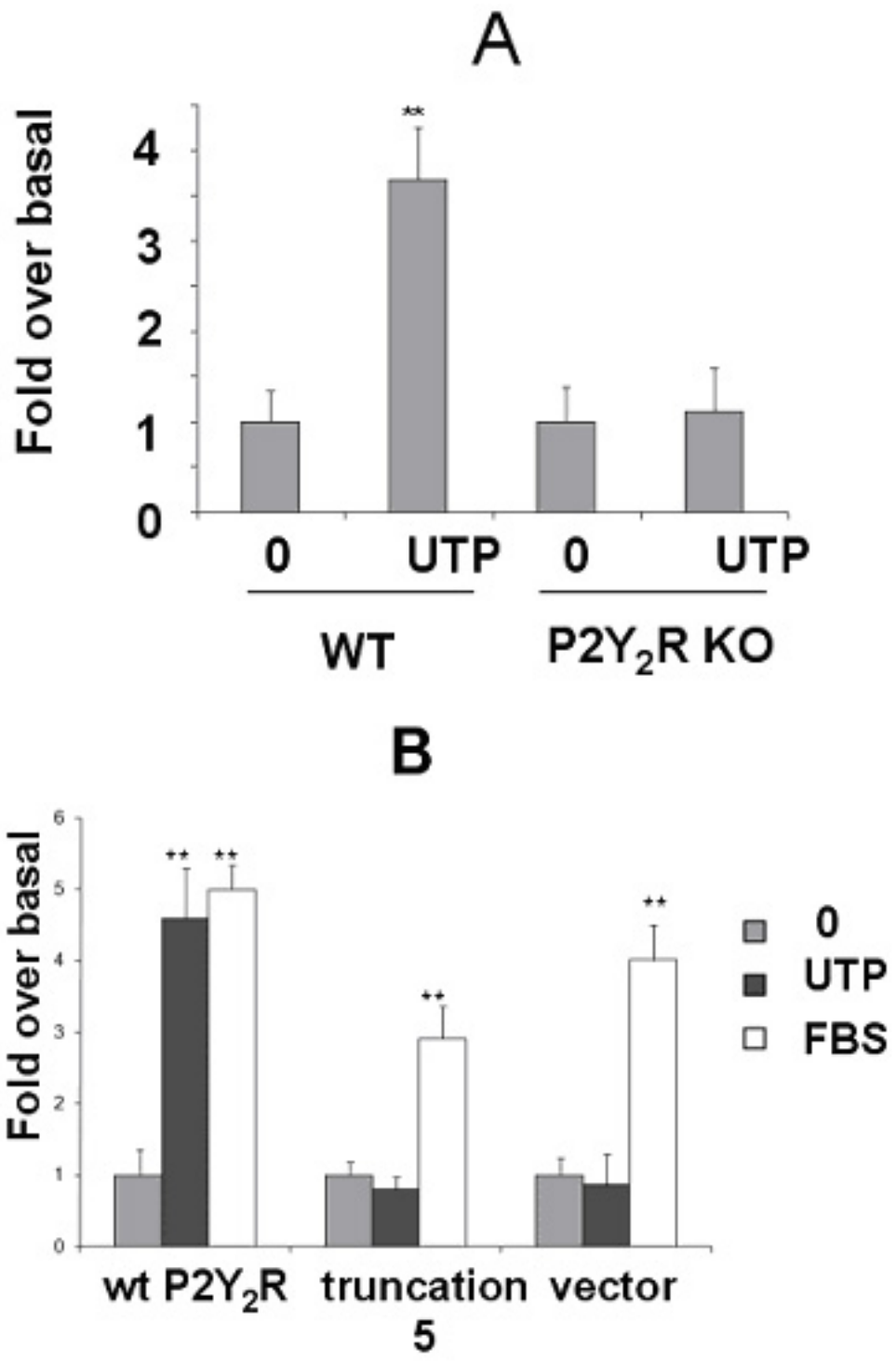
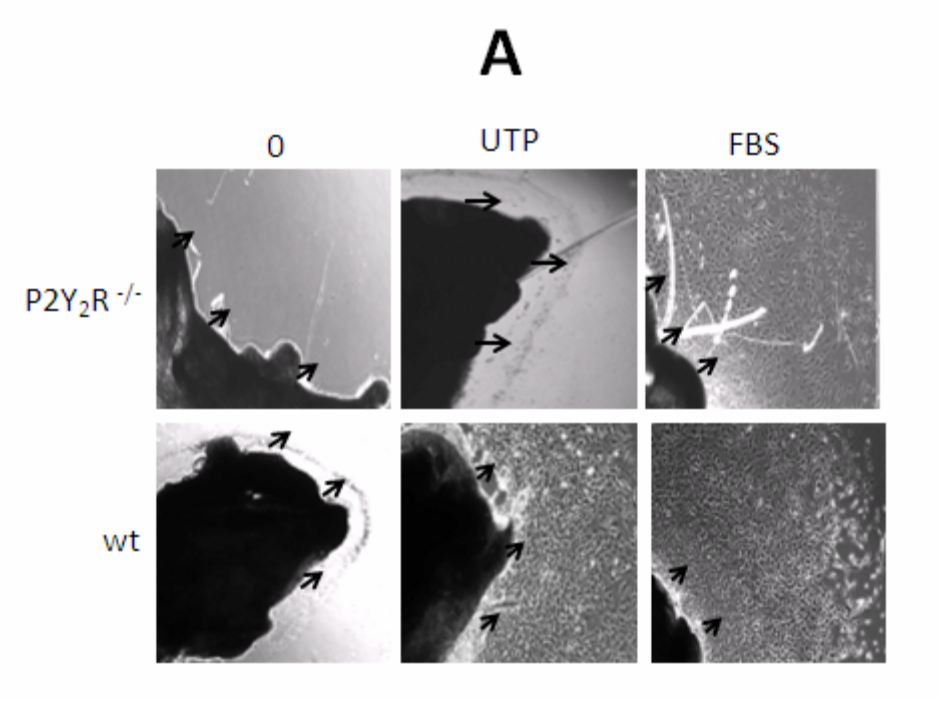
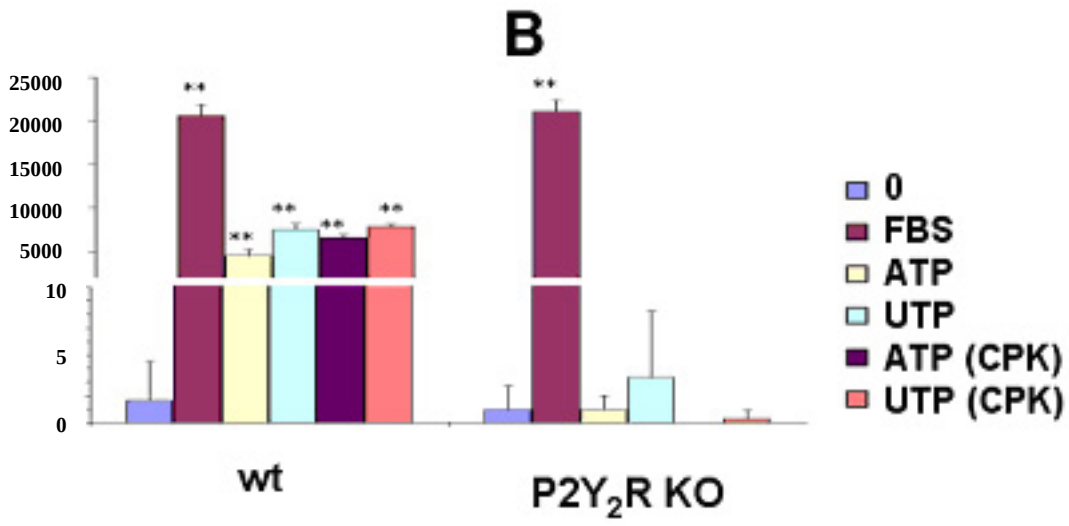

A
Fold over basal
4
3
2
1
0
**
0
UTP
0
UTP
WT
P2Y2R KO
B
Fold over basal
6
5
4
3
2
1
0
++
++
++
0
UTP
FBS
wt P2Y2R
truncation 5
vector

Figure IV-6. Migration of SMC out of mouse aortic explants.

Aortic explants were obtained from wild type (WT) and $P2Y_2$ receptor KO mice, as described in "Experimental Procedures", and cultured for 4 h in DMEM with or without ATP or UTP (10 μM) or 5% (v/v) FBS. Some explants also were cultured for 4 h with a nucleotide regenerating system (CPK), as described in "Experimental Procedures". Then, medium was replaced daily with DMEM alone and after 5 days, cells growing out of the aortic explants were visualized with an Olympus IX70 inverted microscope. *A,* Micrographs from a representative experiment are shown. Arrows indicate the direction of cell migration. Magnification: 20X. *B,* SMC migrating from aortic explants treated as above were removed after five days by incubation with a trypsin-EDTA solution. The number of cells migrating from explants was determined in three independent experiments performed in triplicate, as described in the "Experimental Procedures". Data with ATP, UTP, FBS and CPK are expressed as fold increase over untreated control and represent means ± S.E. of results from three independent experiments, where ** indicates $P<0.01$, as compared to untreated cells.


A
0
UTP
FBS
P2Y2R -/-
wt


B
25000
20000
15000
10000
5000
10
5
0
**
wt
P2Y2R KO
0
FBS
ATP
UTP
ATP (CPK)
UTP (CPK)

Chapter V

Ongoing Project: The $P2Y_2$ Nucleotide Receptor Regulates Tight Junction Complex Assembly and Endothelial Cell Permeability Properties

Introduction

Leukocyte recruitment and expression of pro-inflammatory cytokines occur early during atherogenesis. Decreases in inflammatory mediator levels can inhibit macrophage accumulation and formation of atheromatous lesions (Smith *et al*., 1995; Rajavashisth *et al*., 1998). The extravasation of leukocytes from the luminal to interstitial compartment is a hallmark of the inflammatory response and is associated with the pathogenesis of many cardiovascular diseases (Libby, 2002). Prior to their migration through the vessel wall, the circulating leukocytes start rolling along the vessel endothelium by interaction with low affinity selectins (*e. g.* E-, L- and P-selectin). Then, the leukocytes express integrins α_4 and β_2 that mediate their firm adhesion to the endothelium (Springer, 1994). Though there have been a lot of studies on leukocyte rolling and firm adhesion, the mechanism of leukocyte transendothelial migration is unclear.

The endothelium is a monolayer of endothelial cells that are connected to each other through intercellular junctions. Electron microscopy studies revealed that leukocyte

transmigration is mostly through the junctions between pairs of adjacent cells (*i.e.*, the paracellular pathway) (Marchesi, 1961; Hurley and Xeros, 1961). At least three types of endothelial junctions have been described: gap junctions, tight junctions (TJ), and adherens junctions (AJ) (Reviewed by Bazzoni and Dejana, 2001). These junctions are defined morphologically and contain specific transmembrane proteins. Although TJ are localized closest to the luminal surface among the three types of junctions and are strategically placed to govern leukocyte extravasation, the functions of TJ are not well understood.

Freeze fracture electron microscopy has shown that TJ are connected to actin filaments, suggesting that cytoskeleton is involved in regulation of tight junction structure and function (Madara, 1987). The transmembrane proteins occludin and claudins have been demonstrated to contribute to the paracellular seal in cultured cells and they localize to the discrete membrane contact sites in the freeze fracture micrograph. These proteins are in turn linked to the cytosolic protein ZO-1, which can target actin filaments to TJ (reviewed by Bazzoni and Dejana, 2003). It has been found that leukocyte permeability of TJ is affected by the organization of perijunctional actin. For example, activation of Rho family small GTPases, which regulate actin cytoskeletal reorganization, increases paracellular permeability of leukocytes (Breslin and Yuan, 2004). A schematic representation of the structures of TJ and AJ can be found in Fig. I-3.

Nucleotides are released at sites of atherosclerotic and restenotic lesions and can induce inflammatory responses (Pearson and Gordon, 1979; Grierson and Meldolesi, 1995). The effects of nucleotides are mediated by P2 receptors, which can be classified into P2X ligand-gated ion channels and P2Y G-protein coupled receptors. Seven P2X and

eight P2Y receptors have been cloned and their pharmacology and functions have been studied. Although multiple P2 receptors are present in vascular cells, it has been found that $P2Y_2$ receptor mRNA is specifically up-regulated in two vascular injury models, balloon angioplasty and perivascular placement of a silicone collar, suggesting the potential dominance of the $P2Y_2$ receptor, as compared to other P2 receptor subtypes, in atherosclerotic lesion formation (Seye *et al*., 1997; 2002). Activation of the $P2Y_2$ receptor causes intracellular calcium mobilization mediated by $G_{q/11}$ and phospholipase C and RhoA small GTPase activation, suggesting that these responses participate in increased leukocyte permeability of TJ (Lustig *et al*., 1992; Chapter III). Indeed, it was found that activation of the $P2Y_2$ receptor causes an increase of the number of CD68+ macrophages in the intima, indicating that $P2Y_2$ receptor activation facilitates leukocyte extravasation (Seye *et al*., 2002).

The present study focuses on the mechanism of $P2Y_2$ receptor regulation of reorganization and leukocyte permeability of TJ. Results show that the $P2Y_2$ receptor is physically associated with TJ upon activation through binding to ZO-1. Sustained activation of the $P2Y_2$ receptor also causes a decrease in protein levels of occludin and claudins.

Experimental Procedures

Materials

The anti-occludin, anti-claudin 1, anti-claudin 7, anti-ZO-1 and anti-ZO-2 antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-HA affinity matrix was from Roche Applied Science (Indianapolis, IN). Anti-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human microvascular endothelial cells (HMVEC) were purchased from Cambrex (East Rutherford, NJ).

Immunoblotting and Immunoprecipitation

HMVEC or 1321N1 cells transfected with HA-tagged $P2Y_2$ receptor were washed with ice-cold PBS and lysed in lysis buffer B containing 0.5% (v/v) Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 0.1% (w/v) protease inhibitor mixture. Protein concentrations were measured using a Lowry assay. Then, 500 mg of the protein lysate were and incubated with anti-HA affinity matrix overnight at 4 °C. The immunoprecipitates were collected by brief centrifugation and washed three times with modified RIPA buffer. Bound proteins were eluted by boiling in Laemmli buffer, resolved by SDS-PAGE, and immunoblotting was performed with the antibodies indicated in the figure legends.

Results

UTP Induces a Decrease in Tight Junction Protein Levels

Incubation of serum-starved HMVEC with 10 μM UTP caused a dose-dependent decrease in the protein levels of occludin, claudin 1, and claudin 7 (Fig. V-1). Occludin levels decrease within 1 h after UTP stimulation and continue to decline over 8 h.

UTP Induces Association Between the $P2Y_2$ Receptor and ZO-1/ZO-2 in 1321N1 Astrocytoma Cells

To determine whether the $P2Y_2$ receptor is physically linked to tight junction proteins, an HA-tagged $P2Y_2$ receptor was transfected into HMVEC. Serum-starved cells were stimulated with UTP for the indicated time, and immunoprecipitated by anti-HA affinity matrix. Co-precipitated ZO-1 and ZO-2 proteins were analyzed by immunoblotting. We detected an association between the $P2Y_2$ receptor and ZO-1/ZO-2 as early as 5 minutes after UTP stimulation (Fig. V-2).

Figure V-1. UTP stimulation causes a decrease in tight junction protein levels in HMVEC.

Serum-starved HMVEC were stimulated with or without 10 μM UTP for the indicated time or VEGF for 2 h at 37 ^{o}C. Cells were then lysed with Laemmli buffer and lysate was resolved by SDS-PAGE and analyzed by immunoblotting with anti-occludin (1:1000), anti-claudin 1 (1:250), anti-claudin 7 (1:500), and anti-actin (1:1000) antibodies. Results are representative of three independent experiments.

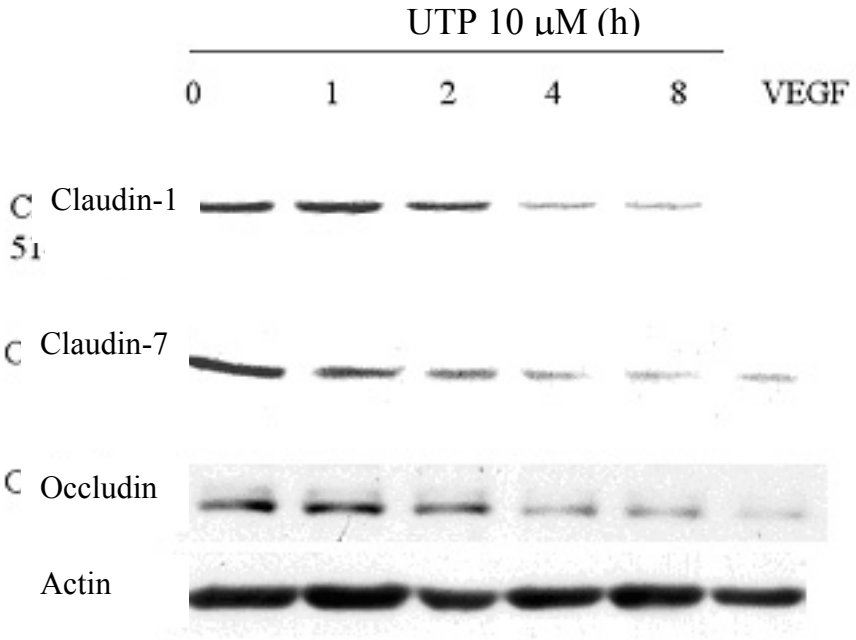
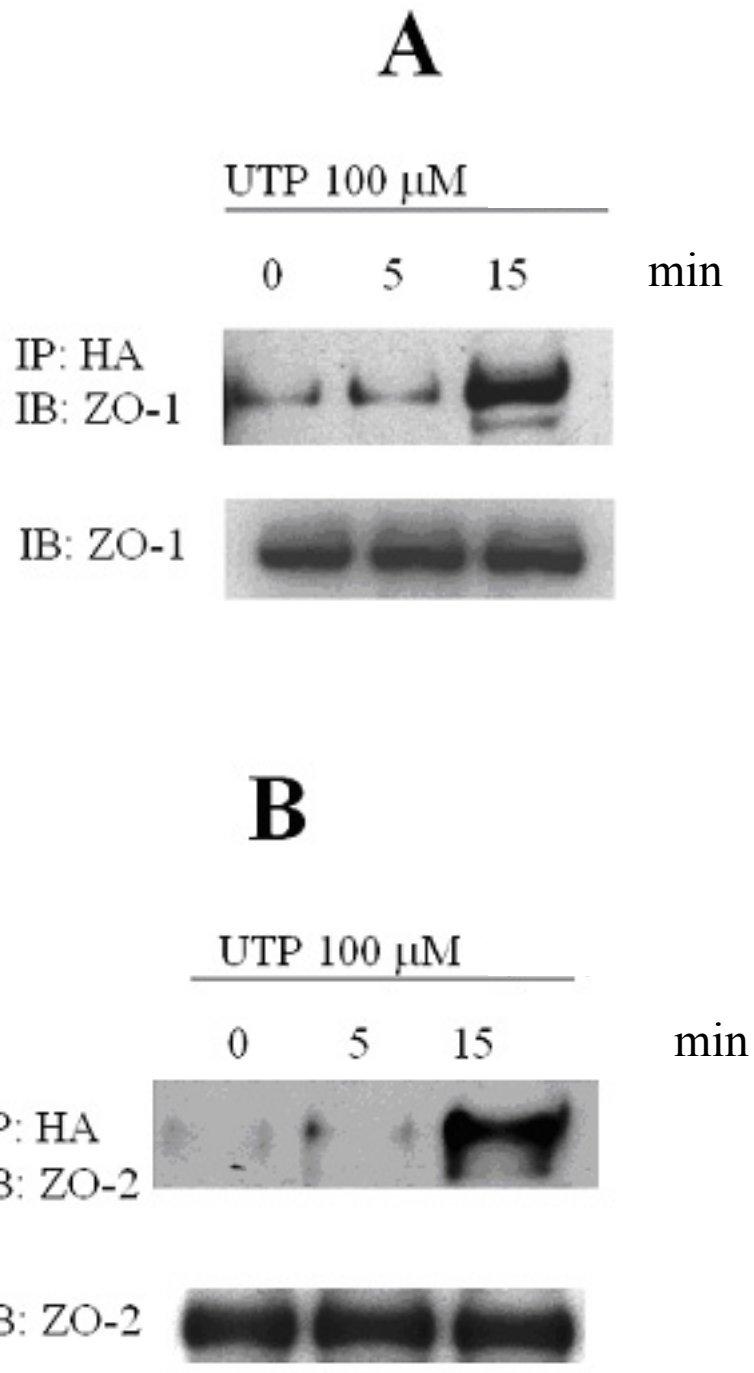

UTP 10 μM (h)
0
1
2
4
8
VEGF
Claudin-1
Claudin-7
Occludin
Actin

Figure V-2. The $P2Y_2$ receptor interacts with ZO-1/ZO-2 upon UTP stimulation.

1321N1 astrocytoma cells stably transfected with the HA-tagged $P2Y_2$ receptor were serum-starved and then stimulated with or without 100 μM UTP for the indicated time. Cell lysates were prepared in RIPA buffer, and subjected to immunoprecipitation with anti-HA affinity matrix. Co-immunoprecipitated ZO-1 (*A*) or ZO-2 (*B*) was analyzed by immunoblotting with anti-ZO-1 and anti-ZO-2 antibodies, respectively. Results shown are representative of three independent experiments.


A
UTP 100 μM
0
5
15
min
IP: HA
IB: ZO-1
IB: ZO-1
B
UTP 100 μM
0
5
15
min
IP: HA
IB: ZO-2
IB: ZO-2

Chapter VI

Summary and General Discussion

Normal blood vessels consist of three layers: intima, which includes the endothelium and underlying extracellular matrix, media which mostly contains SMC, and adventitia which has fibroblasts, collagen bundles, and proteoglycans. In this dissertation, the contribution of the $P2Y_2$ receptor to vascular inflammation has been elucidated from three aspects: endothelial adhesiveness to monocytes, migration of SMC, and endothelial dysfunction.

It was speculated that the $P2Y_2$ receptor was a potential regulator of atherosclerosis and postangioplasty restenosis because of the observed up-regulation of $P2Y_2$ receptors in vascular injury models (Seye *et al*., 1997; 2002). In the perivascular collar placement model, $P2Y_2$ receptor mRNA was significantly up-regulated after three days of collar placement. Activation of the $P2Y_2$ receptor *in situ* increased intimal hyperplasia and macrophage infiltration into the intima (Seye *et al*., 2002), indicating that the $P2Y_2$ receptor regulates mechanisms that lead to vascular smooth muscle cell proliferation/migration and leukocyte extravasation.

In HCAEC, UTP and ATP can induce the expression of VCAM-1, but not ICAM-1, with similar efficacy, indicating a role for $P2Y_2$ receptors (Figs. II-1, II-2, II-3). This response was dependent on increases in $[Ca^{2+}]_i$, and G_o (Figs. II-4, II-5). The role of the

$P2Y_2$ receptor in UTP-induced VCAM-1 expression was confirmed using $P2Y_2$ receptor anti-sense oligonucleotide to block $P2Y_2$ receptor expression in HCAEC (Fig. II-5B and C). In P2Y receptor deficient 1321N1 cells, UTP was unable to induce VCAM-1 expression, unless the cells were transfected with $P2Y_2$ receptor cDNA (Fig. II-6). Notably, VCAM-1, an endothelial cell adhesion protein, is known to bind to integrins on monocytes and T lymphocytes (Reviewed by Libby, 2002), raising the possibility that the $P2Y_2$ receptor induces leukocyte extravasation mediated by VCAM-1. Indeed, VCAM-1 expression in endothelial cells induced by ATP/UTP is required for monocyte binding *in vitro* (Fig. II-8). Transient UTP stimulation (≤1 min) caused the tyrosine phosphorylation of VEGFR-2 in both HCAEC and 1321N1 cells transfected with $P2Y_2$ receptor cDNA (Fig. III-1). The role of VEGFR-2 in VCAM-1 expression was clearly demonstrated with VEGFR-2 inhibitor and VEGFR-2 siRNA, which completely suppressed UTP-induced VCAM-1 expression (Fig. III-2, III-3). The $P2Y_2$ receptor and VEGFR-2 were found to physically associate upon UTP stimulation (Fig. III-4). Interestingly, the association between the $P2Y_2$ receptor and VEGFR-2 induced by UTP was transient (<10 min), although VEGFR-2 remained phosphorylated for at least 30 min. It was reported that proline rich SH3 binding sites in the C-terminal tail of $P2Y_2$ receptor mediate transactivation of the EGFR and PDGFR (Liu *et al*., 2004). Similarly, mutation or deletion of the SH3 binding sites suppressed UTP induced VEGFR-2 phosphorylation and VCAM-1 expression (Fig. III-5, III-6). Importantly, RhoA and Rac1, members of Rho small GTPases that regulate vascular cytoskeletal organization, were activated by UTP stimulation in HCAEC (Fig. III-8). However, VCAM-1 expression was inhibited by transfection with a dominant negative mutant of RhoA, but not Rac1. Our group has

identified an RGD sequence in the first extracellular loop of the $P2Y_2$ receptor that promotes its binding to $\alpha_v\beta_3/\beta_5$ integrins (Erb *et al*., 2001). Since $\alpha_v\beta_3/\beta_5$ integrins mediate monocyte adherence to endothelium/epithelium (Beekhuizen and van Furth, 1993), the RGD sequence in the $P2Y_2$ receptor may be involved in monocyte adherence to endothelium that occurs in vascular disease.

The $P2Y_2$ receptor can regulate cytoskeletal organization and migration of SMC. The long intracellular C-terminus of $P2Y_2$ receptor has multiple sites that are potentially active in protein binding and signal transduction. Screening for protein-protein interactions using the $P2Y_2$ receptor C-terminal tail identified an actin-binding protein, FLNa. $P2Y_2$ receptor and FLNa interaction was confirmed using co-immunoprecipitation and GST-fusion protein pull down assays. We mapped the FLNa-interaction site to amino acids 322-333 of the $P2Y_2$ receptor and generated a receptor mutant that was FLNa-binding deficient. We also used aortic SMC from $P2Y_2$ receptor knockout mice to directly evaluate whether loss of $P2Y_2$ receptor expression affects FLNa related responses activated by UTP. Transfection of aortic SMC from $P2Y_2$ receptor KO mice with wild type $P2Y_2$ receptor cDNA enabled UTP-induced tyrosine phosphorylation of FLNa, whereas UTP had no effect in cells transfected with the mutant $P2Y_2$ receptor defective in FLNa binidng. Phosphorylation of FLNa on serine residues by $P2Y_2$ receptor activation has also been observed (data not shown). In addition, UTP significantly stimulated the spreading (on collagen I) and migration of aortic SMC isolated from wild type but not $P2Y_2$ receptor KO mice. Expression of the wild type $P2Y_2$ receptor in SMC isolated from $P2Y_2$ receptor KO mice restored UTP-induced cell spreading and migration.

However, expression of the $P2Y_2$ receptor defective in FLNa-binding did not restore UTP-induced spreading and migration of SMC.

Vascular diseases such as atherosclerosis and restenosis after angioplasty are initiated by vascular injury and characterized by formation of atherosclerotic plaques and narrowed/blocked arteries. The pathogenesis of vascular disease has been attributed to dyslipidemia, such hypercholesterolemia, until about fifteen years ago, when vascular inflammation and the underlying molecular/cellular mechanisms have gained more interest (Libby, 2002).

Large quantities of nucleotides are released from damaged cells by stresses of a mechanical or chemical nature, including hypoxia/ischemia or shear stress, and from other sources such as aggregating platelets, and nerves around the arteries (Pearson and Gordon, 1979; Di Virgilio *et al.*, 2001). In addition, balloon angioplasty damages the vascular endothelium and causes nucleotide release into the vessel wall. Recent studies showed that certain pathological conditions and chronic hypoxic exposure may cause suppression of nucleotide degradation in endothelial cells (Robson *et al.*, 1997; Gerasimovskaya *et al.*, 2002), indicating that vascular injury contributes to the local elevation of nucleotide concentrations in the arterial wall.

The $P2Y_2$ receptor is up-regulated by tissue injury caused by ligation of the salivary gland duct, activation of thymocytes, and balloon angioplasty and perivascular collar placement in arteries (Koshiba *et al.*, 1997; Seye *et al.*, 1997; 2002; Turner *et al.*, 1998). In the collared rabbit carotid artery model, our group reported that activation of the $P2Y_2$ receptor by *in vivo* delivery of UTP caused monocyte/macrophage infiltration into the vessel wall and intimal hyperplasia, two important processes in the vascular

pathophysiology. This dissertation has partially delineated the signaling mechanisms of $P2Y_2$ receptor-induced macrophage infiltration (Chapter II and III) and intimal hyperplasia (Chapter IV).

The $P2Y_2$ receptor possesses an RGD domain in an extracellular domain, which can bind to the integrins $\alpha_v\beta_3/\beta_5$ (Erb *et al.*, 2001; Hynes, 1992). It has been reported that monocytes adhere to endothelium and epithelium via integrin $\alpha_v\beta_3$ (Beekhuizen and van Furth, 1993). Thus, monocytic integrin $\alpha_v\beta_3$ may also mediate monocyte binding to endothelium by interaction with the $P2Y_2R$ expressed in endothelial cells.

The SH3 binding motifs in the C-terminal tail of the $P2Y_2R$, which were previously reported to mediate transactivation of the EGFR and PDGFR in 1321N1 astrocytoma cells (Liu *et al.*, 2004), also mediate VEGFR-2 activation in endothelial cells. The ligand-independent activation of VEGFR-2 represents a mechanism of initiation of vascular inflammation in the presence of nucleotides released during vascular injury. Such cross-talk between the $P2Y_2R$ and growth factor receptors is relevant to drug design targeting the $P2Y_2R$ and/or VEGF/VEGFR-2 pathway to prevent vascular inflammation.

Vascular inflammation occurs in many diseases such as atherosclerosis, hypertension, diabetes and stroke. This research has shown that the $P2Y_2$ receptor plays a role in vascular inflammation during atherogenesis. Further delineation of the signaling pathways activated by the $P2Y_2$ receptor may help in the prevention and treatment of these diseases by limiting $P2Y_2$ receptor up-regulation or responses to $P2Y_2$ receptor activation.

References:

Abe K, Rossman KL, Liu B, Ritola KD, Chiang D, Campbell SL, Burridge K, Der CJ. Vav2 is an activator of Cdc42, Rac1, and RhoA. *J Biol Chem*. 2000;275:10141-9.

Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. *J Cell Biochem.* 1996;61:514-23.

Allen S, Khan S, Al-Mohanna F, Batten P, Yacoub M. Native low density lipoprotein-induced calcium transients trigger VCAM-1 and E-selectin expression in cultured human vascular endothelial cells. *J Clin Invest*. 1998;101:1064-75.

Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science*. 2003;300:1430-4.

Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol.* 1995;269:G467-75.

Andersen HR, Maeng M, Thorwest M, Falk E. Remodeling rather than neointimal formation explains luminal narrowing after deep vessel wall injury: insights from a porcine coronary (re)stenosis model. *Circulation*. 1996;93:1716-24.

Assoian RK, Marcantonio EE. The extracellular matrix as a cell cycle control element in atherosclerosis and restenosis. *J Clin Invest*. 1996;98:2436-9.

Ayyanathan K, Webbs TE, Sandhu AK, Athwal RS, Barnard EA, Kunapuli SP. Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. *Biochem Biophys Res Commun.* 1996;218:783–788

Bagchi S, Liao Z, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, Erb L. The $P2Y_2$ nucleotide receptor interacts with alphav integrins to activate Go and induce cell migration. *J Biol Chem*. 2005;280:39050-7.

Balda MS, Gonzalez-Mariscal L, Matter K, Cereijido M, Anderson JM. Assembly of the tight junction: the role of diacylglycerol. *J Cell Biol*. 1993;123:293-302.

Balda MS, Anderson JM, Matter K. The SH3 domain of the tight junction protein ZO-1 binds to a serine protein kinase that phosphorylates a region C-terminal to this domain. *FEBS Lett*. 1996a;399:326-32.

Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and

disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol*. 1996b;134:1031-49.

Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. *Embo J*. 2000;19:2024-33.

Baricordi OR, Ferrari D, Melchiorri L, Chiozzi P, Hanau S, Chiari E, Rubini M, Di Virgilio F. An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. *Blood*. 1996;87:682-90.

Bazzoni G, Dejana E, Lampugnani MG. Endothelial adhesion molecules in the development of the vascular tree: the garden of forking paths. *Curr Opin Cell Biol*. 1999;11:573-81.

Bazzoni G, Martinez-Estrada OM, Orsenigo F, Cordenonsi M, Citi S, Dejana E. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *J Biol Chem*. 2000a;275:20520-6.

Bazzoni G, Martinez-Estrada OM, Mueller F, Nelboeck P, Schmid G, Bartfai T, Dejana E, Brockhaus M. Homophilic interaction of junctional adhesion molecule. *J Biol Chem*. 2000b;275:30970-6.

Bazzoni G, Dejana E. Pores in the sieve and channels in the wall: control of paracellular permeability by junctional proteins in endothelial cells. *Microcirculation*. 2001;8:143-52.

Bazzoni G. The JAM family of junctional adhesion molecules. *Curr Opin Cell Biol*. 2003;15:525-30.

Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev.* 2004 Jul;84(3):869-901.

Beatch M, Jesaitis LA, Gallin WJ, Goodenough DA, Stevenson BR. The tight junction protein ZO-2 contains three PDZ (PSD-95/Discs-Large/ZO-1) domains and an alternatively spliced region. *J Biol Chem*. 1996;271:25723-6.

Beekhuizen H, van Furth R. Monocyte adherence to human vascular endothelium. *J Leukoc Biol*. 1993;54:363-78.

Behrens J, Birchmeier W, Goodman SL, Imhof BA. Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-arc-1: mechanistic aspects and identification of the antigen as a component related to uvomorulin. *J Cell Biol*. 1985;101:1307-15.

Bergfeld GR, Forrester T. Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovasc Res*. 1992;26:40-7.

Boarder MR, Hourani SM. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. *Trends Pharmacol Sci*. 1998;19:99-107.

Bochaton-Piallat ML, Ropraz P, Gabbiani F, Gabbiani G. Phenotypic heterogeneity of rat arterial smooth muscle cell clones. Implications for the development of experimental intimal thickening. *Arterioscler Thromb Vasc Biol.* 1996;16:815-20.

Bodin P, Burnstock G. Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells. *Experientia*. 1995;51:256-9.

Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998;394:894-7.

Boyer JL, Downes CP, Harden TK. Kinetics of activation of phospholipase C by P2Y purinergic receptor agonists and guanine nucleotides. *J Biol Chem*. 1989;264:884-90.

Braun M, Pietsch P, Felix SB, Baumann G. Modulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on human coronary smooth muscle cells by cytokines. *J Mol Cell Cardiol*. 1995;27:2571-9.

Breier G, Breviario F, Caveda L, Berthier R, Schnürch H, Gotsch U, Vestweber D, Risau W, Dejana E. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood.* 1996;87:630-41.

Breslin JW, Yuan SY. Involvement of RhoA and Rho kinase in neutrophil-stimulated endothelial hyperpermeability. *Am J Physiol Heart Circ Physiol*. 2004;286:H1057-62.

Burnstock G, Totowa, NJ, Human Press. In: The P2 Nucleotide Receptors, edited by J. T. Turner, G. A. Weisman, and J. S. Fedan. 1998;3-42.

Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell*. 2004;116:167-79.

Calderwood DA, Shattil SJ, Ginsberg MH. Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. *J Biol Chem*. 2000;275:22607-10.

Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A*. 2001;98:9742-7.

Cario-Toumaniantz C, Loirand G, Ladoux A, Pacaud P. $P2X_7$ receptor activation-induced contraction and lysis in human saphenous vein smooth muscle. *Circ Res*. 1998;83:196-203.

Carpenter RC, Miao L, Miyagi Y, Bengten E, Zhang JH. Altered expression of P2 receptor mRNAs in the basilar artery in a rat double hemorrhage model. *Stroke*. 2001;32:516-22.

Cerione RA, Zheng Y. The Dbl family of oncogenes. *Curr Opin Cell Biol*. 1996;8:216-22.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, Livi GP. A G protein-coupled receptor for UDP-glucose. *J Biol Chem.* 275:10767–10771

Chang K, Hanaoka K, Kumada M, Takuwa Y. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J Biol Chem*. 1995;270:26152-8.

Chaulet H, Desgranges C, Renault MA, Dupuch F, Ezan G, Peiretti F, Loirand G, Pacaud P, Gadeau AP. Extracellular nucleotides induce arterial smooth muscle cell migration via osteopontin. *Circ Res*. 2001;89:772-8.

Clowes AW, Clowes MM, Reidy MA. Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels. *Lab Invest*. 1986;54:295-303.

Cockcroft S, Stutchfield J. ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. *FEBS Lett*. 1989;245:25-9.

Communi D, Pirotton S, Parmentier M, Boeynaems JM. Cloning and functional expression of a human uridine nucleotide receptor. *J Biol Chem.* 1995;270:30849-30852

Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. *Biochem Biophys Res Commun.* 1996;222:303–308

Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J Biol Chem.* 1997; 272:31969–31973

Communi D, Janssens R, Suarez-Huerta N, Robaye B, Boeynaems JM. Advances in signalling by extracellular nucleotides. the role and transduction mechanisms of P2Y receptors. *Cell Signal*. 2000;12:351-60.

Communi D, Gonzalez NS, Detheux M, Brezillon S, Lannoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to G_i. *J Biol Chem*. 2001;276:41479-85.

Cooper DM, Rodbell M. ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. *Nature*. 1979;282:517-8.

Cotran RS, Kumar V, Collins T, Robbins SL. Robbins Pathologic Basis of Disease W.B. Saunders Company, 072167335X

Cowen DS, Lazarus HM, Shurin SB, Stoll SE, Dubyak GR. Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. *J Clin Invest*. 1989;83:1651-60.

Cunningham CC, Gorlin JB, Kwiatkowski DJ, Hartwig JH, Janmey PA, Byers HR, Stossel TP. Actin-binding protein requirement for cortical stability and efficient locomotion. *Science*. 1992;255:325-7.

Cunningham CC. Actin polymerization and intracellular solvent flow in cell surface blebbing. *J Cell Biol*. 1995;129:1589-99.

Cybulsky MI, Gimbrone MA Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science.* 1991;251:788-91.

Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin Invest*. 2001;107:1255-62.

de Boer OJ, van der Wal AC, Teeling P, Becker AE. Leucocyte recruitment in rupture prone regions of lipid-rich plaques: a prominent role for neovascularization? *Cardiovasc Res.* 1999;41:443-9.

De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Jr., Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest*. 1995;96:60-8.

De Meyer GR, Van Put DJ, Kockx MM, Van Schil P, Bosmans R, Bult H, Buyssens N, Vanmaele R, Herman AG. Possible mechanisms of collar-induced intimal thickening. *Arterioscler Thromb Vasc Biol*. 1997;17:1924-30.

de Villiers WJ, Fraser IP, Hughes DA, Doyle AG, Gordon S. Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function. *J Exp Med.* 1994;180:705-9.

Dejana E, Corada M, Lampugnani MG. Endothelial cell-to-cell junctions. *FASEB J.* 1995;9:910-8.

Dejana E, Bazzoni G, Lampugnani MG. Vascular endothelial (VE)-cadherin: only an intercellular glue? *Exp Cell Res*. 1999;252:13-9.

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood*. 2001;97:587-600.

Di Virgilio F, Solini A. P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol*. 2002;135:831-42.

Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest.* 1998;102:145-52.

Dubyak GR, el-Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol*. 1993;265:C577-606.

Dufourcq P, Louis H, Moreau C, Daret D, Boisseau MR, Lamaziere JM, Bonnet J. Vitronectin expression and interaction with receptors in smooth muscle cells from human atheromatous plaque. *Arterioscler Thromb Vasc Biol*. 1998;18:168-76.

Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell.* 1990;60:577-84.

Ellsworth ML, Forrester T, Ellis CG, Dietrich HH. The erythrocyte as a regulator of vascular tone. *Am J Physiol*. 1995;269:H2155-61.

Elmaleh DR, Narula J, Babich JW, Petrov A, Fischman AJ, Khaw BA, Rapaport E, Zamecnik PC. Rapid noninvasive detection of experimental atherosclerotic lesions with novel 99mTc-labeled diadenosine tetraphosphates. *Proc Natl Acad Sci U S A*. 1998;95:691-5.

Erb L, Garrad R, Wang Y, Quinn T, Turner JT, Weisman GA. Site-directed mutagenesis of P2U purinoceptors. Positively charged amino acids in transmembrane helices 6 and 7 affect agonist potency and specificity. *J Biol Chem*. 1995;270:4185-8.

Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Perez LI, Gonzalez FA, Gresham HD, Turner JT, Weisman GA. An RGD sequence in the $P2Y_2$ receptor interacts with $\alpha_v\beta_3$ integrins and is required for G_o-mediated signal transduction. *J Cell Biol*. 2001;153:491-501.

Erlinge D, Yoo H, Edvinsson L, Reis DJ, Wahlestedt C. Mitogenic effects of ATP on vascular smooth muscle cells vs. other growth factors and sympathetic cotransmitters. *Am J Physiol*. 1993;265:H1089-97.

Erlinge D, You J, Wahlestedt C, Edvinsson L. Characterisation of an ATP receptor mediating mitogenesis in vascular smooth muscle cells. *Eur J Pharmacol*. 1995;289:135-49.

Erlinge D, Hou M, Webb TE, Barnard EA, Moller S. Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by quantitative RT-PCR. *Biochem Biophys Res Commun*. 1998a;248:864-70.

Erlinge D. Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen Pharmacol*. 1998b;31:1-8.

Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. The Tight Junction Protein ZO-1 Establishes a Link between the Transmembrane Protein Occludin and the Actin Cytoskeleton. *J Biol Chem.* 1998; 273:29745-53.

Faruqi RM, DiCorleto PE. Mechanisms of monocyte recruitment and accumulation. *Br Heart J*. 1993;69:S19-29.

Fox JW, Lamperti ED, Eksioglu YZ, Hong SE, Feng Y, Graham DA, Scheffer IE, Dobyns WB, Hirsch BA, Radtke RA, Berkovic SF, Huttenlocher PR, Walsh CA. Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. *Neuron*. 1998;21:1315-25.

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol.* 1993;123(6 Pt 2):1777-88.

Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and –2: Novel Integral Membrane Proteins Localizing at Tight Junctions with No Sequence Similarity to Occludin. *J Cell Biol.* 1998;141:1539-50

Gabbiani G, Kocher O, Bloom WS, Vandekerckhove J, Weber K. Actin expression in smooth muscle cells of rat aortic intimal thickening, human atheromatous plaque, and cultured rat aortic media. *J Clin Invest*. 1984;73:148-52.

Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest.* 1994;94:2493-503.

Garcia-Guzman M, Stühmer W, Soto F. Molecular characterization and pharmacological properties of the human $P2X_3$ purinoceptor. *Brain Res Mol Brain Res.* 1997a;47(1-2):59-66.

Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund PE, Stühmer W. Characterization of recombinant human $P2X_4$ receptor reveals pharmacological differences to the rat homologue. *Mol Pharmacol.* 1997b;51(1):109-18.

Garrad RC, Otero MA, Erb L, Theiss PM, Clarke LL, Gonzalez FA, Turner JT, Weisman GA. Structural basis of agonist-induced desensitization and sequestration of the $P2Y_2$ nucleotide receptor. Consequences of truncation of the C terminus. *J Biol Chem*. 1998;273:29437-44.

Geisenheimer J, Han SS. A quantitative electron microscopic study of desmosomes and hemidesmosomes in human crevicular epithelium. *J Periodontol.* 1971;42(7):396-405.

Gerasimovskaya EV, Ahmad S, White CW, Jones PL, Carpenter TC, Stenmark KR. Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth. Signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor. *J Biol Chem*. 2002;277:44638-50.

Gibbons GH, Dzau VJ. The emerging concept of vascular remodeling. *N Engl J Med*. 1994;330:1431-8.

Gordon JL. Extracellular ATP: effects, sources and fate. *Biochem J*. 1986;233:309-19.

Gorlin JB, Yamin R, Egan S, Stewart M, Stossel TP, Kwiatkowski DJ, Hartwig JH. Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J Cell Biol.* 1990;111:1089-105.

Grierson JP, Meldolesi J. Shear stress-induced $[Ca^{2+}]_i$ transients and oscillations in mouse fibroblasts are mediated by endogenously released ATP. *J Biol Chem*. 1995;270:4451-6.

Gschwind A, Zwick E, Prenzel N, Leserer M, Ullrich A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene*. 2001;20:1594-600.

Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998;2:275-81.

Gumbiner B, Lowenkopf T, Apatira D. Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc Natl Acad Sci U S A.* 1991;88:3460-4.

Gumbiner BM. Signal transduction of beta-catenin. Curr Opin Cell Biol. 1995;7:634-40.

Guns PJ, Van Assche T, Fransen P, Robaye B, Boeynaems JM, Bult H. Endothelium-dependent relaxation evoked by ATP and UTP in the aorta of $P2Y_2$-deficient mice. *Br J Pharmacol*. 2006;147:569-74.

Hall A. Rho GTPases and the actin cytoskeleton. *Science*. 1998;279:509-14.

Hamada K, Takuwa N, Yokoyama K, Takuwa Y. Stretch activates Jun N-terminal kinase/stress-activated protein kinase in vascular smooth muscle cells through mechanisms involving autocrine ATP stimulation of purinoceptors. *J Biol Chem*. 1998;273:6334-40.

Hartley SA, Kato K, Salter KJ, Kozlowski RZ. Functional evidence for a novel suramin-insensitive pyrimidine receptor in rat small pulmonary arteries. *Circ Res*. 1998;83:940-6.

Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J Cell Biol.* 1998;141:199-208.

Haudenschild CC, Schwartz SM. Endothelial regeneration. II. Restitution of endothelial continuity. *Lab Invest*. 1979;41:407-18.

Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schönbeck U. Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. *Circulation.* 2001;104:1899-904.

Hill BJ, Wamhoff BR, Sturek M. Functional nucleotide receptor expression and sarcoplasmic reticulum morphology in dedifferentiated porcine coronary smooth muscle cells. *J Vasc Res*. 2001;38:432-43.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature*. 2001;409:202-7.

Howarth AG, Hughes MR, Stevenson BR. Detection of the tight junction-associated protein ZO-1 in astrocytes and other nonepithelial cell types. *J Physiol.* 1992;Am262; C461-9.

Howarth AG, Stevenson BR. Molecular environment of ZO-1 in epithelial and non-epithelial cells. *Cell Motil Cytoskel.* 1995;31(4):323-32.

Hurley JV, Xeros N. Electron microscopic observations on the emigration of leucocytes. *Aust J Exp Biol Med Sci*. 1961;39:609-23.

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*. 1992;69:11-25.

Iademarco MF, Barks JL, Dean DC. Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF-alpha in cultured endothelial cells. *J Clin Invest*. 1995;95:264-71.

Ikeda M, Kohno M, Yasunari K, Yokokawa K, Horio T, Ueda M, Morisaki N, Yoshikawa J. Natriuretic peptide family as a novel antimigration factor of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1997;17(4):731-6.

Jesaitis LA, Goodenough DA. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein. *J Cell Biol.* 1994;124:949-61.

Jiang WG, Martin TA, Matsumoto K, Nakamura T, Mansel RE. Hepatocyte growth factor/scatter factor decreases the expression of occludin and transendothelial resistance (TER) and increases paracellular permeability in human vascular endothelial cells. *J Cell Physiol.* 1999;181(2):319-29.

Johnson-Leger C, Aurrand-Lions M, Imhof BA. The parting of the endothelium: miracle, or simply a junctional affair? *J Cell Sci*. 2000;113 (Pt 6):921-33.

Jou TS, Schneeberger EE, Nelson WJ. Structural and Functional Regulation of Tight Junctions by RhoA and Rac1 Small GTPases. *J Cell Biol.* 1998;142:101-15.

Katoh Y, Periasamy M. Growth and differentiation of smooth muscle cells during vascular development. *Trends in Cardiovascular Medicine*. 1996:100-106.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276:7614-20.

Kocher O, Gabbiani F, Gabbiani G, Reidy MA, Cokay MS, Peters H, Huttner I. Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening. Biochemical and morphologic studies. *Lab Invest*. 1991;65:459-70.

Koshiba M, Apasov S, Sverdlov V, Chen P, Erb L, Turner JT, Weisman GA, Sitkovsky MV. Transient up-regulation of $P2Y_2$ nucleotide receptor mRNA expression is

an immediate early gene response in activated thymocytes. *Proc Natl Acad Sci U S A*. 1997;94:831-6.

Kozma R, Ahmed S, Best A, Lim L. The GTPase-activating protein n-chimaerin cooperates with Rac1 and Cdc42Hs to induce the formation of lamellipodia and filopodia. *Mol Cell Biol*. 1996;16:5069-80.

Kuhns DB, Wright DG, Nath J, Kaplan SS, Basford RE. ATP induces transient elevations of $[Ca^{2+}]_i$ in human neutrophils and primes these cells for enhanced O2-generation. *Lab Invest*. 1988;58:448-53.

Kumari R, Goh G, Ng LL, Boarder MR. ATP and UTP responses of cultured rat aortic smooth muscle cells revisited: dominance of $P2Y_2$ receptors. *Br J Pharmacol*. 2003;140:1169-76.

Lafont A, Guzman LA, Whitlow PL, Goormastic M, Cornhill JF, Chisolm GM. Restenosis after experimental angioplasty. Intimal, medial, and adventitial changes associated with constrictive remodeling. *Circ Res*. 1995;76:996-1002.

Lampugnani MG, Caveda L, Breviario F, Del Maschio A, Dejana E. Endothelial cell-to-cell junctions. Structural characteristics and functional role in the regulation of vascular permeability and leukocyte extravasation. *Baillieres Clin Haematol.* 1993;6(3):539-58.

Lampugnani MG, Dejana E. Interendothelial junctions: structure, signalling and functional roles. *Curr Opin Cell Biol*. 1997;9:674-82.

Lazarowski ER, Homolya L, Boucher RC, Harden TK. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem*. 1997;272:24348-54.

Le KT, Paquet M, Nouel D, Babinski K, Seguela P. Primary structure and expression of a naturally truncated human P2X ATP receptor subunit from brain and immune system. *FEBS Lett*. 1997;418:195–199

Leon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C. The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett*. 1997;403:26-30.

Li H, Cybulsky MI, Gimbrone MA Jr, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler Thromb.* 1993;13:197-204.

Li MG, Serr M, Edwards K, Ludmann S, Yamamoto D, Tilney LG, Field CM, Hays TS. Filamin is required for ring canal assembly and actin organization during Drosophila oogenesis. *J Cell Biol*. 1999;146:1061-74.

Liao Z, Seye CI, Weisman GA, Erb L. The $P2Y_2$ nucleotide receptor requires interaction with alpha v integrins to access and activate G_{12}. *J Cell Sci*. 2007;120:1654-62.

Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest*. 1991;64:5-15.

Libby P, Sukhova G, Lee RT, Galis ZS. Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J Cardiovasc Pharmacol*. 1995;25 Suppl 2:S9-12.

Libby P. Multiple mechanisms of thrombosis complicating atherosclerotic plaques. *Clin Cardiol*. 2000;23 Suppl 6:VI-3-7.

Libby P, Simon DI. Inflammation and thrombosis: the clot thickens. *Circulation*. 2001;103:1718-20.

Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868-74.

Liu BP, Burridge K. Vav2 activates Rac1, Cdc42, and RhoA downstream from growth factor receptors but not beta1 integrins. *Mol Cell Biol*. 2000;20:7160-9.

Liu J, Liao Z, Camden J, Griffin KD, Garrad RC, Santiago-Perez LI, Gonzalez FA, Seye CI, Weisman GA, Erb L. Src homology 3 binding sites in the $P2Y_2$ nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. *J Biol Chem*. 2004;279:8212-8.

Lustig KD, Erb L, Landis DM, Hicks-Taylor CS, Zhang X, Sportiello MG, Weisman GA. Mechanisms by which extracellular ATP and UTP stimulate the release of prostacyclin from bovine pulmonary artery endothelial cells. *Biochim Biophys Acta*. 1992;1134:61-72.

Lynch KJ, Touma E, Niforatos W, Kage KL, Burgard EC, van Biesen T, Kowaluk EA, Jarvis MF. Molecular and functional characterization of human $P2X_2$ receptors. *Mol Pharmacol.* 1999;56(6):1171-81.

Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, Luster AD. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest*. 1999;104:1041-50.

Madara JL. Intestinal absorptive cell tight junctions are linked to cytoskeleton. *Am J Physiol*. 1987;253:C171-5.

Madara JL, Parkos C, Colgan S, Nusrat A, Atisook K, Kaoutzani K, Kaoutzani P. The movement of solutes and cells across tight junctions. *Ann N Y Acad Sci.* 1992;664:47-60.

Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM. PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol*. 1990;111:2149-58.

Malam-Souley R, Campan M, Gadeau AP, Desgranges C. Exogenous ATP induces a limited cell cycle progression of arterial smooth muscle cells. *Am J Physiol*. 1993;264:C783-8.

Malam-Souley R, Seye C, Gadeau AP, Loirand G, Pillois X, Campan M, Pacaud P, Desgranges C. Nucleotide receptor P2u partially mediates ATP-induced cell cycle progression of aortic smooth muscle cells. *J Cell Physiol*. 1995;166:57-65.

Malmsjo M, Adner M, Harden TK, Pendergast W, Edvinsson L, Erlinge D. The stable pyrimidines UDPβS and UTPγS discriminate between the P2 receptors that mediate vascular contraction and relaxation of the rat mesenteric artery. *Br J Pharmacol*. 2000;131:51-6.

Malmsjo M, Hou M, Pendergast W, Erlinge D, Edvinsson L. Potent $P2Y_6$ receptor mediated contractions in human cerebral arteries. *BMC Pharmacol*. 2003a;3:4.

Malmsjo M, Hou M, Pendergast W, Erlinge D, Edvinsson L. The stable pyrimidines UDPβS and UTPγS discriminate between contractile cerebrovascular P2 receptors. *Eur J Pharmacol*. 2003b;458:305-11.

Marchesi VT. The site of leucocyte emigration during inflammation. *Q J Exp Physiol Cogn Med Sci*. 1961;46:115-8.

Martìn-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol.* 1998;142:117-27.

McClellan G, Weisberg A, Rose D, Winegrad S. Endothelial cell storage and release of endothelin as a cardioregulatory mechanism. *Circ Res.* 1994;75:85-96.

Meredith JE, Jr., Schwartz MA. Integrins, adhesion and apoptosis. *Trends Cell Biol*. 1997;7:146-50.

Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol*. 2003;24:327-34.

Nagano T, Yoneda T, Hatanaka Y, Kubota C, Murakami F, Sato M. Filamin A-interacting protein (FILIP) regulates cortical cell migration out of the ventricular zone. *Nat Cell Biol*. 2002;4:495-501.

Narumiya S, Ishizaki T, Watanabe N. Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett*. 1997;410:68-72.

Nawroth PP, Stern DM, Dietrich M. Thromboxane production by perturbed bovine aortic endothelial cells in culture. *Blut.* 1985;50:7-11.

Nguyen T, Erb L, Weisman GA, Marchese A, Heng HH, Garrad RC, George SR, Turner JT, O'Dowd BF. Cloning, expression, and chromosomal localization of the human uridine nucleotide receptor gene. *J Biol Chem.* 1995;270:30845–30848

Nicholas RA, Watt WC, Lazarowski ER, Li Q, Harden K. Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol*. 1996;50:224-9.

North RA, Surprenant A. Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol*. 2000;40:563-80.

Ohta Y, Hartwig JH. Phosphorylation of actin-binding protein 280 by growth factors is mediated by p90 ribosomal protein S6 kinase. *J Biol Chem*. 1996;271:11858-64.

Ohta Y, Suzuki N, Nakamura S, Hartwig JH, Stossel TP. The small GTPase RalA targets filamin to induce filopodia. *Proc Natl Acad Sci U S A*. 1999;96:2122-8.

Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995;75:487-517.

Pal Sharma C, Goldmann WH. Phosphorylation of actin-binding protein (ABP-280; filamin) by tyrosine kinase p56lck modulates actin filament cross-linking. *Cell Biol Int*. 2004;28:935-41.

Parker JC, Snow RL. Influence of external ATP on permeability and metabolism of dog red blood cells. *Am J Physiol*. 1972;223:888-93.

Parker AL, Likar LL, Dawicki DD, Rounds S. Mechanism of ATP-induced leukocyte adherence to cultured pulmonary artery endothelial cells. *Am J Physiol*. 1996;270:L695-703.

Parr CE, Sullivan DM, Paradiso AM, Lazarowski ER, Burch LH, Olsen JC, Erb L, Weisman GA, Boucher RC, Turner JT. Cloning and expression of a human $P2_U$ nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc Natl Acad Sci U S A*. 1994;91:3275-9.

Pearson JD, Gordon JL. Vascular endothelial and smooth muscle cells in culture selectively release adenine nucleotides. *Nature*. 1979;281:384-6.

Perez-Moreno M, Fuchs E. Catenins: keeping cells from getting their signals crossed. *Dev Cell.* 2006;11:601-12.

Pierce KL, Luttrell LM, Lefkowitz RJ. New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene*. 2001;20:1532-9.

Pillois X, Chaulet H, Belloc I, Dupuch F, Desgranges C, Gadeau AP. Nucleotide receptors involved in UTP-induced rat arterial smooth muscle cell migration. *Circ Res*. 2002;90:678-81.

Poole JC, Florey HW. Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits. *J Pathol Bacteriol.* 1958;75:245-51.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 1999;402:884-8.

Pulvirenti TJ, Yin JL, Chaufour X, McLachlan C, Hambly BD, Bennett MR, Barden JA. P2X (purinergic) receptor redistribution in rabbit aorta following injury to endothelial cells and cholesterol feeding. *J Neurocytol*. 2000;29:623-31.

Radisavljevic Z, Avraham H, Avraham S. Vascular endothelial growth factor up-regulates ICAM-1 expression via the phosphatidylinositol 3 OH-kinase/AKT/Nitric oxide pathway and modulates migration of brain microvascular endothelial cells. *J Biol Chem*. 2000;275:20770-4.

Rajavashisth T, Qiao JH, Tripathi S, Tripathi J, Mishra N, Hua M, Wang XP, Loussararian A, Clinton S, Libby P, Lusis A. Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor- deficient mice. *J Clin Invest*. 1998;101:2702-10.

Ralevic V, Burnstock G. Roles of P2-purinoceptors in the cardiovascular system. *Circulation*. 1991;84:1-14.

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev*. 1998;50:413-92.

Rassendren F, Buell GN, Virginio C, Collo G, North RA, Surprenant A. The permeabilizing ATP receptor, $P2X_7$.Cloning and expression of a human cDNA. *J Biol Chem.* 1997;272:5482–5486

Renault MA, Jalvy S, Belloc I, Pasquet S, Sena S, Olive M, Desgranges C, Gadeau AP. AP-1 is involved in UTP-induced osteopontin expression in arterial smooth muscle cells. *Circ Res*. 2003;93:674-81.

Renault MA, Jalvy S, Potier M, Belloc I, Genot E, Dekker LV, Desgranges C, Gadeau AP. UTP induces osteopontin expression through a coordinate action of NFkappaB, activator protein-1, and upstream stimulatory factor in arterial smooth muscle cells. *J Biol Chem*. 2005;280:2708-13.

Rice GE, Munro JM, Bevilacqua MP. Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *J Exp Med*. 1990;171:1369-74.

Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 1992;70:389-99.

Robson SC, Kaczmarek E, Siegel JB, Candinas D, Koziak K, Millan M, Hancock WW, Bach FH. Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J Exp Med*. 1997;185:153-63.

Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801-9.

Rubin LL. Endothelial cells: adhesion and tight junctions. *Curr Opin Cell Biol*. 1992;4:830-3.

Rubin LL, Staddon JM. The cell biology of the blood-brain barrier. *Annu Rev Neurosci*. 1999;22:11-28.

Saiag B, Milon D, Allain H, Rault B, Van den Driessche J. Constriction of the smooth muscle of rat tail and femoral arteries and dog saphenous vein is induced by uridine triphosphate via 'pyrimidinoceptors', and by adenosine triphosphate via P2x purinoceptors. *Blood Vessels*. 1990;27:352-64.

Saiag B, Milon D, Shacoori V, Allain H, Rault B, Van Den Driessche J. Newly evidenced pyrimidinoceptors and the P2x purinoceptors are present on the vascular smooth muscle and respectively mediate the UTP- and ATP-induced contractions of the dog maxillary internal vein. *Res Commun Chem Pathol Pharmacol*. 1992;76:89-94.

Salvemini D, Korbut R, Anggård E, Vane J. Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by Escherichia coli lipopolysaccharide. *Proc*

Natl Acad Sci U S A. 1990;87:2593-7. Erratum in: *Proc Natl Acad Sci U S A* 1990;87:6007.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Vaillant N, Gadeau AP, Desgranges C, Scalbert E, Chardin P, Pacaud P, Loirand G. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am J Physiol Heart Circ Physiol*. 2000;278:H1751-61.

Schmidt A, Utepbergenov DI, Krause G, Blasig IE. Use of surface plasmon resonance for real-time analysis of the interaction of ZO-1 and occludin. *Biochem Biophys Res Commun*. 2001;288:1194-9.

Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol.* 2004;286:C1213-28.

Schneider A, Panzer U, Zahner G, Wenzel U, Wolf G, Thaiss F, Helmchen U, Stahl RA. Monocyte chemoattractant protein-1 mediates collagen deposition in experimental glomerulonephritis by transforming growth factor β. *Kidney Int.* 1999;56:135-44.

Schwartz SM, deBlois D, O'Brien ER. The intima. Soil for atherosclerosis and restenosis. *Circ Res*. 1995;77:445-65.

Schwartz RS. Pathophysiology of restenosis: interaction of thrombosis, hyperplasia, and/or remodeling. *Am J Cardiol*. 1998;81:14E-17E.

Seta K, Sadoshima J. Phosphorylation of tyrosine 319 of the angiotensin II type 1 receptor mediates angiotensin II-induced trans-activation of the epidermal growth factor receptor. *J Biol Chem*. 2003;278:9019-26.

Seye CI, Gadeau AP, Daret D, Dupuch F, Alzieu P, Capron L, Desgranges C. Overexpression of $P2Y_2$ purinoceptor in intimal lesions of the rat aorta. *Arterioscler Thromb Vasc Biol*. 1997;17:3602-10.

Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, Gonzalez FA, Weisman GA. Functional $P2Y_2$ nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation*. 2002;106:2720-6.

Seye CI, Yu N, Jain R, Kong Q, Minor T, Newton J, Erb L, González FA, Weisman GA. The $P2Y_2$ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J Biol Chem.* 2003;278: 24960-5.

Seye CI, Yu N, González FA, Erb L, Weisman GA. The $P2Y_2$ nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). *J Biol Chem.* 2004;279:35679–86.

Shen BQ, Lee DY, Zioncheck TF. Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/Flk-1 receptor and a protein kinase C signaling pathway. *J Biol Chem*. 1999;274:33057-63.

Shen J, Seye CI, Wang M, Weisman GA, Wilden PA, Sturek M. Cloning, up-regulation, and mitogenic role of porcine $P2Y_2$ receptor in coronary artery smooth muscle cells. *Mol Pharmacol*. 2004;66:1265-74.

Sherr CJ. Mitogenic response to colony-stimulating factor 1. *Trends Genet.* 1991;7(11-12):398-402.

Shimokawa H, Flavahan NA, Vanhoutte PM. Natural course of the impairment of endothelium-dependent relaxations after balloon endothelium removal in porcine coronary arteries. Possible dysfunction of a pertussis toxin-sensitive G protein. *Circ Res*. 1989;65:740-53.

Simionescu M. Structural biochemical and functional differentiation of the vascular endothelium. In: Morphogenesis of Endothelium, edited by W. Risau and G. M. Rubanyi. Amsterdam: Harwood Academic. 2000:1-21.

Simon AM, Goodenough DA. Diverse functions of vertebrate gap junctions. *Trends Cell Biol*. 1998;8:477-83.

Smith RE, Strieter RM, Zhang K, Phan SH, Standiford TJ, Lukacs NW, Kunkel SL. A role for C-C chemokines in fibrotic lung disease. *J Leukoc Biol*. 1995;57:782-7.

Soltoff SP. Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled $P2Y_2$ receptor. Phorbol ester or $[Ca^{2+}]_i$ elevation can substitute for receptor activation. *J Biol Chem*. 1998;273:23110-7.

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med*. 2003;9:347-51.

Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 1994;76:301-14.

Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, Yeung YG. Biology and action of colony--stimulating factor-1. *Mol Reprod Dev.* 1997;46(1):4-10.

Steinberg TH, Newman AS, Swanson JA, Silverstein SC. ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J Biol Chem*. 1987;262:8884-8.

Stevenson BR, Siliciano JD, Mooseker M, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 1986;103:755-66.

Stewart P, e.b.W.R.a.G.M.R.A.H.A. Development of the blood-brain barrier. In: Morphogenesis of Endothelium, edited by W. Risau and G. M. Rubanyi. 2000:109-122.

Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS. Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol*. 2001;2:138-45.

Suh BC, Kim JS, Namgung U, Ha H, Kim KT. $P2X_7$ nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *J Immunol*. 2001;166:6754-63.

Sukhova GK, Schönbeck U, Rabkin E, Schoen FJ, Poole AR, Billinghurst RC, Libby P. Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation.* 1999;99:2503-9.

Sun B, Li J, Okahara K, Kambayashi J. $P2X_1$ purinoceptor in human platelets. Molecular cloning and functional characterization after heterologous expression. *J Biol Chem*. 1998;273:11544-7.

Takeichi M. Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol.* 1993;5:806-11. *FASEB J.* 1995;9:910-8.

Tanimoto T, Jin ZG, Berk BC. Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). *J Biol Chem*. 2002;277:42997-3001.

Thomas TH, Advani A. Inflammation in cardiovascular disease and regulation of the actin cytoskeleton in inflammatory cells: the actin cytoskeleton as a target. *Cardiovasc. Hematol. Agents Med. Chem.* 2006 Apr;4(2):165-82.

Tigges U, Koch B, Wissing J, Jockusch BM, Ziegler WH. The F-actin cross-linking and focal adhesion protein filamin A is a ligand and in vivo substrate for protein kinase C alpha. *J Biol Chem*. 2003;278:23561-9.

Thuringer D, Maulon L, Frelin C. Rapid transactivation of the vascular endothelial growth factor receptor KDR/Flk-1 by the bradykinin B2 receptor contributes to

endothelial nitric-oxide synthase activation in cardiac capillary endothelial cells. *J Biol Chem*. 2002;277:2028-32.

Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol*. 2001;2:285-93.

Turner JT, Park M, Camden JM, Weisman GA. Salivary gland nucleotide receptors. Changes in expression and activity related to development and tissue damage. *Ann N Y Acad Sci*. 1998;842:70-5.

Urano T, Nishimori H, Han H, Furuhata T, Kimura Y, Nakamura Y, Tokino T. Cloning of P2XM, a novel human P2X receptor gene regulated by p53. *Cancer Res.* 1997;57:3281–3287

Vadlamudi RK, Li F, Adam L, Nguyen D, Ohta Y, Stossel TP, Kumar R. Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat Cell Biol*. 2002;4:681-90.

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G. A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature*. 1994;371:516-9.

Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev*. 1997;11:2295-322.

Ventura MA, Thomopoulos P. ADP and ATP activate distinct signaling pathways in human promonocytic U-937 cells differentiated with 1,25-dihydroxy-vitamin D3. *Mol Pharmacol*. 1995;47:104-14.

Vial C, Evans RJ. $P2X_1$ receptor-deficient mice establish the native P2X receptor and a $P2Y_6$-like receptor in arteries. *Mol Pharmacol*. 2002;62:1438-45.

Viola A, Gupta N. Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. *Nat Rev Immunol.* 2007;7(11):889-96.

Viswanathan M, Stromberg C, Seltzer A, Saavedra JM. Balloon angioplasty enhances the expression of angiotensin II AT1 receptors in neointima of rat aorta. *J Clin Invest*. 1992;90:1707-12.

von Kugelgen I, Starke K. Evidence for two separate vasoconstriction-mediating nucleotide receptors, both distinct from the P2x-receptor, in rabbit basilar artery: a receptor for pyrimidine nucleotides and a receptor for purine nucleotides. *Naunyn Schmiedebergs Arch Pharmacol*. 1990;341:538-46.

von Kugelgen I, Wetter A. Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362:310-23.

Walpola PL, Gotlieb AI, Cybulsky MI, Langille BL. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *Arterioscler Thromb Vasc Biol.* 1995;15:2-10. Erratum in: *Arterioscler Thromb Vasc Biol.* 1995;15:429.

Wang L, Andersson M, Karlsson L, Watson MA, Cousens DJ, Jern S, Erlinge D. Increased mitogenic and decreased contractile P2 receptors in smooth muscle cells by shear stress in human vessels with intact endothelium. *Arterioscler Thromb Vasc Biol*. 2003;23:1370-6.

Warny M, Aboudola S, Robson SC, Sevigny J, Communi D, Soltoff SP, Kelly CP. $P2Y_6$ nucleotide receptor mediates monocyte interleukin-8 production in response to UDP or lipopolysaccharide. *J Biol Chem*. 2001;276:26051-6.

Weidinger FF, McLenachan JM, Cybulsky MI, Gordon JB, Rennke HG, Hollenberg NK, Fallon JT, Ganz P, Cooke JP. Persistent dysfunction of regenerated endothelium after balloon angioplasty of rabbit iliac artery. *Circulation*. 1990;81:1667-79.

Weksler BB, Ley CW, Jaffe EA. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A 23187. *J Clin Invest.* 1978;62:923-30.

Whitehead IP, Campbell S, Rossman KL, Der CJ. Dbl family proteins. *Biochim Biophys Acta*. 1997;1332:F1-23.

Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A*. 1989;86(8):2839-43.

Winkles JA, Alberts GF, Brogi E, Libby P. Endothelin-1 and endothelin receptor mRNA expression in normal and atherosclerotic human arteries. *Biochem Biophys Res Commun*. 1993;191:1081-8.

Wolburg H, Lippoldt A. Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul Pharmacol*. 2002;38:323-37.

Woo MS, Ohta Y, Rabinovitz I, Stossel TP, Blenis J. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. *Mol Cell Biol*. 2004;24:3025-35.

Xie Z, Xu W, Davie EW, Chung DW. Molecular cloning of human ABPL, an actin-binding protein homologue. *Biochem Biophys Res Commun.* 1998;251:914-9.

Xu W, Xie Z, Chung DW, Davie EW. A novel human actin-binding protein homologue that binds to platelet glycoprotein Ib_{α}. *Blood.* 1998;92:1268-76.

Yamamoto K, Yamamoto M. Pathophysiology of restenosis: interaction of thrombosis, hyperplasia, and/or remodeling muscle cells in culture. *Exp Cell Res*. 1994;214:258-63.

Zalewski A, Shi Y, Johnson AG. Diverse origin of intimal cells: smooth muscle cells, myofibroblasts, fibroblasts, and beyond? *Circ Res*. 2002;91:652-5.

Zeng H, Sanyal S, Mukhopadhyay D. Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J Biol Chem*. 2001;276:32714-9.

Zeng H, Zhao D, Mukhopadhyay D. KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. *J Biol Chem*. 2002;277:46791-8.

Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma F, Jr. $P2Y_{13}$: identification and characterization of a novel $G\alpha_i$-coupled ADP receptor from human and mouse. *J Pharmacol Exp Ther*. 2002;301:705-13.

Zohn IM, Campbell SL, Khosravi-Far R, Rossman KL, Der CJ. Rho family proteins and Ras transformation: the RHOad less traveled gets congested. *Oncogene*. 1998;17:1415-38.

VITA

Ningpu Yu was born on February 3rd 1979 in Tianjin, China. After attending public schools, he received his Bachelor of Science degree from the Department of Biochemistry and Molecular Biology of Nankai University in 2001. Currently he is finishing his Ph.D degree in the Department of Biochemistry of the University of Missouri-Columbia.