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Universität Freiburg (Schweiz)

**Regulation of Vascular Endothelial Function by  
RhoA/Rho-Kinase Pathway:  
Implications for Cardiovascular Diseases**

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*Commit to the Lord whatever you do, and all your plans will succeed.*

*Proverb 16:3*

*If I have seen further than others, it is by standing upon the shoulders of giants.*

*Issac Newton, Father of Modern Science. 1642-1727.*

*Dedicated to my parents*

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## Abbreviations

5-HT	Serotonin/5-hydroxytryptamine
AA	Amino acid
ABCA-1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
ACh	Acetylcholine
Adm	Adrenomedulin
ADP	Adenosine diphosphate
ANP	Atrial natriuretic peptide
AP	Alkaline phosphatase
ApoA-1	Apolipoprotein A1
ApoE	Apolipoprotein E
ApoE <sup>-/-</sup>	Apolipoprotein E-deficient
ATP	Adenosine triphosphate
BCIP/NBT	Bromo-chloro-indonyl phosphate/Nitro Blue Tetrazolium
BH <sub>4</sub>	Tetrahydrobiopterin
BSA	Bovine serum albumin
CAD	Coronary artery disease
CaM	Calmodulin
cAMP	cyclic adenosine monophosphate
CAT	Catalytic
CD	Cluster of differentiation
cDNA	complementary DNA
CETP	Cholesterol ester transfer protein
cGMP	cyclic guanine monophosphate
CM	Chylomicron
CMR	Chylomicron remnant
CMV Prom	Cytomegalovirus Promoter
CMV	Cytomegalovirus
CNP	C-type natriuretic peptide
d.n.	Dominant negative
EC	Endothelial cells
ECGS	Endothelial Cell Growth Supplement
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor



EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
FMN	Flavin mononucleotide
FS	Fluvastatin
GAPs	GTPase activating proteins
GDI	GDP dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGPP	Geranylgeranylpyrophosphate
GST-TRBD	Glutathione S-transferase Rhotekin Rho-binding domain
GTP	Guanosine triphosphate
HA	Hemagglutinin
HDL-C	High-density lipoprotein cholesterol
HL	Hepatic lipase
HMG CoA	Hydroxymethylglutaryl Coenzyme A
hSMCs	Human smooth muscle cells
hsp90	Heat shock protein 90
HUVECs	Human umbilical vein endothelial cells
i.p.	Intraperitoneal
IDL	Intermediate-density lipoprotein
KD	Kinase domain
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LDLR	LDL receptor
L-NAME	L-Nitro arginine methyl ester
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	LDL related protein
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid

Myr	Myristoylation
NA	Noradrenaline
NADH	reduced Nicotinamide adenine dinucleotide
NADPH	reduced Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOSIP	eNOS interacting protein
Ox-LDL	Oxidized low density lipoprotein
P receptors	Purinergic receptors
Palm	Palmytoylation
PAR-1	Protease-activated receptor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-BB	Platelet derived growth factor-BB
PGI <sub>2</sub>	Prostaglandin I <sub>2</sub>
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3'-kinase
PI3P	Phosphotidylinositol 3'-phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLTP	Phospholipid transfer protein
PMSF	Phenylmethylsulphonyl fluoride
PROCAM	Prospective Cardiovascular Münster
PS	Phosphatidylserine
PTCA	Percutaneous transluminal cardioangiography
rAd	Recombinant adenovirus
RBD	Rho binding domain
rCD2	rat cytoplasmic domain 2
RCT	Reverse Cholesterol Transport
rHDL	reconstituted HDL
S2	serotonergic receptor
SDS-PAGE	Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SMC	Smooth muscle cells

SOD	Superoxide dismutase
SR A	Scavenger receptor A
SR-B1	Scavenger receptor-class B type 1
TF	Tissue factor
TLC	Thin layer chromatography
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
t-PA	tissue-type plasminogen activator
TXA <sub>2</sub> /PGH <sub>2</sub>	Thromboxane A <sub>2</sub> /Prostaglandin H <sub>2</sub>
VEGF	Vascular endothelial growth factor
WB	Western blot
WT	Wild type

## Summary

The **vascular endothelium** plays an important role in the maintenance of normal cardiovascular functions. It prevents smooth muscle contraction, inhibits smooth muscle cell proliferation and /or migration and exerts anti-thrombotic effects. One of the most important factors derived from endothelial cells is **nitric oxide (NO)** which is produced via activation of endothelial NO synthase (**eNOS**). eNOS metabolizes the substrate **L-arginine** to NO in the presence of co-factors under physiological conditions. However, under disease conditions, such as atherosclerosis, diabetes, hypertension, aging, etc., NO bioavailability is decreased, which is implicated in initiation and evolution of the disease process. The decreased NO bioavailability is either attributed to a decreased eNOS gene expression or decrease in enzyme activity or inactivation of bioactive NO by oxidative stress.

**RhoGTPase** is a member of the Ras superfamily of small GTP-binding proteins which is not only involved in regulation of cellular functions associated with cytoskeleton reorganisation such as platelet aggregation, smooth muscle contraction and cell migration, but also in regulation of gene expression. Studies in recent years demonstrate that RhoA suppresses eNOS gene expression in endothelial cells by destabilizing eNOS mRNA. Inhibition of RhoA by the HMG-CoA reductase inhibitors or statins upregulates eNOS expression. These may importantly contribute to the beneficial effects of the drugs in patients with coronary heart diseases.

*In the first part of the project we further confirmed the role of the RhoGTPase, RhoA in **down-regulation of eNOS gene expression** in human endothelial cells. This effect is mediated by down-stream effector Rho-kinase (ROCK). Most importantly, we established a role of RhoA/ROCK pathway in **inhibition of Akt/eNOS cascade**, i.e. activation of RhoA/ROCK inhibits PKB/Akt activity and subsequently activation or phosphorylation of eNOS at S-1177.*

*In the second part* of the project we explored another important role of Rho/ROCK pathway in regulation of eNOS activity namely **stimulation of arginase** in endothelial cells. Arginase competes with eNOS for the substrate L-arginine. It metabolizes L-arginine to urea, thus depletes intracellular L-arginine for NO production. We showed that overexpression of RhoA or ROCK active mutants enhances arginase activity in endothelial cells. Interestingly, in ApoE<sup>-/-</sup> atherosclerotic mice aorta, RhoA expression was much more pronounced as compared to the wild type animals, which is correlated with a higher arginase activity in ApoE<sup>-/-</sup> mice aortas. In atherosclerotic ApoE<sup>-/-</sup> mice aortas, L-arginine caused a more pronounced contraction as compared to wild type mice. This response was reversed to a greater relaxation by an arginase inhibitor L-norvaline in ApoE<sup>-/-</sup> mice compared to the wild type animals. The results demonstrate that an increased arginase activity mediated by Rho/ROCK pathway is involved atherosclerotic endothelial dysfunction.

*In the third part* of the project, we demonstrated that Rho/ROCK pathway together with p38<sup>mapk</sup> increases expression of **endothelial tissue factor** which is critical in thrombus formation and acute coronary syndromes. In this part of the project, we showed that high density lipoprotein (HDL) on one hand activates PI3K/Akt/eNOS pathway and on the other hand inhibits tissue factor expression. The inhibition of endothelial tissue factor expression by HDL is mediated through inhibition of RhoA and activation of PI3K, independent of the downstream enzymes, Akt/eNOS. Taken together, our results demonstrate multi-functional roles of Rho/ROCK pathway in endothelial dysfunction, which may be importantly involved in the pathogenesis of cardiovascular disease.

## **Zusammenfassung**

Das Gefäßendothel spielt eine wichtige Rolle bei der Aufrechterhaltung normaler kardiovaskulärer Funktionen. Es verhindert die Kontraktion glatter Muskulatur, hemmt die Proliferation und/oder Migration der glatten Muskelzellen und übt eine antithrombotische Wirkung aus. Einer der wichtigsten Faktoren endothelialer Herkunft ist Stickstoffmonoxid (NO), das durch Aktivierung der endothelialen NO-Synthase (eNOS) produziert wird. eNOS setzt als Substrat L-Arginin unter physiologischen Bedingungen und in Anwesenheit von Cofaktoren zu NO um. Im Verlauf bestimmter Krankheiten, z.B. bei Arteriosklerose, Diabetes und Bluthochdruck, oder im Alter ist die biologische Verfügbarkeit von NO jedoch verringert, was mit der Entstehung und dem Fortschreiten dieser Prozesse in Verbindung gebracht wird. Diese verringerte biologische Verfügbarkeit von NO wird entweder auf eine geringere eNOS-Expression oder einen Rückgang der Enzymaktivität bzw. eine Inaktivierung von aktivem NO durch oxidativen Stress zurückgeführt.

Die RhoGTPase ist ein Mitglied der Ras-Superfamilie kleiner GTP-bindender Proteine. Sie ist nicht nur an der Regulation mit Cytoskelettorganisation verbundener zellulärer Prozesse wie Blutplättchenaggregation, Kontraktion glatter Muskulatur und Zellmigration, sondern auch an der Regulation der Genexpression beteiligt. In den letzten Jahren haben Studien ergeben, dass RhoA eNOS-Expression in endothelialen Zellen durch Destabilisierung der eNOS-mRNA unterdrückt. Hemmung von RhoA durch HMG-CoA-Reduktaseinhibitoren oder Statine erhöht eNOS-Expression. Dies kann zu den positiven Effekten der Medikation bei Patienten mit koronarer Herzkrankheit beitragen.

Im ersten Teil des Projekts haben wir die Rolle der RhoGTPase RhoA bei der Herunterregulierung der eNOS-Expression in menschlichen Endothelzellen bestätigt. Diese Wirkung wird durch den Effektor Rho-Kinase (ROCK) vermittelt. Insbesondere haben wir eine Beteiligung des RhoA/ROCK-Signalwegs an der Hemmung der Akt/eNOS-Kaskade

nachgewiesen, d.h., die Aktivierung von RhoA/ROCK hemmt PKB/Akt-Aktivität und verhindert infolgedessen Aktivierung oder Phosphorylierung von eNOS an S-1177.

Im zweiten Teil des Projekts haben wir eine weitere wichtige Funktion des RhoA/ROCK-Signalwegs in der Regulation der eNOS-Aktivität, die Stimulation des Enzyms Arginase in Endothelzellen, untersucht. Die Arginase setzt L-Arginin zu Harnstoff um, wodurch intrazelluläres L-Arginin, das für die NO-Bildung eingesetzt wird, abgezogen wird. Wir konnten zeigen, dass die Ueberexpression aktiver Formen von RhoA oder ROCK die Arginaseaktivität in Endothelzellen erhöht. Interessanterweise war die RhoA-Expression in den Aortae arteriosklerotischer ApoE<sup>-/-</sup> Mäuse deutlich höher als in denen der Wildtyp-Mäusen. Dies korrelierte mit einer erhöhten Arginaseaktivität in den ApoE<sup>-/-</sup> Mäusen. In den Aortae der arteriosklerotischen Mäuse rief L-Arginin eine stärkere Kontraktion als in denen der Wildtyp-Mäuse hervor. Diese Reaktion wurde bei Behandlung mit dem Arginaseinhibitor L-Norvalin in eine stärkere Relaxation der ApoE<sup>-/-</sup>-Aortae gegenüber den Wildtypgefäßen umgekehrt. Diese Ergebnisse zeigen, dass die durch den RhoA/ROCK-Signalweg erhöhte Arginaseaktivität an arteriosklerotischer Fehlfunktion des Endothels beteiligt ist.

Im dritten Teil des Projekts konnten wir nachweisen, dass der RhoA/ROCK-Signalweg zusammen mit p38<sup>mapk</sup> die Expression des endothelialen Tissue-Faktors erhöht. Tissue-Faktor ist kritisch für die Thrombusbildung und spielt eine wichtige Rolle bei akuten koronaren Symptomen. In diesem Teil des Projekts zeigten unsere Resultate, dass das High-Density-Lipoprotein (HDL) einerseits den PI3K/Akt/eNOS-Signalweg aktiviert und andererseits die Expression des Tissue-Faktors hemmt. Die Hemmung der Expression des endothelialen Tissue-Faktors durch HDL ist auf zwei Mechanismen zurückzuführen: Hemmung von RhoA und Aktivierung der PI3K, wobei die weiter abwärts im PI3K-Signalweg liegenden Enzyme Akt und eNOS nicht involviert sind.

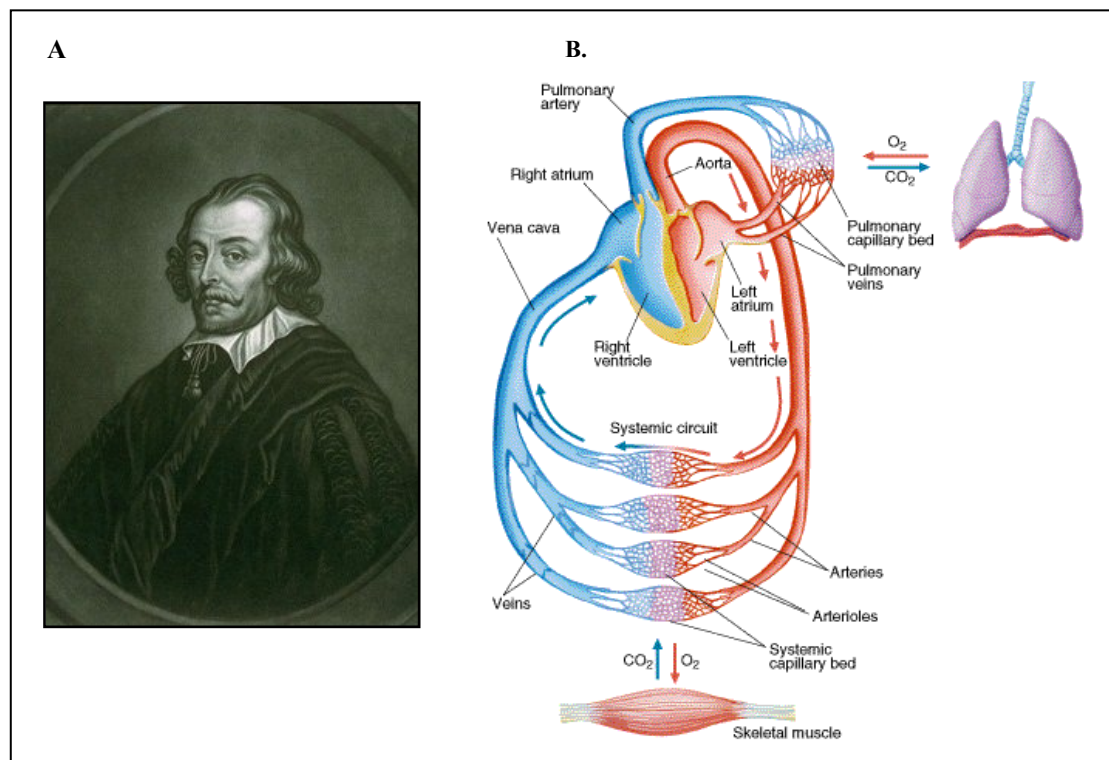
Zusammenfassend demonstrieren unsere Ergebnisse eine multifunktionelle Beteiligung des RhoA/ROCK-Signalwegs an endothelialer Fehlfunktion. Dies spielt möglicherweise eine wichtige Rolle bei der Pathogenese kardiovaskulärer Erkrankungen.



## I. INTRODUCTION

### 1. THE CARDIOVASCULAR CIRCULATION SYSTEM: OVERVIEW

In the 17th century, the English physician **William Harvey** (**Figure 1A**) demonstrated that all venous blood moves from the periphery of the body to a single center, the heart which acted as a pump forcing blood to the periphery of the body through arteries and returning it to the heart through veins and was recycled. Harvey's discovery led to the basic knowledge of the cardiovascular system as a closed loop in which blood is circulating.



**Figure 1: (A) William Harvey (1578 – 1657). (B) The pulmonary circulation and the systemic circulation.**

The heart pumps blood through two separate vascular systems, one to the lung, the low pressure **pulmonary circulation** in which gas exchange occurs, and one to all other organs of the body, the **systemic circulation**, which delivers blood to individual organs, matching supply to metabolic demand (**Figure 1B**). Arteries branch and diverge as they move away from the heart. As they form smaller and smaller

divisions, they become a meshwork of capillaries. The veins channel blood from capillaries back to the heart. The capillary meshwork is the site of gas, nutrient and waste exchange between the blood and the respiring tissues. It is the connecting site of arteries and veins, which William Harvey could not see by eye.

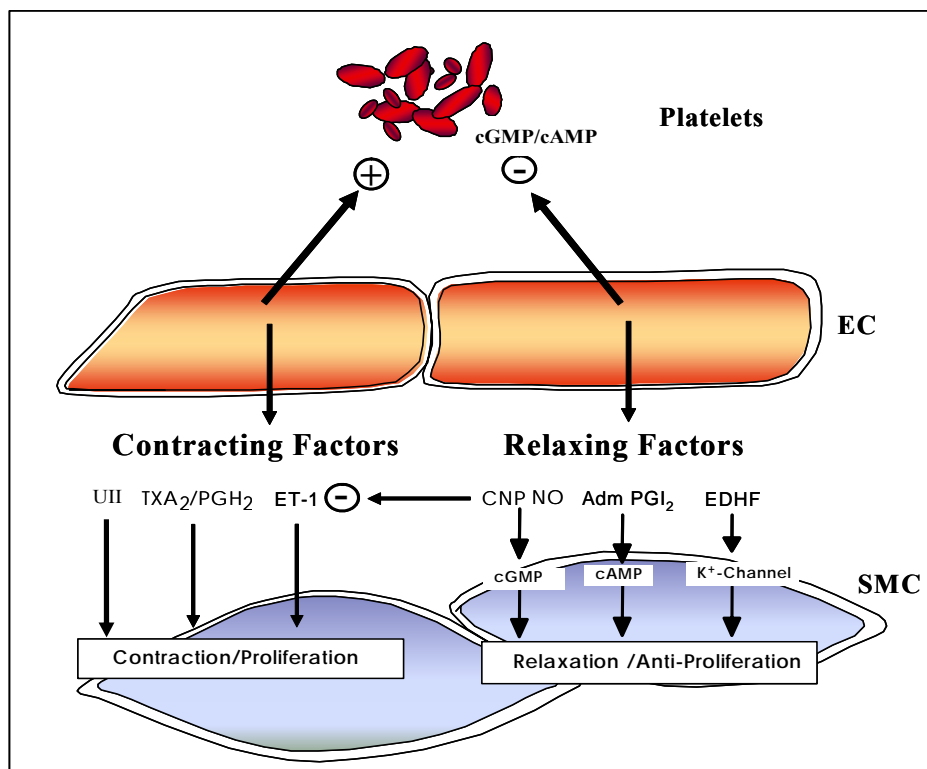
## 2. THE VASCULAR ENDOTHELIUM: IMPORTANT MODULATOR OF CARDIOVASCULAR FUNCTION

The blood flow to individual organs is tightly regulated by **neuronal** and **hormonal** factors (**Table 1**). For example, the sympathetic nervous activity stimulates cardiac function and vasoconstriction by releasing neurotransmitter catecholamines i.e. noradrenaline and adrenaline from the synaptic endings via activation of adrenergic  $\beta$ -1 in the heart and  $\alpha$ -1 receptors on vascular smooth muscle cells (SMC). The effects of the sympathetic nervous activity are counteracted by parasympathetic nervous system via neurotransmitter, acetylcholine. Moreover, circulating hormones including vasoconstrictors (i.e. angiotensin II, serotonin, vasopressin etc.) and vasodilators (i.e. ANP, bradykinin, adenosine etc.) also play important roles in the regulation of blood flow to organs (**Table 1**).

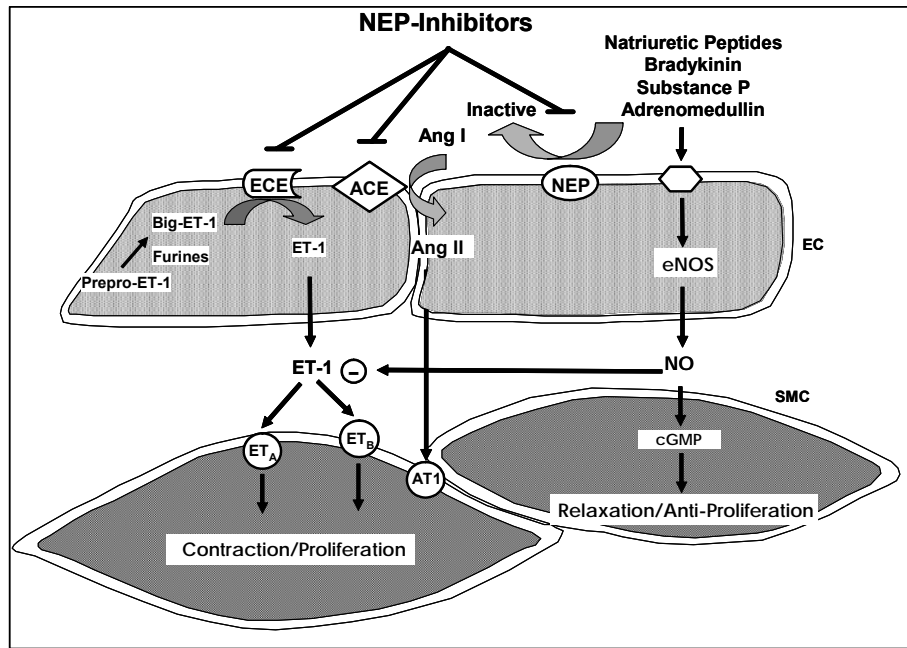
Vasoconstriction	Vasorelaxation
<i>Sympathetic Nervous System</i>	<i>Parasympathetic Nervous System</i>
Angiotensin II	Atrial natriuretic peptide (ANP)
Serotonin	Adenosine
Vasopressin	Bradykinin
Endothelin-1	Prostacyclin
Thromboxanes	Adrenomedullin

**Table 1:** A vast list of various vasoactive factors are known to regulate blood flow to the organs. The list is growing steadily.

Research in the past two decades provides firm evidence demonstrating that the monolayer of the **endothelium** is not only a simple diffusion barrier between the intravascular and extravascular space of blood and lymph vessels, but also an active organ synthesizing, releasing and metabolizing various vasoactive hormones (**Figure 2, Figure 3**). A number of **relaxing** and **contracting factors** derived from the endothelium have been characterized (**Figure 2**).

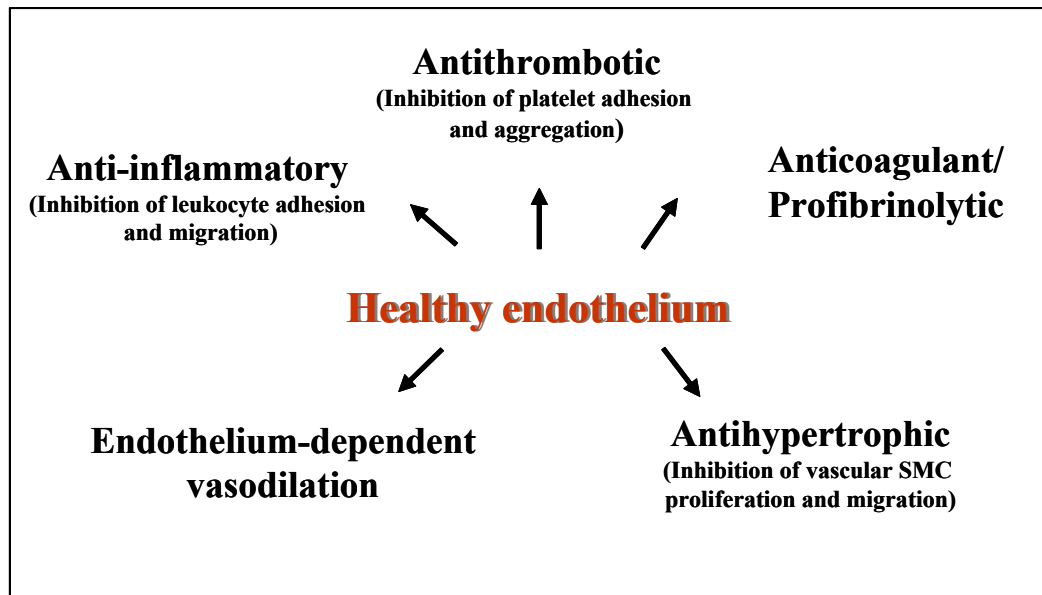


**Figure 2:** The endothelium as an active organ releases various vasoactive substances including relaxing and contracting factors. The endothelium-derived relaxing factors shown on the right relax smooth muscle; inhibit cell proliferation and platelet aggregation via second messengers (cGMP or cAMP) or via opening of K<sup>+</sup>-channels. The endothelium-derived contracting factors shown on the left cause smooth muscle contractions stimulate cell proliferation or platelet aggregation. The production of contracting factors could be inhibited by relaxing factors under physiological conditions. TXA<sub>2</sub>/PGH<sub>2</sub> = thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub>, ET-1 = endothelin-1, CNP = C-type natriuretic peptide, NO = nitric oxide, Adm = adrenomedullin, PGI<sub>2</sub> = prostaglandin I<sub>2</sub>, EDHF = endothelium-derived hyperpolarizing factor, cGMP = cyclic guanosine monophosphate, cAMP = cyclic adenosine monophosphate, UII = urotensin II, EC = endothelial cell, SMC = smooth muscle cell, ⊕ stimulation, ⊖ inhibition.



**Figure 3: Activation and inactivation of vasoactive hormones by endothelial cells:** Endothelial cells express endothelin-converting enzyme (ECE) and angiotensin-converting enzyme (ACE) which converts big-ET-1 and angiotensin I into active ET-1 and angiotensin II, respectively causing potent vasoconstriction and promoting smooth muscle cell growth. Endothelial cells also express neutral endopeptidase (NEP) which inactivates various vasoactive substances known to release NO or PGI<sub>2</sub>. Combined inhibitors of NEP, ACE and/or ECE demonstrate a powerful strategy to improve endothelial function. ET<sub>A</sub>/ET<sub>B</sub>=endothelin receptors, AT1=angiotensin receptor type 1, eNOS= endothelial nitric oxide synthase, cGMP= cyclic guanosine monophosphate.

The vascular endothelium is also an important source of **thrombogenic** and **anti-thrombogenic factors** which regulate platelet-vessel wall interaction. Finally, it **directly** (by producing growth promoters and inhibitors) or **indirectly** (by inhibiting activation of platelets which are rich in growth factors for SMC) **modulates SMC migration and proliferation** (Figure 2). The multiple functions of the endothelium under physiological conditions are summarized in **Figure 4**. However, under pathological conditions, an imbalance between these factors occurs as a result contracting factors, thrombogenic factors and growth promoters become dominant. **Among them, endothelial nitric oxide (NO) is one of the most important factors in the regulation of vascular homeostasis.**



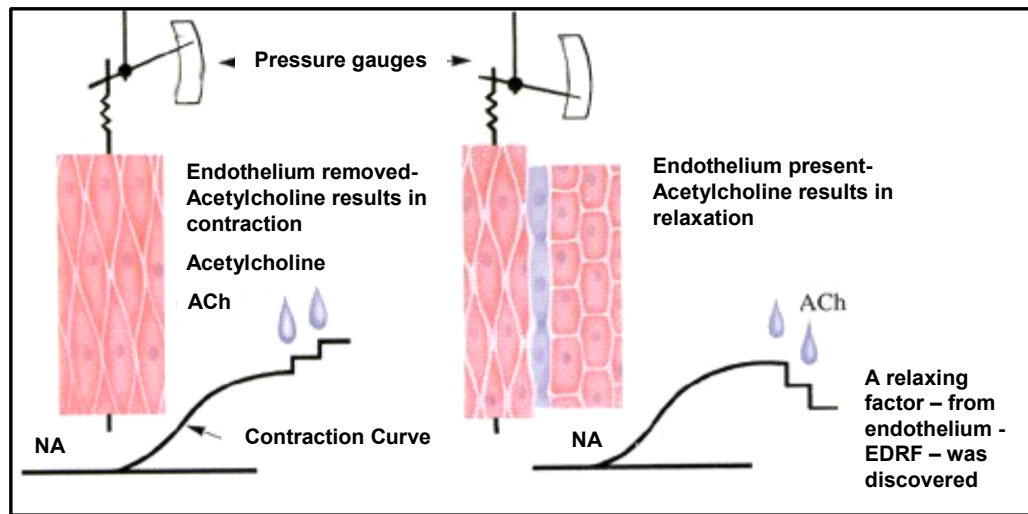
**Figure 4:** The healthy endothelium not only mediates endothelium-dependent vasodilation, but also actively suppresses thrombosis, vascular inflammation, and hypertrophy (from Landmesser U, *Circulation*. 2004;109, Suppl 1:II27-33).

## 2.1 The Discovery of Endothelium-Derived NO

In 1978, the group of Murad demonstrated that the vasodilatory effects of nitroglycerine and other nitrates (that had been used for decades to treat patients with angina pectoris without knowing their mechanism of action) were mediated by a common degradation product, NO with a very short half life of 3-5 seconds. This class of compounds causes vascular relaxation via activation of soluble guanylyl cyclase in SMC of blood vessels.

In 1980, Furchgott observed that relaxations evoked by acetylcholine (ACh) in rabbit aorta and other arteries require an intact endothelial layer (Furchgott et al., 1980). Indeed, in arteries, in which the endothelium was gently removed either mechanically or enzymatically, the relaxations in response to ACh were absent (**Figure 5**). With a simple but elegant so-called ‘sandwich preparation’ bioassay, a layering of two strips of arteries with (sandwiched) and without endothelial layer between the strips (**Figure 5**), Furchgott demonstrated convincingly that the relaxations induced by ACh in the arteries are mediated by a diffusable relaxing

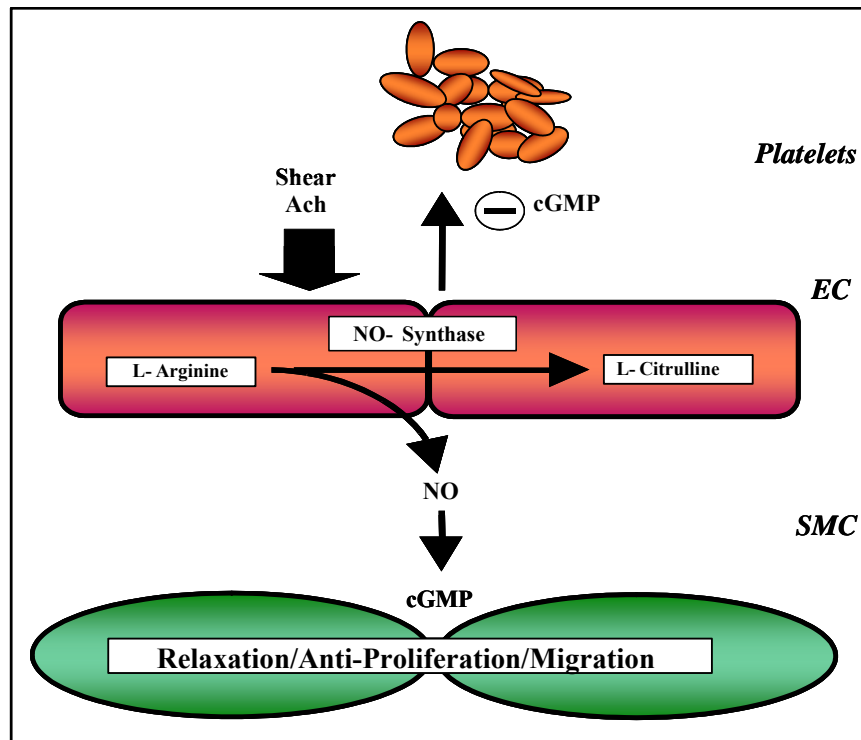
substance(s) produced by the endothelium. This factor(s) was named endothelium-derived relaxation factor (EDRF).



**Figure 5: Robert F Furchgott showed that acetylcholine-induced relaxation of blood vessels was dependent on the endothelium. His "sandwich" experiment set the stage for future scientific development. He used two different pieces of the aorta; one had the endothelial layer intact, in the other it had been removed. NA = Noradrenaline. (from Furchgott RF, *Nature*. 1980. 288:373-6).**

Within a few years, this observation was expanded to different species including vertebrates, bony fish and amphibia, mammals and humans in different vascular beds, suggesting an ancestral phenomenon preserved during evolution.

During the following years, it appeared that there were chemical similarities between NO and EDRF. Besides, both compounds exerted vasodilation by means of cyclic GMP synthesis. Finally, the group of Ignarro proposed in 1987 that **EDRF was nitric oxide** (Ignarro et al., 1987). Soon, the group of Moncada (1987) obtained the first results supporting that proposal (Palmer et al., 1987). They demonstrated that endothelial cells produce NO in sufficient amounts to explain the relaxation observed (Radomski et al., 1987; Ignarro et al., 1987) and that **nitric oxide** is produced from the precursor, **L-arginine** via endothelial nitric oxide synthase (eNOS) (**Figure 6**).



**Figure 6: Endothelial L-arginine /NO pathway.** (Abbr: EC = endothelial cells, SMC = smooth muscle cells, ⊖ inhibition)

For the discovery of **endothelial L-arginine/NO pathway** Drs. Furchgott, Murad and Ignarro were awarded the 1998 Nobel Prize in Medicine and Physiology (**Figure 7**).



**Figure 7: RF Furchgott, F Murad and LJ Ignarro: Nobel Laureates in Medicine and Physiology, 1998.**

## 2.2 Functions of Endothelial NO

In cardiovascular system, NO is an important determinant of basal vascular tone, prevents platelet activation, limits leukocyte adhesion to the endothelium, regulates myocardial contractility and inhibits SMC migration and proliferation (**Figure 6**). **Overproduction of NO** plays a role in cardiovascular disorders, such as septic shock (Isobe et al., 2001) and in a major portion of neural damage following vascular stroke (Rao et al., 1999). **Decreased NO** function is involved in the pathogenesis of many cardiovascular disorders such as hypertension, atherosclerosis, venous bypass graft disease, diabetic vascular disease etc. (see 2.3).

## 2.3 Risk Factors for Endothelial Dysfunction

As mentioned above, **endothelial dysfunction**, represented as impaired function of endothelial L-arginine/NO pathway is a common mechanism for various cardiovascular disorders. In the past years, a vast list of risk factors for cardiovascular disease has been reported to cause endothelial dysfunction (**Figure 8**).

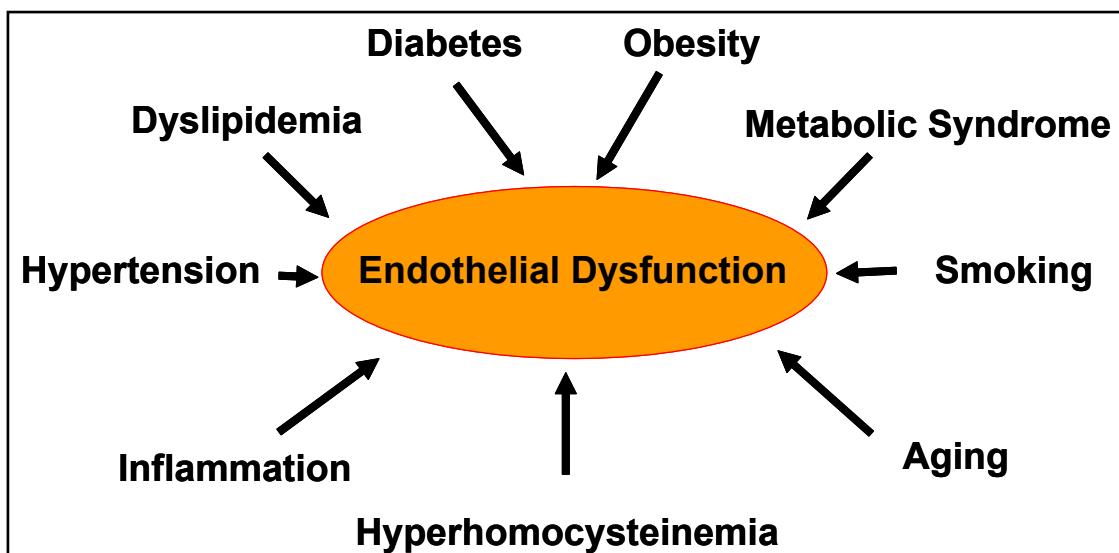
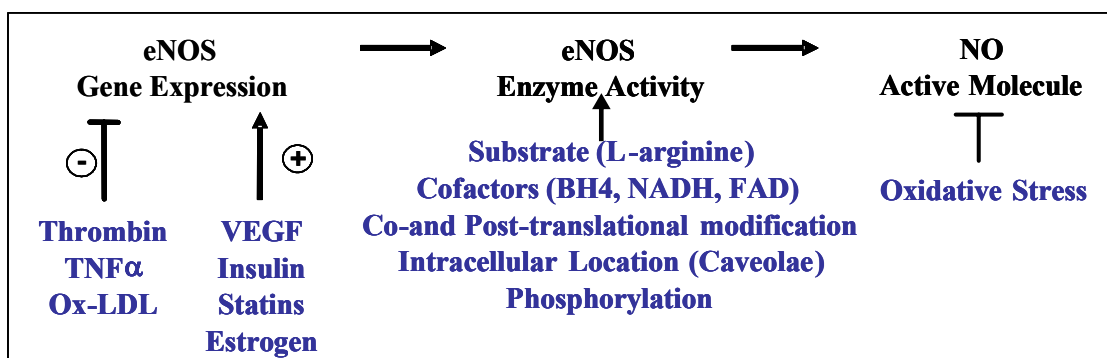


Figure 8: Risk factors that impair endothelial function.



## 2.4 Regulatory mechanism of endothelial L-arginine/NO pathway

The underlying mechanisms of endothelial dysfunction caused by the risk factors could be summarized at **three different levels** (Figure 9): (1) regulation of eNOS gene expression (2) regulation of eNOS enzyme activity and (3) regulation of NO bioavailability.



**Figure 9: Regulatory mechanism of endothelial L-arginine/NO pathway.** (Abbr:  $\text{TNF}\alpha$  = tumor necrosis factor- $\alpha$ ,  $\text{BH}_4$  = tetrahydrobiopterin, VEGF = vascular endothelial growth factor, ox-LDL = oxidized LDL,  $\ominus$  = inhibition,  $\oplus$  = stimulation)

### 2.4.1. Regulation of eNOS gene expression

Three isoforms of eNOS could be distinguished: NOS1 (also named as neuronal NOS, nNOS, or NOS I), NOS2 (inducible NOS, iNOS, or NOS II), and finally NOS3 (endothelial NOS, eNOS, or NOS III) (Forstermann et al., 1995) (**Table 2**). All these subtypes use L-arginine as substrates and oxygen, NADPH, FMN, FAD, tetrahydrobiopterin and heme as cofactors to produce NO. Although, NOS1 was initially found in the brain, NOS2 in macrophages and NOS3 in the endothelium, they have later been found in many other cells of the organism with different level of expressions. Some of the important characteristics of NOS isoforms are summarized in **Table 2**.

Human NOS	Cell Types	Size	Subcellular Site	Regulation of activity	NO output	Co-factors
<b>Constitutive neuronal</b> ( <i>nNOS</i> , <i>NOS1</i> )	Neuronal, skeletal muscle cells, cardiac muscle cells	161 kDa	Cytosol	Regulatable	Low (pM)	BH <sub>4</sub> , NADPH, FAD, FMN, heme
<b>Constitutive, endothelial</b> ( <i>eNOS</i> , <i>NOS3</i> )	Endothelial cells	133 kDa	Membrane>>cytosol	Regulatable	Low (pM)	BH <sub>4</sub> , NADPH, FAD, FMN, heme
<b>Inducible NOS</b> ( <i>iNOS</i> , <i>NOS2</i> )	Macrophage, many other cell types	131 kDa	Cytosol>>Membrane	Non-regulatable	High (μM)	BH <sub>4</sub> , NADPH, FAD, FMN, heme

**Table 2: Characteristics of different isoforms of nitric oxide synthase. Abbr: BH<sub>4</sub> = tetrahydrobiopterin, NADPH = nicotinamide adenine dinucleotide phosphate reduced, FAD = flavin adenine dinucleotide, FMN = flavin mononucleotide.**

eNOS was originally proposed as a constitutively expressed enzyme in vascular endothelial cells. However, studies in the past provide evidence that the expression level of eNOS could be regulated by several mechanisms. mRNA levels represent the balance between gene transcription and mRNA degradation. The kinetics of mRNA degradation is dependent partly, on nucleotide sequence motifs, which are usually located in the 3' untranslated region of the gene. Possible interactions of specific proteins to these sequences may render the mRNA more or less susceptible to endonucleolytic cleavage. Two motifs often implicated in mRNA destabilization are present at the 3' end of the eNOS mRNA (Marsden et al., 1993). In accordance, some stimuli affect eNOS mRNA stability. **TNF-α** destabilizes eNOS mRNA, which is suggested to be mediated by the increased binding of regulatory cytosolic proteins to the 3' untranslated region of the eNOS mRNA (Alonso et al., 1997). Other stimuli that have been reported to decrease eNOS mRNA stability include **lipopolysaccharides** (endotoxins) (Lu et al., 1996), **hypoxia** (Michel et al.,

1997), and **oxidized LDL** (Liao et al., 1995). Such mechanisms could also be involved in decreased eNOS expression in inflammatory models of renal injury such as necrotizing crescentic glomerulonephritis (Heeringa et al., 1998). On the other hand, certain conditions of **shear stress** upregulate eNOS mRNA levels through posttranscriptional events (Ziegler et al., 1998). **VEGF-** as well as **hydrogen peroxide**-induced eNOS upregulation is also dependent on an enhanced stability of eNOS mRNA. Thus, eNOS expression is affected by various stimuli, which modify eNOS regulation at the mRNA level by inducing changes in transcription kinetics and stability of the eNOS mRNA.

#### ***2.4.2. Regulation of eNOS Enzymatic Activity***

##### *Co-factors*

eNOS consists of a flavin-containing reductase domain and a heme-containing oxidase domain. NADPH reduces the flavin component of the reductase domain, but electron transfer to heme will not occur until **Ca<sup>2+</sup>/calmodulin** is present (Craig et al., 2002). In the presence of Ca<sup>2+</sup>/calmodulin, there is an electron transfer from NADPH to the heme group. In the presence of L-arginine, electrons flow to the heme moiety to reduce oxygen which is used to oxidize L-arginine to NO and citrulline. Another cofactor, tetrahydrobiopterin or **BH<sub>4</sub>**, has been postulated to play an important role in whether the electron flow in the enzyme can be directed to L-arginine (Heitzer et al., 2000; Shinozaki et al., 1999). Indeed, in the (neural) NOS I isoform or nNOS, depletion of BH<sub>4</sub> results in uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide and subsequently hydrogen peroxide. Regarding recombinant eNOS, it is confirmed that addition of BH<sub>4</sub> increases NO production and reduces superoxide generation by eNOS (Xia et al., 1998). The exact mechanisms by which BH<sub>4</sub> exerts these effects are not known. In nNOS, BH<sub>4</sub> appears to play a major

role in stabilizing the NOS in its active dimeric form (Noguchi et al., 2001). However, this allosteric role of BH<sub>4</sub> appears to be less prominent for recombinant eNOS.

### *Protein-protein Interactions*

Considerable attention has been paid to the mechanisms by which the localization of eNOS to caveolae affects the function of the enzyme, including the interaction of eNOS with other resident proteins such as the caveolae coat protein, **caveolin**.

*In vitro* studies and experiments with eNOS and caveolin-1 overexpression in COS-7 cells revealed that both N- and C-terminal domains of caveolin interact directly with the eNOS oxygenase domain and inhibit eNOS catalytic activity (Ju et al., 1997; Michel et al., 1997). *In vitro* manipulations further indicated that Ca<sup>2+</sup>-calmodulin may disrupt the interaction between eNOS and caveolin, leading to enhanced enzymatic activity (Michel et al., 1997). N-myristoylation and post-translational palmitoylation target newly synthesized eNOS to the caveolae. Agonist-induced activation of eNOS is accompanied by depalmitoylation mediated via a calcium/calmodulin dependent acyl hydrolase. Re-formation of the caveolin-1/eNOS heterodimer is facilitated by re-palmitoylation, by a process that remains poorly understood (Weis et al., 2004).

Bucci et al. demonstrated that a chimeric peptide with a cellular internalization sequence fused to the caveolin-1 scaffolding domain was efficiently incorporated into blood vessels and endothelial cells, resulting in selective inhibition of acetylcholine-induced vasodilation and NO production (Bucci et al., 2000). Caveolin-1-deficient mice moreover, demonstrated a higher eNOS activity when eNOS signalling was measured by monitoring the response of aortic rings to various stimuli. They also have a loss of caveolin-2 protein expression, defects in the endocytosis of a known

caveolar ligand, i.e. fluorescein isothiocyanate-albumin and a hyperproliferative phenotype. Caveolin-1-deficient mouse smooth muscle cells proliferated approximately 2-fold faster and to higher saturation densities than their wild-type counterparts (Hassan et al., 2004). The reason to this observation is still to be investigated. Importantly, these phenotypic changes are reversed by recombinant expression of the caveolin-1 cDNA, indicating that caveolin-1 expression is required to stabilize the caveolin-2 protein product, to mediate the caveolar endocytosis of specific ligands, to negatively regulate the proliferation of certain cell types and to provide tonic inhibition of eNOS activity in endothelial cells (Razani et al., 2001).

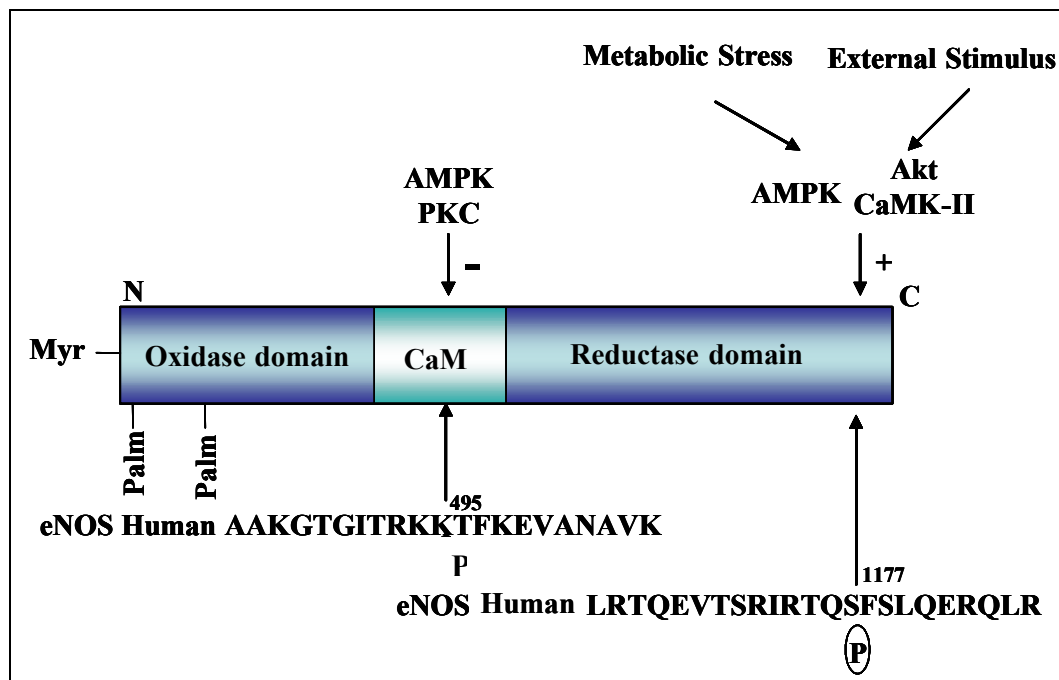
There is also evidence of other protein-protein interactions that modify eNOS function. Ju and colleagues have demonstrated that the activity of the enzyme is negatively regulated by its association with the C-terminal domain of G protein-coupled receptors such as the bradykinin B2 receptor, which is mobilized to the caveolae upon agonist stimulation (Ju et al., 1998). B2 receptors are coupled to the activation of recombinant eNOS expressed in adventitial fibroblasts (Tsutsui et al., 2000). In addition, stimulation of endothelial cells by histamine, vascular endothelial growth factor (VEGF), or shear stress leads to the binding of heat shock protein 90 (**hsp90**) to eNOS, which causes allosteric activation of the enzyme (Brouet et al., 2001). Furthermore, yeast two-hybrid screening has recently identified a novel 34-kDa protein designated **NOSIP** (eNOS interacting protein) that binds to the C-terminal region of the eNOS oxygenase domain and promotes translocation of the enzyme from caveolae to intracellular sites, resulting in the attenuation of NO production (Dedio et al., 2001)

*Post-translational Regulation*

In addition to modulation by protein-protein interaction, multiple signal transduction pathways converge to regulate eNOS by a **phosphorylation process**. The activation of the enzyme in response to multiple hormone agonists such as estradiol, bradykinin and VEGF occurs in association with elevations in cytosolic calcium concentrations (Goetz et al., 1999; Prabhakar et al., 1998; Papapetropoulos et al., 1997). In contrast, eNOS activation by shear stress and isometric vessel contraction occurs independently of changes in intracellular calcium levels (Fisslthaler et al., 2000; Fleming et al., 1999). Shear stress-induced enzyme activation is regulated by potassium channels, and it is prevented by tyrosine kinase inhibition, indicating that the process also entails tyrosine phosphorylation (Fleming et al., 1998; Fleming et al., 1997; Thorsgaard et al., 2003).

In addition to regulation by calcium and via tyrosine phosphorylation, multiple protein kinases modify eNOS activity through effects on serine phosphorylation **at position 1177 in human endothelial cells** and serine phosphorylation at 1179 in bovine endothelial cells. These kinases include AMP-activated protein kinase, PKC, cAMP-dependent protein kinase (PKA) and the serine/threonine kinase Akt, which is also known as protein kinase B (PKB). Factors that activate eNOS through PKB/Akt-mediated phosphorylation of Ser-1177 include estradiol (Haynes et al., 2000), shear stress (Dimmeler et al., 1999), VEGF (Dimmeler et al., 2000) and insulin (Montagnani et al., 2001). In contrast to the activation that occurs with phosphorylation of Ser-1177, phosphorylation of the threonine at position 497 yields attenuated eNOS activity (Harris et al., 2001). There seems to be evidence of coordinated regulation of eNOS activity by agonists such as VEGF, which cause both phosphorylation of Ser-1177 and dephosphorylation of Thr-497 (Michell et al., 2001).

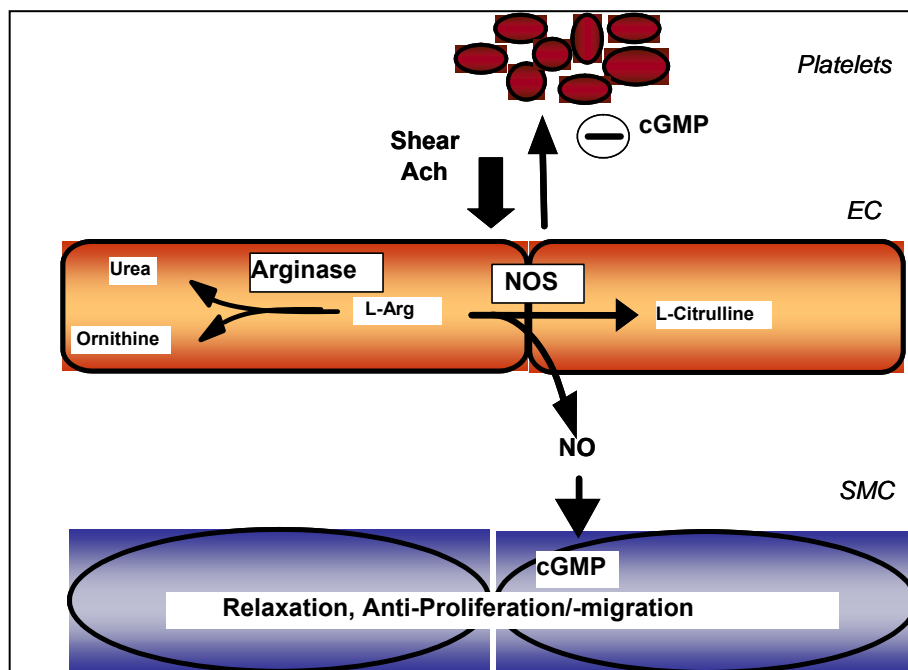
Recent work has further demonstrated that PKA signaling leads to eNOS phosphorylation at Ser-1179 in bovine endothelial cells and dephosphorylation of Thr-497, thereby enhancing enzymatic activity, whereas PKC promotes both the dephosphorylation of Ser-1177 and the phosphorylation at Thr-497, resulting in attenuated enzyme activity (Michell et al., 2001). Furthermore, the dephosphorylation processes are mediated by phosphatases PP2A and PP1 acting selectively at Ser-1177 and Thr-497, respectively. Thus eNOS activity is regulated by a complex combination of protein-protein interactions and signal transduction cascades involving regulation and phosphorylation events (**Figure 10**).



**Figure 10: Regulation of eNOS enzymatic activity by phosphorylation.** A number of protein kinases cause activation or inhibition of eNOS indicated by a plus or minus, respectively, by phosphorylating Ser1177 or Thr495. Palm = palmitoylation; Myr = myristoylation, CaM = CaM-binding domain, N = amino terminus, C = carboxyl terminus, P = phosphorylation. (from Michell BJ, Curr. Biol. 12, 845-848).

### Arginine Substrate and NO Production

L-arginine is the major substrate of eNOS for NO production. Emerging evidence demonstrates that **arginase**, an enzyme in the urea cycle, competes with NOS for the substrate L-arginine and thus reduces NO production as been shown in macrophages and vascular endothelial cells (Hallemesch et al., 2002, Chang et al., 1998) (**Figure 11**). This mechanism has been recently implicated in diabetic erectile dysfunction, aging-associated endothelial dysfunction, allergen-induced deficiency of NO and airway hyperresponsiveness in asthma, pulmonary artery hypertension and aortic coarctation-induced hypertension (Bivalacqua et al., 2001; Berkowitz et al., 2003; Meurs et al., 2002, Xu et al., 2004; Zhang et al., 2004). Two types of mammalian arginase namely arginase I and II encoded by different genes exist (Vockley et al., 1996).



**Figure 11: Role of arginase in the modulation of NO production. Arginase hydrolyses L-arginine (L-Arg) to L-ornithine and urea; thereby decreasing intracellular availability for NO production. Ach = acetylcholine, EC = endothelial cells, SMC = smooth muscle cells, ⊖ = inhibition.**



Arginase I, located in the cytoplasm, is expressed most abundantly in the liver, while arginase II is a mitochondrial enzyme and is mostly expressed in extrahepatic tissues (Jenkinson et al., 1996; Gotoh et al., 1997; Morris et al., 1997). Recent studies demonstrate that the activity of arginase could be regulated in many cell types including vascular endothelial cells, smooth muscle cells and macrophages by various cytokines.

Depending on cell types studied, activity or gene expression of the two isozymes are either constitutively expressed or induced, which regulates NO production. In porcine coronary and rat aortic endothelial cells, arginase I is constitutively expressed (Zhang et al., 2001; Buga et al., 1996), whereas arginase II is induced in response to lipopolysaccharide (Buga et al., 1996). In human diabetic corpus cavernosum, arginase II expression is significantly increased and inhibition of this enzyme enhanced NO-dependent relaxation of penile corpus cavernosum smooth muscle (Bivalacqua et al., 2001). These observations suggest a potential role of arginase II in negative regulation of NO production. However, the exact type of the isozymes expressed in human vascular endothelial cells has not yet been elucidated. Moreover, the regulatory mechanisms of arginase activity in endothelial cells and in atherosclerotic blood vessels are unknown.

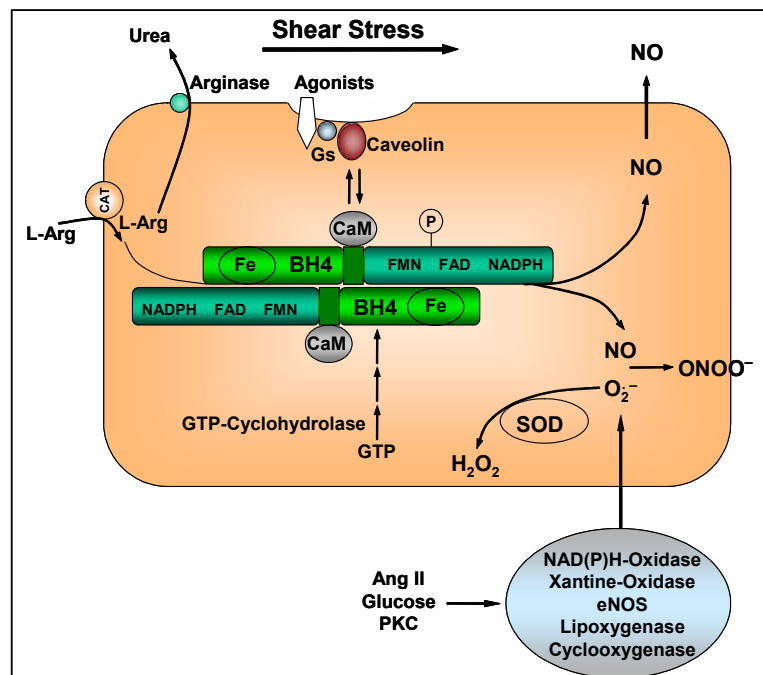
#### ***2.4.3. Reactive Oxygen Species and NO Bioavailability***

The principle source of superoxide anion production in the vascular endothelial cells and smooth muscle cells seems to be **NAD(P)H oxidases and xanthine oxidase**. NAD(P)H oxidases are expressed in endothelial cells as well as in SMC (Griendling et al., 2000; Rajagopalan et al., 1996) and regulated by various factors. The activity and expression of subunit p22-phox have been shown to be regulated by angiotensin II (Laursen et al., 1997). Studies have shown that in cultured

vascular smooth muscle cells angiotensin II markedly increases NADH and NADPH oxidase activity (Rajagopalan et al., 1996). Vessels from rats made hypertensive by chronic infusion of angiotensin II, produce increased amounts of superoxide, show increased NADH/NADPH oxidase activity and have abnormal endothelium-dependent vasodilation (Ushio-Fukai et al., 1996), which can be improved by treatment of the animals with superoxide dismutase (SOD). Under certain conditions, NOS may generate superoxide instead of NO, a process called NOS uncoupling (i.e., uncoupling of NADPH oxidation and NO synthesis) (Pou et al., 1992). In spontaneous hypertension and aging, the production of NO is increased, but inefficient, due to increased inactivation. In salt-induced hypertension NO production may be impaired. In atherosclerosis, an enhanced degradation of NO by superoxide radicals may explain the reduced endothelium-dependent relaxations. Superoxide generation by eNOS is mediated via the heme group of its oxygenase domain (Stroes et al., 1998) and is dependent on the presence of its substrate, arginine and its cofactor, BH<sub>4</sub> (Vasquez-Vivar et al., 1998; Wever et al., 1997). When there is an abundance of both factors, eNOS produces NO. When the concentration of one of these factors is relatively low, eNOS generates superoxide.

Indeed, there are other important sources of superoxide anion generation. In human blood vessels, the NAD(P)H oxidase system is the principal source of superoxide and is functionally related to clinical risk factors and systemic endothelial dysfunction. Furthermore, the C242T polymorphism in the NAD(P)H oxidase p22phox subunit is associated with significantly reduced superoxide production in patients carrying the 242T allele, suggesting a role for genetic variation in modulating vascular superoxide production (Cahilly et al., 2000).

In vessels from patients with diabetes mellitus with endothelial dysfunction, NAD(P)H oxidase activity and protein subunits are significantly increased compared with matched non-diabetic vessels (Endemann et al., 2004). Furthermore, the vascular endothelium in diabetic vessels is a net source of superoxide rather than NO production, due to dysfunction of endothelial NO synthase (eNOS). This deficit is dependent on the eNOS cofactor, BH<sub>4</sub>, and is in part mediated by protein kinase C signalling. These studies suggest an important role for both the NAD(P)H oxidases and endothelial NOS in the increased vascular superoxide production and endothelial dysfunction in human vascular disease states. As a free radical, NO is inherently reactive and mediates cellular toxicity by damaging critical metabolic enzymes and by reacting with superoxide to form an even more potent oxidant, peroxynitrite ONOO<sup>-</sup> (Zou et al., 2004) (**Figure 12**).



**Figure 12:** Transportation of the eNOS substrate L-arginine (L-Arg) into the cells and activation of eNOS enzyme and interaction of NO with superoxide anion generated from different metabolic enzymes which produce peroxynitrite, ONOO<sup>-</sup>. Abbr: BH<sub>4</sub> = tetrahydrobiopterin, SOD=superoxide dismutase, Fe=ferum, Gs=stimulatory G proteins, CaM=calmodulin, CAT=cation transporter, AngII=angiotensin II, PKC=protein kinase C.

An increased production of  $\text{ONOO}^-$  has been demonstrated in various cardiovascular diseases such as diabetes, atherosclerosis, hypertension and aging (Sucu et al., 2003; Suarez-Pinzon et al., 2001; Ma et al., 2001; van der Loo et al., 2000).

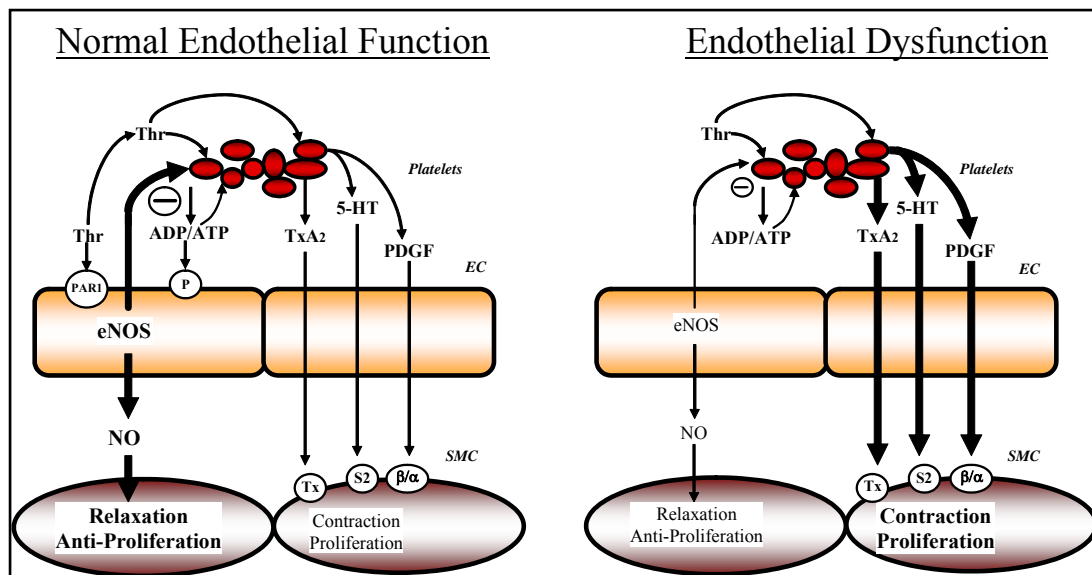
## **2.5 Anti-thrombotic and Anti-coagulative Effects of Endothelial Cells**

### **2.5.1. *Anti-thrombotic Effects of Endothelial Cells***

The intimal surface of healthy endothelium is both anti-coagulant and anti-thrombotic. The endothelial cells secrete a variety of molecules important for the regulation of blood coagulation and platelet functions. Vessel damage or exposure to certain cytokines or proinflammatory stimuli shifts the balance towards a pro-coagulant/pro-thrombotic phenotype of the endothelial cells. Many of these functions of endothelial cells could be attributed to the function of NO (**Figure 13**). Under physiological conditions, unstimulated platelets circulate in blood for approximately 10 days in a passive manner, but they possess mechanism for recognition of injury sites, presence of fissured atheromous plaques, or vessels which have been traumatized or severed to which they adhere, spread and undergo activation. Platelets release a number of vasoconstrictive factors such as thromboxane  $\text{A}_2$  and serotonin (5-HT) which possess profound effects both on platelets and vascular wall. Platelets also release potent growth factors, such as PDGF stimulating smooth muscle cell proliferation and migration leading to neointimal formation.

Platelets also stimulate NO release from the endothelium by released adenosine diphosphate (ADP) and triphosphate (ATP) (Yang et al., 1991). Hence, in the healthy arteries with intact endothelium, platelets exhibit endothelium-dependent relaxations and inhibition of platelet function at sites where platelets are activated (**Figure 13**, Yang et al., 1991); this tends to flush away and disaggregate evolving

platelet clots and thus, preventing the progression of thrombosis and inhibiting neointimal formation. In contrast, in blood vessels with injured endothelial cells such as after PTCA or in atherosclerotic arteries where NO or PGI<sub>2</sub> production is impaired or in the venous bypass grafts where the endothelium releases little amount of NO (Lüscher et al., 1988; Yang et al., 1997), platelets evoke only contractions induced by platelet-derived serotonin and TxA<sub>2</sub> on smooth muscle cells, in particular when platelets are stimulated by thrombin (**Figure 13**, Yang et al., 1991, Yang et al., 1994). Therefore, thrombin is an important amplifier of platelet-vessel wall interaction.

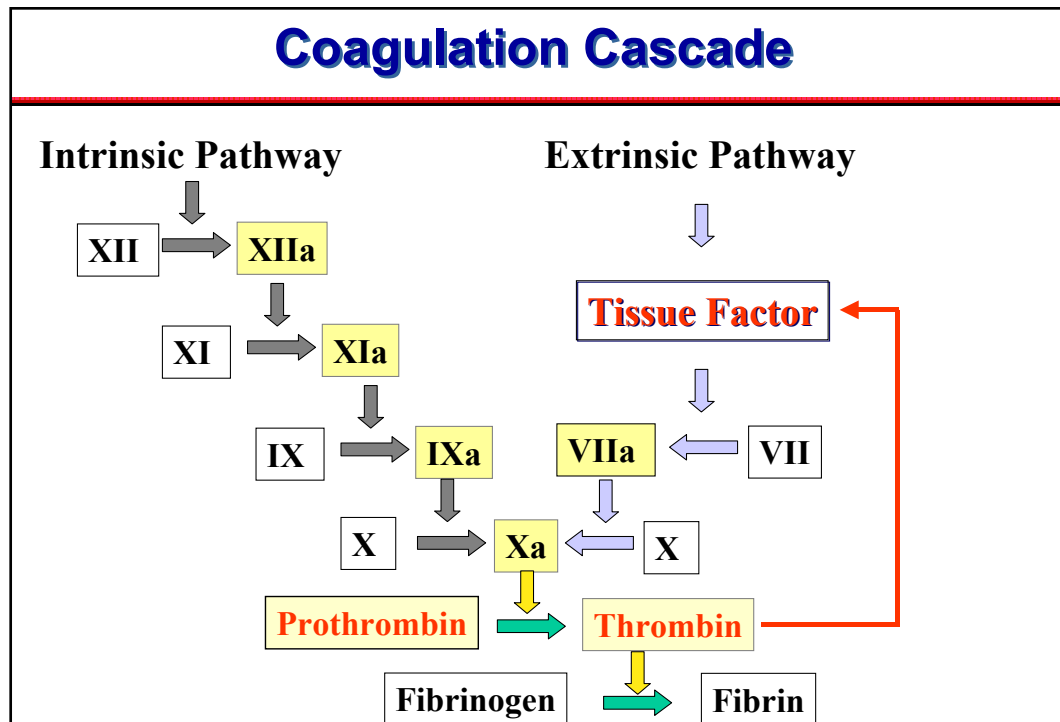


**Figure 13. Role of the endothelium in modulation of platelet-vessel wall interaction:** Under the condition of normal endothelial function (left) platelets release adenine nucleotides activating purinergic (P) receptors and thrombin activates its receptor on endothelial cells (PAR1) linked to the formation of NO. This mechanism leads to vasodilation, inhibition of platelet aggregation and inhibition of smooth muscle proliferation/migration in response to the growth factor platelet-derived growth factor (PDGF) released from activated platelets and thereby plays an important protective role to maintain local blood flow and vascular structure integrity. In contrast, under the condition of endothelial dysfunction, much less NO is produced and the contractile effects of thromboxane A2 (TxA<sub>2</sub>) and serotonin (5-HT; 5-hydroxytryptamine) released from platelets particularly after stimulation by thrombin and smooth muscle proliferation/migration in response to PDGF are dominant. ADP=adenosine diphosphate; Tx=TxA<sub>2</sub> receptor; S2=5HT<sub>2</sub>-serotonergic receptor. O=receptor. α/β = receptor subunits of PDGF.

### ***2.5.2. Pro-coagulative Effects of Activated Endothelial Cells: Production of Tissue Factor***

In the quiescent state, endothelial cells maintain blood fluidity by promoting the activity of numerous **anticoagulant pathways**, such as the protein C/protein S pathway. Activated protein C inactivates two cofactors essential for blood coagulation: factors VIIIa and Va. To be effective, protein C must form a complex with protein S, which is synthesized by endothelial cells (Stern et al., 1991; Esmon, 2000). Moreover, the endothelial cell surface is rich in heparin-like glycosaminoglycans, providing the main site for inactivation of active thrombin by providing a rich source of antithrombin, which is bound to these glycosaminoglycans. Endothelial cells also synthesize tissue factor pathway inhibitor (Kato, 2002). Finally, receptor-mediated endocytosis of factor Xa by endothelial cells participates to its elimination from blood.

The endothelium also participates to **fibrinolysis** by releasing tissue-type plasminogen activator (t-PA) and urokinase, allowing the transformation of plasminogen into plasmin, which degrades thrombi by digesting fibrin network. Unlike extravascular cells, endothelial cells do not normally express the primary trigger of the coagulation system, **tissue factor**. However, when exposed to thrombin, cytokines, or LPS, endothelial cells synthesize and express tissue factor at their surface (Nawroth et al., 1986; Mackman, 1997).



**Figure. 14:** The coagulation cascade of secondary hemostasis is traditionally divided into three parts: the final common pathway, the intrinsic pathway and the extrinsic pathway.

The coagulation factors are involved in an amplification cascade of proteolytic reactions (**Figure 14**). Small concentrations of initiator substances lead to the generation of ever-increasing quantities of the clotting factors lower down the cascade, culminating in the formation of thrombin (Schenone, 2004; Norris, 2003). Thrombin converts soluble plasma fibrinogen into molecules of fibrin which polymerise and form fibrin clot. Fibrin enmeshes the platelet aggregates at the sites of vascular injury and stabilises the haemostatic plug. To enable the coagulation reactions to proceed, clotting factors need to bind to negatively-charged phospholipid surfaces allowing the enzymes and their substrates and cofactors to come into close proximity. Disrupted platelet membranes provide the main binding surfaces but reactions can also occur on the membranes of red and white cells and damaged blood vessel endothelial cells.

In short, the coagulation cascade is initiated by FVII which becomes activated upon binding to tissue thromboplastin (tissue factor-TF). Activated FVII directly activates FX. Activated FX with the aid of FV converts prothrombin to thrombin which is generated in insufficient concentrations to convert fibrinogen to fibrin clot. Activation of FIX is enhanced by the action of FXI activation of which is one of the positive feedback reactions of thrombin. In addition to this effect, thrombin could also induce TF expression in endothelial cells (Eto et al., 2002) amplifying the coagulation cascade.

### ***2.5.3. The Role of Tissue Factor (TF) in Cardiovascular Diseases***

In human coronary atherectomy specimens, concentrations of TF antigen and activity were found to be higher in plaques taken from patients with *de novo* angina, unstable angina or myocardial infarction as compared to stable patients or patients with re-stenosis (Misumi et al., 1998; Annex et al., 1995). In addition, thrombus was detected only in TF-positive plaques and fibrin deposition was mainly observed around the massive infiltration of TF-positive macrophages in patients with unstable angina. These findings suggest a role of TF in the progression to unstable coronary syndromes.

Many studies have investigated the induction of TF-mediated procoagulant activity in cultured human endothelial cells, and several stimulating factors have been identified including interleukins, tumor necrosis factor- $\alpha$ , endotoxin, and shear stress (Haslinger et al., 2003; Mazzolai et al., 2002; Chu et al., 2001). In some studies no or only minimal TF activity at the (apical) endothelial cell surface was detected after stimulation with tumor necrosis factor. TF expression was more prominently localized at the subendothelial matrix, at the basolateral surface, or it remained intracellular in the endothelial cells. This suggests a tightly regulated TF expression in endothelial



cells which may only gain access to the blood after disruption of the endothelial monolayer (Mulder et al., 1994).

Some pathways of TF expression described for monocytes–macrophages and/or vascular smooth muscle cells, have also been identified in endothelial cells. Oxidized low-density lipoprotein is a potent stimulus for TF expression in cultured human endothelial cells (Cui et al., 1999; Wang et al., 2000). Ligation of the CD40 receptor (expression of which might be upregulated after exposure with interferon- $\gamma$ ) by T cells or activated platelets increases TF expression on endothelial cells (Slupsky et al., 1998). Platelets have also been shown to stimulate TF production in human endothelial cells (Becker et al., 2000). Therefore, **TF expression in activated endothelial cells may importantly contribute to the atherothrombotic events in patients with coronary artery disease.**

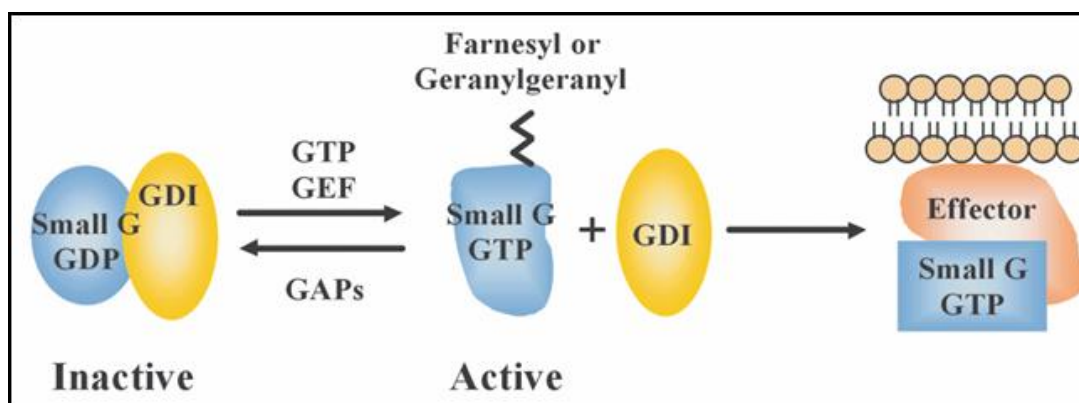
#### ***2.5.4. Regulatory Mechanisms of Endothelial TF Expression***

Intracellular regulatory mechanisms of endothelial TF expression have not been completely elucidated. Recent studies have demonstrated that endothelial TF expression is **positively regulated** by the small G-protein, **RhoA** (Eto et al., 2002; Ishibashi et al., 2003) also by the serine/threonine kinase **p38<sup>mapk</sup>** in different vascular cell types (O'Reilly et al., 2003; Eto et al., 2002; McGilvray et al., 2002). Casani et al. has demonstrated recently that in lipopolysaccharide-stimulated monocytes TF expression was blunted partially by the inhibition of the translocation of RhoA to the platelet membrane (Casani et al., et al., 2004). There are also demonstrations of a **reciprocal relationship** between procoagulant activity and the **PI3K-Akt signaling** pathway or a negative regulation by Akt (Blum et al., 2001; Kim et al., 2002).

The small G protein RhoA exerts a wide spectrum of functions including regulation of gene expression, cell proliferation, cell migration, cytoskeletal

rearrangement, and intracellular vesicle trafficking and protein nucleocytoplasmic transportation, acting as a molecular switch (Matozaki et al., 2000), cycle between inactive GDP-bound and active GTP-bound forms (**Figure 15**).

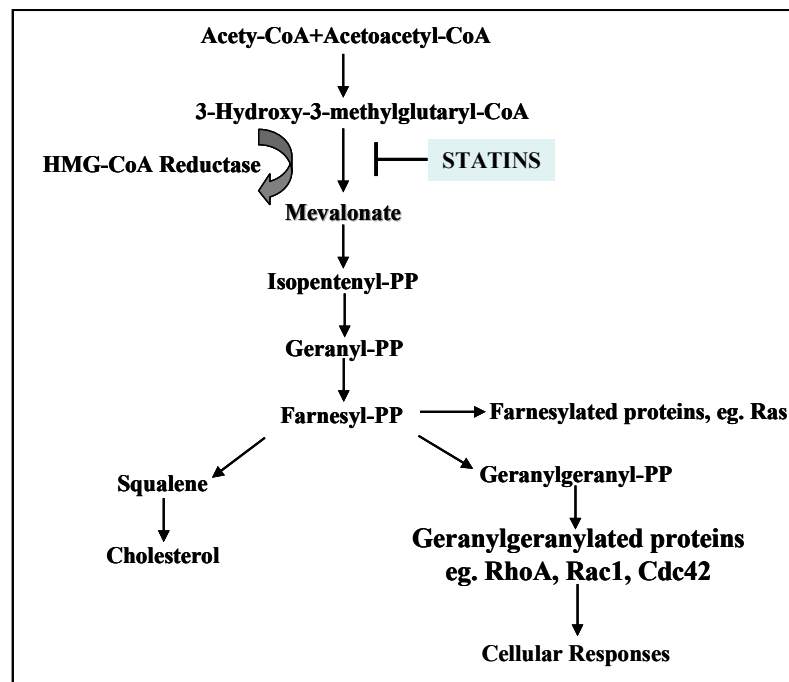
The exchange of GDP from the inactive form for GTP is on one hand stimulated by protein regulators named guanine nucleotide exchange factors (GEFs) upon stimulation by various agents or hormones (i.e. growth factors, cytokines, integrins and G-protein-coupled receptor ligands). On the other hand, the GDP/GTP exchange reaction is inhibited by another type of regulator namely GDP dissociation inhibitors (GDI) that prevent GDP dissociation from the GDP-bound form and keep the small G protein inactive.



**Figure 15: General model of regulation of small G-protein activity.** Abbr: GDP=guanosine diphosphate, GTP=guanosine triphosphate, GDI=GDP-dissociation inhibitor, GAPs=GTPase-activating proteins, GEF= Guanine nucleotide exchange factor.

Multiple downstream effectors of RhoA have been identified. They serve as important signal transduction molecules transmitting extracellular signal into the cells and regulating multiple cellular functions and also plays a major role in the regulation and control of cardiovascular diseases. Activated RhoA, for example associates with the membrane where it interacts with the Ser/Thr kinase, **Rho-kinase** to initiate signalling cascades regulating cellular responses. The binding of RhoA.GTP to Rho-kinase leads, through a conformational change to autophosphorylation and activation

of the kinase (Kaibuchi et al., 1999). Activation of Rho-kinase leads to smooth muscle contraction, cell migration and cancer metastasis via the regulation of myosin II and myosin light chain phosphatase (Somlyo et al., 2000). Many of the vascular protective effects of HMG-CoA reductase inhibitors or statins are attributed to inhibition of RhoA, which are independent of cholesterol synthesis (**Figure 16**). Inhibition of mevalonate (an important precursor in the biosynthesis of cholesterol) synthesis by statins prevents the synthesis of important isoprenoid intermediates such as geranylgeranylpyrophosphate (GGPP) which is important in the activation of RhoA (**Figure 16**).



**Figure 16:** Inhibition of HMG-CoA reductase by statins has multiple downstream effects, including inhibition of cholesterol biosynthesis and of isoprenoid intermediates such as Farnesyl-pyrophosphate and Geranylgeranyl-pyrophosphate. Geranylgeranylation and farnesylation of proteins such as the small GTPases RhoA, Rac and Ras are important for activation of the signalling molecules. Abbr: PP= pyrophosphate.

## 2.6 High-Density Lipoprotein (HDL) and Cardiovascular Protection

### 2.6.1. Epidemiological Evidence for the Protective Effects of HDL

Prospective epidemiological studies have consistently demonstrated that plasma HDL cholesterol concentration is inversely related to the incidence of coronary artery disease (CAD). The Framingham Heart Study reveals that HDL-C levels are a strong, graded and independent cardiovascular risk factor. The protective effects of an elevated HDL-C level persist until age 80 years (Gordon et al., 1977). Conversely, a low HDL-C level is associated with increased CAD risk even with "normal" ( $<5.2$  mmol/L) total plasma cholesterol levels.

Coronary disease risk is increased by 2% in men and 3% in women for every 1 mg/dL (0.026 mM) reduction in HDL cholesterol, and in the majority of studies, this relationship remains statistically significant after adjustment for other lipid and non-lipid risk factors (Maron DJ, 2000). The ability of HDL-cholesterol to predict CAD independently of LDL-cholesterol is exemplified by data from the Prospective Cardiovascular Münster (PROCAM) study, where incidence of CAD rises in relation to both decreasing HDL levels, as well as increasing LDL levels (Assmann et al., 1992). Incidence of myocardial infarction in 10 years is reported to be significantly higher according to baseline triglyceride level and low-density lipoprotein cholesterol (LDL-C) level in patients with a low high-density lipoprotein cholesterol (HDL-C) level ( $<45$  mg/dL) in the PROCAM study (**Figure 17**). Therefore, low levels of HDL ( $<0.9$  mmol/l) have specifically been related to an increase in coronary mortality, particularly in diabetic subjects, and increased risk of death due to CAD, whether total cholesterol concentration is below or above 5.2 mmol/l. Despite the strong epidemiological link between reduced HDL levels and the incidence of CAD, it is

unclear whether HDL plays a direct or indirect role in the pathogenesis of atherosclerosis.

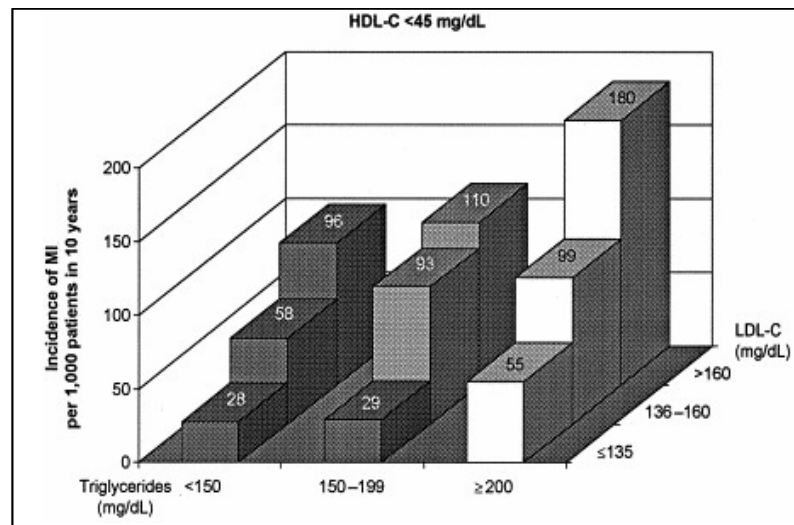


Figure 17: Incidence of myocardial infarction (MI) in 10 years according to baseline triglyceride level and low-density lipoprotein cholesterol (LDL-C) level in patients with a low high-density lipoprotein cholesterol (HDL-C) level (<45 mg/dL) in the Prospective Cardiovascular Münster study. There were 206 coronary events in 2,490 men aged 35 to 65 years (Assmann G; Am J Cardiol. 2001).

#### 2.6.2. Cardiovascular Protective Effects of HDL

The cardioprotective effects of HDL are attributed mainly to the two categories of functions that are the **reverse cholesterol transport** and **cholesterol-independent effects** (Figure 18).

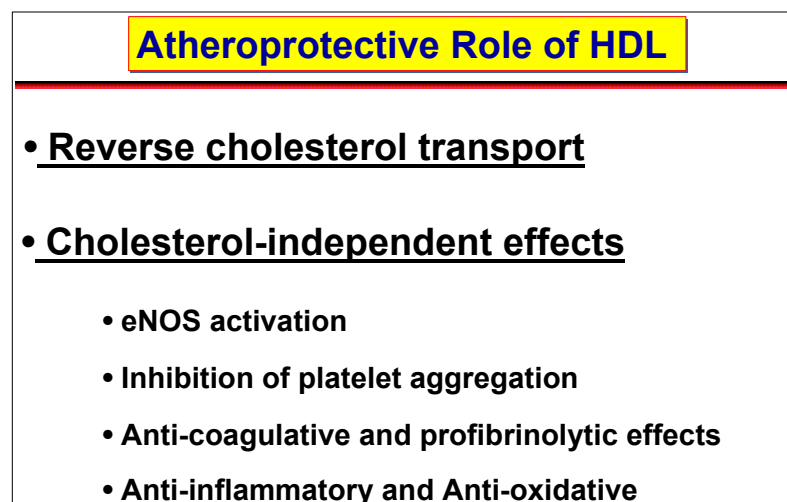


Figure 18: General mechanisms of HDL on vascular function.

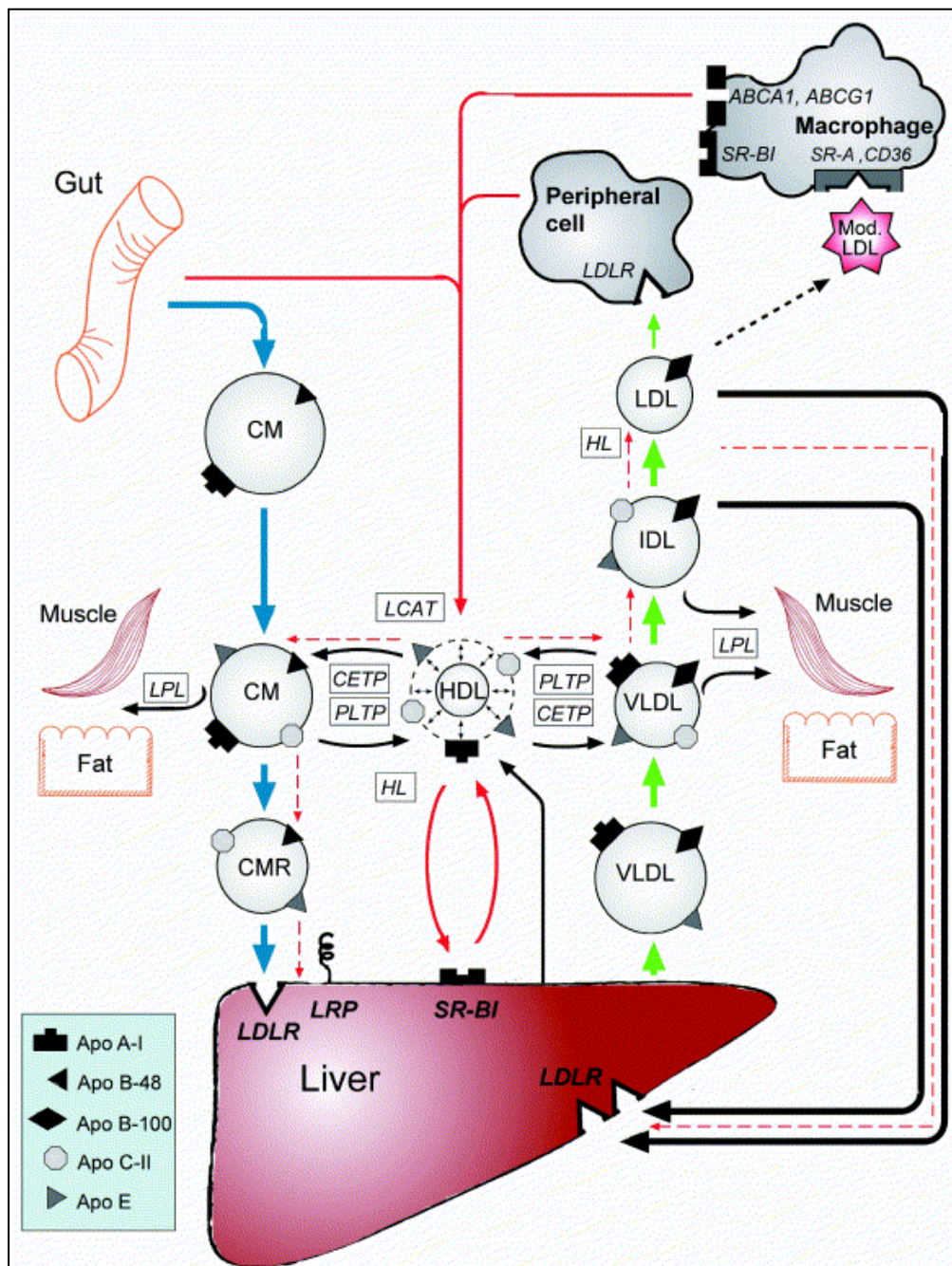
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*Cholesterol-lowering Effect: The Reverse Cholesterol Pathway (RCT)*

The metabolism of HDL involves sophisticated dynamics in its own pathway as well as balances with other lipoproteins. RCT appears to be the primary mechanism by which excess peripheral cholesterol is delivered back to the liver for disposal as bile salts or biliary cholesterol and to steroidogenic organs for the conversion into steroid hormones.

The RCT pathway regulates the formation, conversion, transformation, and degradation of HDL (Rader et al., 2003) (**Figure 19**). Excess cholesterol in the macrophages results in the upregulation of the ATP-binding cassette A1 (ABCA-1) transporter, which picks up the free cholesterol and phospholipids within the cells and transports them to cell surface. The hepatically secreted apoA-I becomes associated with phospholipids and forms the discoidal pre- HDL, the nascent form of HDL in plasma. This form of HDL, also termed lipid-poor ApoA-1, is involved in the removal of cholesterol and phospholipids from the peripheral tissues.

Inadequately lipidated ApoA-1 and nascent HDL are rapidly catabolized and cleared from serum, resulting in hypoalphalipoproteinemia. Cellular exposure to ApoA-1 increases the delivery of vesicles enriched with cholesterol from the Golgi apparatus to the plasma membrane, resulting in increased cholesterol efflux.



**Figure 19: The reverse cholesterol transport contributes to atheroprotective effects of HDL.** Abbr: CM = Chylomicron, LPL = lipoprotein lipase, CMR = chylomicron remnant, LDLR = LDL receptor, LRP = LDL related protein, SR-B1 = scavenger receptor class B type 1, SR A = scavenger receptor A, HL = hepatic lipase, PLTP = phospholipid transfer protein, CETP = cholesterol ester transfer protein, IDL = intermediate density lipoprotein, ABCA1/G1 = ATP-binding cassette A1/G1 transporter, Apo A1 = apolipoprotein A1.

Free cholesterol on the surface of HDL is esterified by the activity of lecithin: cholesterol acyltransferase (LCAT), forming larger HDL particles. Lipid-free apo A-I or lipid-poor pre- $\beta$ -HDL particles produced in the intestine or liver or shed during lipolysis of triglyceride-rich lipoproteins initiate efflux of phospholipids and cholesterol from cell membranes in a process facilitated by phospholipids transfer protein (PLTP).

The HDL particles finally transfer cholesterol to the liver by at least two distinct processes (**Figure 19**): through an exchange of cholesteryl ester for triglycerides in an interaction with triglyceride-rich lipoproteins, mediated by cholesteryl ester transfer protein (CETP) or through docking to the scavenger receptor Class B type 1 (SR-B1), expressed on the hepatocyte cell membrane. Cholesteryl esters readily move to the core of HDL particles, producing a steady gradient of free cholesterol and enabling HDL to accept cholesterol from various donors. The reciprocal exchange of cholesteryl ester for triglycerides mediated by CETP moves the bulk of the cholesteryl esters to lipoprotein remnant particles, which are subsequently cleared by the liver.

At the same time, HDL becomes enriched with triglycerides, which are substrates for hepatic lipase (HL). The concerted action of CETP-mediated cholesteryl ester transfer and HL-mediated hydrolysis of triglycerides and phospholipids helps to form the smaller HDL particles that are the preferred binding partners for SR-B1, the major HDL receptor on hepatocytes. The binding of HDL with SR-B1 mediates the selective uptake of cholesteryl esters that have not undergone CETP-mediated transfer to apo B-containing particles (intermediate-density lipoprotein [IDL] and low-density lipoprotein [LDL]). These Apo B containing particles are also taken up by peripheral tissues such as fat or muscles by

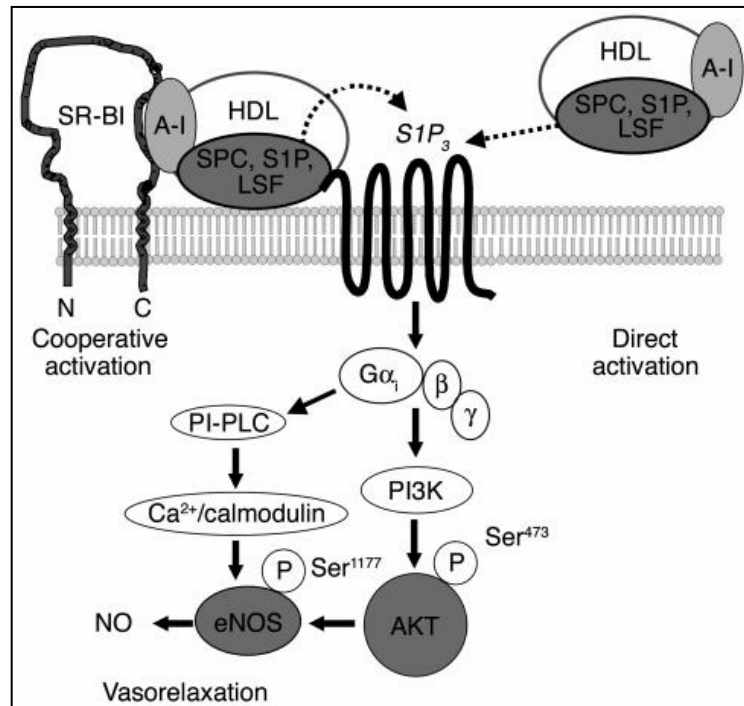


the action of lipoprotein lipase (LPL). Finally, by hydrolyzing triglyceride and phospholipids, HL regenerates smaller HDL and lipid-free ApoA-I, which in turn are rapidly reloaded with cellular cholesterol and phospholipids to form new HDL particles.

### *Cholesterol Independent Effects*

As shown in **Figure 18**, the pleiotropic effects or cholesterol independent effects of HDL on protection of vascular function have been explored in recent years. Much interest has been focused on the endothelial function in the most recent years. HDL exerts multiple biological actions on the vascular endothelium. Incubation of cultured endothelial cells with HDL activates eNOS in a process that involves the binding of ApoA-I to the SR-BI (Yuhanna et al., 2001). The mechanism by which HDL activates eNOS is not clear.

As mentioned previously, the enzymatic activity of eNOS is regulated by a variety of mechanisms, including membrane localization, intracellular calcium and ceramide, and phosphorylation (Shaul et al., 2002). On one hand, HDL interaction with SR-BI modifies membrane cholesterol distribution and morphology, thus potentially influencing eNOS activity. This same interaction leads to an intricate activation of kinases (tyrosine kinase, PI3K, Akt and mitogen-activated protein kinase, MAPK) cascades and ultimately resulting in eNOS phosphorylation and activation (**Figure 20**) (Li et al., 2002; Mineo et al., 2003). HDL also enhances eNOS expression in cultured human endothelial cells (Kuvin et al., 2002).



**Figure 20: Model of HDL-induced eNOS activation and vasodilation by the lysophospholipid receptor S1P<sub>3</sub>.** Abbr: SR-B1=scavenger receptor class B type 1; A-I=ApoA-I; SPC=Sphingosylphosphorylcholine; S1P=Sphingosine-1-phosphate; LSF=Lysosulfatide; PI-PLC=phosphatidylinositol-specific phospholipase C; NO=Nitric oxide; G=G-proteins; S1P<sub>3</sub>=lysophospholipid receptor; N and C, amino- and carboxy-terminus of SR-B1. (Nofer JR et al. 2003).

*In vivo* studies provide additional support to the concept that HDL prevents endothelial dysfunction by promoting endothelial NO production. Early investigations by quantitative coronary angiography and intravascular ultrasound analysis showed a positive correlation between plasma HDL concentration and NO-dependent coronary vasodilation. A short-term treatment with niacin in patients with low HDL causes an elevation of plasma HDL with a parallel increase of NO-mediated vasodilation. Even more striking, the intravenous infusion of reconstituted HDL (rHDL) in hypercholesterolemic subjects rapidly restores the altered endothelium-dependent vasodilation by increasing NO bioavailability. (Spieker et al., 2002; Bisioendial et al., 2003) Direct inhibition of platelet aggregation by HDL or rHDL has also been shown (Aviram et al., 1983; Nofer et al., 1998; Higashihara et al., 1991). HDL has been

known to inhibit activation of coagulation factor X by factor VIIa and TF purified from placenta (Carson et al., 1981). These studies suggest a modulatory role of HDL or rHDL in hemostasis. HDL was also reported to enhance the anticoagulant activity of activated protein C and protein S. The observation that the infusion of rHDL into human volunteers limits the procoagulant state associated with endotoxemia supports the concept that HDL may exert a significant anticoagulant effect in vivo in humans. Other pleiotropic effects of HDL such as anti-inflammatory and anti-oxidative effects (**Figure 18**) have also been reported.

## **2.7 Aims of the Thesis**

The thesis aims to investigate

- (1) the potential role of RhoA/ROCK pathway in the regulation of eNOS gene expression and enzymatic activity and the underlying mechanism
- (2) the role of RhoA/ROCK in endothelial TF expression
- (3) the effects of HDL on eNOS activation and TF expression and the underlying mechanisms.

## II. MATERIAL AND METHODS

### 1. Materials

Thrombin was from Calbiochem; L- $\alpha$ -phosphatidyl-L-serine, L- $\alpha$ -phosphatidylinositol 4,5-diphosphate sodium salt and platelet derived growth factor-BB (PDGF-BB) from Sigma; Redivue [ $\gamma$ - $^{32}$ P]ATP and L-[U- $^{14}$ C] arginine monohydrochloride from Amersham; Thin-layer-chromatography (TLC) plates (silica-60, WF<sub>2545</sub>) from Merck; endothelial cell growth supplement (ECGS) from PromoCell GmbH; anti-eNOS monoclonal antibody from BD Transduction Laboratory company; anti-human tissue factor from American Diagnostica, anti-RhoA monoclonal antibody, protein A/G PLUS-agarose and Glutathione-agarose from Santa Cruz Biotechnology, Inc.; anti-p38<sup>mapk</sup>, anti-phospho-p38<sup>mapk</sup>, anti-phospho-GSK3, anti-Akt, anti-Phospho-Akt (Ser473 and Thr308) antibodies, anti-phospho-eNOS (Ser1177) antibody and Akt kinase assay kit from Cell Signaling Technology; anti-phosphotyrosine (4G10) agarose conjugate from Upstate Biotechnology; Anti-mouse and anti-rabbit IgG (H+L) alkaline phosphatase (AP) conjugate and Western Blue<sup>TM</sup> stabilized substrate for AP from Promega. The recombinant glutathione S-transferase (GST)-TRBD (Rhotekin Rho binding domain 7-89 amino acid) fusion protein was purified (Ming et al., 1998). All the cell culture materials were purchased from Gibco. Fluvastatin was provided by Novartis (Basel, Switzerland); anti-RhoA monoclonal antibody from Santa Cruz Biotechnology, Inc. (Nunningen, Switzerland); anti-arginase I antibody from BD Transduction Laboratory; anti-arginase II rabbit polyclonal antibody was generated by Eurogentech, S.A, by immunization with a synthesized peptide containing the 16 C-terminal amino acid (AA 339 –354) of human arginase II; anti- $\alpha$ -tubulin antibody from Sigma; anti-mouse and – rabbit IgG alkaline phosphatase (AP) conjugate were purchased from

Promega (Wallisellen, Switzerland); BCIP/NBT solution was purchased from Upstate Biotechnology Inc. (Lucerne, Switzerland). Endothelial cell growth supplement (ECGS) pack from PromoCell GmbH (Allschwill, Switzerland); all cell culture media and materials were purchased from Gibco BRL (Basel, Switzerland).

## **2. Animals and Organ Chamber**

13 male apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice and 13 male C57BL/6J wild type control mice (4-weeks-old) were obtained from Jackson Laboratory (USA). To accelerate lesion formation, animals were fed a Western type diet (Harlan TD88137, 21.2% total fat and 0.2% cholesterol) for 4 months (Barton M et al., 1998). At 5 months of age, animals were anesthetized with 7 pentobarbital (100 mg/kg i.p.), the entire aorta from the heart to the iliac bifurcation were removed, placed into cold (4°C) Krebs bicarbonate solution, dissected free from fat and adhering perivascular tissue. The isolated aorta was cut into parts as required for different analyses for protein expression by Western blot. The isolated vessel was snap-frozen in liquid nitrogen. The tissues were homogenized and sonicated in arginase lysis buffer for arginase activity assay or in extraction buffer for analysis of protein expression by Western blot (Ming et al., 2002). Protein concentration was determined by Bradford method (Bio-Rad). Animal handling and experimentation were approved by local animal ethical committee.

## **3. Vascular reactivity studies**

The isolated descending thoracic aortas with intact endothelium from wild type and ApoE<sup>-/-</sup> mice were cut into rings (3 mm in length) which were suspended in a Multi-Myograph System (Model 610M, Danish Myo Technology A/S, Denmark) (Viswambharan et al., 2003). To study the role of arginase in modulation of eNOS

function, aortic rings with endothelium in parallel were incubated with or without the arginase inhibitor L-norvaline (20 mmol/L) for 1 hour (Chang et al., 1998, Wu et al., 1998, Zhang et al., 2001, Bivalacqua et al., 2001, Berkowitz et al., 2003) and then contracted with norepinephrine (0.3  $\mu$ mol/L). L-arginine (1 mmol/L) was added on top of the contraction.

#### **4. Cultivation of Human Umbilical Vein Endothelial Cells (HUVECs)**

Endothelial cells were isolated from human umbilical veins (Eto et al., 2001). Fresh blood vessels were harvested in cold sterile phosphate-buffered saline (PBS) with antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). The vessels were incubated with collagenase type II 75 U/ml for 20 minutes in PBS. Cell pellets were then collected by centrifugation, seeded in culture dishes coated with 1% gelatin and cultured in RPMI 1640 supplemented with 20 mmol/L L-glutamine, HEPES buffer solution, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml ECGS, 25  $\mu$ g/ml heparin and 5% fetal calf serum (FCS). Endothelial cells are characterized by the typical cobblestone and non-overlapping appearance and in-direct immunofluorescence staining using specific antibodies against von Willebrand factor. Human smooth muscle cells (hSMCs) were isolated from human saphenous veins (Yang et al., 1998).

#### **5. Measurement of arginase activity**

Arginase activity in the cells and aortic tissue lysates was measured by colorimetric determination of urea formed from L-arginine (Chang et al., 2000). To prepare cell lysate for arginase assay, cells were first rinsed with ice-cold Dulbecco's PBS twice after each specified treatment and then scraped into 300  $\mu$ l of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 1  $\mu$ g/ml

leupeptin, 1 µg/ml aprotinin, and 0.1 mM PMSF. Cells were lysed by sonication at 20 kHz (B.Braun Labsonic Model 1510, Melsungen, Switzerland) for 30 s (10 s/cycle). Arginase activity in the cell lysates was measured. The cell lysate (50 µl) was added to 50 µl of Tris-HCl (50 mM; pH 7.5) containing 10 mM MnCl<sub>2</sub>. Macrophage arginase was then activated by heating this mixture at 55–60°C for 10 min. The hydrolysis reaction of L-arginine by arginase was conducted by incubating the mixture containing activated arginase with 50 µl of L-arginine (0.5 M; pH 9.7) at 37°C for 1 h and was stopped by adding 400 µl of the acid solution mixture (H<sub>2</sub>SO<sub>4</sub>:H<sub>3</sub>PO<sub>4</sub>:H<sub>2</sub>O=1:3:7). For colorimetric determination of urea - isonitrosopropiophenone (25 µl, 9% in absolute ethanol) was added, and the mixture was heated at 100°C for 45 min. After placing the sample in the dark for 10 min at room temperature, the urea concentration was determined spectrophotometrically by the absorbance at 550 nm measured with a microplate reader (BioRad Microplate Reader Model 680). The amount of the urea produced was used as an index for arginase activity.

## **6. Generation of recombinant adenoviruses (rAd) expressing different mutants.**

### ***Recombinant Adenovirus expressing PI3K and PKB mutants***

Expression plasmid encoding an active PI3K mutant (pEF-BOS-rCD2-p110) was obtained from DA Cantrell (Imperial Cancer Research Fund, London, UK). The active PI3K mutant (rCD2-p110) is a chimeric molecule in which the cytoplasmic domain of the rat CD2 (rCD2) cell-surface antigen was replaced with the catalytic subunit of PI3K, p110α. This targets PI3K catalytic subunit at the membrane and thereby confers PI3K constitutively active (**Figure 21**). Cell surface expression of the

rCD2-p110 was confirmed by flow cytometric immunofluorescence analysis with an anti-rCD2 monoclonal antibody (Reif et al., 1996).

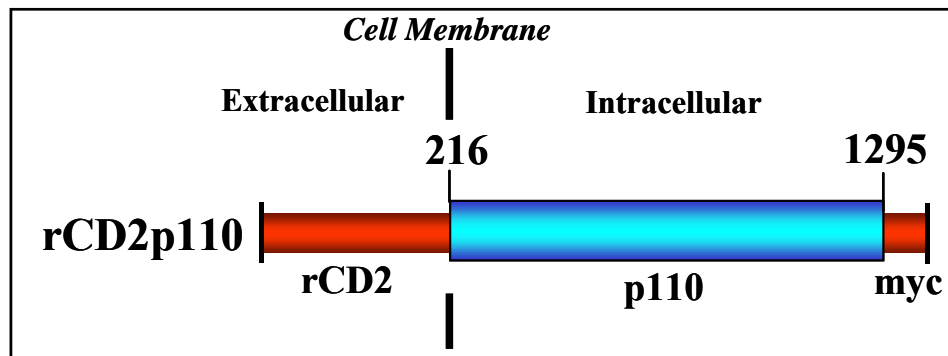


Figure 21: Schematic representation of the active mutant of PI3K employed.

pCMV constructs encoding a hemagglutinin (HA) epitope-tagged membrane-targeted active PKB (pCMV-m/p-HA-PKB $\alpha$ ) and an inactive PKB (pCMV-HA-PKB- $\alpha$ -K179A) were from Dr. B. Hemmings (Andjelkovic et al., 1997) (Figure 22).

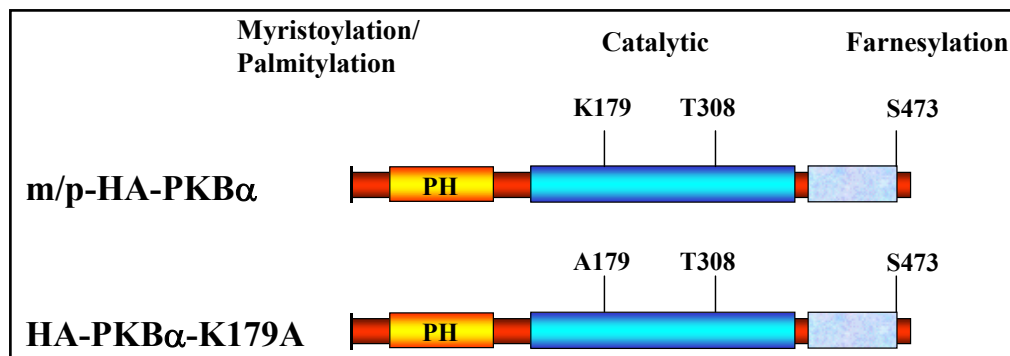


Figure 22: Schematic representation of Akt mutants employed. PH, pleckstrin homology domain. All the PKB constructs are HA epitope tagged at the N-terminus.

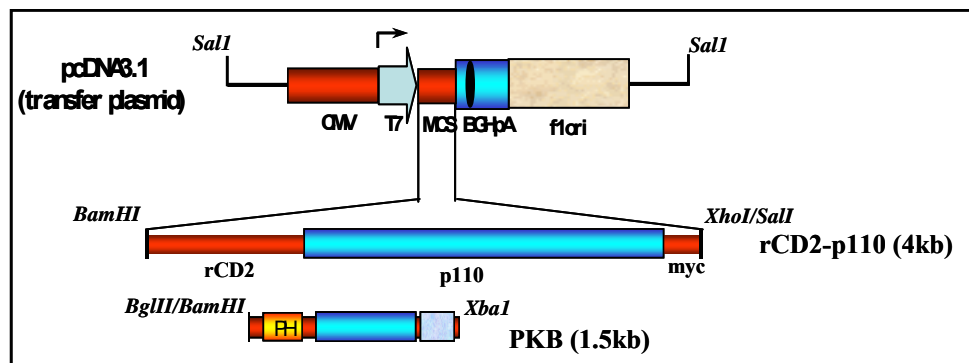
The active mutant of Akt was achieved by membrane localization of the mutant by attaching a myristoylation/palmitoylation signal at the N-terminal via its PH domain, since both of these modifications have been shown to be sufficient to localize many cytosolic proteins to the plasma membrane. Membrane-targeted PKB is constitutively active by an increased phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup>. The kinase-deficient mutant is made inactive by a point mutation in its ATP-binding site (lysine



at 179 to alanine). In this mutant the level of phosphorylation for Ser<sup>473</sup> was much lower. Membrane attachment of the mutants was confirmed by subcellular localization of proteins expressed in 293 cells by immunofluorescence using the anti-hemagglutinin (anti-HA) antibody (Andjelkovic et al., 1997).

The generation of recombinant adenovirus expressing rCD2-p110 or PKB mutants driven by the cytomegalovirus (CMV) promoter was carried out through homologous recombination between co-transformed adenoviral backbone virusmid (VmAdcDNA3,  $\Delta$ E1 $\Delta$ E3) and the corresponding adenoviral transfer plasmid (pcDNA3.1 from Invitrogen) in *E.coli* (Heider et al., 2000).

The rCD2-p110 and PKB mutants cDNA were first subcloned into the adenoviral transfer plasmid pcDNA3.1 (**Figure 23**) allowing the insertion of the transgene into the E1 region of a cloned  $\Delta$ E1 $\Delta$ E3 adenoviral backbone (vmAdcDNA3) bearing an empty expression cassette homologous to the one of the pcDNA3.1.



**Figure 23:** Subcloning ofThe rCD2-p110 and PKB mutant cDNA were subcloned into adenoviral transfer plasmids. Abbr: T7 Prom=T7 promoter.

The transfer plasmid pcDNA3.1-rCD2-p110 was then digested with SalI, and co-transformed with SwaI-linearised vmAdcDNA3 into a recombinant proficient *E.coli* strain (BJ5183) by electroporation to allow homologous recombination in BJ5183. The plasmid pcDNA3.1-m/p-HA-PKB $\alpha$  and pcDNA3.1-HA-PKB $\alpha$ -K179A were

digested with *StuI* and *SspI*, and co-transformed with *SwaI*-linearised vmAdcDNA3 into BJ5183 (Figure 24).

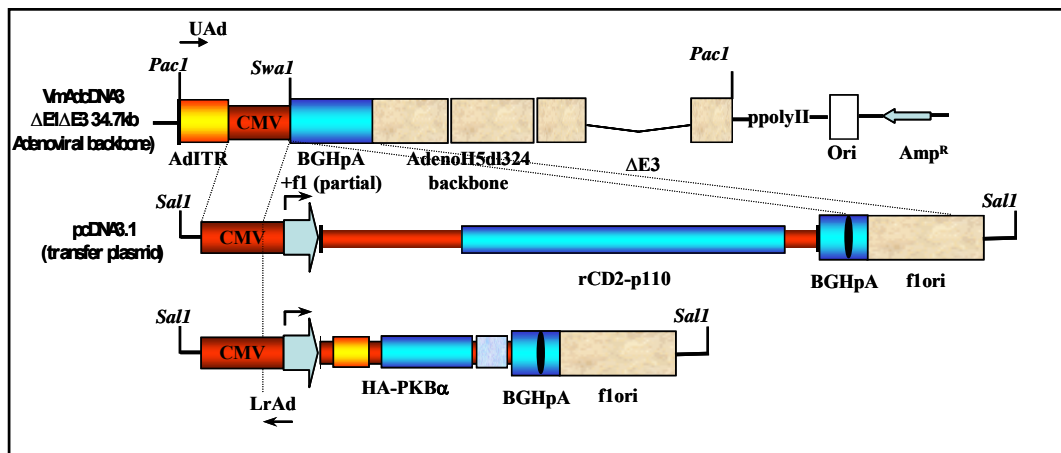


Figure 24: Generation of recombinant adenovirus expressing PI3K and PKB mutants by homologous recombination in a recombinant proficient *E.coli* strain BJ5183. Abbr: CMV=CMV promoter.

The candidate recombinants were subsequently screened by colony PCR using primer UAd and LrAd (Figure 25).

UAd sequence: 5'- GCCCAGGTGTTTTCTCAGTG -3'

LrAd sequence: 5'- ATGGGGTGGAGACTTGGAATC -3'

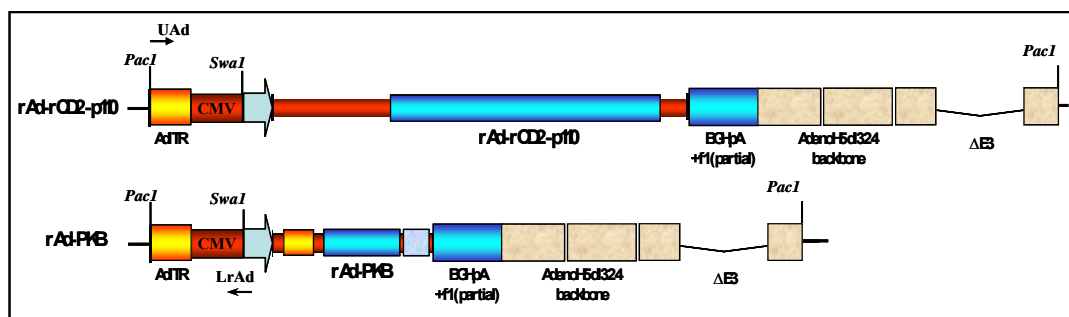


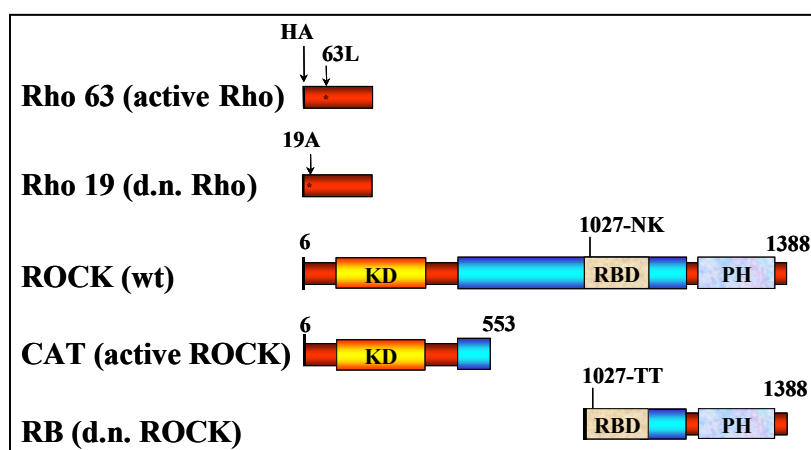
Figure 25: Candidate recombinant adenoviruses were screened by colony PCR using primer pair UAd and LrAd. Product size (opt.  $T_H$ : 57°C, 2mM  $MgCl_2$ ): 488bp.

The positive clones were then re-transformed into another *E.coli* strain HB101 allowing higher plasmid yield. The resultant recombinant virusmids were digested with *PacI* and transfected into E1-complementing packaging cells (HER911) to generate recombinant adenovirus. The primary crude lysates of the recombinant

adenoviruses were prepared as viral stocks and used to infect HER911 cells for scale up of the viral preparation. The viral titer was determined by plaque assay.

### ***Recombinant Adenovirus expressing Rho and ROCK mutants***

Expression plasmid encoding an active RhoA (pcDNA3.1-Rho63L) and a dominant negative RhoA (pcDNA3.1-Rho19N) were obtained from Dr Joseph J. Baldassare (Hu et al., 1999) (**Figure 26**). Mammalian expression vector, pEF-BOS constructs encoding a myc-tagged constitutively active ROCK (pEF-BOS-myc-CAT) and a dominant negative form of ROCK (pEF-BOS-myc-RB/PH (TT)) were from Dr. K.Kaibuchi (Amano et al., 1998).



**Figure 26:** Schematic representation of RhoA and ROCK mutants employed. Abbr: KD=kinase domain, RBD=Rho binding domain, PH=Pleckstrin homology domain, d.n.=dominant negative, wt=wild type, CAT=catalytic.

The active mutant of RhoA was made constitutively active by glutamine-to-leucine substitution at position 63, leading to structural modification of RhoA. This modification lacks an amide group due to leucine substitution. Lack of amide group at position 63 of Rho (mimicking deamination) inhibits both intrinsic GTP hydrolysis and impairs RhoGAP-mediated GTP hydrolysis (abolishing GTPase activity), thus keeping it in the active form. N19RhoA acts as a dominant negative mutant due to its

decreased binding affinity to GTP and an increased affinity to RhoGEFs, thus acting as a competitive inhibitor of the endogenous Rho (Schmidt et al., 1997).

Rho-kinase is composed of NH<sub>2</sub>-terminal catalytic coiled-coil, the inhibitory COOH-terminal portion consisting of Rho-binding (RB) and PH domains. In the resting state, the C-terminal domain interacts with the catalytic domain to maintain it inactive. Upon stimulation, the active form of Rho interacts with the RB domain, alters the conformation of ROCK and thereby, relieves the inhibition by the RB and PH domains. The active mutant of ROCK was made by deletion of the inhibitory COOH-terminal portion. This renders the NH<sub>2</sub>-terminal catalytic domain as constitutively active. The dominant negative mutant of ROCK contains only C-terminal portion consisting of the RB and PH domains, in which point mutations (Asn-1027 and Lys-1028 to Thr) were introduced to abolish the Rho-binding activity and thereby constitutively inhibit ROCK activity (Amano et al., 1999).

Generation of recombinant adenovirus expressing HA-tagged RhoA and ROCK mutants driven by the cytomegalovirus (CMV) promoter was carried out through homologous recombination between co-transformed virusmid (VmRL-CMV1) and the corresponding adenoviral transfer plasmid (pSTCO-HA.mcs) in *E. coli* (Heider et al., 2000).

The Rho and ROCK mutants cDNAs were first subcloned into the adenoviral transfer vector pSTCO-HA.mcs allowing the insertion of the transgene into the E1 region of a cloned  $\Delta$ E1 $\Delta$ E3 adenoviral backbone (vmRL-CMV1 or vmAdcDNA3, respectively) bearing an empty expression cassette homologous to the one of the pSTCO-HA.mcs (**Figure 27**).

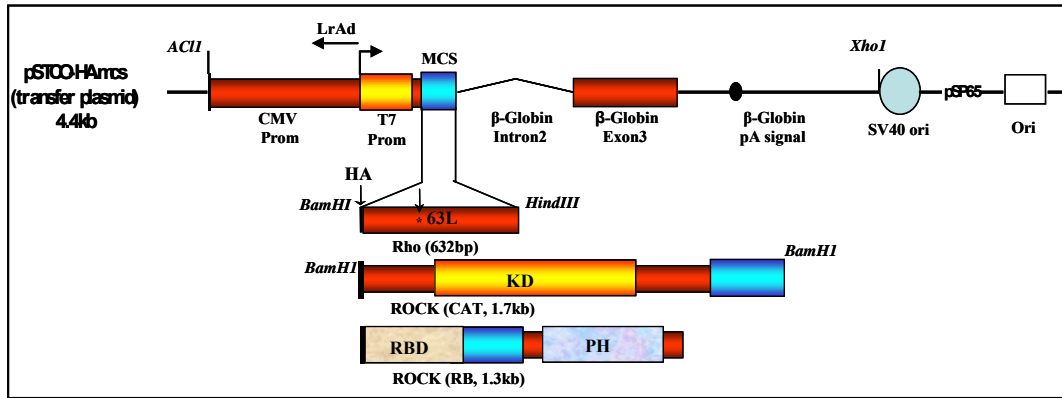


Figure 27: The Rho and ROCK mutants cDNAs were first subcloned into the adenoviral transfer vector pSTCO-HA.

The transfer plasmid pSTCO-HA-Rho63L, -Rho19N, -CAT and -RB/PH (TT) was then digested with *AclI* and *XhoI*, and co-transformed with *SwaI*-linearized vmRL-CMV1 into a recombination proficient *E.coli* strain (BJ5183) by electroporation to allow homologous recombination in BJ5183 (Figure 28).

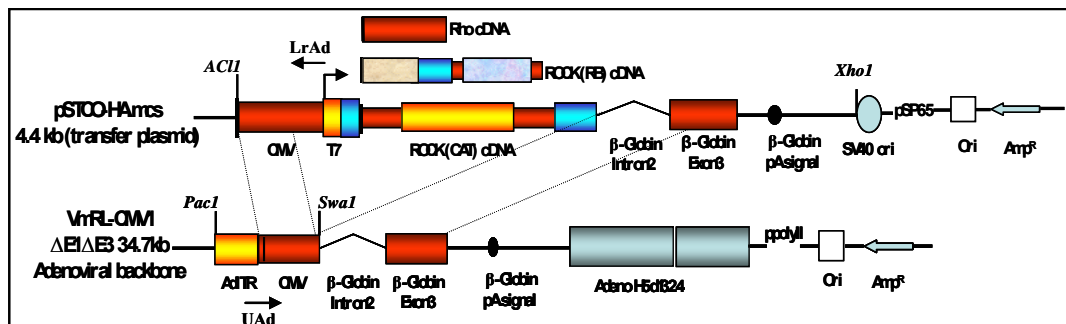


Figure 28: Expression vector pSCT1 and adenoviral backbone recipient vector Vm-CMV1. Generation of recombinant adenovirus expressing RhoA and ROCK mutants by homologous recombination in a recombinant proficient *E.coli* strain BJ5183. Abbr: CMV=CMV promoter, T7=T7 promoter.

The candidate recombinants were subsequently screened by colony PCR using primers UAd and LrAd (Figure 29). The positive clones were re-transformed into another strain HB101 allowing higher plasmid yield. The resultant recombinant virusmids were digested with *PacI* and transfected into E1-complementing packaging cells (HER911) to generate recombinant adenovirus. The primary crude lysates of the recombinant adenoviruses were prepared as viral stocks and used to infect HER911

cells for scale-up of the viral preparation. The viral titer was determined by plaque assay. The control recombinant adenovirus expressing HA-tagged LacZ and GFP (rAd-HA-LacZ and rAd-GFP) was also prepared (Heider et al., 2000).

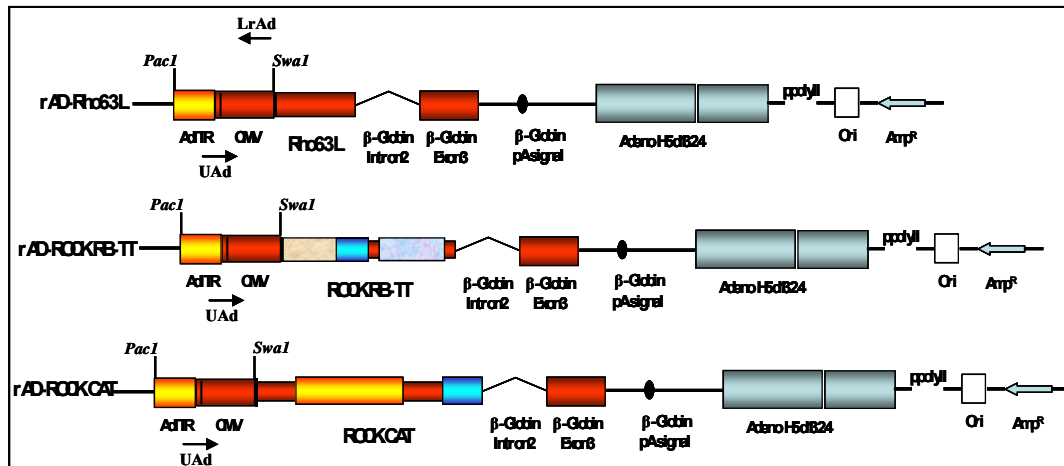


Figure 29: Resultant recombinant adenoviruses were screened by colony PCR.

### Plaque assay

Plaque assay was carried out as follows: Recombinant virus was diluted in DMEM containing 2% FCS in 10-fold serial dilution ranging from  $10^{-4}$  to  $10^{-9}$ . Diluted viral preparations were infected in duplicates to HEK293 cells cultured in 6-well plates and incubated with the same medium for 16 hours. Medium containing the virus was then aspirated and the cells were overlaid with 2 ml of agar overlay solution (0.2% agar in DMEM/2% FCS) per well. The agar was allowed to solidify at room temperature for 30 minutes and the plates were then returned to the incubator. Four days following the initial overlay another 2ml of agar overlay solution was added to the each well and incubated further till the 9<sup>th</sup> day. Plaques were counted under a light microscope in each well. The titre of the adenoviral stock was expressed as plaque forming units/ml (pfu/ml).

## **7. Adenoviral infection.**

HUVECs were maintained in complete RPMI-1640 supplemented with 5% FCS and ECGS at 37°C in an atmosphere of 5% CO<sub>2</sub> and were passaged when they reached confluence. Cells were seeded at a density of  $2 \times 10^5$  / 6 cm dish in complete RPMI-1640 supplemented with 5% FCS and ECGS. Two days later, cells were infected with the recombinant adenovirus at titers ranging from 100 to 150 MOI and further incubated in 0.2% FCS RPMI-1640 supplemented with ECGS for 18 hours or 48 hours before treatment and extraction.

## **8. Western blot (WB)**

Cell extracts were prepared by lysing cells in extraction buffer: 120 mM NaCl, 50 mM Tris, pH 8.0, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 1 mM EGTA, 1 mM sodium pyrophosphate, 30 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 1% NP-40 and 0.1 M PMSF (Ming et al., 1994). 40 µg extracts were subjected to SDS-PAGE and electrophoretically transferred to an Immobilon-P membrane (Millipore), and the resultant membrane was incubated overnight with the corresponding first antibody at 4°C with gentle agitation after blocking with 5% skimmed milk. The protein was decorated with a corresponding anti-mouse or anti-rabbit AP-conjugated secondary antibody and detected using Western Blue™ stabilized substrate. Quantification of the signals was performed using NIH Image 1.62 software. In all the plotted graphics, each point represents the average value from three to eight independent experiments.

## **9. PKB/Akt kinase assay**

PKB/Akt kinase activity was analysed by non-radioactive immunoprecipitation-kinase assay using the Akt kinase assay kit from Cell Signaling

Technology. 30 µg cell extracts (200 µl) were incubated 2 hours with immobilized Akt 1G1 monoclonal antibody. After extensive washing the kinase reaction was performed at 30°C for 30 min in the presence of 200 µM cold ATP and GSK-3 substrate. Phosphorylation of GSK-3 was measured by western blot using phospho-GSK-3 $\alpha/\beta$  (Ser21/9) antibody.

#### **10. PI3-Kinase activity assay**

Cell extracts were prepared by lysing cells on ice for 15 min by the addition of 250 µl of lysis buffer containing 20 mM Tris.HCl, 138 mM NaCl, 2.7 mM KCl, pH 8.0 supplemented with 5% glycerol, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM sodium-o-vanadate, 20 µM leupeptin, 18 µM pepstatin, 1% NP-40, 5 mM EDTA and 20 mM NaF. Cell debris and nuclei were removed by centrifugation at 10,000 x g for 10 min at 4°C, and 40 µg of the cell lysate was then immunoprecipitated with 2 µl of anti-PI3-K p85 antibody and protein A/G PLUS-agarose for 4 h at 4°C. The agarose beads containing the anti-PI3-K p85 immune complexes were washed twice with wash buffer (0.1 M Tris-HCl, pH 7.4 / 0.5 M LiCl) and twice with kinase buffer (20 mM HEPES, pH 7.4 / 5 mM MgCl<sub>2</sub>), and then resuspended in 60 µl of kinase buffer containing 10 µCi of [ $\gamma$ -<sup>32</sup>P]ATP, 60 µM ATP and 10 µg each of phosphatidylserine (PS) and phosphatidylinositol (PI). The kinase reaction was allowed to continue for 10 min at 30°C. The reaction was then stopped by addition of 200 µl of 1N HCl. The lipid reaction products were extracted with 400 µl CH<sub>3</sub>Cl/Methanol (1:1) and dried, and then resuspended in 40 µl of spotting solution (CH<sub>3</sub>Cl/Methanol 2:1) and analysed on TLC silical gel plate by using CH<sub>3</sub>Cl/Methanol/H<sub>2</sub>O/NH<sub>4</sub>OH (45:35:8.5:1.5 v/v) as the separation solvent. The product of PI3P was quantified with



a phosphorImager densitometry (Bio-Rad) by using ImageQuant software Quantity One (Bio-Rad).

### **11. Measurement of eNOS activity (L-Citrulline assay)**

eNOS activity was measured by L-citrulline assay (Heller et al., 1999). Adenovirus-infected semi-confluent HUVECs were incubated at 37°C for 30 minutes in HEPES buffer pH 7.4 (in mM): 10 HEPES, 145 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 10 glucose, 1.5 CaCl<sub>2</sub> containing 0.25% BSA. 0.5 µCi/ml L-[<sup>14</sup>C] arginine was added to each dish for one hour before the reaction was stopped with cold phosphate-buffered saline (PBS) containing 5mM L-arginine and 4mM EDTA. The cells were then denatured in 96% ethanol. After evaporation the soluble cellular components were dissolved in 20mM HEPES-Na (pH 5.5) and applied to well-equilibrated DOWEX (Na<sup>+</sup> form) column. The pellets were dissolved in western blot extraction buffer for protein concentration determination. The [<sup>14</sup>C]-citrulline content of the eluate was quantified by liquid scintillation counting. Citrulline production by the different infections was expressed in pmol/µg cellular protein obtained after subtracting the basal citrulline release. The basal release was determined from the L-Nitro-arginine methyl ester (L-NAME) 100mM, 30min pre-incubation-inhibitable radioactivity in non-infected cells.

### **12. Pull-down assay of GTP-RhoA**

The activation of RhoA was assessed by a pull-down assay (Ren et al., 1999). HUVECs were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 µg/ml each of leupeptin and aprotinin, and 1 mM PMSF). 200 µg of cell lysates were incubated with 10 µg GST-TRBD beads at 4°C

for 60 min. The beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 µg/ml each of leupeptin and aprotinin, and 0.1 mM PMSF). Bound Rho proteins were then detected by Western blot using a monoclonal antibody against RhoA (Santa Cruz Biotechnology). The total amount of RhoA in cell lysates was used as a control for the cross-comparison of Rho activity (level of GTP-bound Rho).

### **13. Statistics**

In all experiments 'n' equals the number of experiments or animals. Relaxations to stimulus are expressed as percentage of decrease in tension of the precontraction to norepinephrine (10<sup>-7</sup>M). All data is expressed as mean ± SEM. Statistical analysis was performed with unpaired t test or ANOVA with Bonferroni's post-test. Differences in mean values at p<0.005 is considered to be significant in all experiments in the study.

### III. RESULTS

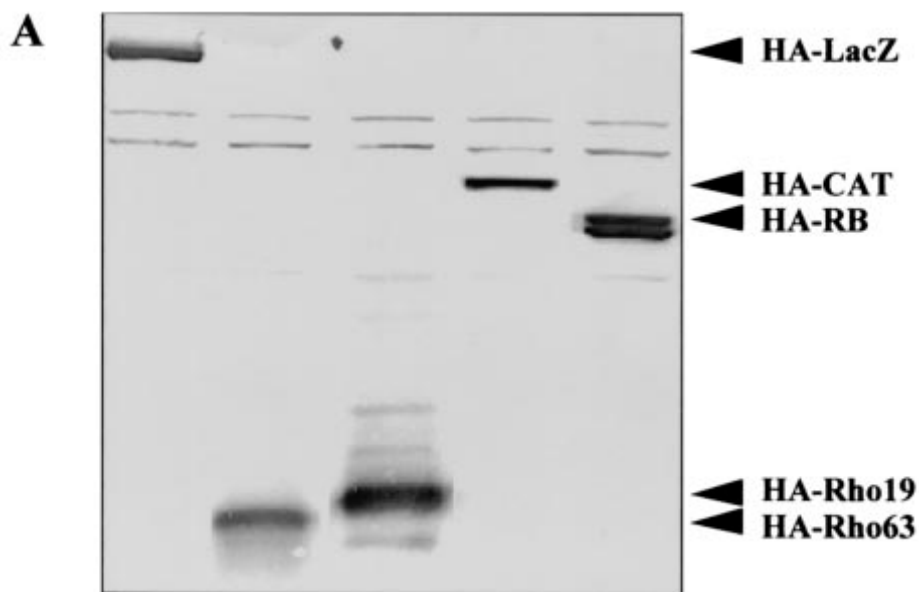
#### **Part 1: RhoA/ROCK Suppresses eNOS Gene Expression and Enzyme Activation via Inhibition of Phosphorylation.**

The **Rho family of GTPases** plays important roles in regulating a diverse set of biological activities (Aspenstrom et al., 1999, Seasholtz et al., 1999). The role of RhoA in the regulation of vascular functions has been gaining increasing attention in cardiovascular research (Fukata et al., 2001, Laufs et al., 2000). Decreased **bioavailability of endothelium-derived NO** is a common feature of several cardiovascular disorders (Wever et al., 1998). Studies with statins which indirectly inhibits RhoA have been shown to increase NO production, at least partly by regulation of eNOS gene expression (Hernandez-Perera et al., 1998). However, statins have also been shown to activate protein kinase B (PKB) which is a direct upstream effector of eNOS. These results prompted us to hypothesize that a cross-talk between **RhoA/ROCK** and the **PKB/eNOS pathway** might exist. The aims of this part of the study are to investigate whether Rho/ROCK negatively regulates eNOS gene expression and enzymatic activity and if so what the underlying mechanisms are.

##### **1. Effects of Rho/ROCK on eNOS gene expression.**

A previous study demonstrated that thrombin-mediated downregulation of eNOS gene expression via decreasing the eNOS mRNA stability could be prevented either by the Rho GTPase inhibitor C3 exoenzyme or by the Rho kinase or ROCK inhibitor Y-27632 in cultured HUVECs (Eto et al., 2001). This suggests that Rho/ROCK pathway plays an important role in eNOS gene expression. To further investigate the role of Rho/ROCK in the regulation of eNOS gene expression and the underlying mechanism, effects of an active or a dominant negative form of Rho or

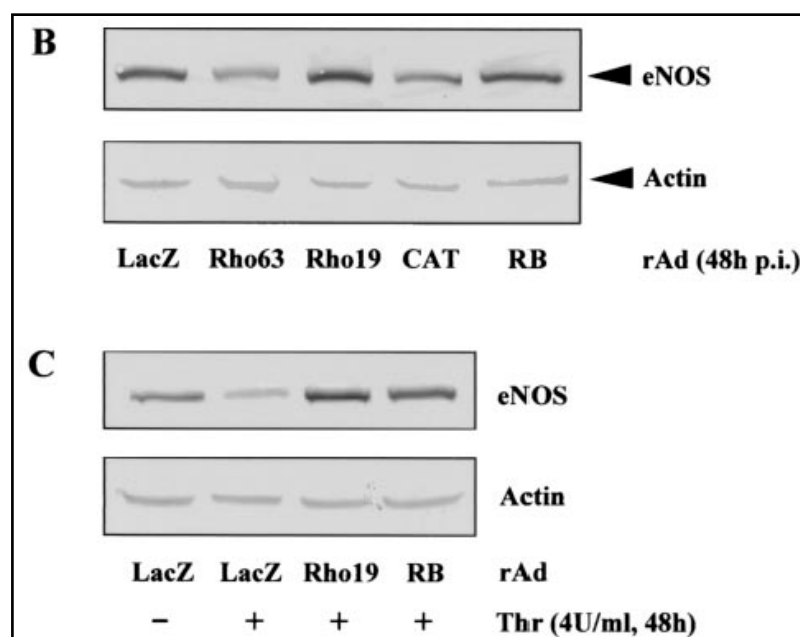
ROCK on eNOS expression in HUVECs were analyzed using adenovirus-mediated gene transfer. For this purpose, the infectivity of HUVECs was first determined by infecting the cells with rAd-GFP. The expression of GFP was detected in about 100% of the HUVECs. Next, the HUVECs were infected with recombinant adenovirus expressing various transgenes and their effects on eNOS gene expression were analyzed by Western blot. All the transgenes were expressed as detected by Western blot using monoclonal anti-HA antibody 12CA5 (**Figure. 1A**).



**Figure 1: Effects of Rho/ROCK on eNOS gene expression.** (A) HUVECs were infected with rAd expressing HA-tagged LacZ, active Rho (Rho63), dominant negative Rho (Rho19), active ROCK (CAT) and dominant negative ROCK (RB) as indicated, at titers ranging from 100 to 150 MOI, and incubated in 0.2% FCS RPMI-1640 supplemented with ECGS for 48 h. Cells were then extracted without any treatment and the expression of the transgenes were analyzed by Western blot (WB) using anti-HA antibody 12CA5.

The effect of the transgenes on eNOS expression was analyzed by WB using anti-eNOS antibody. **Figure 1B** shows that ectopic expression of either active Rho (Rho63) or active ROCK (CAT) led to a decrease in eNOS expression, whereas ectopic expression of dominant negative mutant of Rho (Rho19) or ROCK (RB) abrogated downregulation of eNOS by thrombin (4U/ml, 48 h) (**Figure 1C**). These

data support our previous observation that the Rho/ROCK pathway plays an important role in eNOS expression.

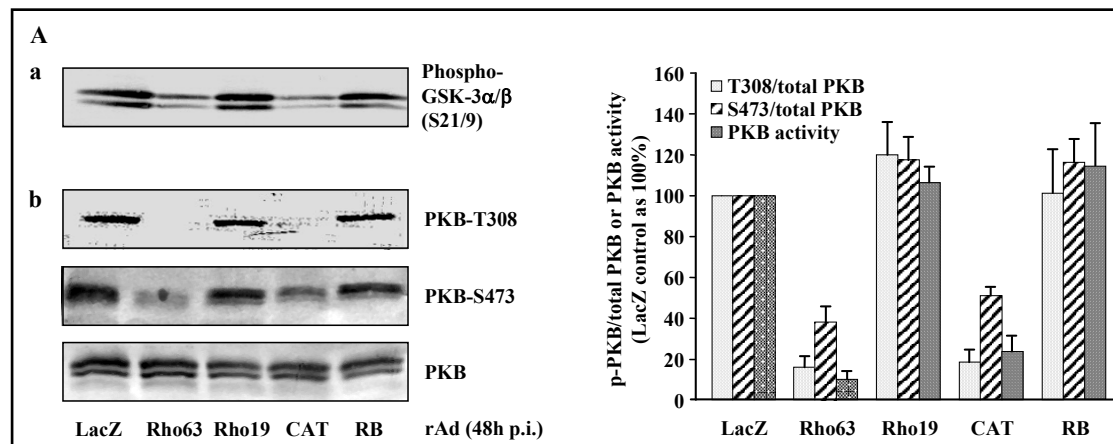


**Figure 1: (B)** The effect of the various transgenes on eNOS expression was examined by WB using anti-eNOS antibody. **(C)** HUVECs were infected with rAd as indicated and serum-starved in 0.2% FCS culture medium for 24 hours followed by treatment with thrombin (4U/ml) for 48 hours. The expression of eNOS was detected by WB. Actin in lower panels served as loading control.

## 2. Inactivation of PKB by Rho/ROCK.

Furthermore, the mechanism by which the Rho/ROCK pathway downregulates expression of eNOS gene was investigated. It has been known that HMG CoA reductase inhibitors or statins plays an important role in the studies involving the pathway. Recently, simvastatin, an indirect inhibitor of Rho (Laufs et al., 2000), was shown to activate PKB (Kureishi et al., 2000), implicating a possible cross-talk between Rho and PKB pathway. It was therefore interesting to examine whether Rho/ROCK can inactivate PKB resulting in downregulation of eNOS expression. To do so, cultured HUVECs were infected with rAd expressing active or dominant negative mutant of Rho or ROCK and their effects on PKB activity were assessed by *in vitro* nonradioactive immunoprecipitation-kinase assay (**Figure. 2A, panel a**), as

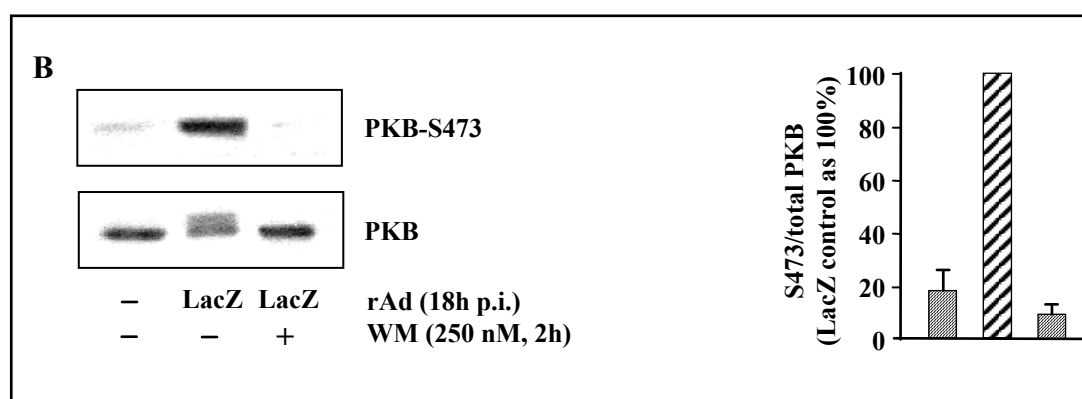
well as by monitoring the phosphorylation of PKB at Thr-308 and Ser-473 (**Figure 2A, panel b**).



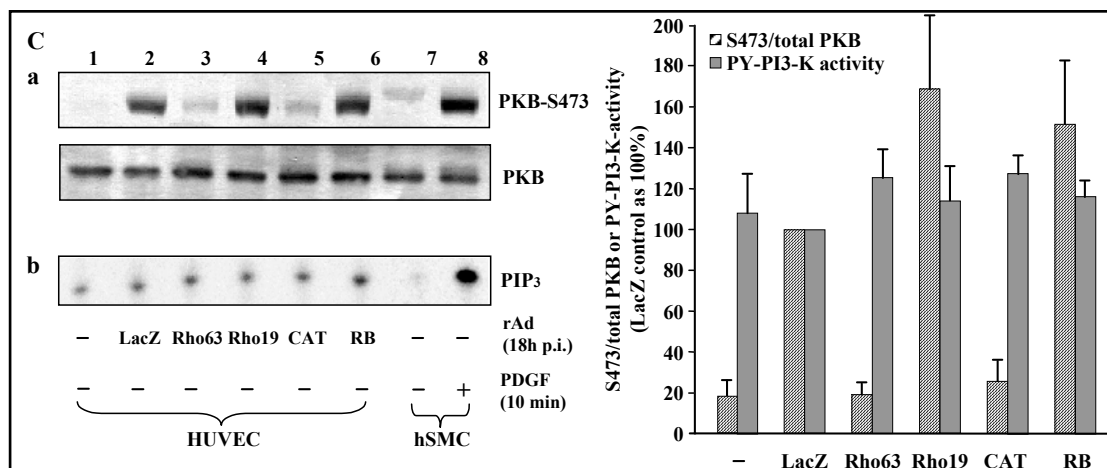
**Figure 2: Active Rho or ROCK inhibits phosphorylation and activity of PKB without affecting p85-PI3K activity.** (A) HUVECs were infected and extracted as described in Figure. 1A. Panel a shows the effect of Rho or ROCK mutants on PKB activity. 30 µg of lysates were used for immunoprecipitation with immobilized Akt 1G1 monoclonal antibody, and subjected to *in vitro* kinase assay using GSK-3 as substrate. Phosphorylation of GSK-3 was measured by western blot using phospho-GSK-3α/β (Ser21/9) antibody. Panel b: The extent of PKB phosphorylation was analyzed by WB using antibodies specific for phosphorylated Thr-308 or Ser-473 of PKB as indicated, whereas total PKB expression was detected by WB using antibodies that recognize PKB regardless of their phosphorylation status (lower panel). Shown are representative WB of five independent experiments.

Quantification of the data is shown in the right panel. The extent of PKB phosphorylation was detected with an anti-Phospho-Akt (Thr-308 or Ser473) antibody. Ectopic expression of active Rho or ROCK decreased both PKB activity (**Figure. 2A, panel a**) and PKB phosphorylation at Thr-308 and Ser-473 without affecting PKB protein level (**Figure. 2A, panel b**), whereas the dominant negative mutant of Rho or ROCK did not significantly affect PKB activity or phosphorylation. These data provide evidence that there is indeed a cross-talk between Rho/ROCK and PKB pathway and that Rho/ROCK pathway negatively regulates PKB activation. As monitoring Ser-473 reflects PKB activity (**Figure. 2A**), the effects on PKB activity were therefore assessed by only monitoring Ser-473 for further studies.

Evidence has been presented that adenovirus infection triggers activation of Phosphatidylinositol 3-kinase (PI3-K) pathway via interaction of the adenoviral penton base capsid with  $\alpha/v$  integrins (Li et al., 1998, Li et al., 2000, Nemerow et al., 1999). In order to demonstrate that the PKB activation upon adenoviral infection shown in **Figure. 2A** is PI3-K dependent, PI3-K inhibitor wortmannin was used to block PI3-K activation. In agreement with the established role of PI3-K in PKB activation, integrin-mediated PKB activation upon control adenoviral infection was effectively inhibited by wortmannin treatment (**Figure. 2B**)



**Figure 2: (B)** HUVECs were infected as indicated and extracted 18 h post infection (p.i). To inhibit PI3-K, cells were treated with 250 nM wortmannin (WM) for 2 h before extraction. Cell lysates were assayed for PKB phosphorylation at Ser-473.



**Figure 2:** (C) Lanes 1 to 6, HUVECs were either left uninfected or were infected as indicated and extracted at 18 h p.i. Cell lysates were assayed for PKB phosphorylation at Ser-473 (panel a) and *in vitro* PI 3-kinase activity in anti-phosphotyrosine (PY) immunoprecipitates (panel b) as described in Materials and Methods. Quantification of signal intensities by using the average of five independent experiments is shown in the right panels. Lanes 7 and 8, cell lysates prepared from untreated and PDGF-treated (50ng/ml of PDGF-BB for 10 min) hSMCs were included as positive control for PI 3-kinase activation.

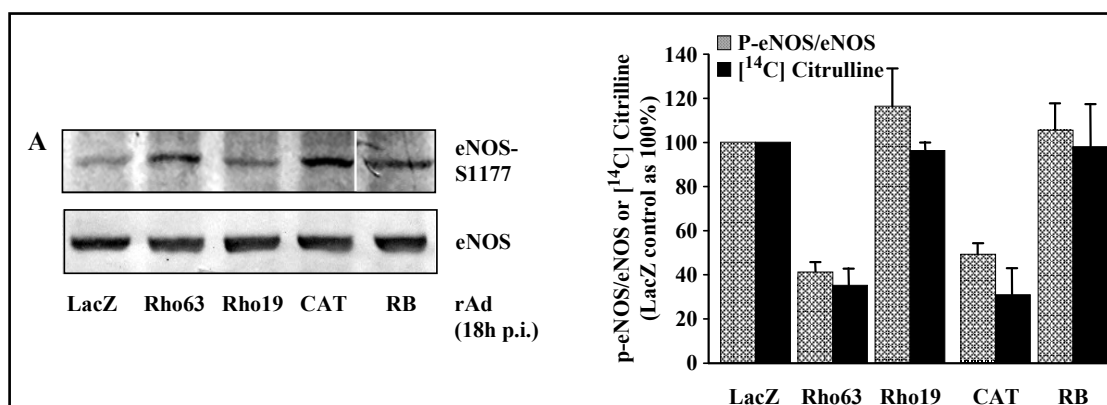
Next, was to determine whether activation of Rho/ROCK pathway inhibits PI3-K activation by analysing the effect of active Rho or ROCK on PI3-K activity in an *in vitro* PI 3-kinase assay in anti-phosphotyrosine immunoprecipitates (**Figure. 2Cb**). Surprisingly, in contrast to the strong phosphorylation or activation of PKB upon adenoviral infection (**Figure. 2Ca**, compare lane 1 and lane 2) and inhibition of PKB phosphorylation or activation by Rho or ROCK (**Figure. 2Ca**, compare lane 3 and 5 with lane 2), no obvious corresponding changes in PI 3-kinase activity could be observed, only a low basal PI 3-kinase activity could be detected (**Figure. 2Cb**, lanes 1 to 6). To ensure that the whole procedures from cell extraction to immunoprecipitation with anti-phosphotyrosine antibody and lipid kinase reaction work properly to allow a detection of a PI 3-kinase activation, cell lysates were prepared from PDGF-stimulated hSMCs and included as a positive control for PI 3-kinase activation (**Figure. 2Cb**, lanes 7 and 8). Given that the inhibition of PI 3-



kinase with wortmannin resulted in inhibition of PKB phosphorylation or activation (**Figure. 2B**), these data suggest that in HUVECs and under our experimental condition, the basal PI 3-kinase activity is required for the PKB activity; however, PKB activation is unlikely to result from an activation of PI 3-kinase but may be from other mechanism such as inhibition of PTEN, inhibition of phosphatase 2A or even other unknown novel mechanism. The yet-to-be-clarified mechanism responsible for PKB activation would be therefore the target of Rho/ROCK to negatively regulate PKB activation shown here.

### **3. Effect of Rho/ROCK on eNOS phosphorylation.**

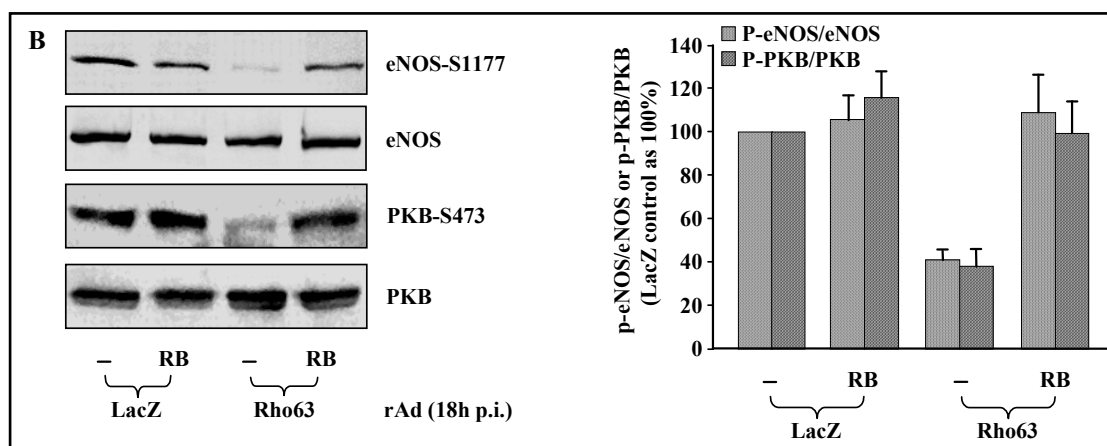
The fact that PKB activates eNOS by phosphorylating the enzyme at Ser-1177 (Dimmeler et al., 1999, Fulton et al., 1999, Michell et al., 1999), and that Rho/ROCK suppresses PKB activation (**Figure. 2**), suggested that Rho/ROCK might negatively regulate eNOS activation, besides suppressing its own gene expression. To test this hypothesis, the effect of Rho or ROCK on eNOS phosphorylation at Ser-1177 was analyzed. Indeed, in parallel to the inhibition on PKB phosphorylation and activation (**Figure. 2**), eNOS phosphorylation was also suppressed upon expression of active Rho or ROCK (**Figure. 3A**).



**Figure 3: Rho inhibits phosphorylation of PKB and eNOS in parallel via its downstream target ROCK.** Cells were infected as indicated and extracted 18 hours post infection (p.i.). A representative Western Blot from five (A) independent experiments is shown under various conditions as indicated. Quantification of the data is shown in the corresponding right panels. (A) The extent of eNOS phosphorylation was analyzed by WB using antibodies specific for phosphorylated Ser-1177 of eNOS (upper panel), whereas eNOS expression was analyzed by WB using antibodies that recognize eNOS regardless of its phosphorylation status (lower panel). In parallel, under the same experimental conditions, eNOS activity was monitored by analyzing the cellular NO production measured as the formation of its co-product [ $^{14}\text{C}$ ]-citrulline.

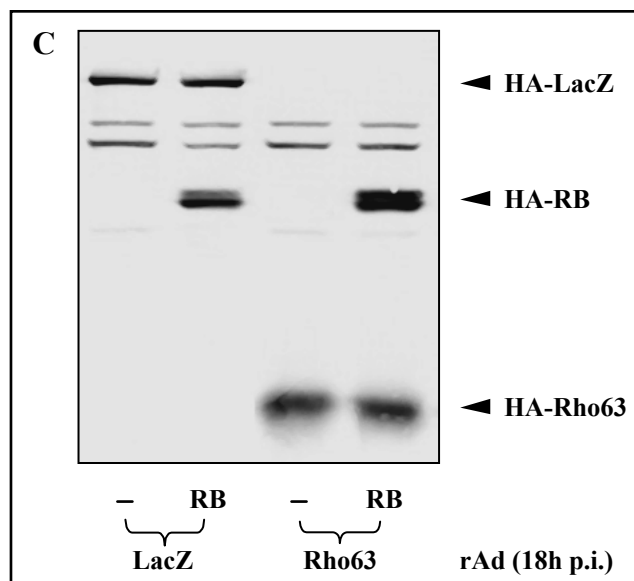
To verify that eNOS enzyme activity parallels its phosphorylation, eNOS activity was measured in parallel under the same experimental conditions by *in vivo* L-citrulline assay. In agreement with the previously demonstrated role of eNOS phosphorylation at Ser-1177 in activation of eNOS enzyme activity (Dimmeler et al., 1999, Fulton et al., 1999, Michell et al., 1999), eNOS activity correlates well with the eNOS phosphorylation (**Figure 3A**, right panel), indicating that the modulation of the Rho/ROCK on eNOS phosphorylation indeed reflects their effects on eNOS activity. In contrast to the experiments shown in **Figure 1B** where cell lysates were prepared at 48 hours post infection (p.i) and eNOS expression was clearly downregulated upon active Rho or ROCK expression, in these experiments the cell lysates were prepared at 18 hours p.i to ensure that the suppression of eNOS phosphorylation is not a consequence of downregulation of eNOS expression by Rho or ROCK. As shown in **Figure 3A** lower panel, there was no significant effect on eNOS expression yet at this

time point. Moreover, co-expression of the dominant negative ROCK (RB) prevented Rho63-mediated decrease in both PKB phosphorylation and eNOS phosphorylation (**Figure. 3B**).



**Figure 3: (B)** The extent of eNOS and PKB phosphorylation was analyzed as described in Figure 3A and Figure 2, respectively.

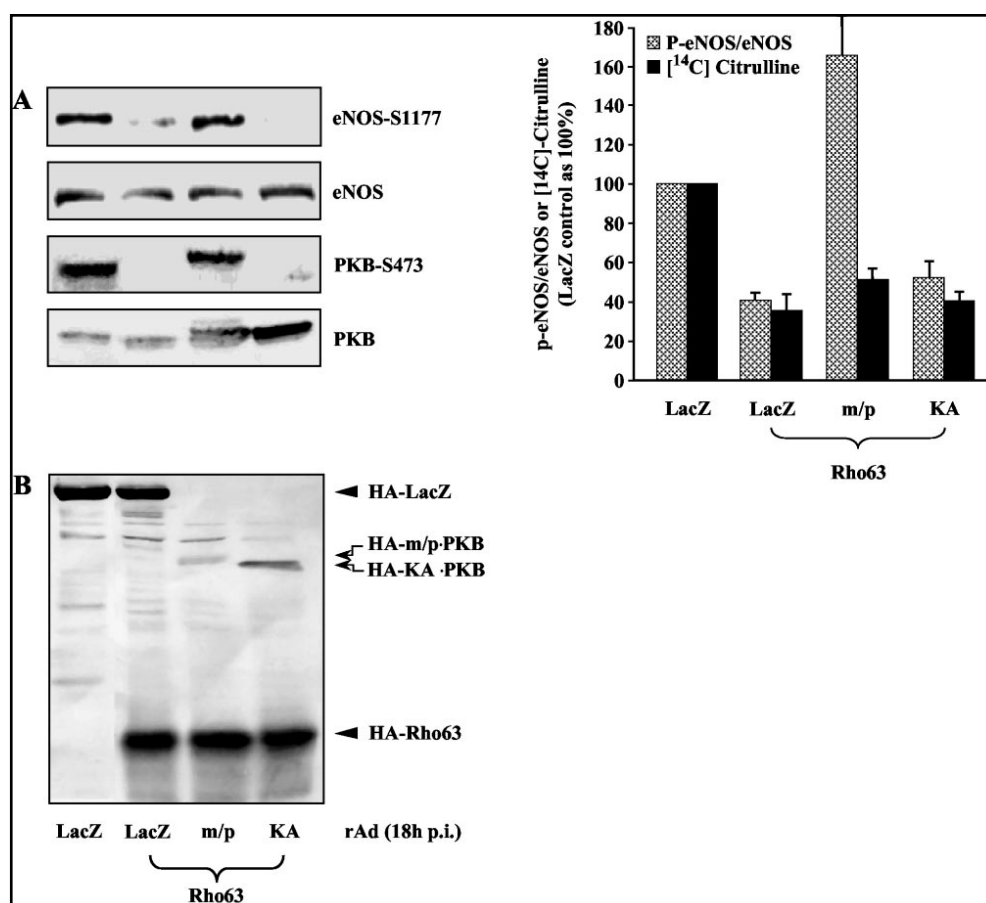
To rule out the possibility that the reversal effect of RB on phosphorylation of PKB and eNOS is due to unexpected transcriptional effects on Rho63, the expression of Rho63 in the absence or presence of RB was determined by Western blot. No obvious difference was observed under this condition (**Figure. 3C**). We conclude that Rho not only downregulates eNOS expression (**Figure. 1**) (Eto et al., 2001, Laufs et al., 1999, Laufs et al., 1998), but it also inhibits eNOS phosphorylation and activity via its downstream target ROCK.



**Figure 3: (C)** The expression of HA-tagged LacZ, RB and Rho63 was detected by Western blot using monoclonal anti-HA antibody 12CA5.

#### **4. Role of PKB in Rho-mediated downregulation of eNOS phosphorylation and expression.**

From the results above, it was of interest to determine whether Rho/ROCK inhibits eNOS gene expression and phosphorylation via inhibition of PKB. Accordingly, cells were co-infected with recombinant adenoviruses expressing a constitutively active or inactive mutant of PKB, and their effects on Rho63-mediated downregulation of eNOS phosphorylation and cellular NO production reflected by L-citrulline production (extracts prepared at 18 hours p.i, **Figure. 4A**), as well as eNOS expression (extracts prepared at 48 hours p.i, **Figure. 4C**) were assessed. Ectopic expression of the active PKB (m/p), but not the inactive PKB (KA) restored eNOS phosphorylation in the presence of Rho63 (**Figure. 4A**). To ensure that the rescuing effect of m/p-PKB on eNOS phosphorylation in the presence of Rho63 is not due to unexpected transcriptional effects on Rho63, control Western blots were performed.

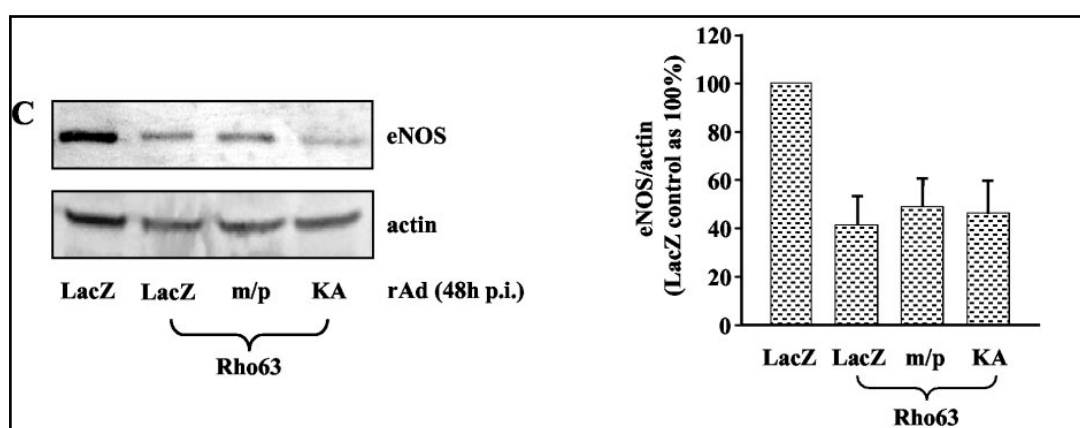


**Figure 4:** Active PKB reversed Rho63-mediated inhibition of phosphorylation, but not of the expression of eNOS. Cells were infected as indicated and the cell lysates were prepared 18 hours post infection for (A)/(B) or 48 hours p.i for (C). A representative Western Blot from five independent experiments is shown under various conditions as indicated. Quantification of the data using average of five independent experiments is shown in the corresponding right panels. (A) Effect of PKB on Rho63-mediated dephosphorylation of eNOS and PKB, and on cellular NO production measured as the formation of [ $^{14}\text{C}$ ]-citrulline. (B) The expression of HA-tagged LacZ, active PKB (m/p), inactive PKB (KA) and Rho63 detected by Western blot using monoclonal anti-HA antibody 12CA5.

As shown in **Figure. 4B**, no inhibitory effect of m/p-PKB on the expression of Rho63 was observed; suggesting that inactivation of PKB is indeed responsible for negative regulation of eNOS phosphorylation by Rho/ROCK pathway.

Of note, in contrast to the effect of Rho/ROCK constructs on eNOS phosphorylation and cellular NO production which parallels each other as shown in **Figure. 3A** right panel, **Figure. 4A** reveals that active PKB restored eNOS

phosphorylation without restoring cellular NO production. As eNOS activity was monitored by measuring L-citrulline production in living cells with L-[ $^{14}\text{C}$ ]arginine, these data suggest that Rho/ROCK pathway may also downregulate other factors that affect cellular NO production such as the availability of the substrate arginine or eNOS cofactor through PKB-independent pathway. Moreover, in contrast to its rescuing effect on eNOS phosphorylation, m/p-PKB could not restore eNOS gene expression in the presence of Rho63 (**Figure. 4C**).

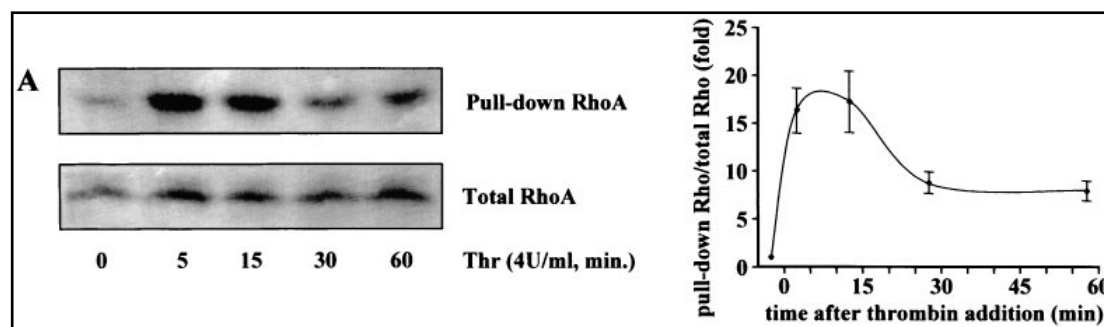


**Figure 4: (C) Effect of PKB on Rho63-mediated downregulation of eNOS gene expression.**

These observations indicate that Rho/ROCK pathway negatively regulates eNOS phosphorylation through inhibition of PKB pathway, whereas it downregulates eNOS gene expression via another yet to be identified pathway.

## 5. Role of Rho and PKB pathways in thrombin-mediated downregulation of eNOS phosphorylation and expression.

Finally, it was investigated whether the conclusions drawn above would also hold true for thrombin, a risk factor in the pathogenesis of cardiovascular disease that activates the Rho/ROCK pathway (Eto et al., 2001) (**Figure. 5A**).



**Figure 5: Role of Rho and PKB in thrombin-mediated downregulation of eNOS phosphorylation and expression. (A)** Time course of Rho activation by thrombin. HUVECs were serum-starved for 24 hours and then treated with 4U/ml thrombin for indicated time. The cell lysates were subjected to pull-down assay of GTP-Rho as described in method.

Treatment of HUVECs with thrombin (4U/ml) for 15 minutes decreased phosphorylation of eNOS and PKB in parallel without significantly affecting the protein level of eNOS or PKB (**Figure. 5B**). Moreover, inhibition of phosphorylation of eNOS or PKB by thrombin was reversed by ectopic expression of the dominant negative Rho (Rho19) or the active PKB (m/p) (**Figure. 5B**), suggesting that thrombin inhibits eNOS phosphorylation via RhoA which in turn inactivates PKB pathway. Consistent with the results shown in **Figure 2B** that active Rho and ROCK inhibited PKB phosphorylation and activity without having an effect on PI3-K activity, activation of Rho by thrombin inhibited PKB phosphorylation without suppression of PI3-K activity (**Figure. 5B**). Remarkably, thrombin even caused a slight activation of PI3-K and is not influenced by dominant mutant of Rho (**Figure. 5B**). These data support the hypothesis that inhibition of PKB phosphorylation and activity by

Rho/ROCK pathway is not resulted from inhibition of its upstream PI3-K. Furthermore, in agreement with previous observations above, down-regulation of eNOS expression by thrombin (4U/ml, 48 hours) was prevented by the dominant negative Rho, but not by the active PKB (Figure. 5C).

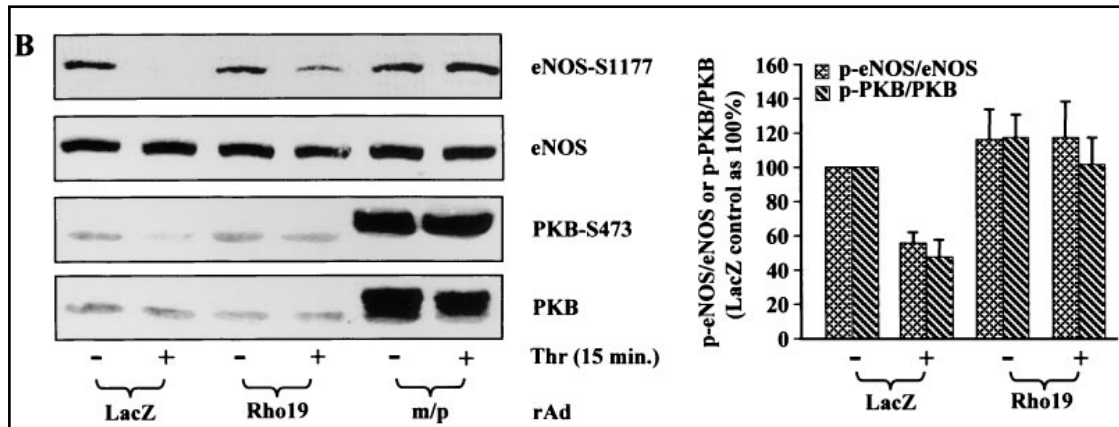


Figure 5: (B) Effect of dominant negative Rho (Rho19) and active PKB (m/p) on thrombin-induced dephosphorylation of eNOS, PKB and p85-PI3K activity. Cells were infected and serum-starved for 24 hours. The cells were then either untreated (-) or treated with thrombin (+) 15min.

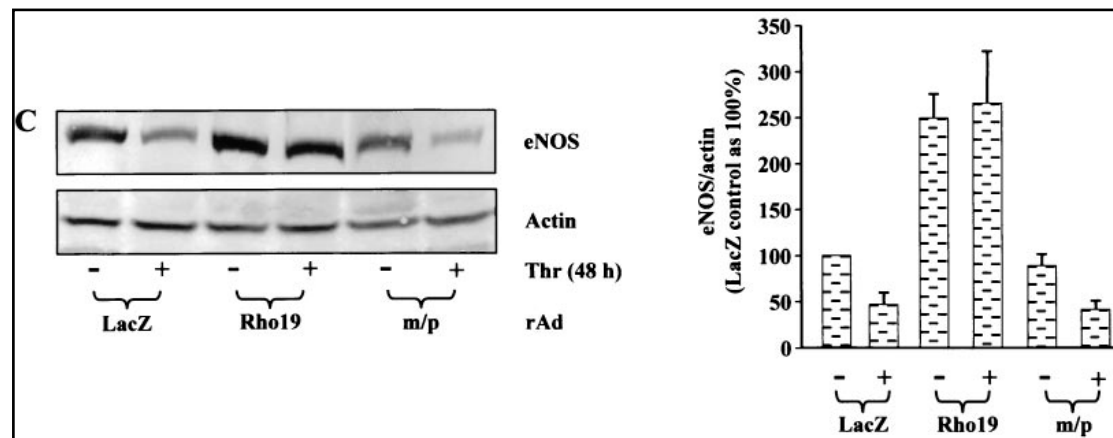


Figure 5: (C) Effect of Rho19 and m/p-PKB on thrombin-mediated downregulation of eNOS gene expression. Cells were infected and treated with thrombin for 48h. Shown are representative blots from three (5A) or five (5B and 5C) independent experiments. Quantification of the data is shown in the right panel.

Taken together, the results confirm the observation obtained with Rho mutants and demonstrate that thrombin downregulates both eNOS expression and phosphorylation via Rho pathway, which inhibits PKB and subsequently eNOS phosphorylation but not eNOS expression.



## DISCUSSION (PART1)

In this study, it is demonstrated that Rho/ROCK not only downregulates eNOS expression, but it also inhibits eNOS phosphorylation and its activity. Moreover, there is evidence that inactivation of PKB by Rho/ROCK is responsible for the inhibition of eNOS phosphorylation but not the downregulation of eNOS gene expression. Furthermore, these conclusions are reinforced by the results obtained with thrombin. Concomitant with its ability to activate Rho/ROCK, thrombin suppressed the phosphorylation of PKB and eNOS, in addition to its downregulatory effect on eNOS expression. Although inhibition of both expression and phosphorylation of eNOS by thrombin is mediated by Rho/ROCK pathway, only the suppression of eNOS phosphorylation could be attributed to inactivation of PKB by thrombin.

Rho GTPase has been shown to regulate a wide spectrum of cellular function (Van Aelst et al., 1997). Increasing evidence suggests a role of Rho-regulated cellular function in mediating cardiovascular pathologies such as smooth muscle hypercontraction (Shimokawa H et al., 2000), smooth muscle cell growth and migration that account for neointimal formation of stenosis (Seaholtz et al., 1999, Eto et al., 2000) and platelet aggregation (Missy et al., 2001, Nishioka et al., 2001). Moreover, in recent years, much has been learned about the role of Rho in modulating endothelial function. In the context of endothelial dysfunction, Rho has been reported to downregulate eNOS expression (Eto et al., 2001, Laufs et al., 1999, Laufs et al., 1998) and is essential for the basal production of endothelin (ET)-1 (Hernandez-Perera et al., 2000), a potent vasoconstrictor and mitogen that regulates vascular tone and remodeling (Levin ER 1995). In agreement with previous reports, it is demonstrated here that activation of Rho is sufficient to downregulate eNOS expression and is required for thrombin-mediated decrease in eNOS expression

(**Figure.1**). Moreover, in this study, we provide additional evidence for an important role of Rho in modulating PKB/eNOS pathway. The activation of Rho was shown either by ectopic expression of active mutant of Rho or by a pathologic stimulus thrombin, dramatically inhibited phosphorylation of eNOS in parallel to that of PKB (**Figure. 2 and 3**). Of interest, while thrombin-induced dephosphorylation of eNOS occurs within 15 min (**Figure. 5B**), it takes hours to days to achieve downregulation of eNOS expression (**Figure. 5C** and (Eto et al., 2001, Laufs et al., 1999, Laufs et al., 1998)). The same is true with the constructs expressing active Rho or ROCK. Due to the fact that it takes at least more than 8 hours to express the transgenes, it is not known exactly how rapidly the dephosphorylation was induced upon Rho63 expression. However, eNOS dephosphorylation could be already observed at 18 hours post-infection, at a time point when eNOS expression was not yet significantly affected (**Figure. 3 and 4A**). In this respect, signals such as thrombin would cause endothelial dysfunction via the activation of Rho that in turn leads to dephosphorylation or inactivation of eNOS and suppression of its gene expression. This mechanism may play an important role in acute coronary syndromes in patients with heart disease.

To date, a number of downstream effector proteins of Rho have been identified (Aspenstrom 1999). Amongst, ROCK is the most extensively and well-studied. Many of the Rho-regulated cellular functions have been shown to be mediated by ROCK, such as smooth muscle contraction (Amano et al., 1996, Kimura et al., 1996, Kureishi et al., 1997), cytoskeletal rearrangement and c-fos expression (Chihara et al.,1997), and cytokinesis (Yasui et al.,1998). We show here that Rho-induced dephosphorylation of PKB and eNOS is also mediated by ROCK. This conclusion is based on the observations that the active ROCK led to

dephosphorylation of both PKB and eNOS (**Figure. 2** and **Figure. 3A**), the dominant negative ROCK prevented Rho63-mediated inhibition of PKB and eNOS phosphorylation (**Figure. 3B**). Important evidence we provide in this report is the cross-talk between Rho/ROCK and PKB. The importance of this finding should be underscored by the fact that PKB serves as a multifunctional regulator of cell survival, growth and glucose metabolism (Datta et al., 1999). With respect to its cardiovascular functions, PKB plays a critical role in endothelial cell survival (Fujio et al., 1999, Gerber et al., 1998) and promotes angiogenesis (Dimmeler et al., 1999, Kureishi et al., 2000) mediated by vascular endothelial growth factor (VEGF), in addition to its involvement in controlling vasomotor activity via regulating eNOS pathway as mentioned above. Here we demonstrate that the cross-talk between Rho/ROCK and PKB pathways is responsible for Rho-mediated eNOS dephosphorylation. Two lines of evidence supported the involvement of PKB in Rho/ROCK-mediated inhibition of eNOS phosphorylation. The first evidence came from experiments using constitutively active mutants. The data in **Figure 2** and **3** show that dephosphorylation of PKB and eNOS by active Rho or ROCK closely parallel one another. The second piece of evidence is provided by the rescue experiment in which an active PKB, but not an inactive PKB, restored phosphorylation of eNOS in the presence of active Rho (**Figure. 4A**). Since evidence has been presented that eNOS phosphorylation by PKB lead to an increased NO production, the active PKB restored eNOS phosphorylation in the presence of active Rho mutant without restoring cellular NO production (**Figure. 4A**). Taking into account that eNOS activity was monitored by measuring the cellular NO production in living cells with L-[<sup>14</sup>C]arginine, and that the effects of Rho and ROCK mutants on eNOS phosphorylation and cellular NO production parallel each other (**Figure. 3A**), a probable explanation would be that Rho/ROCK

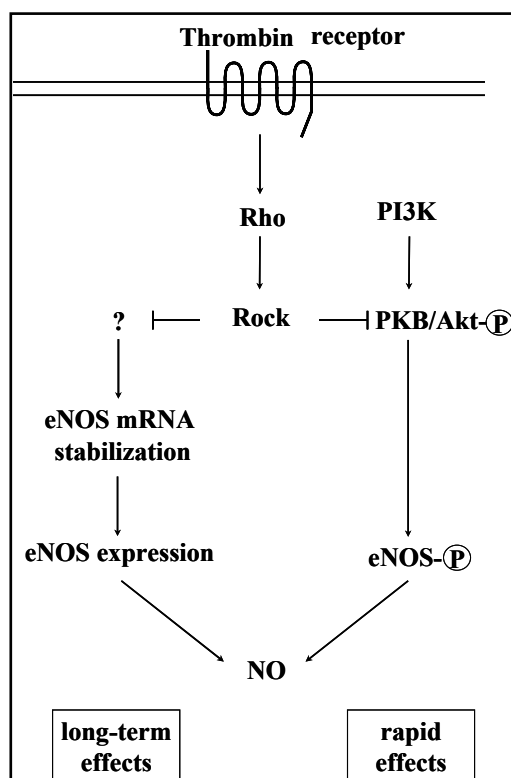
pathway may also downregulate other factors such as the availability of the substrate arginine or eNOS cofactor that are necessary for a normal cellular NO production, but through other pathway(s) independent of PKB.

While the role of PKB in eNOS phosphorylation leading to an increased NO production is well-documented and established, little is known about an involvement of PKB in eNOS gene expression. In view of the fact that PI3-K was reported to be involved in eNOS gene expression (Cieslik et al., 2001, Kuboki et al., 2000) and PKB is a well-characterized downstream signaling of PI3-K, it is important to point out that active PKB did not rescue Rho63- or thrombin-mediated eNOS gene downregulation (**Figure. 4C and 5C**), indicating that PKB is not required for Rho63 or thrombin to downregulate eNOS expression. However, this does not rule out a role of PKB in the regulation of eNOS gene expression. One possibility could be that PKB does not play a role in the regulation of eNOS mRNA stability by thrombin or Rho, yet is involved in eNOS transcriptional regulation as reported in one study regarding regulation of eNOS gene expression by PI3-K (Cieslik et al., 2001).

Regarding the role of PI3-kinase in Rho/ROCK-mediated downregulation of PKB phosphorylation and activity by active mutants of Rho/ROCK, the data suggest that the inhibition of PKB activity by Rho/ROCK is unlikely to result from inhibition of PI3-kinase activity since the PKB activation upon adenoviral infection or the inhibition of PKB activation by active Rho/ROCK is not accompanied by corresponding changes in PI3-kinase activity (**Figure 2C**). This is noteworthy in view of the fact that PI3-kinase is strongly activated upon adenoviral infection in the human colon carcinoma cell line SW480 (Li et al., 1998, Li et al., 2000). Thus, although the basal PI3-kinase activity is required for PKB phosphorylation and activity in HUVECs under this experimental conditions (**Figure 2B**), the PKB

activation probably did not result from PI3-kinase activation but may be from other mechanisms such as inactivation of PTEN, inactivation of phosphatase 2A, or even some other unknown novel mechanism. Which mechanism accounts for the PKB activation and hence be the target of Rho/ROCK to negatively regulate PKB activation as reported in this study remains unknown.

The data presented here are summarized as follows: a stimulus that activates Rho/ROCK pathway would lead to dephosphorylation of eNOS while simultaneously suppressing its expression by decreasing its mRNA stability to achieve rapid and long-term decrease in NO production (**Figure 6**).



**Figure 6:** Activation of RhoA/ROCK for example, by thrombin, on one hand is responsible for the downregulation of eNOS gene expression by destabilizing eNOS mRNA, independently of PKB inhibition, which persists through a long period of time. In addition to this effect, it also inhibits phosphorylation of eNOS via the inactivation of PKB giving rise to a rapid effect. Both of these chronic and acute effects lead to a decrease in NO production.

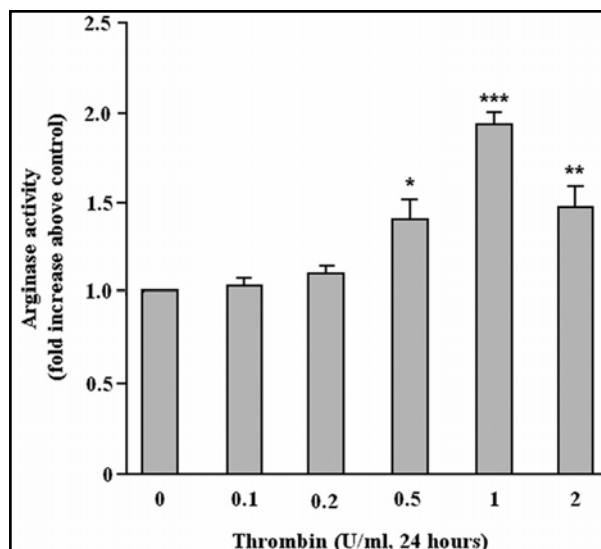
Whereas inhibition of PKB by Rho/ROCK is responsible for the negative regulation of eNOS phosphorylation, the downregulation of eNOS expression is attributed to another yet-to-be-identified pathway. An important task will be to further elucidate the mechanism by which Rho/ROCK inactivates PKB. In addition, the impact of the cross-talk between the two pathways on other PKB-regulated cellular functions should be a goal worthwhile to follow-up for future research.

## **Part 2: RhoA/ROCK Pathway Stimulates Human Endothelial Arginase Activity: Implications for Atherosclerotic Endothelial Dysfunction.**

Besides co-factors, gene expression level, post-translational modifications, the availability of the **substrate L-arginine** is also involved in the regulation of NO bioavailability (Hallemesch et al., 2002). Increasing evidences have demonstrated that arginase; an enzyme in the urea cycle competes with NOS for the substrate L-arginine, which leads to a reduction in NO production (Chang et al., 1998, Zhang et al., 2001). An **increased arginase activity** has been implicated in hypertension, aging-associated endothelial dysfunction, diabetic erectile dysfunction and pulmonary artery hypertension (Bivalacqua et al., 2001, Berkowitz et al., 2003). Clinical and animal studies with L-arginine supplementation failed to show consistent results in the improvement of endothelial dysfunction in atherosclerosis (Chen et al., 2003). It is also to notice that eNOS expression in most atherosclerotic animal models either showed no changes or even an increase. We investigated whether arginase could play a role in **endothelial dysfunction** in atherosclerosis. The underlying mechanisms of increased arginase activity were also studied.

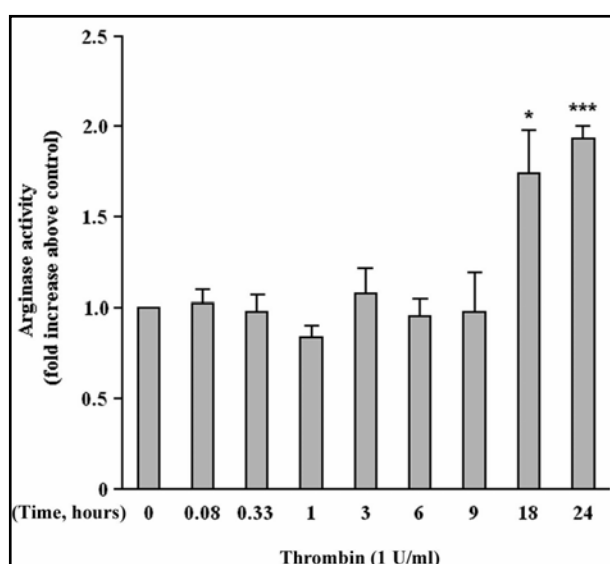
### **1. Thrombin stimulates arginase activity in human endothelial cells**

Stimulation of HUVECs with various concentrations of thrombin (0.1 U/ml to 2 U/ml) for 24 hours enhanced arginase activity with the maximum effect at the concentration of 1 U/ml (1.9-fold increase above control, n=9,  $P<0.001$ , **Figure. 1**). This result demonstrated that thrombin which upregulates RhoA/ROCK could induce arginase activity in human endothelial cells. Since the maximum effect was observed at 1U/ml, this concentration of thrombin was used in the following experiments.



**Figure 1.** Thrombin dose-dependently stimulates arginase activity in HUVECs. HUVECs were serum starved for 24 h, followed by treatment with various concentrations of thrombin as indicated for 24 h. Cells were then subjected to arginase activity assay. Columns represent mean  $\pm$  SEM of 9 experiments (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. untreated cells, i.e. dose zero).

The cells were then stimulated with thrombin (1 U/ml) at different time points from 5 minutes to 24 hours. As demonstrated in **Figure. 2**, a significant increase in arginase activity was observed after 18 to 24 hours exposure of the cells to thrombin with a maximum effect at 24 hours (1.9-fold,  $n=3$ ,  $p$ <0.001).

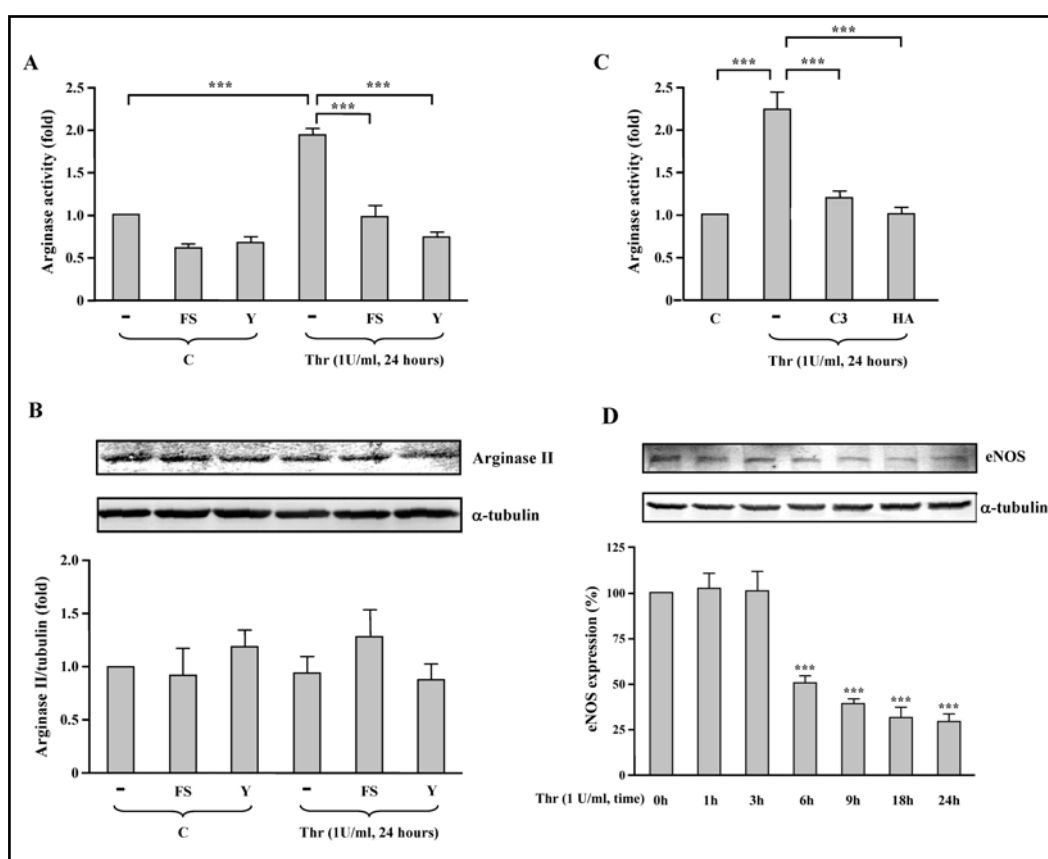


**Figure 2.** Time course of thrombin-induced arginase activity. HUVECs were treated with thrombin (1 U/ml) for different times as indicated, extracted and assayed for arginase activity. Columns represent mean  $\pm$  SEM of 3 experiments (\* $P$ <0.05, \*\*\* $P$ <0.001 vs. untreated cells, i.e. time zero).



## 2. The role of RhoA /ROCK in regulation of arginase activity

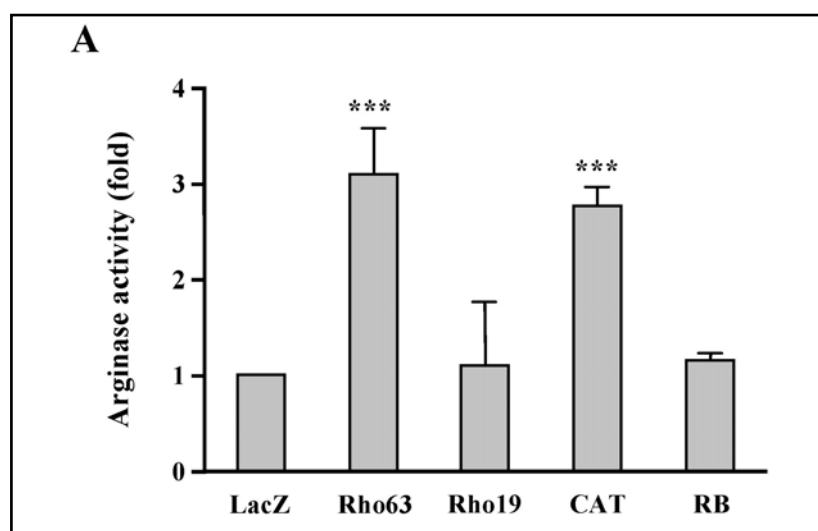
As shown in **Figure. 3A**, stimulation of arginase activity by thrombin (1 U/ml, 24 hours) in endothelial cells was inhibited by HMG CoA reductase inhibitor fluvastatin (1  $\mu$ mol/L) or ROCK inhibitor Y-27632 (10  $\mu$ mol/L) at the concentrations that inhibit RhoA or ROCK as shown by a previous study (Barandier et al., 2003). Neither thrombin nor the inhibitors significantly influenced arginase II protein level (**Figure. 3B**, arginase I was not detectable in the cells).



**Figure 3. Thrombin induces arginase activity via RhoA/ROCK.** (A) stimulation of arginase activity by thrombin (1 U/ml) was inhibited by pretreatment of the cells with fluvastatin (FS, 1  $\mu$ mol/L) or Y-27632 (Y, 10  $\mu$ mol/L) for 1 h 30 minutes or (C) by C3 exoenzyme (20  $\mu$ g/mL, 20 hours) or HA-1077 (10  $\mu$ mol/L, 1 hour). (B) A representative blot revealing arginase II expression was not modified by various substances used in panel A (the expression of arginase I was not detectable in HUVECs). Columns represent mean  $\pm$  SEM of 5 independent experiments. (D) eNOS protein level was significantly decreased after 6 hours of exposure to thrombin (1 U/ml). \*\*\* $P$ <0.001 for comparison between groups as indicated or vs. control (0 h) in panel D.

Stimulation of arginase activity by thrombin was also inhibited by C3 exoenzyme (20  $\mu\text{g/mL}$ ) or by another ROCK inhibitor HA-1077 (10  $\mu\text{mol/L}$ , **Figure. 3C**;  $n=5$ ,  $p<0.001$  vs. thrombin alone), further demonstrating the role of Rho/ROCK pathway in thrombin-stimulated arginase activity in the cells. It is to notice that exposure of the cells to thrombin at 1 U/ml time-dependently suppressed eNOS protein level over 24 hours, an effect that was significant at 6 hours (**Figure. 3D**).

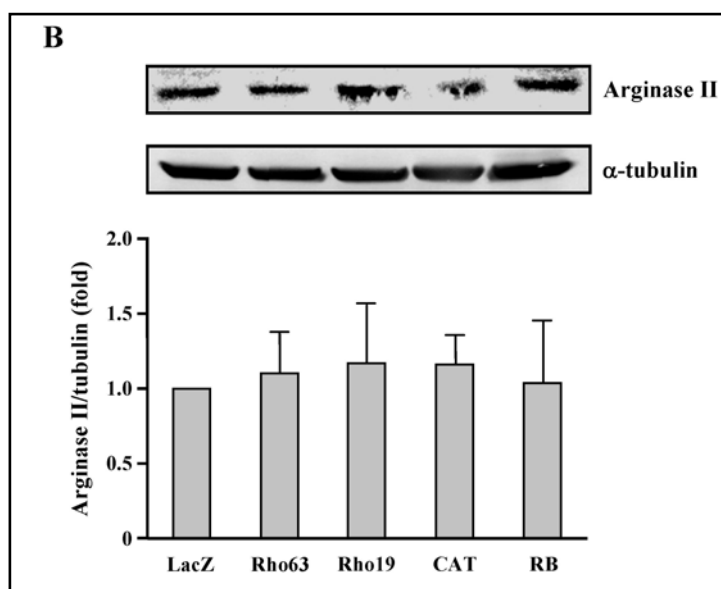
Furthermore, adenovirus-mediated expression of the active mutant of RhoA (Rho63) or ROCK (CAT) enhanced arginase activity in the cells, whereas the negative mutant of RhoA (Rho19) or ROCK (RB) alone had no effect (**Figure. 4A**)



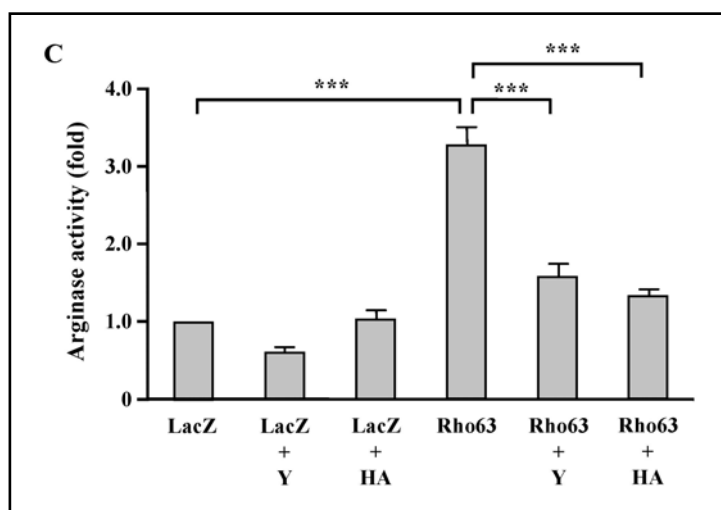
**Figure 4. Active RhoA or ROCK stimulates arginase activity.** (A) HUVECs were infected with recombinant adenovirus (rAd) expressing HA-tagged LacZ, active RhoA (Rho63), dominant negative RhoA (Rho19), active ROCK (CAT) or dominant negative ROCK (RB) as indicated, at titers ranging from 100 to 150 multiplicity of infection (MOI), and incubated in 0.2% FCS RPMI-1640 supplemented with ECGS for 48 h. Cells were then extracted without any treatment and assayed for arginase activity

Again, the arginase II protein level in the cells were not affected by the mutants (**Figure. 4B**,  $n=7$ ). The increase in arginase activity stimulated by the active Rho63 mutant was abolished by the two different ROCK inhibitors Y-27632 (10  $\mu\text{mol/L}$ ) or HA-1077 (10  $\mu\text{mol/L}$ , **Figure. 4C**). The inhibitors alone had no

significant effects on the basal arginase activity (**Figure. 4C**). It was previously shown (Ming et al., 2002) that eNOS protein level is markedly down-regulated by the active RhoA or ROCK mutants 24 hours after transduction.



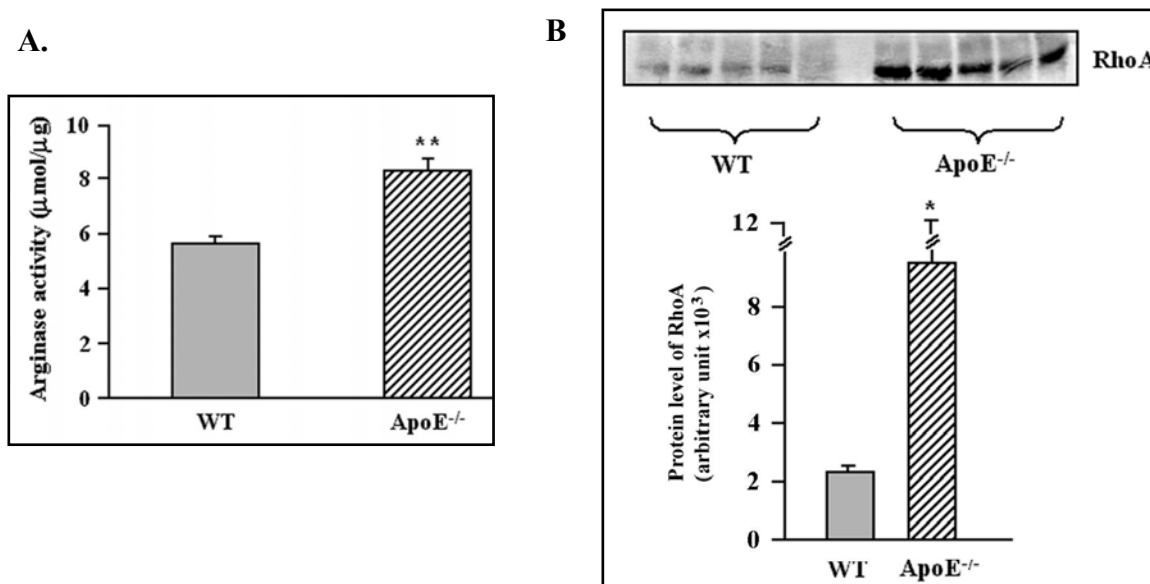
**Figure 4.** Cells stimulated with active RhoA or ROCK subjected to Western blot for arginase expression. A representative blot revealing arginase II expression is shown in B. Columns represent mean  $\pm$  SEM of 7 independent experiments.



**Figure 4.** Active RhoA or ROCK stimulates arginase activity. (C) Rho63 induced activation of arginase activity was inhibited by the ROCK inhibitors Y-27632 (Y) or HA- 1077 (HA). \*\*\* $P < 0.001$  for comparison between groups as indicated or vs. LacZ.

### 3. Increased arginase activity and RhoA expression in atherosclerosis

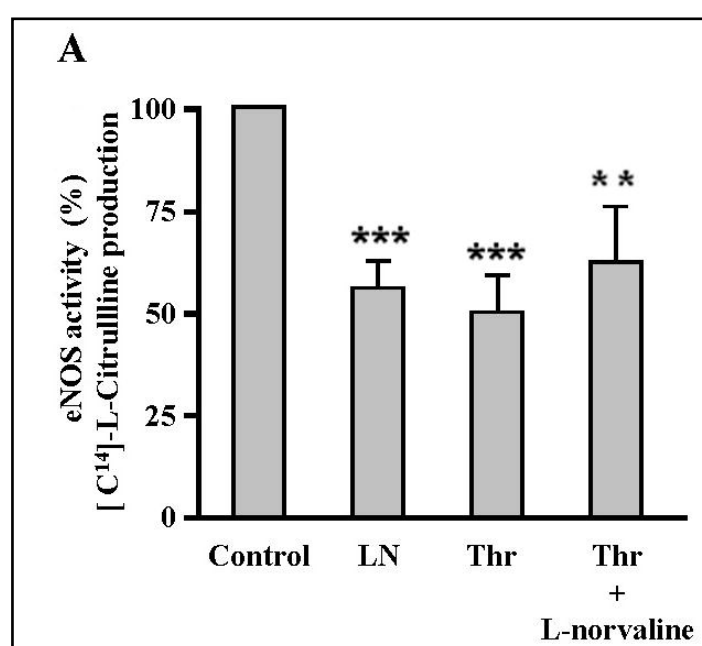
Given the above observations, it was tested whether arginase activity is increased in atherosclerosis. ApoE<sup>-/-</sup> atherosclerotic mice were used for this purpose. Indeed, ApoE<sup>-/-</sup> mice fed with a cholesterol rich diet for 4 months developed atherosclerotic lesions throughout the aorta, (Barton et al., 1998) and showed a significant higher arginase activity than aortas isolated from age-matched wild type mice (1.5-fold increase, n=5, p<0.01, **Figure. 5A**). In addition, the arginase II protein level was comparable in the aortas of the two groups and arginase I protein was not detectable in the mice aortas. Interestingly, RhoA protein level was remarkably increased (4-fold) in the aortas of ApoE<sup>-/-</sup> mice as compared to wild type animals (**Figure. 5B**).



**Figure 5. Increased arginase activity and RhoA level in atherosclerotic aortas of ApoE<sup>-/-</sup> mice:** The isolated aorta was cut in two sections. One section was homogenized and sonicated in arginase lysis buffer for analysis of arginase activity (A), the other section in extraction buffer for analysis of RhoA expression by Western blot (B). \*P<0.05 and \*\*P<0.01 vs. WT.

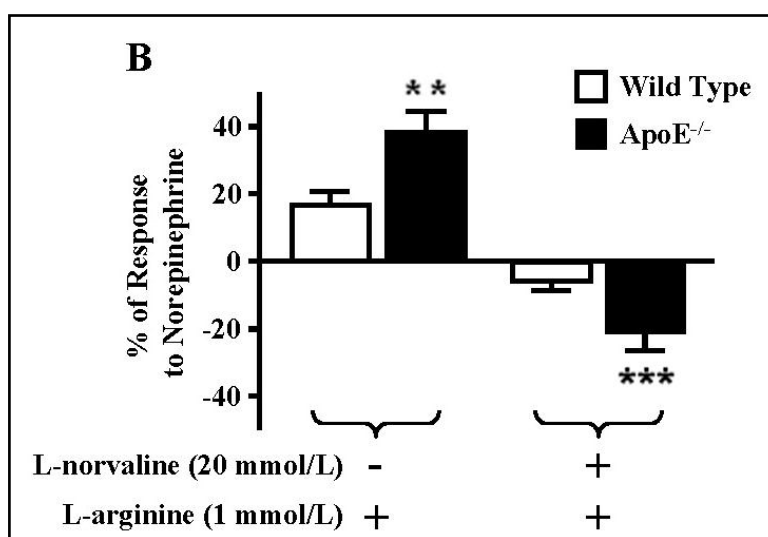
#### 4. Arginase and eNOS activity

The role of arginase in regulating eNOS activity was further investigated. In HUVECs, eNOS activity as measured by L-citrulline production was blunted by eNOS inhibitor L-NAME (0.1 mmol/L) and also by thrombin (1 U/ml, 24 hours,  $n=5$ ,  $50\pm9\%$  decrease,  $P<0.001$ , **Figure. 6A**). Co-treatment of the cells with arginase inhibitor L-norvaline (20 mmol/L, 24 hours) (Chang et al., 1998), however, did not significantly reverse eNOS activity (**Figure. 6A**).



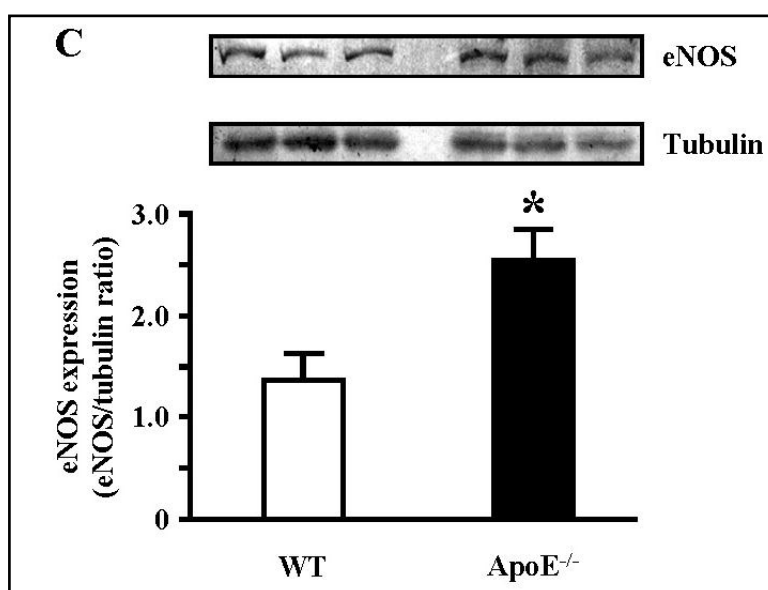
**Figure 6: Regulation of eNOS activity by arginase: (A)** In cultured HUVECs, the eNOS inhibitor L-NAME (LN, 0.1 mmol/L) or thrombin (Thr, 1 U/ml, 24 hours) decreased eNOS activity as measured by L-citrulline production, which was not significantly reversed in the presence of arginase inhibitor L-norvaline (20 mmol/L, 24 hours,  $n=5$ ).

In mouse aortas, L-arginine (1 mmol/L) caused vasoconstriction (in contrast to rats in which L-arginine caused vasorelaxation) that was more pronounced in ApoE<sup>-/-</sup> ( $38\pm6\%$ ) than in wild type animals ( $17\pm4\%$ ,  $n=5$ ,  $p<0.01$ , **Figure. 6B**).



**Figure 6: (B)** In aortic rings pre-contracted with norepinephrine (0.3  $\mu$ mol/L), L-arginine (1 mmol/L) caused a more pronounced vasoconstriction in ApoE<sup>-/-</sup> mice than in wild-type (WT) animals. In the presence of arginase inhibitor L-norvaline the contractions were converted to a greater vascular relaxation in ApoE<sup>-/-</sup> mice than in WT animals (n=5).

However, eNOS expression was significantly higher in ApoE<sup>-/-</sup> than in wild type mice (**Figure. 6C**,  $p < 0.05$ ). Remarkably, the contraction was converted to a more pronounced relaxation by the arginase inhibitor Lnorvaline (20 mmol/L, 1 hour) in atherosclerotic ApoE<sup>-/-</sup> mice ( $-21 \pm 5\%$ ) than in wild type animals ( $-6 \pm 2\%$ , **Figure. 6B**,  $n=5$ ,  $p < 0.001$ ).



**Figure 6: (C)** eNOS expression was significantly higher in ApoE<sup>-/-</sup> mice than in the WT animals. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control or WT.

## DISCUSSION (PART 2)

Emerging evidence demonstrates that arginase is present in various cell types and is involved in negative regulation of NO production as reported in macrophages (Modolell et al., 1995, Morris et al., 1998, Gotoh et al., 1999) and endothelial cells (Zhang et al., 2001, Buga et al., 1996, Li et al. 2001). This study demonstrates that in human endothelial cells, arginase activity is significantly induced by thrombin. The induction of arginase activity can be inhibited by the HMG-CoA reductase inhibitor fluvastatin, which inhibits RhoA activation by geranylgeranylation of the enzyme (Laufs et al. 2000), and also by C3 exoenzyme which inactivates RhoA by ADP ribosylation (Aktories, 1997). Moreover, inhibition of ROCK, a downstream effector of RhoA, either by Y-27632 or HA-1077 abolished the thrombin's effect, suggesting that thrombin stimulates arginase activity in human endothelial cells through RhoA/ROCK pathway. This conclusion is supported by the experiments showing that adenovirus-mediated ectopic expression of the constitutively active mutant of RhoA (Rho63) or ROCK (CAT), but not the negative mutants, significantly enhanced arginase activity in the cells. Furthermore, the effect of Rho63 was abolished by the ROCK inhibitor Y-27632 or HA-1077.

Two isoforms of arginases namely arginase I and II were reported to be expressed in vascular endothelial cells. In endothelial cells of porcine coronary arterioles and rat aortas, arginase I is constitutively expressed, (Zhang et al., 2001), Buga et al., 1996) whereas arginase II is inducible in response to lipopolysaccharide or cytokines (Buga et al., 1996). This study, however, showed abundant basal level of arginase II, whereas arginase I was not detectable in HUVEC, suggesting that arginase II is the major isozyme in HUVECs. It is to note that neither thrombin nor the active mutants of RhoA/ROCK did modulate arginase II expression (arginase I is

not inducible in the cells), suggesting that activation of arginase by thrombin or active RhoA/ROCK mutants occurs at the level of enzyme activity rather than on gene expression. Similar findings were also reported in rat endothelial cells, where expression of arginases was not modified by cytokines, although the enzymatic activity was stimulated (Suschek et al. 2003). It is conceivable that the enzyme might be modified biochemically by RhoA/ROCK pathway, that either alters the enzymatic activity or the affinity of the enzyme to its substrate. A third isoform of arginase might exist in endothelial cells and cannot be ruled out under this experimental condition. This aspect certainly warrants further investigation. Furthermore, it is demonstrated here that arginase enzymatic activity was significantly increased in atherosclerotic aortas of ApoE<sup>-/-</sup> mice. The higher arginase activity in the atherosclerotic aortas was associated with higher RhoA protein level suggesting a role of RhoA in upregulation of arginase activity. A definite characterization of the role of RhoA in stimulation of arginase activity in atherosclerosis *in vivo* could not be performed at this stage due to lack of specific RhoA inhibitors applicable in living mice. Nevertheless, the results obtained from cultured HUVECs support the role of RhoA in stimulation of arginase activity.

Similar to cultured HUVECs, no difference in arginase II expression was observed between control mice and atherosclerotic ApoE<sup>-/-</sup> mice, and arginase I was not detectable in the mice aortas, suggesting an upregulation of arginase II enzymatic activity in atherosclerosis.

Previous studies have indicated an important role of increased arginase activity, even a moderate increase of 1.5 to 2-fold, in endothelial dysfunction in aged rats and in human diabetic erectile dysfunction (Bivalacqua et al. 2001, Berkowitz et al. 2003). Then, the role of increased arginase activity in regulating NO production in



cultured endothelial cells and in atherosclerotic aortas of ApoE<sup>-/-</sup> mice was further investigated. In HUVECs treated with thrombin, eNOS activity was significantly reduced. However, this reduction of eNOS activity was not significantly reversed in the presence of arginase inhibitor L-norvaline. It is most likely due to the fact that eNOS protein level was simultaneously suppressed by thrombin, as demonstrated by **Figure 3D** and also by other previous studies (Eto et al. 2001, Ming et al. 2002). Alternatively, inhibition of co-factors of eNOS by thrombin might also be involved.

Surprisingly, L-arginine induced vasoconstriction in mice aorta, which contrasts the observation in rats (Berkowitz et al. 2003) and humans (Creager et al. 1992), where it evoked vascular relaxation. The contraction induced by L-arginine is much more pronounced in atherosclerotic ApoE<sup>-/-</sup> mice compared to control animals. The results may implicate that in the mice aortas, particularly in the atherosclerotic ApoE<sup>-/-</sup> aortas, L-arginine may be metabolized by arginase to certain vasoconstrictive intermediate products. Most interestingly, the contractions were converted to relaxations in the presence of the arginase inhibitor L-norvaline, an effect which was significantly greater in atherosclerotic ApoE<sup>-/-</sup> mice compared to wild type animals, demonstrating a dominant role of increased arginase activity in regulation of endothelial NO production in atherosclerosis. Most notably, eNOS gene expression is significantly higher in atherosclerotic aortas (see **Figure 6C**), further supporting the concept that endothelial dysfunction in atherosclerosis is due to a decreased NO bioavailability (d'Uscio et al. 2001). The higher eNOS expression in atherosclerosis would make an efficient NO production possible, when arginase is inhibited. This may implicate arginase as a potential therapeutic target for treatment of atherosclerotic vascular disease. It is also important to point out that eNOS protein level was not suppressed in atherosclerotic aortas of ApoE<sup>-/-</sup> mice despite higher

RhoA level in these blood vessels. It is conceivable that the suppressing effect of RhoA on eNOS protein expression might be compensated by other mechanisms in vivo.

In conclusion, this study provides evidence for the role of RhoA/ROCK pathway in stimulation of arginase activity in human endothelial cells. The increased arginase activity is associated with higher RhoA expression and is involved in endothelial dysfunction in atherosclerosis. Targeting arginase in the vasculature may represent a novel therapeutic strategy for treatment of atherosclerosis.

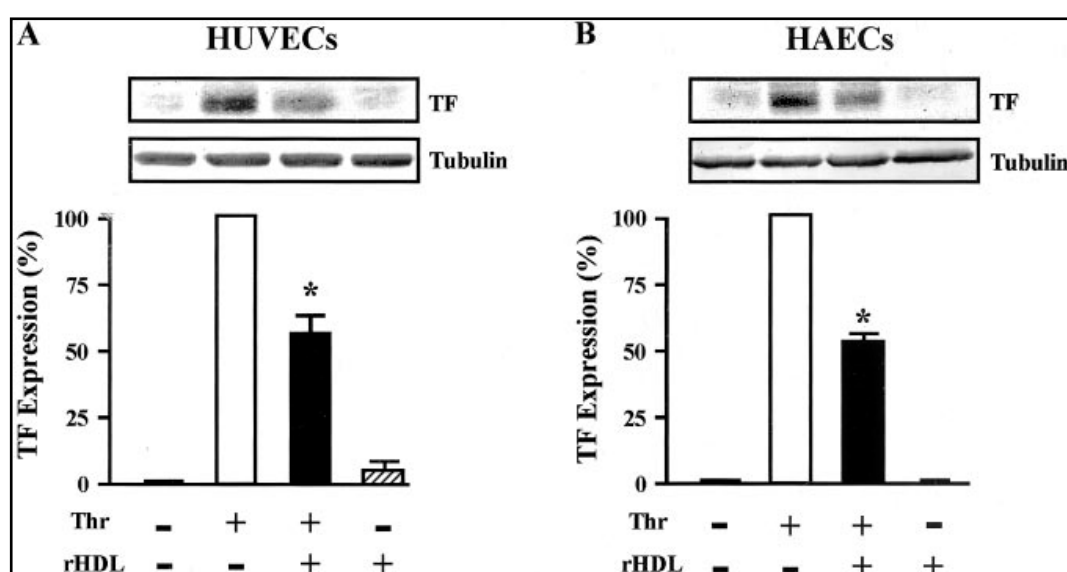
### **Part 3: Rho/ROCK Pathway Stimulates Endothelial Tissue Factor Expression: Inhibition by High-Density Lipoprotein (HDL).**

**Thrombosis** at the site of atherosclerotic plaque disruption is the principal cause of acute coronary syndromes (Moons et al., 2002, Suefuji et al., 1997). **Tissue factor** (TF) plays a crucial role in the initiation of thrombus formation. In atherosclerosis, TF, a 47 kDa integral membrane protein, is expressed in large amounts in macrophages, smooth muscle cells but negligibly in endothelial cells (Wilcox et al., 1989). Studies provide evidence that **RhoA** and **p38<sup>mapk</sup>** pathways positively regulate TF expression (Ishibashi et al., 2003), whereas **PKB/Akt/eNOS** pathway negatively regulates its expression in endothelial cells (Eto et al., 2002).

The protective effects of **high-density lipoprotein** (HDL) are well-documented and considered to be attributed to reverse cholesterol transport. Emerging evidence suggests that HDL exerts its **anti-atherosclerotic effects** also via cholesterol-independent mechanisms including improvement of endothelial function (Yuhanna et al., 2001, Mineo et al., 2003, O'Connell et al., 2001). In isolated *in vitro* reaction assay, HDL has been shown to inhibit activation of coagulation factor X by factor VIIa and TF purified from placenta (Carson, 1981). These studies suggest a modulatory role of HDL in hemostasis. Therefore, this study aimed to investigate if HDL inhibits TF expression in activated endothelial cells by interfering with RhoA/ROCK, p38<sup>mapk</sup> and/or PI3K/PKB/eNOS pathways.

### 1. rHDL inhibits thrombin-induced TF expression.

Stimulation of HUVECs with thrombin (4 U/ml) for 4 hours markedly increased TF protein level (**Figure. 1A**, upper panel, n=7) confirming the results of a previous study by Eto et al. (Eto et al. 2002). The induction of endothelial TF level by thrombin was significantly inhibited by rHDL (0.1 mg/ml, 43% inhibition **Figure. 1A**, lower panel, n=7;  $p<0.001$ ), whereas rHDL alone had no significant effect on the basal TF level in the cells.

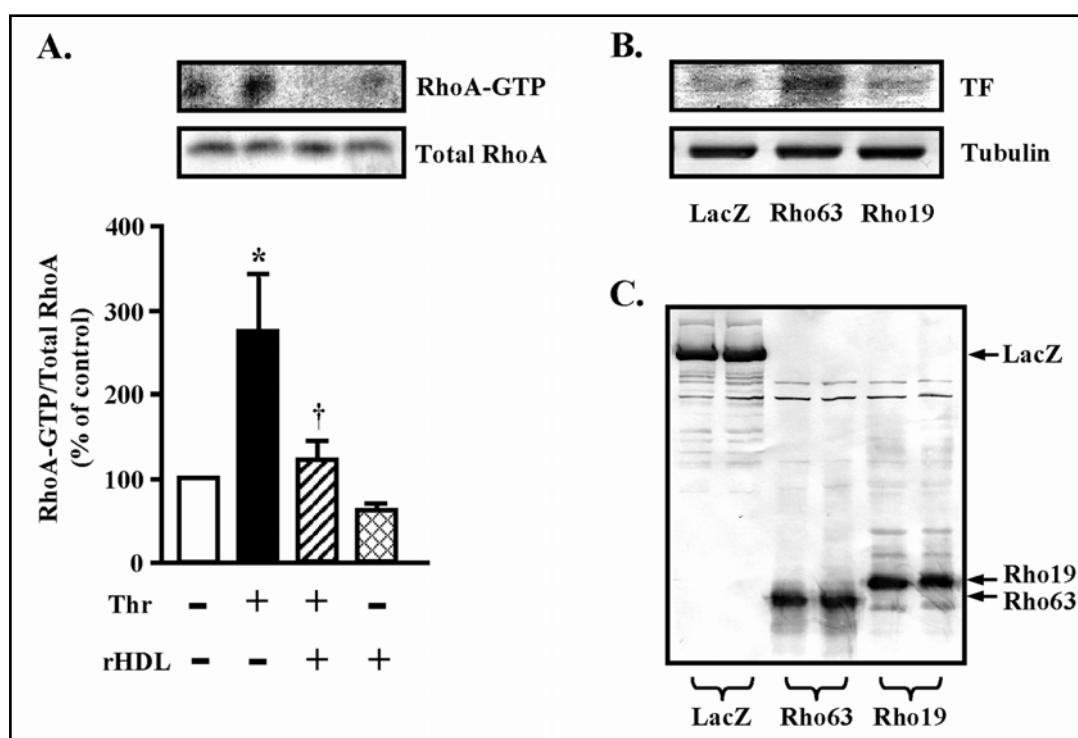


**Figure. 1: rHDL inhibits endothelial tissue factor (TF) expression by thrombin:** In HUVECs (A) as well as human aortic endothelial cells (B, HAEC, n=3) thrombin (Thr, 4 U/ml; 4 hours) up-regulated TF protein level which was significantly inhibited by rHDL (0.1 mg/ml), while rHDL alone had no significant effect on the basal level of TF expression. \*  $p<0.001$  vs. Thr.

HDL isolated from human plasma also inhibits thrombin-induced TF expression. Similar to HUVECs, in cultured human aortic endothelial cells, up-regulation of TF protein level by thrombin (4 U/ml, 4 hours) was also reduced by rHDL (0.1 mg/ml, 47% inhibition, **Figure. 1B**, n=3,  $p<0.001$ ). Due to the greater availability of the material, the subsequent experiments were performed in HUVECs with rHDL.

## 2. Effect of rHDL on thrombin-induced activation of RhoA.

Since RhoA has been demonstrated to be involved in thrombin-mediated induction of TF expression in endothelial cells (Eto et al. 2002, Ishibashi et al. 2003), we analysed whether rHDL interferes with RhoA activation.



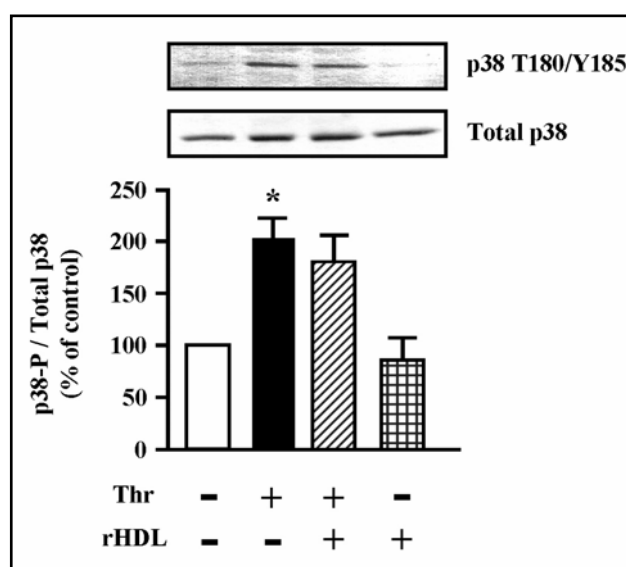
**Figure. 2:** rHDL inhibits endothelial tissue factor (TF) expression via inhibition of RhoA: (A) Pull-down assay shows that thrombin (Thr, 4 U/ml; 15 minutes) activated RhoA, (Rho-GTP) which was blocked by pre-incubation of the cells with rHDL (0.1 mg/ml, n=5). (B) Adenoviral expression of active RhoA mutant Rho63 enhanced endothelial TF expression (n=5). (C) The expression of transgenes of LacZ, Rho63 and Rho19 was shown by Western blot using anti-HA antibody. \*p<0.001 vs. control, †p<0.01 vs. Thr.

Activation of RhoA by thrombin (4 U/ml, 15 minutes) was inhibited by rHDL (0.1 mg/ml, n=5, p<0.01, **Figure. 2A**). The role of RhoA in the induction of TF was further demonstrated by the experiment showing that adenoviral expression of the constitutively active RhoA mutant Rho63 (24 hours post-infection) enhanced endothelial TF expression, while Rho19, the negative mutant had no effect on the

basal TF expression (n=5, **Figure. 2B**). Transgene expression was shown in **Figure 2C**.

### 3. Effect of rHDL on thrombin-induced activation of p38<sup>mapk</sup>.

We analysed whether rHDL reduces endothelial TF protein level by interfering with p38<sup>mapk</sup> which has been shown to be a positive regulatory mechanism of TF expression in endothelial cells (Eto et al. 2002, O'Reilly et al. 2003). Pre-incubation of the cells with rHDL (0.1 mg/ml) for 60 minutes did not significantly affect the activation of p38<sup>mapk</sup> (phosphorylation at T-180/Y-185) in response to thrombin (4 U/ml, 15 minutes, n=5, **Figure. 3**), although rHDL significantly inhibited thrombin-induced TF expression under this condition.

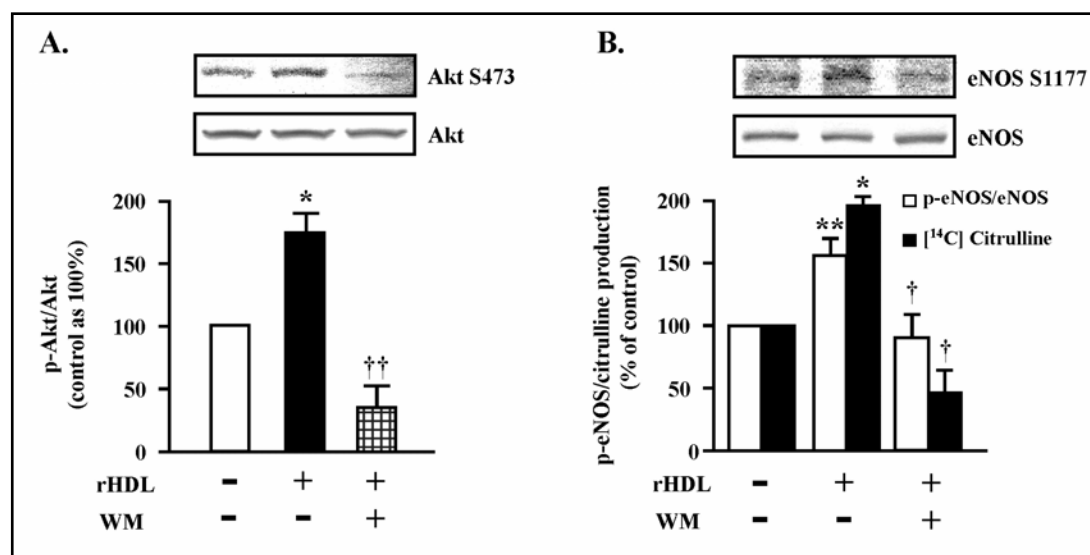


**Figure 3: rHDL did not inhibit activation of p38<sup>mapk</sup>:** Phosphorylation of p38<sup>mapk</sup> was significantly enhanced by thrombin (Thr, 4 U/ml, 15 minutes), which was not affected by rHDL (0.1 mg/ml, n=5). \*p<0.001 vs. control.

### 4. rHDL activates PI3K/Akt/eNOS pathway.

Initial experiments with different concentrations of rHDL (0.01 to 1.0 mg/ml) demonstrated that rHDL at the concentration of 0.1 mg/ml gives the maximal activation of Akt and eNOS. Stimulation of the cells with rHDL (0.1 mg/ml) over 60

minutes induced a 1.7-fold increase in Akt phosphorylation at serine-473 (**Figure. 4A**,  $n=6$ ,  $p<0.05$ ). Activation of Akt by rHDL (0.1 mg/ml, 60 minutes) was abolished by the PI3K inhibitor, wortmannin (0.1  $\mu\text{mol/L}$ , **Figure 4A**,  $p<0.01$ ).

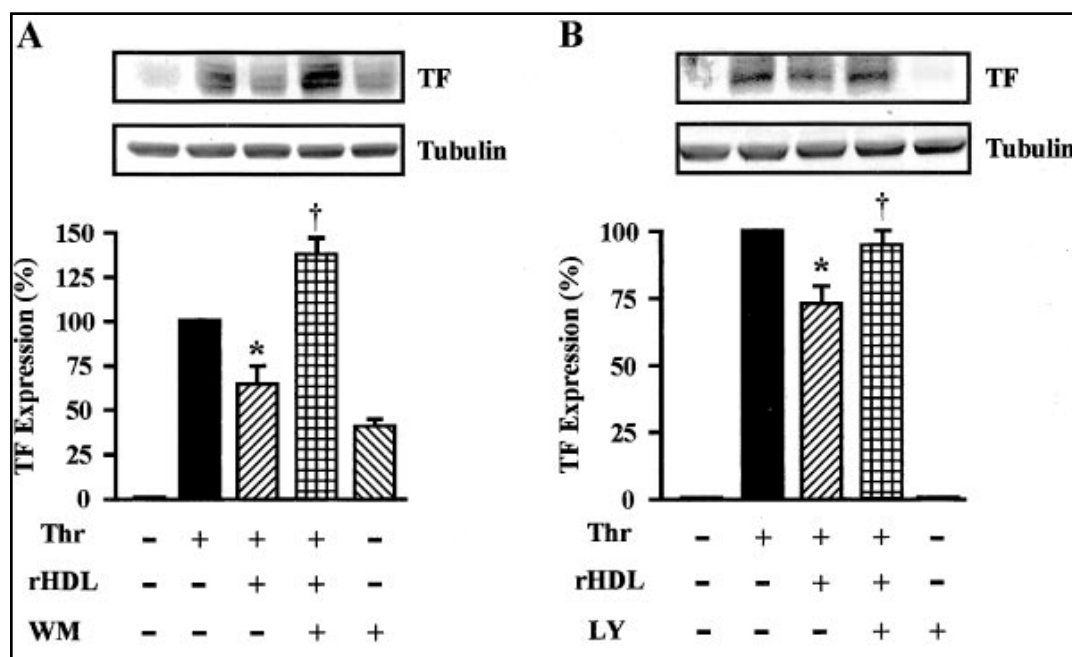


**Figure 4: rHDL activates PI3K/Akt/eNOS pathway: (A)** rHDL (0.1 mg/ml) phosphorylated Akt at serine-473, which was inhibited by the PI3K inhibitor wortmannin (WM, 0.1 mmol/L,  $p<0.05$ ;  $n=6$ ). **(B)** rHDL also stimulated phosphorylation of eNOS at serine-1177 and L-citrulline production, which was blocked by wortmannin (WM, 0.1 mmol/L,  $p<0.01$ ;  $n=6$ ). \* $p<0.05$  vs. control, \*\* $p<0.01$  vs. control, † $p<0.05$  vs. rHDL, †† $p<0.01$  vs. rHDL.

In parallel with Akt activation, rHDL stimulated eNOS phosphorylation at serine-1177 (1.5-fold increase) reaching the maximum at 30 minutes (**Figure. 4B**). eNOS phosphorylation at serine-1177 was correlated with an increased L-citrulline production (1.9-fold,  $n=6$ ,  $p<0.05$ ) which was inhibited by wortmannin (0.1  $\mu\text{mol/L}$ , **Figure. 4B**,  $n=6$ ,  $p<0.01$ ).

## 5. rHDL prevents thrombin-induced TF expression involving PI3K but not Akt/eNOS.

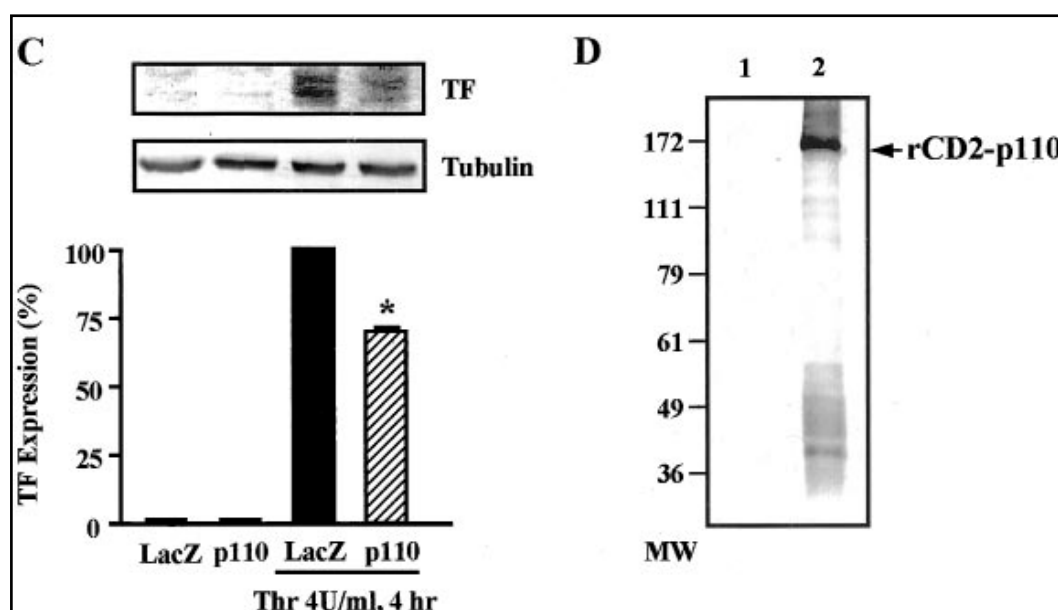
The inhibitory effect of rHDL on TF expression induced by thrombin (4U/ml, 4 hours) was fully reversed by the PI3K inhibitors wortmannin (0.1  $\mu\text{mol/L}$ , **Figure. 5A**,  $n=3$ ,  $p<0.01$ ) or LY-294002 (1  $\mu\text{mol/L}$ ,  $n=5$ ,  $p<0.01$ , **Figure. 5B**).



**Figure 5: rHDL inhibits tissue factor (TF) expression via PI3K:** Inhibition of TF expression in response to thrombin (4 U/ml, 4 hours) by rHDL (0.1 mg/ml) was abolished by the PI3K inhibitors wortmannin (0.1  $\mu$ mol/L, n=3) (A) and LY-294002 (LY, 1 mmol/L, n=5, B).

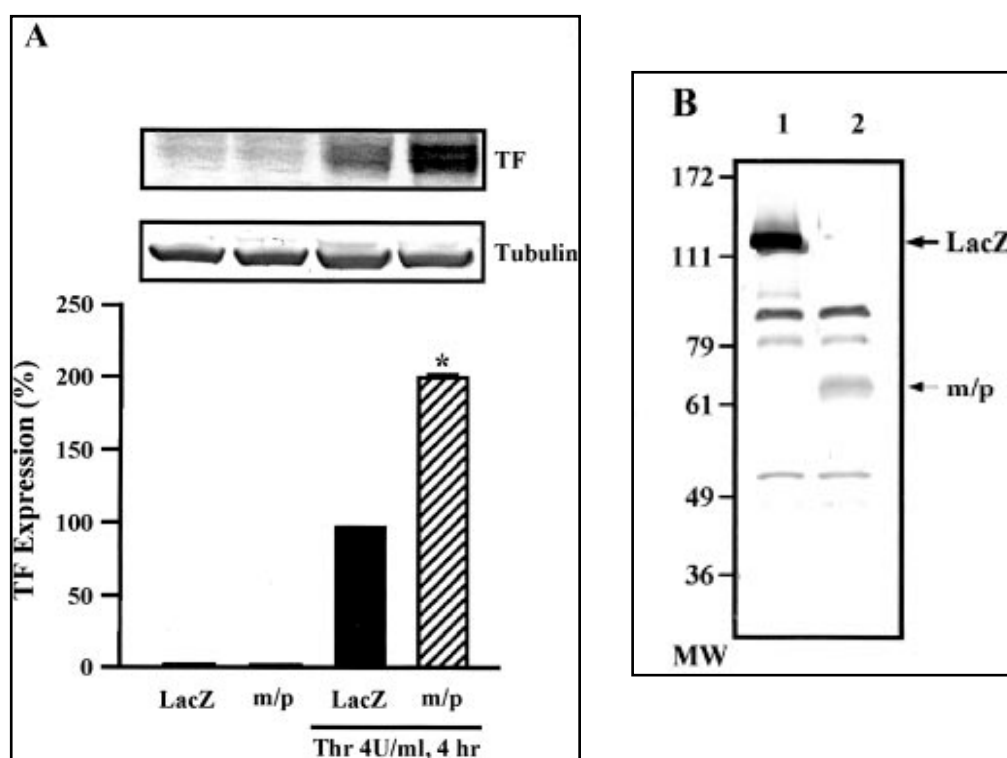
Moreover, adenoviral expression of a constitutively active PI3K (rCD2-p110) significantly reduced TF protein level stimulated by thrombin (**Figure. 5C**, n=4,  $p<0.05$ ). The p110 transgene expression was demonstrated by immunoblotting using anti-CD2 antibody (**Figure. 5D**, lane 2; lane 1 are cells expressing HA-tagged LacZ which is therefore not detectable with anti-CD2 antibody).





**Figure 5:** Adenoviral expression of the PI3K active mutant p110 reduced thrombin-induced TF up-regulation (n=4, C). The transgene expressions of CD2-tagged p110 (rCD2-p110) was demonstrated by immunoblotting using anti-CD2 antibody (D, Lane 2. Lane 1 is the HA-tagged LacZ expressing cell which is not detectable under this condition. p110 appears larger on the SDS-PAGE gel due to the tagged rCD2). \* $p < 0.05$  vs. thrombin, † $p < 0.001$  vs. Thr+rHDL, †† $p < 0.01$  vs. Thr+rHDL.

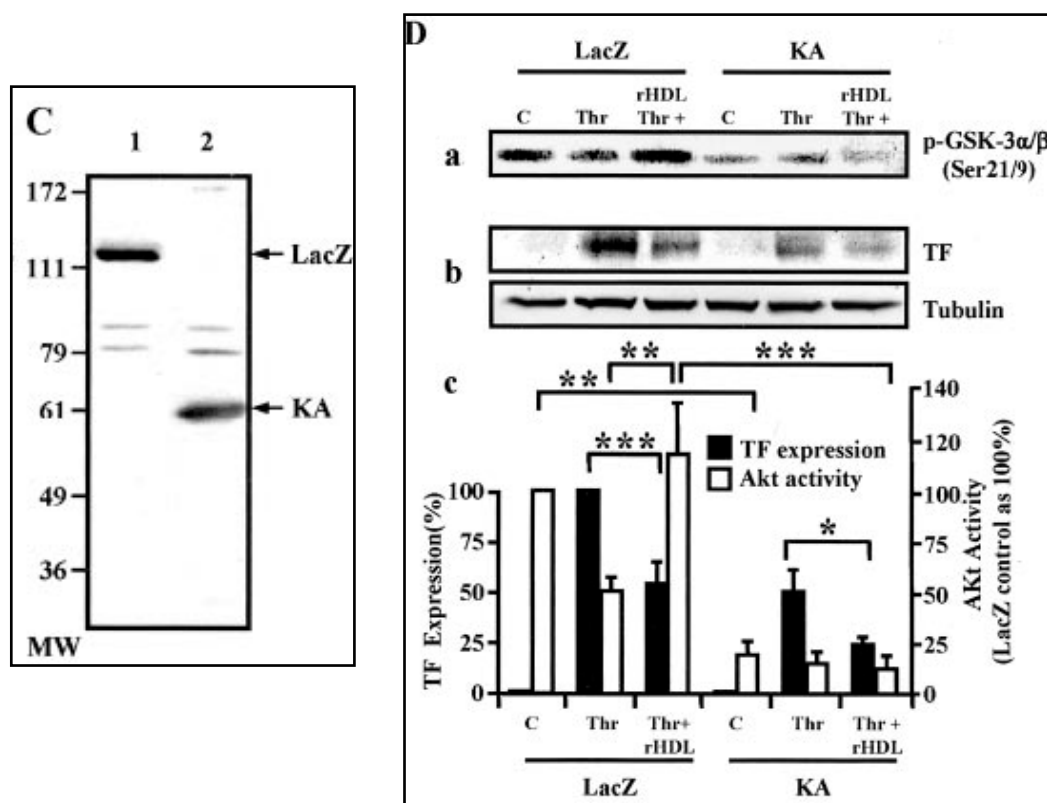
In contrast to the active PI3K mutant, adenoviral expression of a constitutively active Akt mutant (m/p-Akt) enhanced the up-regulation of TF expression stimulated by thrombin (**Figure. 6A**, n=3,  $p < 0.05$ ). The transgene expression of m/p-Akt and LacZ was demonstrated by immunoblotting using monoclonal anti-HA antibody (**Figure. 6B**).



**Figure 6: rHDL inhibits tissue factor (TF) expression independent of Akt/eNOS: (A)** Adenoviral expression of the active Akt mutant (m/p) further enhanced the stimulation of TF level by thrombin. **(B)** The expression of transgenes of HA-tagged LacZ (Lane 1) and m/p (Lane 2) was demonstrated by immunoblotting using anti-HA antibody.

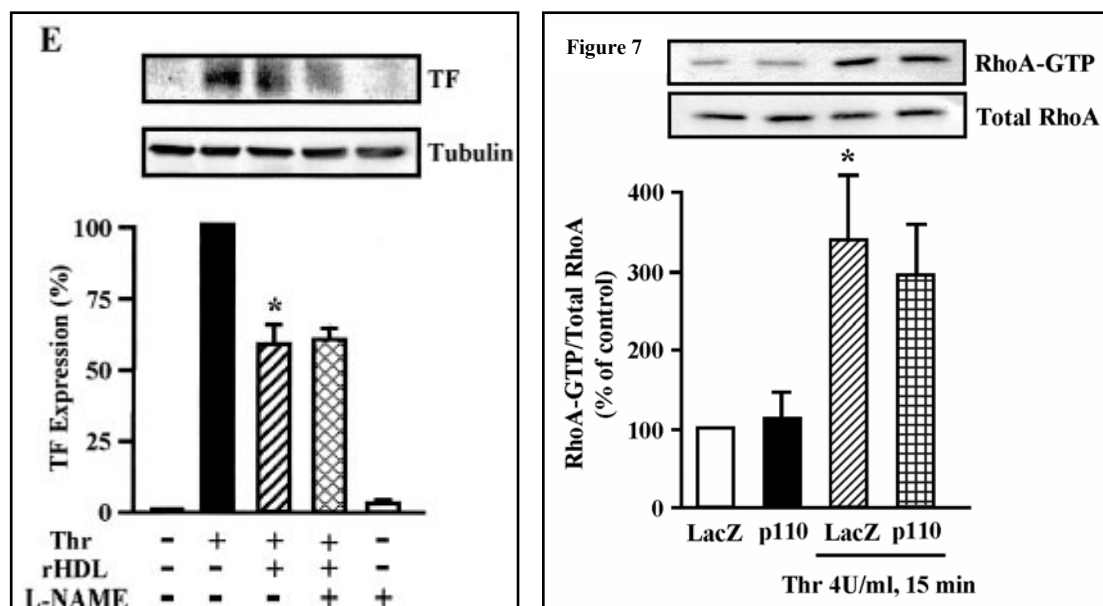
The role of Akt on TF expression was further investigated with the negative Akt mutant, Akt-KA whose expression was demonstrated by immunoblotting on **Figure. 6C**.

An *in vitro* Akt kinase assay revealed that Akt was inhibited by ectopic expression of Akt-KA (**Figure. 6D**, panel a and c), indicating that Akt-KA functions as dominant negative mutant in the cells. It is also important to note that thrombin (4 U/ml, 4 hours) reduced Akt activity which was reversed in the presence of rHDL (0.1mg/ml), and this recovering effect of rHDL on Akt activity was abolished by the dominant negative Akt-KA mutant (**Figure. 6D**, panel a and c).



**Figure 6:** C, Expression of transgenes of HA-tagged LacZ (B and C, lane 1), the active m/p-Akt mutant (B, lane 2), and the negative Akt mutant (KA; C, lane 2) was demonstrated by immunoblotting using anti-HA antibody. Da, In HUVECs expressing LacZ control gene, basal Akt activity (lane 1) measured by phosphorylation of GSK-3 (p-GSK-3) as substrate was suppressed by thrombin (Thr 4 U/mL, 4 hours, lane 2), which was reversed in the presence of rHDL (0.1 mg/mL, lane 3). Expression of the negative Akt-KA mutant inhibited Akt activity (lane 4), which remained low by thrombin treatment (lane 5) and could not be reversed by rHDL (lane 6). Db, In HUVECs expressing LacZ control gene, no significant basal TF expression was observed (lane 1), but it was markedly upregulated by thrombin (4 U/mL, 4 hours, lane 2) reduced by rHDL (0.1 mg/mL, lane 3). Negative Akt-KA mutant alone had no effect on basal TF expression (lane 4) but reduced thrombin's effect (lane 5) and did not reverse rHDL's inhibitory effect on TF expression (lane 6). Tubulin expression was used to ensure equal protein loading. Quantification of the data from Da and Db is shown in Dc.

In line with the effect of the active Akt-m/p mutant which enhanced TF up-regulation by thrombin (**Figure. 6A**), Akt-KA reduced TF up-regulation by thrombin and did not reverse the inhibitory effect of rHDL on TF expression, although neither active nor negative Akt mutants alone exerted any effect on basal TF expression in the cells (**Figure. 6A** and **Figure. 6D**, panel b and c).



**Figure 6:** E, eNOS inhibitor L-NAME, (100  $\mu$ mol/L, n=3) did not affect thrombin's effect on TF expression. \* $P < 0.05$  vs Thr alone in A and E; \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  between the groups in D. **Figure 7:** Adenoviral expression of the active PI3K (rCD2-p110) did not inhibit RhoA activation by thrombin. Infection of HUVECs with the active mutant of PI3K, p110, did not inhibit RhoA activity induced by thrombin (4 U/ml, 15 minutes). \* $p < 0.05$  vs. LacZ.

Moreover, inhibition of eNOS by L-NAME (100  $\mu$ mol/L) did not affect rHDL's effect, and L-NAME alone did not modify basal level of TF expression in the cells (**Figure. 6E**, n=3). Note that viral expression of the active PI3K mutant p110 did not inhibit activation of RhoA by thrombin (4U/ml, 15minutes, n=6, **Figure. 7**).

### DISCUSSION (PART 3)

There is compelling evidence showing that HDL is cardioprotective and low HDL level is an independent risk factor of coronary artery disease (Assmann et al. 2003). The cardioprotective mechanisms of HDL are complex and attributed to multiple biological functions beyond reverse-cholesterol transport (Nofer et al. 2002). Among other mechanisms, protective effects of HDL on endothelial function seem to play an important role (O'Connell et al. 2001, Assmann et al. 2003, Calabresi et al. 2003). The vascular endothelial cells exert a wide spectrum of biological functions as diverse as the control of smooth muscle contraction, platelet aggregation, inflammation, as well as hemostasis (Yang et al. 2002). Alterations of one or more of the above-mentioned physiological roles constitute of endothelial dysfunction that is important in triggering cardiovascular events. Among other factors, TF is importantly involved in atherothrombosis (Ardissino et al. 1997, Moons et al. 2002, Suefuji et al. 1997, Misumi et al. 1998, Annex et al. 1995, Wilcox et al. 1989, Marmur et al. 1996, Toschi et al. 1997). TF is up-regulated in atherosclerotic plaque and the plasma concentration of TF is increased in patients with unstable angina and myocardial infarction (Suefuji et al. 1997, Misumi et al. 1998, Annex et al., 1995). Previous studies showed that thrombin up-regulates TF expression which is associated with an increased cell surface TF activity (Eto et al., 2002, Archipoff et al., 1991). This study further confirmed the stimulating effect of thrombin on TF expression. Most interestingly, the induction of endothelial TF expression by thrombin was significantly inhibited by rHDL. It is important to note that rHDL exerts similar biological functions as plasma HDL and intravenous infusion of rHDL restores HDL plasma concentration and improves endothelial function in ABCA1 heterozygote subjects with isolated low HDL level or in hypercholesterolemic patients (Spieker et

al., 2002, Bisoendial et al., 2003). The results of this study not only implicate a novel cardiovascular protective function of HDL, but also the therapeutic potential of rHDL in patients with coronary heart disease (Spieker et al., 2002, Bisoendial et al., 2003). Indeed, HDL isolated from human plasma, similar to rHDL, was also able to inhibit thrombin-induced endothelial TF expression.

Although the regulatory mechanisms of endothelial TF expression have not been fully elucidated yet, recent research provides compelling evidence that RhoA and p38<sup>mapk</sup> are important positive regulators of TF expression in endothelial cells (Eto et al., 2002, Ishibashi et al., 2003). In current study we demonstrated that adenoviral expression of an active RhoA mutant increased endothelial TF protein level, which further confirms the role of RhoA in up-regulating TF expression in endothelial cells (Eto et al., 2002, Ishibashi et al., 2003). Most interestingly, rHDL was able to inhibit thrombin-induced RhoA activation indicating that rHDL reduces endothelial TF expression by blocking RhoA activation. The mechanisms of RhoA inhibition by rHDL are unclear at this stage. Several possibilities could be postulated. Firstly, rHDL may inhibit upstream mechanisms of RhoA activation, such as guanine nucleotide exchange factors (GEFs) that transform the inactive RhoA.GDP to the active RhoA.GTP form, or inhibit GTPase-activating proteins (GAP) that return RhoA.GTP to the RhoA.GDP form, or rHDL may enhance activity of GDP-dissociation inhibitor that prevents GDP dissociation from the GDP-bound form and keeps the small G-protein inactive. Secondly, rHDL may inhibit HMG-CoA reductase and thereby the production of HMG-CoA/mevalonate intermediates that are required for activation of RhoA (Takemoto et al., 2001). The above-mentioned hypotheses need further investigation.

The fact that TF up-regulation by thrombin was only partially inhibited by rHDL in spite of a full inhibition of RhoA suggests that other mechanism which up-regulates TF expression may not be affected by rHDL. Several studies demonstrated the crucial role of p38<sup>mapk</sup> in TF upregulation in endothelial cells and monocytes (Eto et al., 2002, Blum et al., 2001, O'Reilly et al., 2003, Chu et al., 2001). In this study it was shown that p38<sup>mapk</sup> activation by thrombin remained unaffected by rHDL suggesting that rHDL inhibits endothelial TF expression via interfering with RhoA but not p38<sup>mapk</sup>.

While RhoA and p38<sup>mapk</sup> positively regulate TF expression, PI3K/Akt pathway was shown to inhibit TF expression in human endothelial cells (Eto et al., 2002, Blum et al., 2001). In this study, rHDL activates Akt which is in line with the results of several other studies using plasma HDL (Mineo et al., 2003, Nofer et al., 2001). The effects of PI3K/Akt on thrombin-induced TF up-regulation in human endothelial cells were further investigated here. Two lines of evidence support a role of PI3K in the rHDL-mediated suppression of TF up-regulation by thrombin. Firstly, inhibition of PI3K by two different inhibitors wortmannin or LY-294002 reversed the effect of rHDL suggesting that rHDL inhibits TF up-regulation by thrombin via PI3K. It is to notice that wortmannin alone but not LY-294002, enhanced TF expression indicating that wortmannin may have certain non-specific effects not related to PI3K. Secondly, expression of an active PI3K mutant (rCD2-p110) reduced thrombin-stimulated TF expression (**Figure. 5C**). The function of the active PI3K in the cells was confirmed by the activation of Akt (phosphorylation of Akt at serine-473). Surprisingly, adenoviral expression of the active Akt mutant (m/p) enhanced thrombin-induced TF expression. Moreover, expression of a dominant negative Akt mutant (KA) reduced thrombin's effect and did not reverse the rHDL's inhibitory effect on TF expression

(**Figure. 6D**, panel b and c). These data suggest that PI3K reduces TF expression stimulated by thrombin not via Akt under the experimental condition. Activation of other downstream effectors of PI3K, which is independent of Akt, such as PKC $\xi$ , p70<sup>s6k</sup>, protein tyrosin kinases (Tec family) and Rac47 or even some other yet to be identified effector(s) must play a dominant role in the inhibition of TF expression. Which of these downstream effector(s), that is dominant to Akt, accounts for the negative regulation of PI3K on TF expression, remains to be identified. These results contrast the observation by a recent study showing that expression of a constitutively active Akt mutant reduces VEGF-induced TF expression in human endothelial cells (Blum et al., 2001). The discrepancy between this study (Blum et al., 2001) and those obtained in our study remains obscure. It might be due to the different agents used for the stimulation of TF expression. VEGF was used in that study whereas thrombin was used as a stimulator of TF expression in this condition. It is noteworthy that neither the constitutively active Akt mutant nor the dominant negative mutant alone has any effect on basal TF expression, but they influence thrombin's effect, i.e. the active Akt mutant facilitated and the negative Akt mutant reduced thrombin-induced endothelial TF up-regulation, suggesting that activation of Akt alone is not sufficient to modify TF expression and an interaction between Akt and thrombin-induced signalling mechanisms related to TF expression must exist, which may be different from that of Akt with VEGF. Hence, differential interaction between Akt and the signal transduction pathways stimulated by VEGF or thrombin may explain the different effect of active Akt on TF regulation.

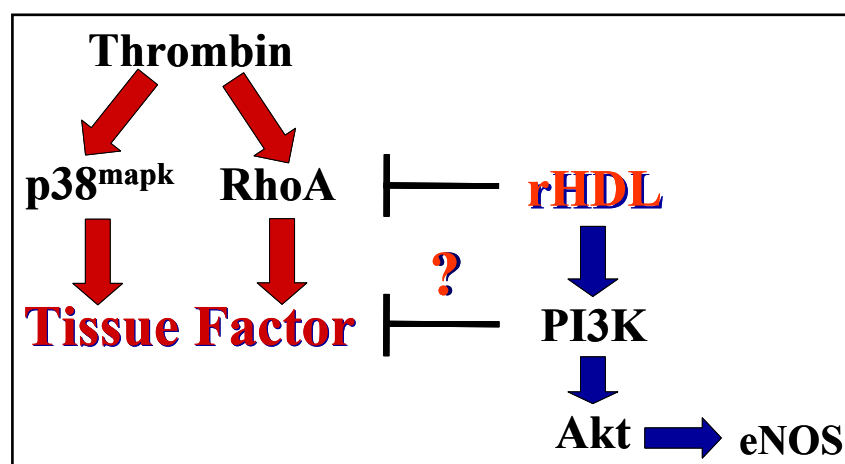
Activation of Akt affects cellular functions involving several downstream targets (Shiojima et al., 2002). In human endothelial cells, Akt phosphorylates eNOS at serine-1177 and enhances eNOS enzyme activity (Fulton et al., 1999, Dimmeler et



al., 1999, Ming et al., 2002). In line with the observation with plasma HDL (Mineo et al., 2003) we also showed in the current study that rHDL stimulates Akt and eNOS. The activation of Akt/eNOS could be abolished by PI3K inhibitor wortmannin. However, inhibition of eNOS by L-NAME did not affect the inhibitory effect of rHDL on thrombin-induced up-regulation of TF expression implicating that eNOS is not involved in the inhibition of endothelial TF expression by rHDL. This result further supports the conclusion that rHDL inhibits thrombin-induced endothelial TF expression through stimulation of PI3K but not Akt/eNOS. Similar observations were obtained in human endothelial cells stimulated with VEGF, a well-known eNOS stimulator (Blum et al., 2001). Indeed, VEGF-induced TF expression in the cells was not modified by L-NAME (Blum et al., 2001). These results contrast with the observations in macrophages and endothelial cells when stimulated with endotoxin or IL-1 $\beta$  (Perez-Ruiz et al., 2002, Yang et al., 2000, Corseaux et al., 1998), where NO is implicated in suppression of TF expression. The discrepancy between this study and those studies may be explained by the fact that large amounts of NO are released from iNOS in macrophages or endothelial cells stimulated with endotoxin, whereas stimulation of eNOS by rHDL is relatively weak that is not sufficient to inhibit TF expression under this experimental condition. Moreover, in the experiments where L-arginine was supplemented to enhance NO production, an inhibition of TF production was observed (Yang et al., 2000, Corseaux et al., 1998). However, a non-NO-mediated role for L-arginine in modulating TF expression cannot be excluded with complete certainty (Loscalzo J, 2000).

A recent study by Gratton demonstrated that adenoviral expression of a constitutively active Akt in endothelial cells causes inactivating phosphorylation of MEKK3 which leads to subsequential inhibition of MKK3/MKK6-p38<sup>mapk</sup> pathway,

and conversely dominant negative Akt mutant decreases inactivating phosphorylation of MEKK3 and therefore, enhances MKK-p38<sup>mapk</sup> activation in response to VEGF (Gratton et al., 2001), suggesting a negative regulation of p38<sup>mapk</sup> pathway by PI3K/Akt pathway. This mechanism, however, does not account for the inhibition of thrombin-induced TF expression by rHDL, since p38<sup>mapk</sup> activation by thrombin was not affected by rHDL (**Figure. 3**). The possibility that rHDL reduces thrombin-induced endothelial TF expression via interfering RhoA activation by PI3K can also be excluded, since adenoviral expression of a constitutively active PI3K mutant did not inhibit thrombin-induced RhoA activation in the cells (**Figure. 7**).



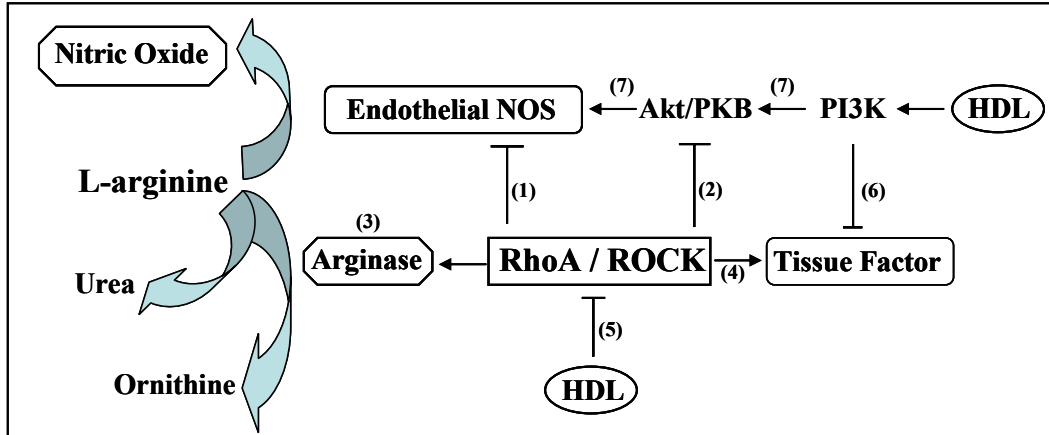
**Figure 8:** Schematic diagram showing mechanisms of inhibition of endothelial tissue factor expression by HDL.

Conversely, active RhoA did not interfere with PI3K activity as reported in a previous study (Ming et al., 2002). Therefore, both inhibition of RhoA and activation of PI3K contribute to the suppression of thrombin-induced TF expression by rHDL. The inhibition of RhoA by rHDL can also explain the result presented on **Figure. 6** showing that rHDL reversed thrombin's inhibitory effect on Akt activation, since RhoA activation induced by thrombin inactivates Akt in the cells as demonstrated by a previous study (Ming et al., 2002).

In conclusion, rHDL inhibits thrombin-induced TF up-regulation via inhibition of RhoA and stimulation of PI3K but not Akt/eNOS (**Figure. 8**). This finding may represent a novel atherosprotective mechanism of HDL in clinical settings and implicate the therapeutic potential rHDL in coronary artery disease.

#### IV. CONCLUSIONS

Taken all the results together, we conclude that Rho/ROCK pathway is activated under atherogenic stimuli, which plays multiple roles in the regulation of endothelial cell function at several levels (details, see **Figure 9**).



**Figure 9:** The multiple roles of RhoA/ROCK pathway in endothelial dysfunction. Atherogenic stimulants activate Rho/ROCK pathway which decreases endothelial NO production via destabilizing eNOS mRNA (1) and inhibiting eNOS enzyme activation through blockade of PKB/Akt (2). Moreover, Rho/ROCK increases arginase activity, which metabolizes L-arginine to urae and ornithine, thereby further decreasing NO production by depletion of the eNOS substrate, L-arginine in the cells (3). Activation of the Rho/ROCK pathway also upregulates tissue factor expression in endothelial cells (4). This effect of Rho/ROCK could be prevented by HDL (5). In addition, HDL suppresses endothelial tissue factor expression via PI3K (6), independent of Akt/PKB/eNOS cascade (7). Activation of the PI3K/PKB/eNOS pathway by HDL leads to an enhanced NO production.

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## VI. CURRICULUM VITAE

**Name** : Hema Viswambharan

**Date of Birth** : January 4th, 1970

**1983 - 1989** : High School attendance at the Methodist Girls' High School, Kuantan, Malaysia

**1990 - 1995** : Bachelor of Science (Hons.) in Genetics, University of Malaya, Malaysia. Project work on 'Growth performance of four strains of *Oreochromis sp.* (Tilapia) in different pond systems.'

**1996 - 1999** : Master of Medical Science in Medical Microbiology, University of Malaya, Malaysia. Project work on 'Detection and characterization of *Chlamydia trachomatis* among women with pelvic inflammatory disease in Malaysia.'

**1999 - 2000** : Research Assistant in Institute of Physiology, University Zurich, Switzerland

**2000 - present**: PhD studentship under the supervision of Prof. Dr. Zhihong Yang at the University of Fribourg, Switzerland.

### **PUBLICATIONS**

- Yang Z, Huerlimann DA, **Viswambharan H**, Luescher TF. Phorbol ester downregulates PDGF beta-receptor via PKC beta1 in vascular smooth muscle cells. *Biochem Biophys Res Commun.* 2001. 286:372-375.
- Quaschnig T, d'Uscio LV, **Viswambharan H**, Ruschitzka FT, Luscher TF. Chronic vasoactive peptide inhibition restores endothelin-converting enzyme activity and normalizes endothelin levels in salt-induced hypertension. *Nephrol. Dial. Transplant.* 2001. 16: 1176-1182.

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- **Viswambharan H**, Ming XF, Zhu S, Hubsch A, Lerch P, Vergeres G, Rusconi S, Yang Z. Reconstituted High-Density Lipoprotein Inhibits Thrombin-Induced Endothelial Tissue Factor Expression Through Inhibition of RhoA and Stimulation of Phosphatidylinositol 3-Kinase but not Akt/Endothelial Nitric Oxide Synthase. Circ Res. 2004. 94:918-925
- Ming XF, Barandier C., **Viswambharan H.**, Kwak BR., Mach F., Mazzolai L., Hayoz D., Ruffieux J., Rusconi S., Montani JP., Yang Z. Thrombin Stimulates Human Endothelial Arginase Enzymatic Activity via RhoA/ROCK Pathway. Implications for Atherosclerotic Endothelial Dysfunction. Circulation. 2004. 110:3708-14.
- Mujynya-Ludunge K., **Viswambharan H.**, Driscoll R., Ming XF., von Segesser LK., Kappenberger L., Yang Z., Vassalli G. Endothelial Nitric Oxide Synthase Gene Transfer restores Endothelial-dependent relaxations and attenuates lesion formation in carotid arteries in apolipoprotein E-deficient mice. Basic Res Cardiol. 2004. 99: 102-111.

### **PRIZES AND AWARDS**

1. Awarded the ‘**Stiftung Pfizer Forschungs-Preis**’ **2005** for the work ‘Reconstituted High Density Lipoprotein Inhibits Endothelial Tissue Factor Expression’.



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## APPENDIX

### ***Publications:***

1. *Rho GTPase/ Rho Kinase negatively regulates eNOS phosphorylation through inhibition of protein kinase B/Akt in human endothelial cells. Mol Cell Biol.* 2002. 22:8467-8477.
2. *Thrombin Stimulates Human Endothelial Arginase Enzymatic Activity via RhoA/ROCK Pathway. Implications for Atherosclerotic Endothelial Dysfunction. Circulation.* 2004. 110:3708-14.
3. *Reconstituted High-Density Lipoprotein Inhibits Thrombin-Induced Endothelial Tissue Factor Expression through Inhibition of RhoA and Stimulation of Phosphatidylinositol 3-Kinase but not Akt/Endothelial Nitric Oxide Synthase. Circ Res.* 2004. 94:918-925.
4. *Phorbol ester downregulates PDGF beta-receptor via PKC beta1 in vascular smooth muscle cells. Biochem Biophys Res Commun.* 2001. 286:372-375.
5. *Chronic vasopeptidase inhibition restores endothelin-converting enzyme activity and normalizes endothelin levels in salt-induced hypertension. Nephrol. Dial. Transplant.* 2001. 16: 1176-1182.
6. *HMG-CoA reductase inhibition improves endothelial cells function and inhibits smooth muscle cells proliferation in human saphenous veins. J Am Coll Cardiol.* 2001. 36:1691-1697.
7. *Enhanced endothelial nitric oxide synthase activity in mice infected with Trypanosoma brucei. Int J Parasitol.* 2003. 33:1099-104.
8. *Endothelial Nitric Oxide Synthase Gene Transfer restores Endothelial-dependent relaxations and attenuates lesion formation in carotid arteries in apolipoprotein E-deficient mice. Basic Res Cardiol.* 2004. 99