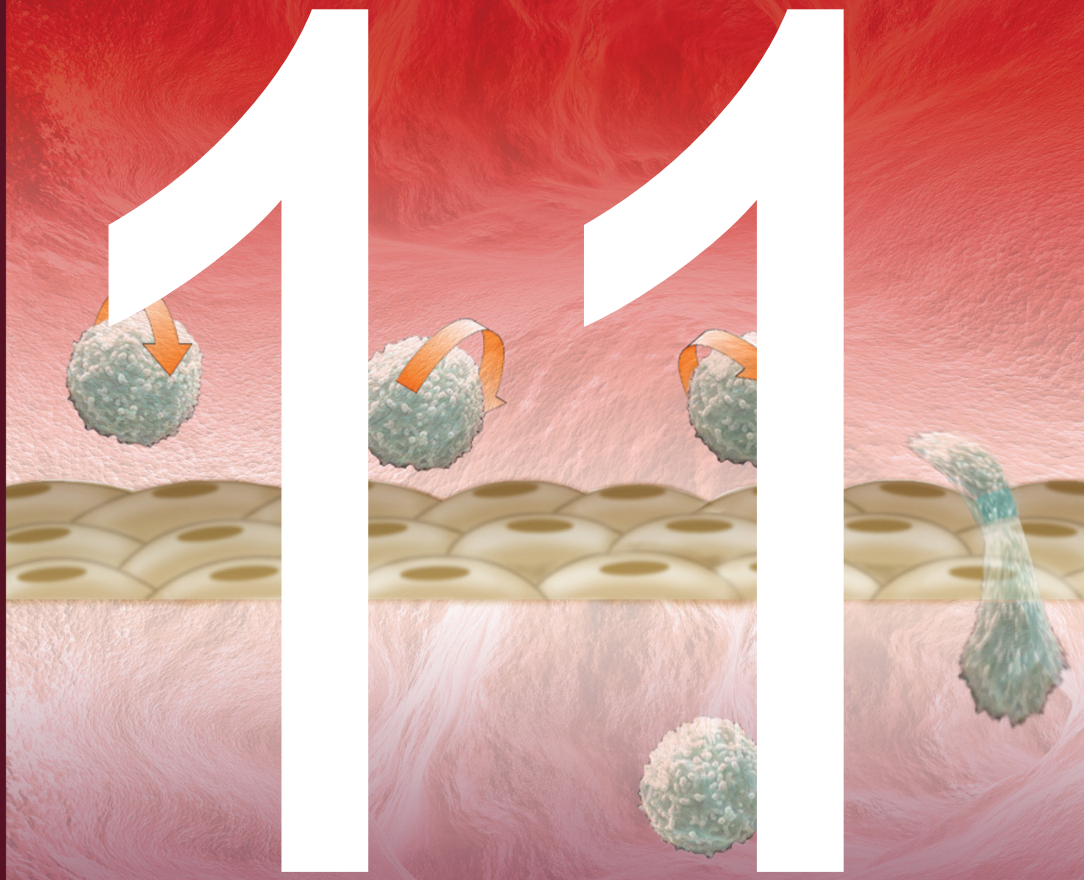
Mechanisms of Vascular Disease:

A Reference Book for Vascular Specialists



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Mechanisms of Vascular Disease

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A Reference Book for Vascular Specialists

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Abbreviation List

a1-PI	a1-protease inhibitor
5-HT	5-Hydroxytryptamine/Serotonin
AAA	Abdominal aortic aneurysm
AAS	Acute aortic syndrome
AAV	Adeno-associated viruses
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ACS	Abdominal compartment syndrome
ACTH	Adrenocorticotropic hormone
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
ALI	Acute lung injury
AMP	Adenosine monophosphate
AMPA	α-amino-3 hydroxy-5-methylisoxazole
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
AOD	Aortic occlusive disease
AP1	Activated protein 1
APC	Activated protein C
APC	Antigen presenting cell
APLAS	Antiphospholipid antibody syndrome
ApoAI	Apolipoprotein AI
ApoE	Apolipoprotein E
APS	Antiphospholipid antibody syndrome
APTT	Activated partial thromboplastin time

ARDS	Acute respiratory distress syndrome
AT	Antithrombin
ATP	Adenosine triphosphate
AVP	Ambulatory venous thrombosis
β2-GPI	β2-glycoprotein Ib
bFGF	Basic fibroblast growth factor
BKCa	Large conductance calcium activated potassium channel
BMPs	Bone morphogenetic proteins
BMS	Bare metal stent
CAD	Coronary artery disease
CaM	Calmodulin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
cGMP	Cyclic guanine monophosphate
CD	Cluster of differentiation
CD40L	Cluster of differentiation 40 ligand
CEA	Carotid endarterectomy
CETP	Cholesteryl ester transfer protein
CFD	Computational fluid dynamics
CG	Cationized gelatin
CGRP	Calcitonic gene regulated peptide
CHD	Coronary heart disease
CI	Confidence interval
CIMT	Carotid intimal-media thickness
c-JNK	c-Jun N-terminal kinase
CK-MB	Creatinine kinase (Myocardial specific)
CNCP	Chronic noncancer pain
cNOS	Constitutive nitric oxygen synthase enzyme
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CROW	Charcot restraint orthotic walker
CRRT	Continuous renal replacement therapy

CRP	C-reactive protein
CRPS	Complex regional pain syndromes
CT	Computational tomography
CTA	Computed tomographic angiography
CTD	Connective tissue disorders
CTGF	Connective tissue growth factor
CYP	Cytochrome P450
CVD	Cardiovascular disease
CVI	Chronic venous insufficiency
DAG	Diacylglycerol
DES	Drug-eluting stent
DRG	Dorsal root ganglion
DNA	Deoxyribonucleic acid
DSA	Digital subtraction arteriography
DTS	Dense tubular system
DVT	Deep vein thrombosis
EC	Endothelial cell
ECM	Extracellular matrix
EDCF	Endothelium-derived contracting factor
EDH	Endothelium-dependent hyperpolarisation
EDS	Ehlers-Danlos syndrome
EET	Epoxyeicosatrienoic acids
ELAM-1	Endothelial-leukocyte adhesion molecule-1
ELG	Endoluminal grafts
ELISA	Enzyme linked immunosorbent assay
E_K	Equilibrium potential
E_M	Membrane potential
eNOS	Endothelial nitric oxide synthase enzyme
EPC	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
ePTFE	Expanded polytetrafluoroethylene
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate

ET	Essential thrombocytosis
ET-1	Endothelin 1
EVAR	Endovascular aortic aneurysm repair
EVLA	Endovenous LASER ablation
FDA	Food and drug administration
FDPs	Fibrin degradation products (soluble)
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor 2
FMN	Flavin mononucleotide
FVL	Factor V Leiden
GABA	Gamma-aminobutyric acid
GABA B	Gamma-aminobutyric acid subtype B
G-CSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte-macrophage colony stimulating factor
GP	Glycoprotein
GPCR	G-protein coupled receptor
GSV	Great saphenous vein
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HIF	Hypoxia inducible factor
HIT	Heparin induced thrombocytopenia
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMG Co-A	Hydroxymethylglutaryl coenzyme-A
HMW	High molecular weight
HPETE	Hydroperoxyeicosatetraenoic acid
HETE	Hydroxyeicosatetraenoic acids
HR	Hazard ratio
hsCRP	High-sensitive C-reactive protein
HSP	Heat shock protein
HUV	Human umbilical vein
IAH	Intra-abdominal hypertension

IAP	Intra-abdominal pressure
IAPP	Intra-abdominal perfusion pressure
ICAM-1	Inter-cellular adhesion molecule-1
ICAM-2	Inter-cellular adhesion molecule-2
ICP	Intra-compartmental pressure
ICU	Intensive care unit
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IHD	Ischemic heart disease
IL	Interleukin
IL-1	Interleukin-1
IL-1α	Interleukin-1 alpha
IL1-β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
ILT	Intraluminal thrombus
IKCa	Intermediate conductance calcium-activated potassium channels
IMH	Intramural haematoma
IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthase enzyme
IP(3)	1,4,5-inositol triphosphate
IRI	Ischemia reperfusion injury
IVIG	Intravenous pooled immunoglobulin
IVUS	Intravascular ultrasound
KGF	Keratinocyte growth factor
KGF-2	Keratinocyte growth factor-2
LAP	Latency associated peptide
LCS	Limb compartment syndrome
LDL	Low density lipoprotein
LDS	Loeys-Dietz syndrome
LLC	Large latent complex
LEC	Lymphatic endothelial cells

LFA-1	Lymphocyte function-associated antigen-1
LO	Lipoxygenase
LOX	Lysyl oxidase
LOPS	Loss of protective sensation
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTGFBP	Latent TGF binding protein
MAC-1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MFS	Marfan syndrome
MHC	Major histocompatibility
MI	Myocardial infarction
MIP-1	Macrophage inflammatory protein-1
MLC_{20}	Myosin light $chain_{20}$
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
MODS	Multiple organ dysfunction syndrome
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MRTA	Magnetic resonance tomographic angiography
MTHFR	Methylenetetrahydrofolate reductase
MT-MMP	Membrane-type MMP
MVPS	Mitral valve prolapse syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor

NFκB	Nuclear factor kappa B
NiTi	Nitinol
NJP	Non-junctional perforators
NMDA	N-methyl-D-aspartate
NNH	Number needed to harm
NNT	Number needed to treat
NO	Nitric oxide
NOS	Nitric oxide synthase enzyme
NSAID	Non-steroidal anti-inflammatory drug
NV	Neovascularisation
OCP	Oestrogen/progesterone contraceptive pill
OPN	Osteopontin
OPG	Osteoprotegerin
OR	Odds ratio
OxLDL	Oxidised low density lipoprotein
PAD	Peripheral arterial disease
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PAR-1	Protease activated receptor-1
PAR-4	Protease activated receptor-4
PAU	Penetrating aortic ulcer
PC	Protein C
PCA	Poly (carbonate-urea) urethane
PCI	Percutaneous coronary intervention (angioplasty)
PCWP	Pulmonary capillary wedge pressure
PDGF	Platelet-derived growth factor
PDGFβ	Platelet-derived growth factor-β
PDS	Polydioxanone
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PEDF	Pigment epithelium-derived factor
PES	Paclitaxel-eluting stent

PET	Positron emission tomography
PF4	Platelet factor 4
PGI_2	Prostacyclin
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
$PGEI_2/PGI_2$	Prostaglandin I_2
PGN	Peptidoglycan
PHN	Postherpetic neuropathy
PHZ	Para-anastomotic hyper-compliant zone
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PLOD	Procollagen lysyl hydroxylase
PMCA	Plasma membrane Ca^{2+} APTases
PMN	Polymorphonuclear leukocyte
POSS	Polyhedral oligomeric silsesquioxanes
PPAR	Peroxisomal proliferation activating receptor
PPI	Proton pump inhibitor
PRV	Polycythaemia rubra vera
PS	Protein S
PSGL-1	P-selectin glycoprotein ligand-1
PT	Prothombin time
PTCA	Percutaneous coronary angioplasty
PTFE	Polytetrafluoroethylene
PTS	Post-thrombotic syndrome
PUFA	Polyunsaturated fatty acid
PVI	Primary valvular incompetence
rAAA	Ruptured AAA
Rac	Ras activated cell adhesion molecule
RANTES	Regulated upon activation, normal T cell expressed and secreted
RAS	Renin angiotensin system
RCT	Randomised controlled trial

RF	Rheumatoid factor
RFA	Radiofrequency ablation
rhAPC	Recombinant human activated protein C
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
RSD	Reflex sympathetic dystrophy
S1P	Sphingosine-1-phosphate
SAPK	Stress-activated protein kinase
SCF	Stem cell factor
SCS	Spinal cord stimulation
ScvO2	Superior vena cava venous oxygen saturation
SDF-1	Stromal-cell-derived factor-1
SERCA	Sarco/endoplasmic reticulum CaATPases
SEP	Serum elastin peptides
SES	Sirolimus-eluting stent
SEPS	Subfascial endoscopic perforator surgery
SFA	Superficial femoral artery
SFJ	Sapheno-femoral junction
SIRS	Systemic inflammatory response syndrome
SKCa	Small conductance calcium-activated potassium channels
SLE	Systemic lupus erythematosus
SMA	Smooth muscle alpha actin
SMC	Smooth muscle cell
SMP	Sympathetically maintained pain
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptors
SNP	Single nucleotide polymorphisms
SNRI	Serotonin/Noradrenaline reuptake inhibitors
SPJ	Sapheno-popliteal junction
SPP	Skin perfusion pressure
SR	Sarcoplasmic reticulum
SSRIs	Selective serotonin re-uptake inhibitors
SSV	Small saphenous vein

SVT	Superficial thrombophlebitis
STIM1	Stromal interacting molecule 1
TαCE	TNFα converting enzyme
TAAD	Thoracic aortic aneurysm disease
TAD	Thoracic aortic dissection
TAFI	Thrombin-activatable fibrinolysis inhibitor
Tc-99 MDP	Technetium-99 methylene diphosphonate
TCA	Tricyclic antidepressant
TCC	Total contact cast
TCR	T-cell receptor
TENS	Transcutaneous electrical nerve stimulation
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF	Transforming growth factor
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TGL	Triglycerides
Th	T helper
TIA	Transient ischemic attack
TIMP	Tissue inhibitors of metalloproteinase
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
TRP	Transient receptor potential
TRPC	Transmembrane receptor potential canonical
TRPV1	Transmembrane receptor potential Vanilloid-type
TXA2	Thromboxane A2
uPA	Urokinase
UT	University of Texas
VCAM	Vascular cell adhesion molecule
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

VEGF-R	Vascular endothelial growth factor receptor
VIP	Vasoactive intestinal peptide
VLA-1	Very late activating antigen-1
VOCC	Voltage operated calcium channels
VPT	Vibratory perception threshold
VSMC	Vascular smooth muscle cells
VTE	Venous thromboembolism
VV	Varicose veins
vWF	von Willebrand factor
XO	Xanthine oxidase

11 • Platelets in the Pathogenesis of Vascular Disease and their Role as a Therapeutic Target

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INTRODUCTION

Platelets are key blood components with a physiological role in the initiation of endogenous haemostasis and effective endothelial repair following vascular injury. Platelets are responsible for the initiation of a series of complex interactions culminating in platelet aggregation and thrombus formation. As such, key platelet functions, such as adherence, activation, aggregation and interaction with coagulation factors, operate in the context of a complex and balanced interplay of receptors and mediators that ensure this process is controlled and specifically targeted to areas of vascular injury. However, in disease states, such as atherosclerosis, the abnormal initiation of platelet functions also contributes to the pathogenesis and propagation of vascular disease. Consequently, targeted therapeutic inhibition of platelets has demonstrated an important clinical role in situations of both pathological and iatrogenic vascular injury, such as atherosclerosis and angioplasty. This chapter will firstly outline the relevant platelet receptors, their agonists and other important structural platelet components and their role in platelet function. Secondly, it will outline the role of these functions in the pathogenesis and propagation of vascular disease. Finally, the mechanism of therapeutic anti-platelet agents will be reviewed along with a description of currently used methods to assess platelet function.

PLATELET FUNCTION – ADHESION AND ACTIVATION

Platelets are enucleated cytoplasmic fragments of bone marrow megakaryocytes with a limited capacity for protein synthesis. Although lacking DNA, platelets do contain megakarocyte mRNA along with components necessary for protein synthesis,[1] and are capable of performing nuclear functions such as pre-RNA splicing.[2] Once in the bloodstream, platelets have a lifespan of 7–10 days. The primary function of platelets is to stop haemorrhage from sites of vascular injury. This is accomplished through the key platelet functional processes of adhesion, activation, cross-linking or aggregation, with the involvement of several important pro-activation mediators.

Platelet adhesion

Platelet adhesion is initiated by tethering of circulating platelets to an area of vascular injury. Usually, the intact endothelium prevents unwanted platelet activation by acting as a physical barrier to underlying thrombogenic substances (such as collagen, tissue factor and von Willebrand factor) and by releasing mediators that inhibit platelet activation (Figure 11.1). This involves three separate pathways, (1) the arachadonic acid-prostacyclin pathway, (2) the L-arginine-nitric oxide pathway and (3) the endothelial ecto-adenosine diphosphatase (ecto-ADPase) pathway.[3] Endothelial cyclooxygenase 1 & 2 (COX-1 & 2) convert arachadonic acid to prostacyclin metabolites (such as prostaglandin I2 (PGI2)) which elevate platelet intracellular cAMP levels and inhibit platelet activation in a process thought to be mediated by protein kinase A.[4,5] Nitric oxide, produced by endothelial cells, passively diffuses into platelets causing an increase in cytosolic cyclic guanine monophosphate (cGMP) levels and activation of cGMP dependant protein kinases with a consequent reduction in intracellular calcium.[6] Ecto-ADPase is a protein constituent of the endothelial cell surface, which upon activation, limits the recruitment phase of platelet reactivity by reducing plasma concentrations of nucleotides, particularly ADP.[7]

Endothelial cells with impairment of the above processes are termed dysfunctional, and express an 'atherogenic' profile of receptors such as P-selectin, E-selectin, ICAM-1 and VCAM-1 (as seen in Figure 11.1), as do endothelial cells which have been activated by exposure to various mediators (such as thrombin, TNF-α and LPS), sepsis, trauma, rapid temperature variations, shear stress and minor alterations to the local micro-environment.[8,9] These features usually also accompany acute vessel injury. However, in the absence of acute injury, an activated or dysfunctional endothelium may result from prolonged exposure to high blood pressure, shear stresses and dyslipidaemia, and consequently result in pathological platelet activation and inflammatory cell recruitment.[10]

Platelet adhesion begins by the exposure of circulating platelets to an activated or dysfunctional endothelium, or to exposed sub-endothelial matrix proteins such as collagen, fibrinogen and von Willebrand factor following endothelial injury.[11] These ligands are capable of binding to receptors on inactivated platelets at high shear rates and tethering them to the site of vascular injury. Collagen binds to glycoprotein VI (GPVI), whilst von Willebrand factor binds to the platelet receptor GPIb-IX-V.[12] In addition, collagen also binds von Willebrand factor, which is a mechanism of facilitating the adhesion of other inactivated platlelets. P selectin on the surface of activated endothelial cells, also binds to GP1bα and PSGL-1 on the platelet surface, facilitating tethering and rolling. Platelet adhesion triggers the process of platelet activation, culminating in the activation of the GPIIb/IIIa receptor, enabling it to bind soluble fibrinogen and von Willebrand factor allowing firm adhesion of the platelet to the endothelium[13] via fibrinogen bound to receptors on the endothelial surface (αvβ3 and ICAM-1). Figure 11.1 illustrates how a tethered platelet becomes activated and firmly adherent via fibrinogen bound surface receptors.

GPIIb/IIIa (αIIbβ3, CD41/CD61) is a member of the superfamily of 'integrin' type receptors, which are transmembrane proteins comprising of various combinations of non-covalently bonded subtypes of α and β subunits. Integrins are involved in intracellular and extracellular signal transduction (in both directions) as well as the mechanical coupling of cytoskeleton proteins to either the extracellular matrix or surface receptors on other cells.[14]

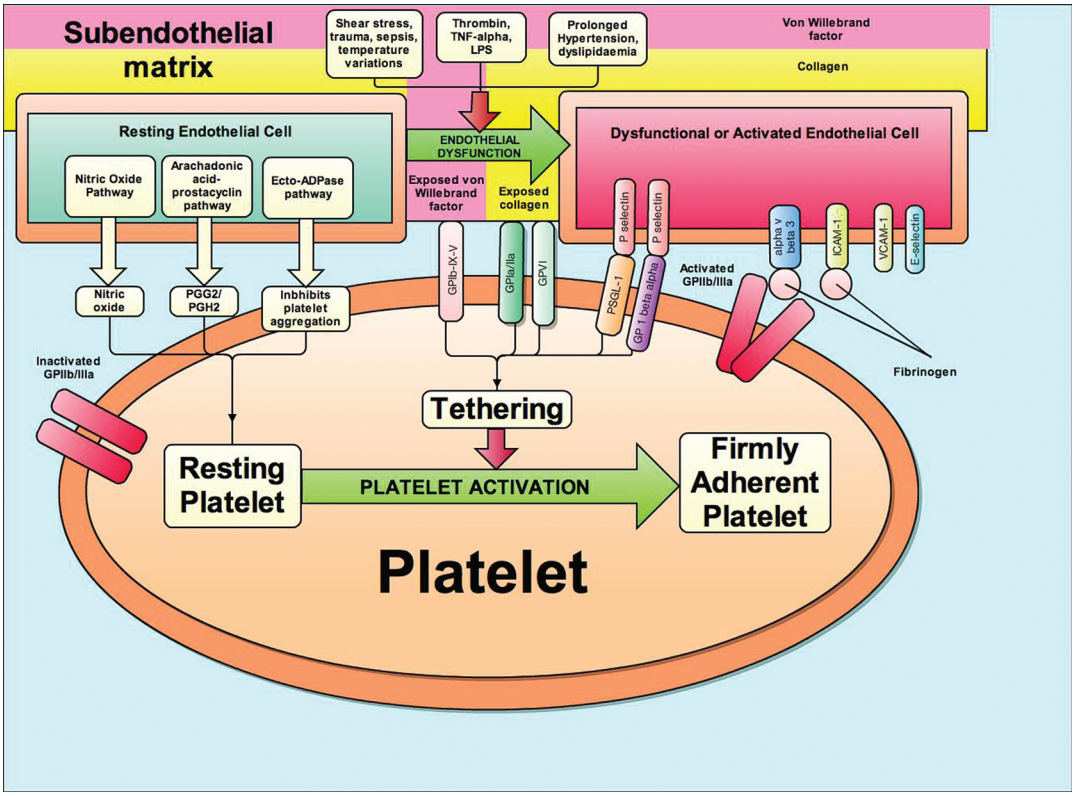

FIGURE 11.1: Mechanisms of platelet adhesion to dysfunctional endothelium. Normal endothelial cells inhibit platelet activation by three primary pathways as shown. Endothelial cells activated by injury, sepsis or inflammation, or dysfunctional (for example, after exposure to prolonged hypertension), express an array of receptors that facilitate tethering of platelet to the vascular wall, removing it from the blood stream. These interactions, particularly with potent platelet stimulators such as collagen, promote activation of the platelet; enabling activated GPIIb/IIIa receptors to bind fibrinogen bound on the surface of endothelial cells by receptors such as αvβ3 and ICAM-1. Platelet-adhesion is also mediated by interactions between platelet integrin receptors and exposed sub-endothelial matrix proteins such as collagen and von Willebrand factor. These mechanisms result in firm adhesion of the activated platelet to the vessel wall.

Platelet activation

In contrast to the relatively passive process of platelet adhesion, platelet activation is a metabolically active process involving several important and generally irreversible, biochemical and physical alterations to the platelet. These processes include the release of preformed mediators, alteration to the surface receptor profile and cytoskeletal changes resulting in a dramatic physical change to the platelet structure. These changes serve to facilitate the incorporation of the activated platelet into a developing platelet thrombus, activate neighbouring platelets via positive feedback mechanisms whilst also playing a key a role in the recruitment of inflammatory cells and the propagation of a broader inflammatory response.

Mediators of platelet activation and 'outside-in' signaling

Various non-chemical stimuli can activate platelets. These include hypothermia, trauma and alterations to acid-base balance. Nonetheless, endogenous molecules mediate the vast majority of platelet activation in both

the physiological and pathological setting, acting via both autocrine and paracrine mechanisms. The most important of these are collagen, thrombin, adenosine diphosphate, adrenaline and thromboxane A2.

'Outside-in signaling' refers to the process of mediators binding to specific receptors on the platelet surface and initiating a secondary messenger response inside the platelet. These processes are mediated via several secondary messenger pathways namely: the phospholipase C and PI-3 kinase pathway, the eicosanoid and arachidonate pathway, protein kinase C and the cAMP and cGMP pathways.[15] 'Inside out signaling' refers to intracellular pathways mediating functional changes to surface receptors, such as the GPIIb/IIIa receptor (as discussed below).

Thrombin and collagen

Collagen and thrombin are the most potent platelet stimulators. Collagen binds directly to GPVI and the integrin $\alpha_2\beta_1$ (also known as GPIa/IIa) and is crucial in the initial tethering of platelets to sites of vascular injury. Other integrin receptors bind collagen bound to von Willebrand factor. Collagen types I, III and VI are the most common type of collagen in the blood vessel subendothelial matrix and they bind directly to GPVI (see Figures 11.1 and 11.3). After binding, a series of intracellular signaling processes result in protein phosphorylation and consequently platelet activation.[16]

Thrombin is generated at sites of vascular injury from its precursor prothrombin, by virtue of the intrinsic, tissue factor-driven pathway of the coagulation cascade. Thrombin binds to G-protein linked protease activated receptors (known as PARs) on the platelet surface. Human platelets express two distinct PAR receptors, PAR1 and PAR4. PAR1 is coupled to Gα12/13, Gαq and Gαi proteins, which mediate cytoskeletal responses, increased intracellular calcium and reduced cAMP respectively, each of which are crucial steps in platelet activation (Figure 11.2). PAR1 appears to be a more potent activator of platelets than PAR4 and has been proposed as a potential target for therapeutic inhibition (atopaxar – see Figure 11.3). Effective inhibition of PAR1 and PAR4 leads to near complete inhibition of platelet activation, even in the presence of high concentrations of thrombin.[17]

Adenosine diphosphate (ADP)

ADP is generated and stored in platelets and red blood cells in dense granules. Platelet activation results in the release of stored ADP granules and activation of nearby platelets – a key amplifying process (Figures 11.2 & 11.3). Several receptors, known as P2 receptors interact with ADP resulting in platelet activation. Present on platelets are the P2X2, P2Y1 and P2Y12 receptors. P2X2 is an intrinsic ion channel, which upon ligand binding allows calcium influx into the platelet, promoting activation.[18] P2Y1 is a G-protein coupled seven transmembrane domain receptor which activates protein phospholipase C causing release of stored intracellular calcium and facilitating conformational change of the platelet. P2Y12 is similarly a G-protein coupled receptor that, upon activation, mediates a reduction in intracellular cAMP (Figure 11.2). Of these receptors, P2Y12 plays the more significant role in amplifying and sustaining the platelet activation process. Platelet function studies have demonstrated that simultaneous activation of both P2Y1 and P2Y12 receptors is needed for the full platelet response to ADP. Both these features have made inhibition of P2Y12 with clopidogrel a very effective therapeutic target.[19]

Thromboxane A2 (TXA2)

Thromboxane A_2 is synthesized from arachadonic acid in activated platelets via

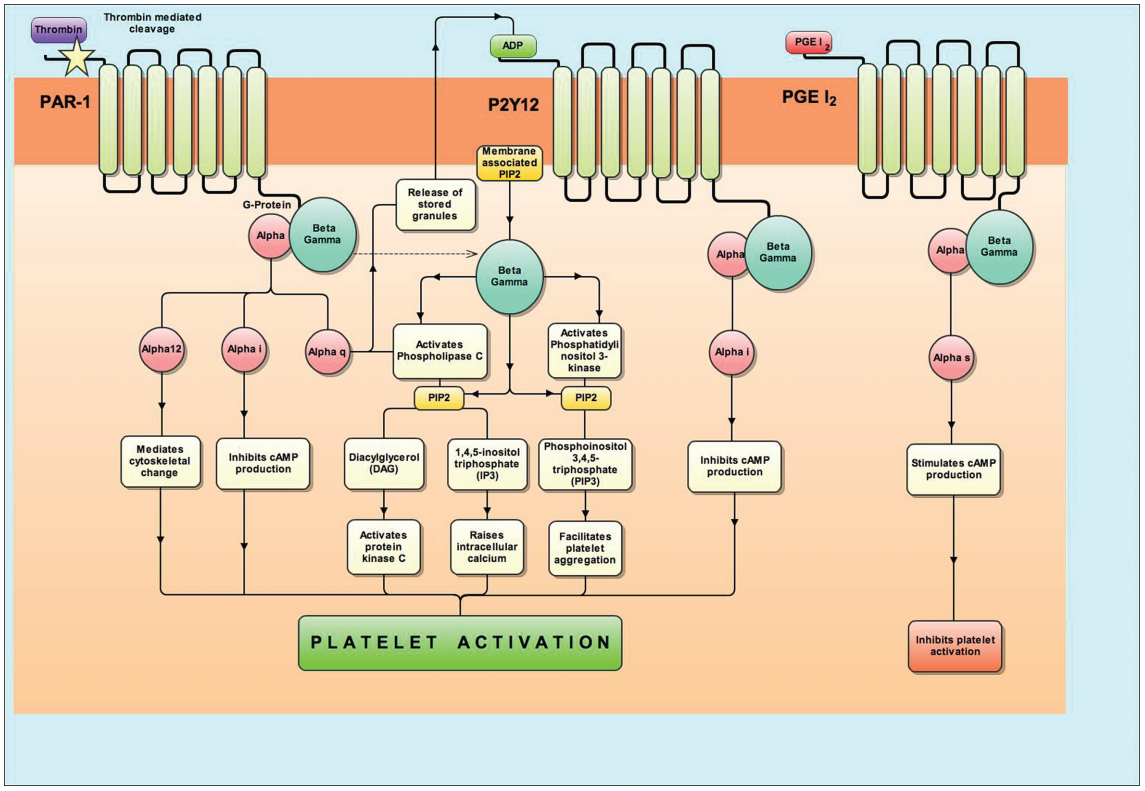

FIGURE 11.2: Intracellular signaling mechanisms involved in platelet activation. The signaling pathways of three platelet receptors are depicted as examples: PAR-1 (protease-activated receptor-1; the main ADP receptor, P2Y12; PGE I2). Each of these receptors is a seven transmembrane domain G-protein coupled receptor. Each G-protein consists of an α and $\beta\gamma$ subunit. The $\beta\gamma$ subunit is involved in the formation of DAG, IP3 and PIP3, which play key roles in platelet activation. The Gα subunit exists in several isoforms. Gα12 mediates platelet shape change by activating cytoskeletal proteins. Gαq plays a pivotal role by activating phospholipase C, and facilitating the formation DAG and IP3, which promote platelet activation by activating protein kinase C and raising cytosolic calcium. In addition, Gαq also promotes release of pre-formed granules containing pro-activating and pro-inflammatory mediators, such as ADP. Gαi and Gαs inhibit and stimulate cAMP production respectively with lower intracellular cAMP concentration favoring activation. Gαs is primarily made available in response to prostaglandin I_2 action, primarily from release of endothelial cells, inhibiting platelet activation. (Adapted from Abrams CS et al 'Platelet Biology' – *UptoDate article* Jan 2010 and Bhatt DL et al 'Scientific and therapeutic advances in antiplatelet therapy' (2003) *Nature Reviews Drug Discovery* 2: 15-18). cAMP = cyclic adenosine diphosphate, PGE I_2 = prostaglandin I_2.

the cyclooxygenase pathway. It freely diffuses across the platelet membrane to activate neighbouring platelets. Thromboxane A_2 binds to Tα or Tβ receptors, which are in turn coupled to G-proteins Gαq, Gα12 or Gα13 – each of which activate phospholipase C – an enzyme which degrades membrane phosphoinositides creating key second messengers inositol triphosphate (IP3) and diacylglycerol (DAG) which facilitate protein kinase C mediated intracellular protein phosphorylation and raised intracellular calcium respectively (Figures 11.2 and 11.3). The G-protein linked receptors aid in amplification of the response to ligand binding as a single receptor may interact with multiple G-proteins.[20]

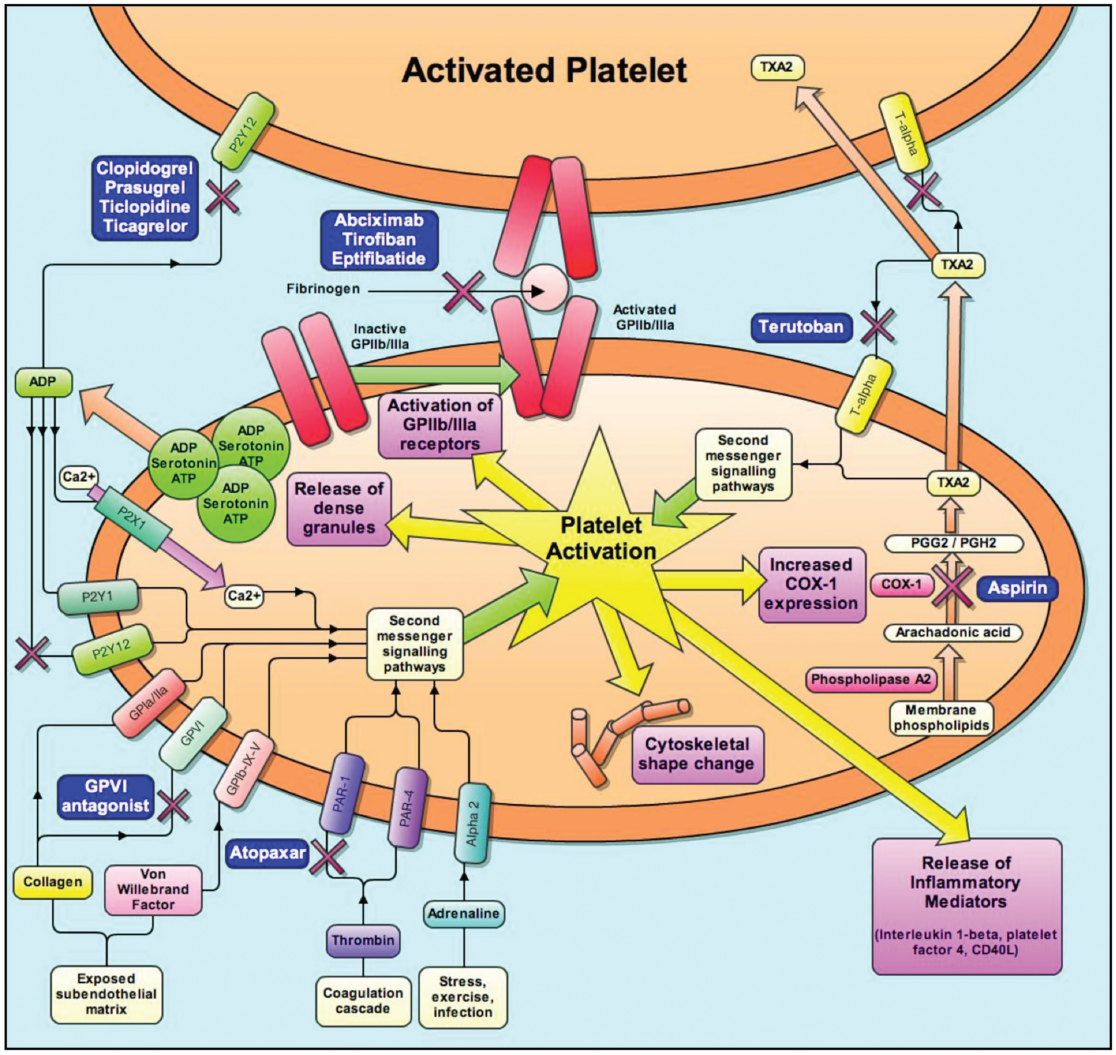

FIGURE 11.3: Mechanisms of platelet activation and platelet inhibition. A wide array of mediators can trigger platelet activation. Thromboxane A2 is produced from membrane derived arachadonic acid in a process inhibited by aspirin. TXA_2 can then freely diffuse across the platelet membrane and activate both its own and neighboring platelets. A similar mechanism of amplification is provided by the release of ADP and the stimulation of P2Y12 receptors. Other mediators act via specific receptors that promote platelet activation by raising intracellular calcium (eg. thrombin, TXA2) or reducing intracellular cAMP concentrations (ADP). These receptors provide useful therapeutic targets for platelet inhibition (shown in blue). Once activated, platelets can interact with neighboring activated platelets via fibrinogen bound to activated GPIIb/IIIa receptors, allowing cross linking and the formation of a platelet thrombus. (Adapted from Hankey GJ et al 'Antiplatelet Drugs' (2003) *MJA* 178(11): 577-8).

Adrenaline

Adrenaline is the least potent physiological stimulator of platelet activation. Surface α2 adrenergic receptors are G-protein linked receptors which are potent inhibitors of cAMP formation, although studies suggest supra-physiological doses are required for activation of platelets by adrenaline alone.[21] Nonetheless, circulating adrenaline release by a stress response, may serve to reduce

overall platelet activation threshold, making platelets more susceptible to lower doses of other platelet activating mediators.

Second messenger systems

Platelet-activating ligands bind to G-protein coupled receptors inducing intracellular second messenger pathways, which then mediate the biochemical and structural changes associated with platelet activation. G-proteins usually consist of α and $\beta\gamma$ subunits. The α subunit exists in several isoforms, each mediating a specific intracellular function.[22] The α_q and $\beta\gamma$ subunits activate phospholipase C (both) and PI3K ($\beta\gamma$) in a manner discussed further below. The α_s and α_i subunits promote or inhibit intracellular cyclic AMP activity respectively – although the exact mechanism involved is as yet unclear. Increased intracellular cAMP activity is associated with inhibition of platelet activation, a process utilized by the anti-platelet agent dypridimole. The α_{12} subunit is involved in mediation of shape change by promoting cytoskeletal reorganization (as discussed further below).[23] Figure 11.2 provides a broad outline of the key intracellular second messenger systems involved in platelet activation by several key receptors.

There are two central intracellular pathways involved in platelet activation – the phosphoinositide hydrolysis pathway and the eicosanoid synthesis pathway. The former is a consequence of the activation of phospholipase C beta (via G alpha-q subunit of G-protein linked receptor proteins) and the activation of phosphoinositol 3-kinase gamma (via G beta-gamma). Once activated phospholipase C hydrolyzes PI-4,5-P(2) (PIP2) to DAG and IP(3). DAG in turn binds to and activates protein kinase C, causing phosphorylation of key enzymes known as protein kinase C iso-enzymes. IP(3) binds to receptors within the intracellular tubular system, resulting the release of sequestered intracellular calcium.[15]

The eicosanoid pathway results in the formation of thromboxane A_2. Platelet activation results in the release of arachidonate from membrane phospholipids by the action phospholipase A_2, which is stimulated by raised intracellular calcium. Arachidonate is then metabolized to thromboxane A_2 by the action of cyclooxygenase-1 (COX-1), by a process inhibited by aspirin.[24]

Physiological consequences of platelet activation

Platelet activation involves four primary features: (1) An important conformational change of the GPIIb/IIIa integrin receptor, allowing it to bind fibrinogen and von Willebrand factor, promoting platelet aggregation and the formation of a platelet thrombus. (2) The release of preformed intracellular granules of ADP and thromboxane A_2 promotes further platelet activation and a local positive feedback of the activation process. (3) Activation results in a conformational change in the platelet itself, by rearrangement of the internal cytoskeletal ultra-structure. (4) It is increasingly recognized that platelet activation augments an inflammatory response by the surface expression of receptors, the recruitment of inflammatory cells, and the release of pro-inflammatory mediators. These functions have an important physiological role in the engagement of repair processes following injury. Nonetheless, inappropriate activation of these processes are crucial elements in pathogenesis of atherosclerosis.

The GP IIb/IIIa receptor and 'inside-out' signaling

The GPIIb/IIIa receptor is from the β_3 subgroup of the integrin receptor super-family.[25]

The receptor consists of two proteins each with a transmembrane domain. GPIIb consists of a heavy (105kD) and light (25kD) chain linked via a disulfide bond. GPIIIa consist of a single (95kD) chain.[26] Once activated, the receptor recognizes RGD (arginine-glycine-aspartic acid) and KQAGDV (glycine-glutamine-alanine-glycine-aspartic acid-valine) peptide sequences present on fibrinogen (both sequences), von Willenbrand factor and fibronectin (RGD sequence) making each of these proteins ligands for the activated receptor – with fibrinogen being the most potent.[27] As shown in Figure 11.3, one fibrinogen molecule can serve to crosslink platelets and augment platelet aggregation.[25]

GPIIb/IIIa receptors are found exclusively on platelets (with the exception of the platelet precursor, the megakaryocyte), with approximately 60-80,000 receptors per platelet comprising 2% of all protein present in the platelet. They have been utilized in the clinical setting as potent anti-platelet targets as discussed further below.

Granule exocytosis

Granule exocytosis is a key consequence of platelet activation, allowing platelet activation to be exponentially amplified and a local inflammatory response to ensue. Microscopically, platelets contain dense, alpha and lysosomal granules. Dense granules contain platelet agonists, which promote platelet activation such as ADP, ATP and serotonin. Alpha granules contain adhesion-promoting proteins including fibrinogen, fibronectin, vitronectin and von Willebrand factor. These mediate platelet aggregation. Lysosomal granules contain glycosidase and proteases, the role of which is unclear. SNARE complex proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are thought to be the primary mechanism regulating the vesicle-membrane interactions. Granule exocytosis is thought to be modified by the presence of aspirin, explaining part of its anti-platelet action.[28]

Activation-induced conformational change of platelets

Once activated, platelets undergo a dramatic physical shape change, losing their discoid shape and developing elongated projections of cytoplasm, called filopods, mediated by reorganization of the cytoskeletal ultra structure. This reorganization involves alterations to three primary components of the platelet cytoskeleton: the cytoplasmic actin network, the cytoskeletal rim and the marginal band. The membrane associated cytoplasmic actin network consists of both filamentous polymers and monomeric globular forms of actin, a 42kDa abundant cytoskeletal protein. Platelet activation results in an increase in the proportion of filamentous or F-actin, and reorganization of the actin network into longer actin filaments promoting conformational change. This process is mediated by phosphatidylinositol produced during platelet activation. F-actin filaments are anchored to the plasma membrane, via actin binding protein via the GPIb/IX complex.[29] The cytoskeletal rim contains multiple components including actin, filamin, talin, vinculin, spectrin (also seen in red blood cells), and alpha actin along with multiple membrane glycoproteins. The interaction of filamin with actin is the key factor in preserving the discoid shape of the resting platelet. Disruption of this interaction occurs in the presence of rising cytosolic calcium concentration, resulting in a loss of tethering of the GPIb to the cytoskeletal ring, promoting conformational change. Shape change is also facilitated by contraction of the tubulin polymers of the marginal band, however the exact biomechanical significance of this is still yet to be determined.[30] The net result of the above processes is the

physical transformation of the platelet from a discoid to a flat, broadened and star-shaped conformation known as spreading – allowing efficient incorporation of the platelet into an evolving platelet thrombus.

PLATELETS AND ATHEROSCLEROSIS

There is increasing evidence that platelets play a crucial role in all stages of the pathogenesis of vascular disease – particularly atherosclerosis. Research over the last 10–15 years has demonstrated that atherosclerosis involves an active inflammatory process rather than the benign accumulation of intra-luminal lipids.[31] Platelets play key roles in the development and progression of atherosclerotic plaques, by the action of released mediators and facilitating interactions with other inflammatory cells. For the subset of atherosclerotic plaques that are unstable or prone to rupture, localized platelet activation and aggregation result in an occlusive platelet thrombus interrupting blood flow and causing distal ischemic injury. This mechanism underpins myocardial infarction and acute coronary syndromes, and explains to some degree the effectiveness of anti-platelet agents in the treatment and prevention of such conditions.[32]

Role of platelets in the initiation of atherosclerosis

Platelets are the first cell to arrive at the developing atherosclerotic lesion. Studies demonstrate that platelets adhere to carotid endothelium of ApoE deficient mice.[33] P-selectin (CD62P) and E-selectin are expressed on the surface of activated endothelial cells (and platelets) which interact with GP1βα, PSGL-1 and the von Willebrand receptor complex receptors on the platelet surface in a loose manner which is insufficient for stable adherence, but instead facilitates the rolling process[34] (Figure 11.1). In addition, soluble von Willebrand factor is secreted by endothelium in response to inflammatory stimuli. Mice deficient in von Willebrand factor demonstrate a reduced propensity towards atherosclerosis.[35]

As platelets roll along the surface of activated endothelium, they become activated, and firm adhesion can occur via the interaction between β_3 integrins present on endothelial cells and the fibrinogen bound GPIIb/IIIa receptor on platelets (Figure 11.1). Once activated, platelets become firmly adherent and are able to recruit other platelets to the area of endothelial injury.[36] Inhibition of platelet activation, such as suppression of COX-1 dependant thromboxane A2 production or activity, has been demonstrated to slow the formation of atherosclerosis in murine models.[37]

Role of platelets in the progression of atherosclerosis

Activated platelets also express P-selectin on their surface, which not only mediates platelet-endothelial interactions, but also stimulates neighboring monocytes and macrophages to release pro-inflammatory mediators. For example, P-selectin mediated signaling between aggregated platelet and monocytes promotes up regulation of COX-2 mRNA and the production of interleukin-1β, which promotes inflammation and further platelet activation.[38]

Firmly attached platelets have also been shown to recruit monocytes from the blood stream to the site of vascular injury in a process described as 'tethering'. Such platelets interact with circulating monocytes via PSGL-1 (P-selectin glycoprotein ligand-1 – on monocytes) and P-selectin expressed by platelets, multiple platelet receptors including

the fibrinogen bound activated GPIIb/IIIa receptor and MAC-1 (on monocytes) and the lymphocyte function associate antigen (LFA-1), which binds to ICAM-2 on platelets. These interactions result in monocyte recruitment to the injured endothelium.[39]

These platelets release an array of pro-inflammatory mediators such as interleukin-1β, platelet factor 4, RANTES (regulated upon activation, normal T cell expressed and secreted) and CD40 ligand.[25] These mediators promote localized inflammation and atherosclerotic development by activating the vascular endothelium to facilitate the chemoattraction, chemotaxis and transmigration of monocytes.

Role of platelets in vulnerable plaques and plaque rupture

Platelets have a well-established role in the development of a thrombus after the rupture of the thin fibrous cap present in vulnerable plaques. Disruption of the thin fibrous cap, usually in the adjoining shoulder region, exposes the highly thrombogenic lipid core to the bloodstream, triggering a cascade of platelet activation and thrombosis.

However, the extent to which platelets interact with an established vulnerable plaque before it undergoes a clinically significant rupture is uncertain. It is circumstantially suggested by the success of antiplatelet therapies in reducing ischemic events.[40] Subclinical plaque rupture is a frequent event with 9% of autopsies on patients not dying from myocardial infarction demonstrating ruptured fibrous caps (22% in patients with cardiovascular risk factors). This suggests that rather than every plaque rupture precipitating an ischemic event, it is likely that the thrombotic response to plaque disruption is dynamic with thrombosis and thrombolysis occurring simultaneously in patients with acute coronary syndrome.[41,42] Consequently, a rupture prone plaque may suffer periodic disruptions in its fibrous cap resulting in ongoing interactions with activated platelets.[43] Thus, in addition to their role in acute plaque rupture, at any given time, activated platelets may be associated with unstable plaques presumably in a number and frequency proportional to the degree of plaque instability. The detection of such activated platelets potentially may allow identification of unstable plaques prior to rupture.[44]

CURRENT AND FUTURE ANTI-PLATELET AGENTS

Given the pivotal role of platelets in atherosclerosis, platelet inhibition provides major benefits in the treatment of atherosclerotic disease, both in the acute and preventative settings. Several targets have proven suitable therapeutic targets. Figure 11.3 schematically illustrates the mechanism of action of these agents.

Aspirin (Salicylic acid)

Aspirin, or salicylic acid, was the earliest known anti-platelet agent, used as early as 500BC. Aspirin irreversibly inhibits COX-1 enzymes stored in platelets, preventing the conversion of arachadonic acid to PGG_2 and PGH_2, which are substrates for formation of thromboxane A_2 – one of the platelet's primary positive feedback system mediating amplification of both intra and inter-platelet activation (Figure 11.3). Aspirin acetylates a key serine residue at the COX-1 catalytic centre, irreversibly corrupting its enzymatic function. Platelets lack the machinery to resynthesize COX-1, thus aspirin leads to irreversible platelet inhibition for the life of the platelet (7-10 days), despite its relatively short plasma half-life of 15 minutes.[45]

Aspirin has a well-established clinical benefit in vascular disease, particularly myocardial infarction and stroke, in both the acute and chronic setting. In acute myocardial infarction, aspirin demonstrated a 23% reduction of mortality at 5 weeks, in addition to thrombolysis – with benefits still measurable at 10 years.[46] Aspirin also reduces death and myocardial infarction in unstable angina, and is associated with reduced acute vessel closure following coronary angioplasty. With regards to secondary prevention, recent metanalyses of patients with previously diagnosed vascular disease (coronary artery disease, TIA, stroke or peripheral vascular disease) demonstrated a 25% reduction in vascular death, myocardial infarction or stroke. In addition, patients with stable angina, intermittent claudication and atrial fibrillation (who cannot be fully anticoagulated) also derive similar benefit from aspirin.[47] However, the benefits are less well established in the primary prevention population as the benefits of a reduced rate of myocardial infarction are tempered by an increased rate of hemorrhagic stroke. A net benefit is likely to be limited only to those patients with known cardiovascular risk factors such as diabetes.[48]

The optimal dose of aspirin had been established as 75-150mg daily, in order to achieve its full platelet inhibitory effect. Higher doses (up to 325mg daily) demonstrate increased gastrointestinal side effects with no additional anti-platelet effect.[48]

Aspirin resistance is a newly recognized phenomenon where aspirin is unable to exert its full antiplatelet action and confer its cardiovascular protection, in certain patients. Aspirin resistance is multifactorial. Pharmacological reasons include: (1) competitive inhibition by co-administration with NSAIDs (reversible COX-1&2 inhibitors); (2) the action of possible inhibitory proteins, such as vitamin D binding protein which may act directly or indirectly; and (3) genetic polymorphisms in cyclooxygenase making the active site less susceptible to the action of aspirin. Non-pharmacological reasons include: (1) an increased reactivity of platelets to other activating factors such as collagen, ADP, von Willebrand factor and adrenaline; (2) an increased rate of platelet formation, associated with myocardial infarction and coronary artery bypass graft surgery, resulting in an increased proportion of uninhibited platelet COX-1; (3) the presence of alternative pathways to thromboxane A2 generation which bypass COX-1. These may include COX-2 (present in the setting of inflammation), which requires a higher dose of aspirin to be effectively inhibited. Furthermore thromboxane A_2 precursors may be acquired from monocytes and endothelial cells, despite inhibition of COX-1. Some forms of aspirin resistance are amenable to an increased dose of aspirin, whilst other forms require a reliance upon other agents to effectively inhibit platelets.[49]

Thienopyridines

Thienopyridines are pro-drugs which, when metabolized to their active form, cause irreversible inhibition of the P2Y12 receptor and inhibit the action of ADP. The three in clinical use are ticlopidine, clopidogrel and prasugrel, of which cloidogrel is currently the most widely used.

Clopidogrel

Once in the bloodstream, clopidogrel undergoes enzymatic alteration via a two-step pathway to produce the short lived active metabolite R-130964. These steps involve hepatic enzymes of the cytochrome P450 family, particularly CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4/5.[50,51] Binding of the active metabolite to the

P2Y12 receptor results in irreversible conformational change, which inhibits ADP binding for the life of the platelet.[52]

As an antiplatelet agent, clopidogrel reduces platelet aggregation, platelet-leukocyte interactions and expression of cellular adhesion molecules such as P-selectin.[53] In addition, clopidogrel's active metabolite has been shown to improve endothelial function possibly through blocking P2Y12 receptor and/or by direct effects independent of its antiplatelet activity.[54] Clopidogrel also limits ADP's role in amplifying platelet activation through other agonists such as thrombin and collagen.[55]

The CAPRIE trial demonstrated that clopidogrel had moderately improved cardiovascular secondary protection in patients with atherosclerotic disease (myocardial infarction, ischemic stroke, peripheral vascular disease), compared to aspirin with similar tolerability. The combination of aspirin and clopidogrel, known as dual antiplatelet therapy, significantly reduces the risk of subacute in-stent restenosis post angioplasty within the first month (bare metal stents) or twelve months (drug-eluting stents), and has an evolving clinical role in the long term management of refractory unstable angina.[56]

Clopidogrel is dosed using a loading and maintenance regime. The loading dose is aimed at obtaining rapid antagonism of the P2Y12 receptor. Greater platelet inhibition is achieved more promptly with a 600mg loading dose (maximum inhibition of 40-45% 2–3 hours after loading dose) compared to a 300mg dose. However a 900mg dose does not lead to further improvements in platelet inhibition or earlier onset of action.[57] The standard maintenance dose is 75mg daily, however recent studies suggest that a 150mg daily dose has a small additional benefit in reducing the risk of stroke, MI, cardiac death and in-stent thrombosis in the subset of patients undergoing PCI for acute coronary syndromes – however the optimal duration of such therapy is yet to be determined.[58,59]

Like aspirin, a proportion of the population displays a degree of resistance to the therapeutic effects of clopidogrel. This is though to be due to polymorphisms in the hepatic enzyme responsible for the conversion of the pro-drug to its active form. The most prevalent is the 681G>A polymorphism (also known as *2 allele) in the CYP2C19 enzyme, which is responsible for first stage of clopidogrel metabolism. This allele is present in roughly 30% of the population with higher rates in the Asian population (up to 50%). Studies have suggested the presence of this polymorphism is associated with a higher rate of in-stent thrombosis, and is associated with increased risk of ischemic stroke, MI and vascular death in patients with the mutation receiving clopidogrel for secondary prevention.[60] Such patients may require an increased dose of clopidogrel, or the use of alternative agents. Genotyping prior to commencing therapy, although available, is not currently routine practice, although studies are ongoing.[58]

Given its reliance upon liver metabolism, there exists the potential for drug interactions to limit the effectiveness of clopidogrel by reducing its bioavailability. In particular, an interaction with proton-pump inhibitors such as omeprazole has been proposed. Although retrospective and observational analyses have suggested an increased rate of death or hospitalization for ACS,[61,62] and in vitro platelet aggregometry studies have suggested an impaired platelet response to clopidogrel in the presence of omeprazole, several randomized clinical trials have failed to identify adverse clinical endpoints attributable to clopidogrel and PPI interaction at this stage.[63,64,65] Such studies may have been limited by power, and further studies are currently in progress.

Prasugrel

Prasugrel is the newest thienopyridine. It has several advantages over clopidogrel. Firstly, its onset of action is significantly quicker than clopidogrel (30 minutes versus 2 hours).[66] Secondly, unlike clopidogrel, prasugrel requires only a single hepatic enzyme step for conversion to its active metabolite. Consequently, this significantly improves its bioavailability compared to clopidogrel.[67] The intermediate form of prasugrel is primarily formed through hydrolysis by the intestinal enzyme hcE2. Subsequently, intestinal (CYP3A, CYP2C9, CYP2C19) and hepatic (CYP3A, CYP2B6, CYP2C9, CYP2C19) cytochromes are involved in conversion from intermediate form to active metabolite.[68]

The decreased reliance on hepatic enzymes for active metabolite formation and its increased bioavailability explain the stronger and more consistent (amongst individuals) inhibition of P2Y12 induced platelet activation compared to clopidogrel.[69] The recent TRITON trial demonstrated a reduction in ischemic events in patients with acute coronary syndromes managed with PCI, and 50% reduction in acute and sub-acute in-stent restenosis in patients on prasugrel compared to clopidogrel. However, this benefit came at the expense of an increased rate of major bleeding.[70]

Ticlopidine

Ticlopidine was the first available thienopyridine. Like clopidogrel, it requires hepatic activation in a two-stage process. It has several disadvantages compared to clopidogrel that limit its clinical usefulness. Firstly, its onset of action is significantly slower than clopidogrel. Secondly, it requires twice daily dosing, which noticeably reduces patient compliance. Importantly, it also has several noticeable side effects including skin rashes, gastrointestinal upset and life threatening blood dyscrasias such as neutropaenia.[71]

Ticagrelor

Ticagrelor is a new non-thienopyridine competitive P2Y12 antagonist, which has recently undergone Phase III clinical trials.[72] Unlike the thienopyridines, ticagrelor is not a pro-drug, but a direct P2Y12 receptor antagonist – requiring no hepatic or intestinal enzymatic activation. It has several advantages over clopidogrel: a more reliable pharmacokinetic profile, faster onset of action, and a lack of susceptibility to genetic based resistance as with thienopyridines. In addition, reversible inhibition allows its antiplatelet effect to cease rapidly after stopping therapy, unlike thienopyridines, which require 7–10 days for a return to normal platelet function. Furthermore, a recent randomized controlled clinical trial comparing ticagrelor with clopidogrel in patients with acute coronary syndromes, found a significantly reduced rate of death from vascular causes, stroke and myocardial infarction.[72] Unlike prasugrel, this benefit was not realized at the expense of increased major bleeding. There are, however, some notable issues: firstly, ticagrelor was associated with a higher rate of procedure related bleeding compared to clopidogrel. Secondly, ticagrelor was associated with idiosyncratic side effects of symptomatic dyspnoea and transient increase in ventricular pauses. Lastly, ticagrelor requires twice daily dosing and would likely reduce patient adherence.[71] Nonetheless, ticagrelor remains an exciting agent, which may address some of the shortcomings of the thienopyridines.

GPIIb/IIIa Antagonists

The GPIIb/IIIa receptor is a useful therapeutic target given its prominent role in platelet

aggregation and thrombus development. Diverse arrays of drugs have been developed to target the GPIIb/IIIa receptor including monoclonal antibodies, cyclic peptides and chemical compounds.

Abciximab is a fully humanised monoclonal antibody specifically targeted to inhibit the GPIIb/IIIa receptor. It consists of the murine generated variable domains linked to human IgG antibody structure which limits the immunogenicity of abciximab.[73] Eptifibatide was developed from a template peptide extracted from the venom of the south-eastern pygmy rattlesnake. It consists of a cyclic heptapeptide with a KGD sequence, which confers particular specificity for the GPIIb/IIIa receptor (as opposed to the RGD sequence in other endogenous ligands).[74] Tirofiban is a small molecular weight non-peptide compound based, again on a snake venom template, which inhibits GPIIb/IIIa receptor.[75]

Of these agents, abciximab demonstrates the highest affinity for the receptor, followed by eptifibatide and tirofiban. Taking into account other pharmacokinetic properties, abciximab has a duration of action of several days, compared to 2-4 hours for eptifibatide and tirofiban. The shorter acting agents are thus preferred in patients likely to undergo cardiac surgery following catheterisation. Profound thrombocytopaenia is well-described complication of GPIIb/IIIa blocker therapy. It is likely mediated by a host immunological response towards neoepitopes exposed after the binding of the GPIIb/IIIa blockers to the receptor.[76]

GPIIb/IIIa antagonists are potent inhibitors of platelet aggregation that improve mortality in patients presenting with acute coronary syndromes, particularly those undergoing percutaneous coronary intervention (PCI).[1,2] Abciximab, has shown a 10-35% reduction in mortality. Similar results, albeit of a smaller magnitude, have been seen with the small-molecule antagonist's tirofiban and eptifibatide, with a 16% to 35% reduction in ischemic events in patients undergoing PCI. A greater benefit was seen in higher acuity patients (patients with elevated troponin levels and/or diabetes).[77] In patients presenting with an ST elevation myocardial infarction undergoing primary angioplasty, GPIIb/IIIa blockade reduced death, subsequent infarction and need for revascularization within 30 days by 46%.[78] The addition of GPIIb/IIIa blockage to thrombolysis therapy has thus far not shown to be beneficial due to an increased bleeding risk.[79]

Interestingly, instead of reducing major ischemic events, long-term oral GPIIb/IIIa inhibitor therapy has uniformly increased the mortality rate. As a potential reason for this, it is postulated that exposure of GPIIb/IIIa to antagonists, which typically mimic the ligand fibrinogen, induce 'outside in signaling', as would be expected for ligand binding to an integrin receptor.[80,83] This can lead to paradoxical platelet activation and aggregation, implying that although GPIIb/IIIa is a good target for platelet inhibition, the ligand-mimetic strategy of receptor blockade is not an ideal pharmacological strategy.[81,82] Allosteric inhibition or selective inhibition of activated GPIIb/IIIa receptors have been recently employed as novel drug developments.[83,84]

Other anti-platelet agents and promising new developments

Dipyridamole is well known antiplatelet agent currently in clinical use for the secondary prevention of stroke. Dipyridamole increases intracellular cyclic AMP by inhibiting enzymes responsible for adenosine breakdown. Raised cAMP suppresses platelet activation, promotes vasodilatation, and stimulates prostacyclin release and coronary artery vasodilation.[85] In combination with

low dose aspirin, it has been shown in one trial to offer additional stroke protection than aspirin alone.[86] It has no demonstrated role in preventing cardiovascular disease.

Cilostazol is a Type III inhibitor of phosphodiesterase in platelets, promoting increased cAMP, and is currently approved for use in peripheral vascular disease. The KAMIR and DECREASE trials have suggested cilostazol confers a modest benefit in patients with coronary artery disease undergoing PCI in addition to standard dual antiplatelet therapy with respect to cardiac death, stroke, MI and instent-thrombosis at 8-12 months.[87,88] However, the outcome of a double-blinded, randomised controlled trial is required before it can be recommended for routine use.

Other new agents to mediate platelet inhibition are currently in development. One such is an oral protease receptor antagonist-1 (PAR-1) antagonist, currently known as atopaxar. PAR-1 is the primary platelet receptor for thrombin and is a potent activating agent. Phase III studies are underway to determine its clinical usefulness, with early Phase II data suggesting it may offer improved clinical outcomes with respect to death, stroke and MI, when added to other antiplatelet agents.[89] Similarly, animal studies have demonstrated that inhibition of the platelet collagen receptor, GPVI, with monoclonal antibodies and Fab fragments, can inhibit collagen induced platelet aggregation in rats. Phase II studies are currently in progress with a view to commence human trials in the near future.[90] Terutoban is an orally active inhibitor of the thromboxane A2 receptor, currently undergoing phase III trials.[91]

A major limitation in the development of new anti-platelet agents is the recurring observation that with increasingly effective platelet inhibition comes an inevitable increased bleeding risk, either tempering or negating the cardiovascular benefit. Novel classes of therapeutic drugs, currently under development, seek to circumvent this problem by selectively targeting activated platelets, for example activation specific GPIIb/IIIa antagonists[84] and PI3 kinase inhibitors.[92] These agents demonstrate potent antiplatelet activity without prolonging bleeding time in animal models. Clinical performance of these agents is still yet to be evaluated, however they remain promising.

PLATELET FUNCTION TESTING

Platelet function testing refers to in vitro measurements of platelets' response to activation in an attempt to quantify the degree of platelet aggregation. It has evolved significantly over the last decade from a laborious laboratory based process to rapid point of care commercially available kits. Despite this, there still remains a surprising lack of standardisation of platelet function testing. Table 11.1 summarises some of the currently available methods, along with their incumbent advantages and limitations.

Light transmission aggregometry

Light transmittance aggregometry (LTA) is currently the gold standard for assessing platelet activation as it is able to measure the functional ability of platelets to aggregate in response to known agonists such as ADP. Platelet aggregation uses the principle that the amount of light transmitted through the sample increases proportional to an increase in platelet aggregation. Lack of standardization, poor reproducibility and spontaneous platelet activation through sample preparation are a few limitations of standard light transmittance aggregometry.[93]

TABLE 11.1: A list of commonly used platelet function tests, their advantages and limitations.

Test	Method	Advantages and Limitations
Classical (or turbidometric) platelet aggregometry	Blood is centrifuged at low force to isolate platelet rich plasma, which is then stirred in a curvette at 37°C and placed between a light source and measuring photocell. Platelet agonists (such as ADP, collagen, adrenaline or ristocetin) are added. As individual platelets aggregate, turbidity of the PRP is reduced, and the increasing light transmission is detected by the photocell.	• Original 'gold standard' technique • Limited to specialised laboratories as requires specialised expertise for accurate performance and interpretation • Labour intensive • Limited sensitivity in detecting small (<100 platelets) aggregates, or preformed aggregates. Thus, limited sensitivity to detect early aggregates in platelet hyperfunction. • Limited ability to detect duration and efficacy of antiplatelet therapy, especially GPIIb/IIIa blockade
Whole blood aggregometry	Whole blood is stirred at 37°C. Platinum electrodes at a fixed distance are added to the blood. Once electrically active, platelet aggregates amass upon the electrodes resulting in a measurable increase in electrical resistance in a manner proportional to the degree of platelet aggregation.	• Comparative accuracy with classical aggregometry • Insensitive to small platelet aggregates • Requires significant technical expertise and expense
The VerifyNow® Assay (Accumetrics Inc, San Diego, California, USA) – previously known as the Ultegra Rapid Platelet Function Assay (RPFA)	A commercially available 'point-of-care' assay. GPIIb/IIIa kit: Whole blood is added to disposable cartridge containing fibrinogen beads and an activating Thrombin Receptor Activating Peptide (TRAP). Platelets activated by TRAP, bind to fibrinogen in a degree proportional to the amount of available receptors. Thus the degree of GPIIb/IIIa blockade can be quantified. Aspirin and Clopidogrel kits: These utilise the same principle using arachadonic acid and ADP as agonists in order to assess the activity of aspirin and clopidogrel respectively.	• Fast and easy to use with results available in 2–3 minutes • Limited to assessing the effectiveness of antiplatelet therapy and no role in assessing platelet activity in disease states • Comparable sensitivity to classical platelet aggregometry • Ideal cut-off points for diagnosing antiplatelet resistance are still a matter of conjecture.

Test	Method	Advantages and Limitations
Flow Cytometry	Flow cytometry uses the principle of light scattering and fluorescence to accurately quantify platelets, blood cells, platelet aggregates and platelet leukocyte aggregates as well as the functional state of platelets.	• Most versatile and best quantitative platelet function measurements • Utilising gating technology, the expression of activation specific markers on platelets can be quantified. This provides very high sensitivity for detecting and quantifying platelet activation. • Can identify platelet/leukocyte aggregates in addition to platelet aggregates • Utilising fluorescence labelled ligands of antibodies such as fibrinogen-FITC or anti-P-selectin monoclonal antibody, direct quantification of receptor blockade can be achieved (for example GPIIb/IIIa blockade) • Requires considerable expertise and specialised laboratory • Time consuming and expensive
Vasodilator Stimulated Phosphoprotein (VASP) Assay	Using flow cytometry, the VASP Assay measures the degree of phosphorylation of VASP which correlates with the degree of activity of the ADP receptor. Phosphorylated VASP correlates with P2Y12 receptor inhibition. The degree of phosphorylation in the presence of ADP, suggests effective antiplatelet activity.	• Best available test for identifying patients with possible clopidogrel resistance • Requires considerable expertise and specialised laboratory • Time consuming and expensive

Whole blood aggregometry

Impendence aggregometry, a newer technique that measures electrical resistance due to aggregation of stimulated platelets on two platinum electrodes, has been shown to have improved reproducibility and sensitivity. The increase in resistance between electrodes is used to determine the amount of platelet aggregation after stimulation by an agonist. Impedance aggregometry correlates well with LTA.[94]

VerifyNow® assay

Verify Now® is a cartridge based rapid point of care test that measures aggregation of platelets via GIIb/IIIa receptors in response to ADP. The extent of platelet aggregation measured by a technique based on light transmittance aggregometry, is expressed as platelet reactivity units (PRU), which is specific to the action of the P2Y12 platelet receptor. Simplicity of technique, reliability of results, lack of reliance on lab equipment

and good correlation with established tests of platelet function are advantages of this test. All point of care tests are limited by low to moderate sensitivity and specificity. However, VerifyNow® P2Y12 assay has been shown to predict clinical adverse outcomes.[95]

Flow cytometry

Flow cytometry enables identification of expression of substances on platelets and leukocytes, particularly formation of platelet-leukocyte aggregates. Fluorophores (substances that fluoresce when stimulated with energy) attached to antibodies which bind to substances of interest; emit light when excited by energy from lasers. These lasers transmit light at a specific frequency aimed at particles flowing past the laser in single file. The intensity of light emitted by the fluorophores allows quantification of the substance targeted by conjugated antibodies. However, accuracy and reproducibility of results vary from one protocol to the next. The ability to assess multiple markers relating to antiplatelet activity at the receptor is the main advantage of flow cytometry. The main disadvantages of this technique is the need for experienced operators, time consuming process for analysis with narrow sample preparation windows, lack of standardized lab protocols and access to lab facilities.

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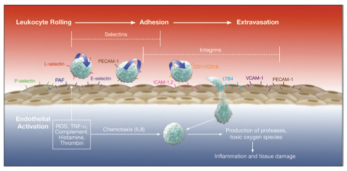

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Mechanisms of Vascular Disease

Edited by Robert Fitridge and Matthew Thompson

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