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NEW DISCOVERIES INVOLVING PLATELETS: BIOENERGETICS AND BIOTECHNOLOGY

presented by

Chia-Jui Ku

has been accepted towards fulfillment
of the requirements for the

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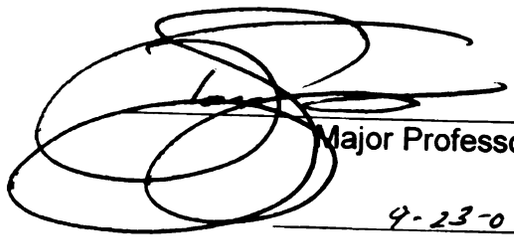

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NEW DISCOVERIES INVOLVING PLATELETS: BIOENERGETICS AND BIOTECHNOLOGY

By

Chia-Jui Ku

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ABSTRACT

NEW DISCOVERIES INVOLVING PLATELETS:
BIOENERGETICS AND BIOTECHNOLOGY

By

Chia-Jui Ku

The circulation is a complex network system comprised of arterioles and capillaries, with a main function of moving oxygen to and from tissues and cells in the body. It is also a complex mixture that includes, but not limited to, cells, macrophages, proteins, and metabolites. Although white cells (leukocytes), red cells (erythrocytes) and platelets have well-defined roles in the bloodstream, there also exist reports suggesting that cells that flow through the circulation may actually participate in other processes in blood vessels.

In this work, it is hypothesized that cells in the blood stream are communicating through NO, which is mediated by the RBC's ability to release ATP. NO is known as the endothelium-derived relaxing factor (EDRF) that not only helps vasodilation, but also regulates platelet activity. In 2006, our group proposed a mechanism to establish a relationship between RBC deformability, RBC-derived ATP, and subsequent endothelium-derived NO production. Although numerous studies have been performed to describe the relationship between RBC-derived ATP and endothelium-derived NO, reports of the synergy between ATP released from RBCs and the ability of platelets to

produce NO are lacking.

Work in this thesis is divided into three concepts; first, to develop a method to measure platelet NO production and release upon stimulation and activation. Secondly, based on the ability of platelets to produce NO, communication between RBCs and platelets were investigated in a capillary flow system. Finally, this newly obtained knowledge was integrated into a microfluidic device developed as a tool to investigate circulation using an *in vitro* format.

The ability to quantitatively determine platelet NO production and release using fluorescence probes provides a useful tool for further biochemical/medical application. Data presented in this work also provides evidence suggesting that the relationship between RBCs and platelets is ATP mediated. Moreover, physiological interactions (adhesion) between cell types could be observed utilizing a microfluidic device with an immobilized endothelium in channels while RBCs and platelets were pumped through. Collectively, communication between three cell types was established in this work, and might be helpful to explain conditions of certain diseases involving hyperactive platelets with either low (e.g., diabetes, hypertension and cystic fibrosis) or high (e.g., sickle cell disease and multiple sclerosis) RBC-derived ATP release.

- My dear family and Chun-Jui

I would not be where I am now
without all of your love and support

獻給 親愛的爸、媽、嘉以及俊睿

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CHAPTER 1 INTRODUCTION

1.1 DISSERTATION INTRODUCTION

The circulation is a complex system comprised of arterioles and capillaries including cells, macrophages, proteins, and metabolites. An understanding of blood flow maintance and cell communication is crucial to improving knowledge of certain diseases. Recently, our group has been focusing on studying aspects of the blood stream, including different cell types (e.g., red blood cells (RBCs), platelets and endothelial cells), and successfully established a relationship between RBC deformability, RBC-derived ATP, and subsequent endothelium-derived nitric oxide (NO) production.[1, 2] However, in this dissertation, studies were focused on describing a relationship between platelets and NO, using microfluidic technology as a circulation mimic. Therefore, in Chapter 1, the platelet will be introduced from a biological point of view, highlighting aspects that are important in our work. NO biochemistry will also be described later in Chapter 1, especially in relation to platelets. In Chapter 2, a method to quantitatively measure platelet NO production and release will be discussed. Furthermore, RBCs were introduced into the same system with the platelets to establish a communication between RBCs and platelets through ATP in Chapter 3. Finally, in Chapter 4, a microfludic device will be employed to mimic the circulation. The development of a microfluidic device was first introduced in

the late 1970's.[3] However, within the past few years, a dramatic increase in microfluidic research has taken place. Although early work in microfluidics ranged from sample purification,[4] amplification[5, 6] to high-throughput screening, applications such as diagnostic testing[7, 8] and single molecule detection[9-11] have also been developed and more information about microfluidics will be described in Chapter 4. A key feature of the work here is emplying the microfluidic device as an *in vitro* platform to mimic *in vivo* process.

1.2 THE PLATELET

Knowledge about the structure, biology, and function of platelets has evolved considerably since 1882, when Giulio Bizzozero linked newly identified, discrete particles in the blood, distinct from red and white blood cells, with the coagulation process.[12-14] For more than 100 years, the dominant role of platelets in hemostasis and thrombosis has been well documented.

Platelets are 2 – 4 μm, anucleate discoid circulating blood particles.[15] They circulate around the body in an inactive state and initiate hemostatic plug formation at a site of vascular injury by promoting coagulation and subsequent wound healing. When platelets adhere to the endothelial defect, they undergo processes such as shape change, granule contents release, and adhesion to form aggregates.[16, 17]

Physiologically, these processes help to limit blood loss; however, inappropriate or excessive platelet activation results in an acute obstruction of blood flow, for example, in acute myocardial infarction (heart attack).[18-20] However, activated platelets also express and release species that stimulate a localized inflammatory response through the activation of leukocytes[21-23] and endothelial cells.[24, 25] It is now clear that platelet function is not only limited to the prevention of blood loss, but also has been implicated in many pathological processes including host defense,[26-28] inflammatory arthritis,[29] adult respiratory distress syndrome,[30, 31] and tumor growth and metastasis.[32]

1.2.1 PLATELET PRODUCTION

The overall process of platelet production begins with the common hematopoietic stem cell (HSC) (Figure 1.1).[33] Two distinct types of blood cell lines are derived: lymphoid, which includes all types of lymphocytes, and myeloid, which includes granulocytes, monocytes, red blood cells and platelets. Polyploid megakaryocytes are the immediate progenitors of platelets.

Megakaryocytes regenerate in human bone marrow[34] at the rate about 10^8 cells per day;[33] and each megakaryocyte can generate more than 5,000 platelets, in turn, 10^{11} of platelets are replenished daily. The physiological number of platelets is $1.5 - 4.0 \times 10^8$ per milliliter of blood.[35] Production of such a number of cells, each with a relatively short

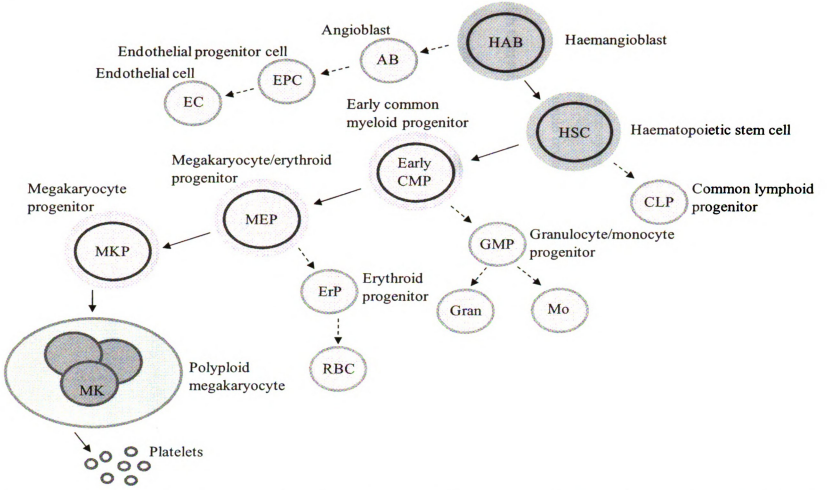


Figure 1.1 Overall scheme of platelet production sequence. Pathways leading to platelet production are indicated by solid arrows, other pathways are indicated by dashed arrows. RBC, red blood cell; Gran, granulocyte; Mo, monocyte.[33]

life span (7 – 10 days), offers an advantage in terms of speed and adaptability to hemostatic challenges.

The megakaryocyte undergoes a series of morphological changes during the 4 – 10 hour process of platelet production (Figure 1.2).[36] Nuclear endomitosis (a process to separate a cell nucleus into two identical nuclei) and organelle synthesis occur first, along with expansion of the cytoplasm. An array of microtubules[37] emerges from centrosomes (the main microtubule organizing center in the cell for regulating cell-cycle procession), which then migrate to the cell periphery.

Aided by sliding of the microtubules, the megakaryocyte's cytoplasm then develops multiple thick pseudopods in preparation for formation of 5 – 10 proplatelets. Organelles and granules migrate along the microtubules to the developing, elongatinig proplatelet ends, where new platelets will form. Proplatelets are 250 – 500 μm[38] long on average and can produce 100 – 200 platelets each. Proplatelets are then released from the cell into the vascular sinus (the cavity in vessel wall), often appearing paired in dumbbell shape. The nucleus is ejected from the mass of platelets, and individual platelets are later released or "budded off" from proplatelet ends. One third of platelets are normally stored in the spleen as an interchangeable pool with circulating cells, and can be pushed into general circulation in times of stress.

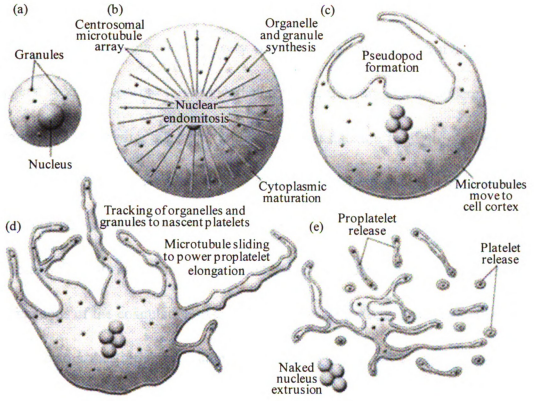


Figure 1.2 Overview of megakaryocyte production of platelets. (a) megakaryocytes; (b) cells first undergo nuclear endomitosis, organelle synthesis, and dramatic cytoplasmic maturation and expansion, while a microtubule array, emanating from centrosomes, is established; (c) centrosomes disassemble and microtubules translocate to the cell cortex. Proplatelet formation commences with the development of thick pseudopods; (d) sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends, where nascent platelets assemble; (e) the entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the mass of proplatelets, and individual platelets are released from proplatelet ends.[36]

1.2.2 PLATELET FUNCTION

Platelets control bleeding (hemostasis) when there is an injury to the blood vessel (Figure 1.3), and the endothelial cell layer is disrupted exposing the underlying extracellular matrix. Platelets are very reactive cells, and upon activation by suitable triggers, such as exposure to subendothelial tissue, they are able to adhere at the site of damage.[39-41] Following adhesion, rapid signal transduction leads to platelet activation, cytoskeletal changes associated with shape change, spreading and secretion, and inside-out activation of integrins that support adhesion and aggregation. During these processes, platelets also assist fibrin formation by providing a surface on which many of the reactions of the coagulation cascade may occur. Many active substances are released: growth factors that influence smooth muscle cells in the vessel wall or tumor growth, serotonin that affects vascular integrity, vasoactive materials that modulate local blood flow, and leukocyte chemoattractants. If these various functions go out of control, a pathological bleeding or thrombotic state may develop.

1.2.2.1 PLATELET ACTIVATION

Platelet activation describes the process that converts the smooth, nonadherent platelet into an adhesive speculated particle that releases and expresses biologically active substances and acquires the ability to bind the plasma protein fibrinogen.[39]

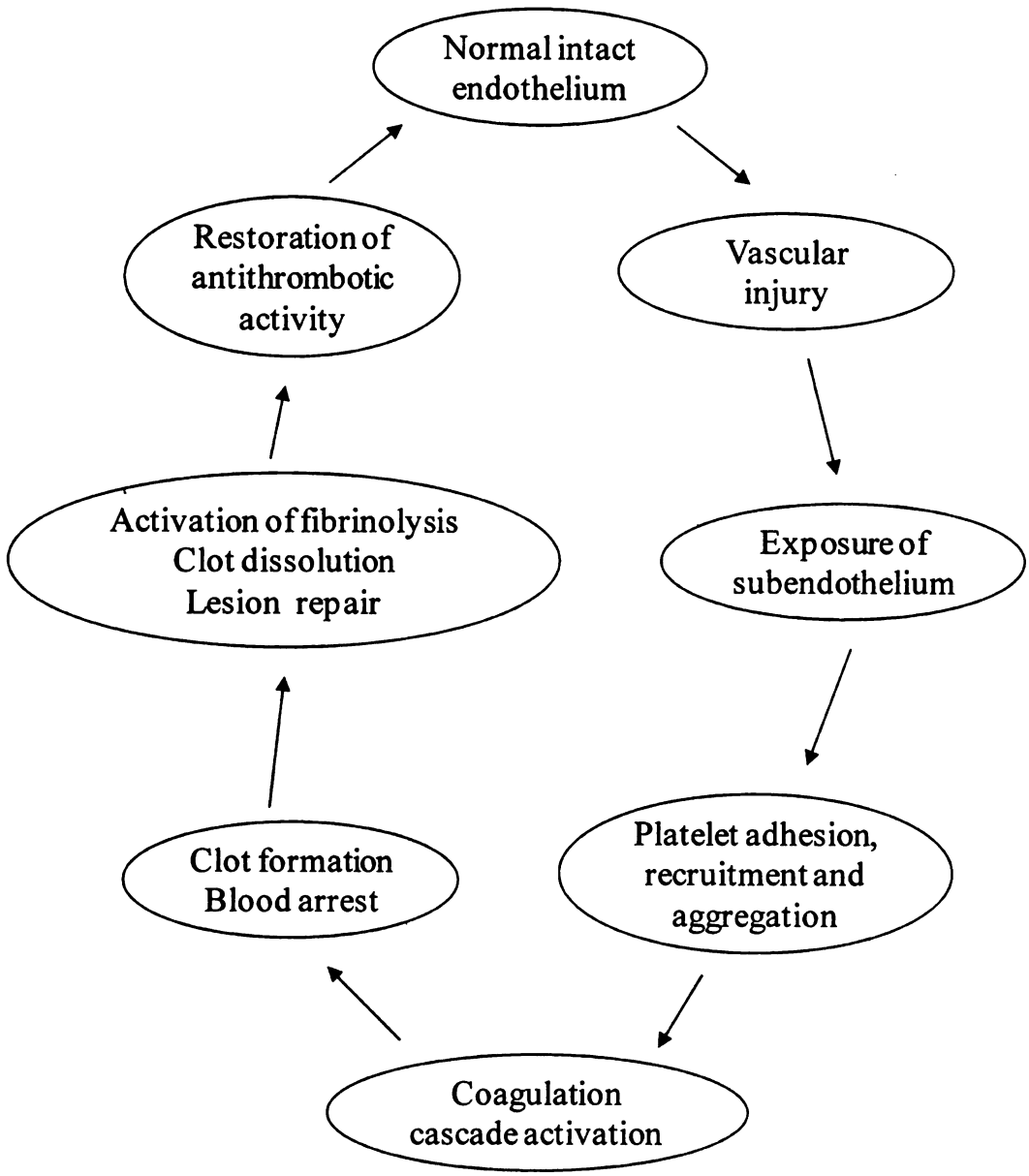


Figure 1.3 A simplified scheme of the hemostatic response

Activation occurs rapidly following exposure to a variety of particulate and soluble substances known as agonists (Table 1.1). Some of substances listed in Table 1.1 result in physiological activation, while others occur in pathological states or are *in vitro* reagents. Activation can also occur as a result of the physical stimulus of high fluid stress, such as that found at the site of a critical arterial narrowing.[41]

On activation, platelets display four basic phenomena: adhesion, shape change, secretion and aggregation. These may not necessarily occur in the same order, and some can occur without others. The various activators elicit different types of response, showing dose-dependence and sometimes synergism.

When the vascular endothelial cell lining becomes disrupted, the subendothelial connective tissues are exposed and platelets rapidly adhere to them, spreading across the site of damage, and changing shape. They then secrete their granular contents, which recruit more platelets to the site, forming an aggregate of cells. Activated platelets also provide a procoagulant surface supporting the reactions leading to thrombin generation and ultimately producing fibrin, which adds mechanical strength to the platelet plug.

1.2.2.2 PLATELET ADHESION

One of the earliest events following blood vessel damage is the adhesion of platelets to areas where subendothelium is exposed.[40, 42] Platelet adhesion requires

Soluble activators	Insoluble activators
Adenosine diphosphate (ADP)	Bacteria
Adrenaline	Collagen
Arachidonate	Glass
Epinephrine	Kaolin
Immune complexs	Latex particles
PAF-acether	Viruses
PG endoperoxides	
Proteolytic enzymes	
Serotonin	
Thrombin	
Thromboxane A_2	
Vasopressin	

Table 1.1 Naturally occurring and artificial activators of platelets

specific structural components of the subendothelium, plasma proteins and receptors (Table 1.2) on the platelet membrane. A number of plasma proteins are candidates for mediators of platelet adhesion to the endothelium, among them von Willebrand factor (vWF),[43] fibronectin, fibrinogen and thrombospondin, the so-called adhesive proteins.[44]

The best studied of this group is vWF and, interestingly, its contribution in platelet adhesion has been studied *in vitro* using a flow chamber and exposed rabbit aorta, and appears to be highly dependent on wall shear rate.[45] At low shear rates, such as large veins (200 s^{-1}), adhesion occurs independently of vWF. However, at high shear rates comparable to those found at the arterial wall (500 -1000 s^{-1}) and in small vessels (>1300 s^{-1}), there is a significant adhesion defect in the absence of vWF. In the high fluid stress environment of flowing arterial blood, initial adherence is mediated primarily by the platelet membrane vWF receptor GP Ib-IX-V.[46-48] Circulating vWF binds to collagen exposed in the subendothelium, allowing it to interact with GP Ib-IX-V.[49] This interaction is reversible and allows the adherent platelets to roll, eventually through clustering of the receptors,[50] results in platelet activation.[51] Platelet number, viscosity and red blood cell count have a linear relationship to adherence, which reflects the rheology of high shear vessels where red blood cells occupy the central core position forcing the platelets to migrate to the periphery of the blood vessel, thus increasing the platelet-vessel wall contact.

Receptor	Ligand
Adhesion	
Integrins	
GP Ia/IIa	Collagen
GP Ic/IIa	Laminin
GP Ic*/IIa	Fibronectin
α_vIIIa	Vitronectin, fibrinogen, vWF, thrombospondin
GP IIb/IIIa	Fibrinogen, fibronectin, vWF, vitronectin
Others	
P-selectin	Selectin counter receptors
GP Ib	vWF
GP IV	Thrombospondin, collagen
Aggregation	
GP IIb/IIIa	Fibrinogen, fibronectin, vWF

Table 1.2 Selected platelet receptors

1.1.2.3 SHAPE CHANGE

Following adhesion to subendothelium, platelets spread, covering the exposed connective tissue matrix, and in doing so change from the circulating discoid form to an irregularly shaped elongated cell with cytoplasmic projections. Platelet pseudopod formation appears to result from rearrangement of the cytoskeletal proteins (actin and myosin) and results in contractile activity, which is analogous to activity seen in muscle cells.[12, 52] Both microfilaments and microtubules are found in pseudopods and it is thought that the latter control recruitment and dissolution of microfilaments. In the early stages of platelet activation, shape change is reversible, but strong stimuli result in the centralization of organelles, degranulation, and release granule contents accompanied by irreversible shape change and aggregation.

1.2.2.4 SECRETION

Platelets release a number of biologically active substances from granules upon activation (Table 1.3). There are three types of granules: the alpha (α-) granule, the dense granule, and lysosomes. α-granules contain platelet-specific proteins, such as β-thromboglobulin and platelet factor 4,[53-56] as well as some proteins which normally circulate in the plasma at relatively high concentrations, e.g. fibrinogen. Dense granules sequester a pool of nucleotides that are not interchangeable with those utilized in the

α-granule	dense granule
α_1-Antitrypsin	Adenosine diphosphate (ADP)
α_2-Macroglobulin	Adenosine triphosphate (ATP)
α_2-Antiplasmin	Ca^{2+}
β-Thromboglobulin	Serotonin
Albumin	
Coagulation factor V	
Fibrinogen	
Fibronectin	
Platelet-derived growth factor	
Platelet factor 4	
P-selectin	
von Willebrand factor	

Table 1.3 Platelet α- and dense granule secretions

general metabolism of the cell. The released ADP provides a feedback loop for further platelet stimulation, and serotonin helps to maintain the integrity of the vascular endothelium.[57] The importance of these two types of granules is well illustrated by the clinical syndromes associated with their deficiency or dysfunction.[58, 59] Lysosomes contain a variety of acid hydrolases such as lysozyme, acid phosphatase and elastase.[60, 61] Unlike α- and dense granules, lysosomes contain enzymes that help digesting such as particles, excess organelles, foreign microbes and do not release their contents while platelet activation.

1.2.2.5 PLATELET AGGREGATION

The process of platelet aggregation describes the ability of platelets to co-adhere with one another in a specific process requiring energy, intracellular processes and initiators. A large number of platelet activators (Table 1.1) are able to cause aggregation. Once tethered to the vessel wall, platelets form irreversible adhesion bonds through the interaction of platelet receptors with specific subendothelial matrix proteins and plasma proteins immobilized at the site of injury. The major platelet integrin $\alpha_{IIb}\beta_3$ (GP IIb/IIIa), binds vWF and/or fibrinogen to facilitate the crosslinking (platelet aggregation) under shear,[41, 62] and further activation of platelets, providing strength and stability to growing the thrombus. The formation of a platelet plug stabilized by an insoluble fibrin network

serves to prevent further blood loss from a damaged vessel.

Although platelet adhesion and aggregation were historically viewed as distinct processes in the formation of a thrombus, it is now clear that the fundamental mechanism is similar,[63] involving the interaction between platelet receptors GP IIb/IIIa and GP Ib-IX-V complex with fibrinogen and vWF in flowing blood.[64-66] For example, an adherent platelet binds fibrinogen and vWF from the circulating blood, creating an ideal surface for the recruitment of further platelets. However, additional receptors such as collagen receptors are required for the stable attachment of platelets to subendothelium structures while shear forces are generated on the platelet by the flowing blood.

1.2.3 PATHWAYS OF PLATELET REGULATION

1.2.3.1 ACTIVATOR REACTIONS

Research over the past 40 years has provided the biochemical rationale for these dramatic alterations in basal behavior of platelets. It is now well established that the formation of a platelet plug is supported by the activity of at least three platelet-derived activator systems.

The release of ADP from platelet α-granules and the interactions of ADP with purinergic receptors form the foundation of the first system. *In vivo*, a platelet-derived pool of ADP may be supplemented with ADP released from red blood cells.[67] The

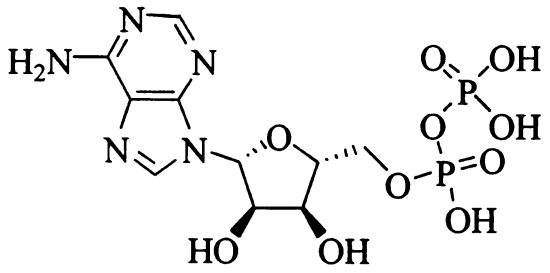
discovery of the mechanism of action of aspirin and its analogs[68-71] formed the foundations for the discovery of the proaggregating metabolites of arachidonic acid in platelets, namely, cyclic endoperoxides and thromboxane A_2 (TXA_2).[72] TXA_2 is synthesized by the sequential action of platelet cyclooxygenase and thromboxane synthase, and once formed, it acts on its receptors to amplify aggregation.[73, 74]

Inhibition of the generation and the action of ADP (e.g., by ticlopidine or clopidogrel) and thromboxane (e.g., by aspirin) is not sufficient to abolish platelet aggregation stimulated by such potent agonists as thrombin. The discovery of matrix metalloproteinase-2 (MMP-2), which is expressed in human platelets, revealed another platelet-derived activator system. The release of MMP-2 is collagen or thrombin stimulated and the subsequent platelet aggregation is in a non-thromboxane-, non-ADP-dependet manner (Figure 1.4).[75] Once released, MMP-2 can remodel platelet surface membranes which enhances aggregation.[76, 77]

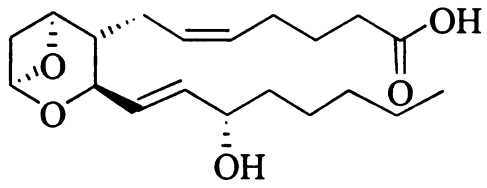
1.2.3.2 INHIBITOR REACTIONS

The vascular endothelium is a major contributor to the inhibitor reactions that control platelet activation. Vane *et al* first discovered that endothelial cells generate prostacyclin,[78] a potent inhibitor of platelet aggregation and stimulator of platelet deaggregation.[79, 80] Prostacyclin is a biological opponent of TXA_2 on platelets

(a)



Adenosine diphosphate



Thromboxane A_2

(b)

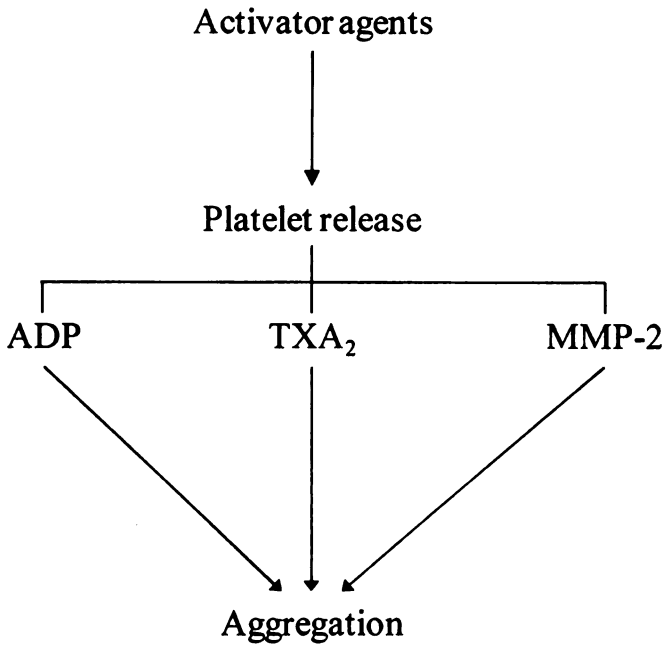


Figure 1.4 (a) Structures of ADP and TXA_2 (b) The activator pathways that amplify platelet aggregation

(Figure 1.5) and the vessel wall resulting in inhibition of platelet aggregation and vasodilation.[73, 81, 82] Prostacyclin binds to its specific receptors present on platelets that are linked to adenylyl cyclase. Stimulation of prostacyclin receptor leads to increased accumulation of intracellular cAMP and downregulation of all pathways involved in the amplification of platelet aggregation.[82] Prostacyclin exerts little influence on the process of platelet adhesion to the subendothelial components of the vessel wall.[83, 84]

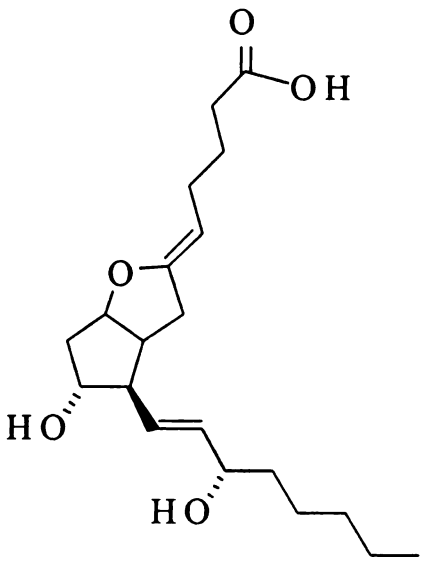
Prostacyclin acts as an indirect inhibitor of platelet activation. It is released close to the endothelial surface in response to stimulation with various vasoactive mediators including angiotensins and bradykinin.[85] Platelets themselves lack the capacity to synthesize prostacyclin; however, they may contribute to the endothelial synthesis of this eicosanoid by generating and releasing arachidonic acid cyclic endoperoxides, which may be taken up by the endothelial cells for prostacyclin synthesis.[81, 86]

1.2.4 ROLE OF PLATELTS IN THROMBUS FORMATION

Hemostasis is the process that maintains the integrity of a closed, high-pressure circulatory system after vascular damage. Vessel-wall injury and the loss of blood from the circulation rapidly initiate events in the vessel wall and in blood that repair lesions.

Thrombi are complex structures that are composed not only of fibrin meshwork, but also contain blood-borne cellular elements like platelets, leukocytes and red blood

(a)



(b)

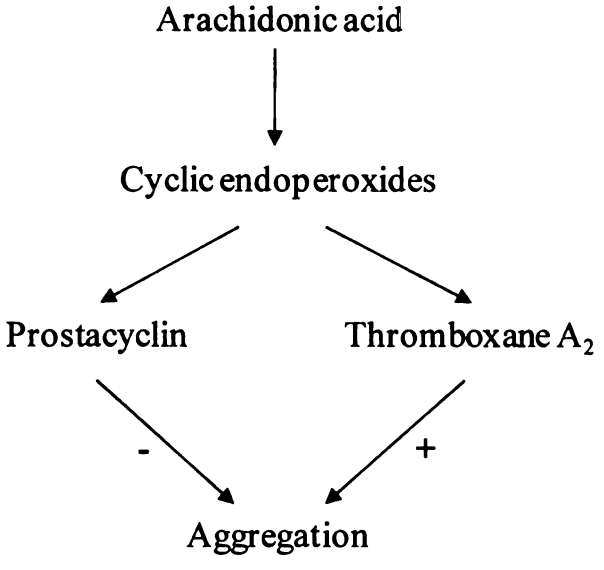


Figure 1.5 (a) The structure of prostacyclin. (b) The prostacyclin-thromboxane balance in regulation of platelet aggregation. + denotes stimulation; - denotes inhibition

cells. Platelets play an essential role in the initial response to vascular injury as they adhere to vessel wall components, become activated, aggregate and secrete mediators that promote further platelet activation and also attract leukocytes. In addition to the plug formation, which transiently stops bleeding, platelets provide a surface for the subsequent steps of the coagulation cascade leading to fibrin formation. Blood coagulation complexes function only in compartments and platelets serve the phospholipid surface for these reactions.[87] These events occur concomitantly, and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially.

There are two distinct pathways acting in parallel or separately that can activate platelets for thrombus formation.[88, 89] In one of these pathways, exposure of subendothelial collagen initiates platelet activation; in the other, thrombin, generated by tissue factor derived from the vessel wall or present in flowing blood is the initiator. Depending on the injury or the disease, one pathway or the other may predominate, but the consequences of platelet activation triggered by these pathways are the same.

When the blood vessel is damaged, platelets are activated by two kinds of interactions, one is the platelet GP VI (a collagen receptor on platelets) with the collagen of the exposed vessel wall and the other is platelet GP Ib-V-IX (a cluster of adhesive receptors for vWF binding on platelets) with collagen-bound vWF. Both interactions result in adhesion of platelets to the site of injury. The relative importance of platelet

GP VI and GP Ib-V-IX in the initial tethering of platelets depends on the shear rate at the vessel wall.[90] However, the interaction of collagen with GP VI is required, as is GP Ib-V-IX with vWF.[88, 91, 92]

Tissue factor (factor III, a protein presents in platelets for initiating the thrombin formation) triggers a second pathway that initiates platelet activation. Platelet activation initiated by this pathway does not require disruption of the endothelium and is independent of vWF[23] and GP VI.[88, 93] Tissue factor forms a complex with factor VIIa, initiating a proteolytic cascade that generates thrombin. Thrombin cleaves protease-activated receptor 4 (Par4) (Par1 in humans) on the platelet surface, thereby activating platelets[94] and causing them to release ADP, serotonin, and TXA_2. In turn, these agonists activate other platelets, and in doing so, amplify the signals for thrombus formation.

A developing thrombus recruits unstimulated platelets,[93] and within the thrombus activation occurs only in a subgroup of the recruited platelets. Others remain loosely associated with the thrombus but do not undergo activation and may ultimately disengage from the thrombus.[93] In short, thrombus formation is a dynamic process in which some platelets adhere to and others separate from the developing thrombus, and in which shear, flow, turbulence, and the number of platelets in the circulation greatly influence the architecture of the clot.

1.3 NITRIC OXIDE

Nitric oxide (NO) is a diffusible, short-lived, diatomic free radical ubiquitously produced by mammalian cells as a biological mediator first identified as the endothelium-deriver relaxing factor (EDRF).[95-97] The half-life of NO is typically on the order of 1 - 2 hours in dilute aqueous solution and approximately 1 - 5 seconds *in vivo* due to the formation of other NO-derived species such as nitrate and nitrite. Although NO is a free radical, it is relatively stable, reacting predominantly with molecules that have molecular orbitals with unpaired electrons such as oxygen, superoxide, and transition metals such as heme iron. The biosynthesis of NO is achieved by sequential oxidation of a terminal guanidino-nitrogen of L-arginine (L-Arg) yielding citrulline[98-100] (Figure 1.6). NO plays a prominent role in controlling a variety of functions in the cardiovascular, immune, reproductive, and nervous systems.[101-104]

1.3.1 NITRIC OXIDE BIOSYNTHESIS

NO is biosynthesized in mammals by the modified urea cycle,[105] which has two important functions: a secretory role to regenerate L-arginine for NO synthesis and an excretory role to eliminate excess nitrogen created by cell metabolism. It is formed by a series of oxidation-reduction mechanisms from the amino acid L-arginine,[106] (Figure 1.6) which is normally present in high concentration in the plasma (80 μM) and at even higher

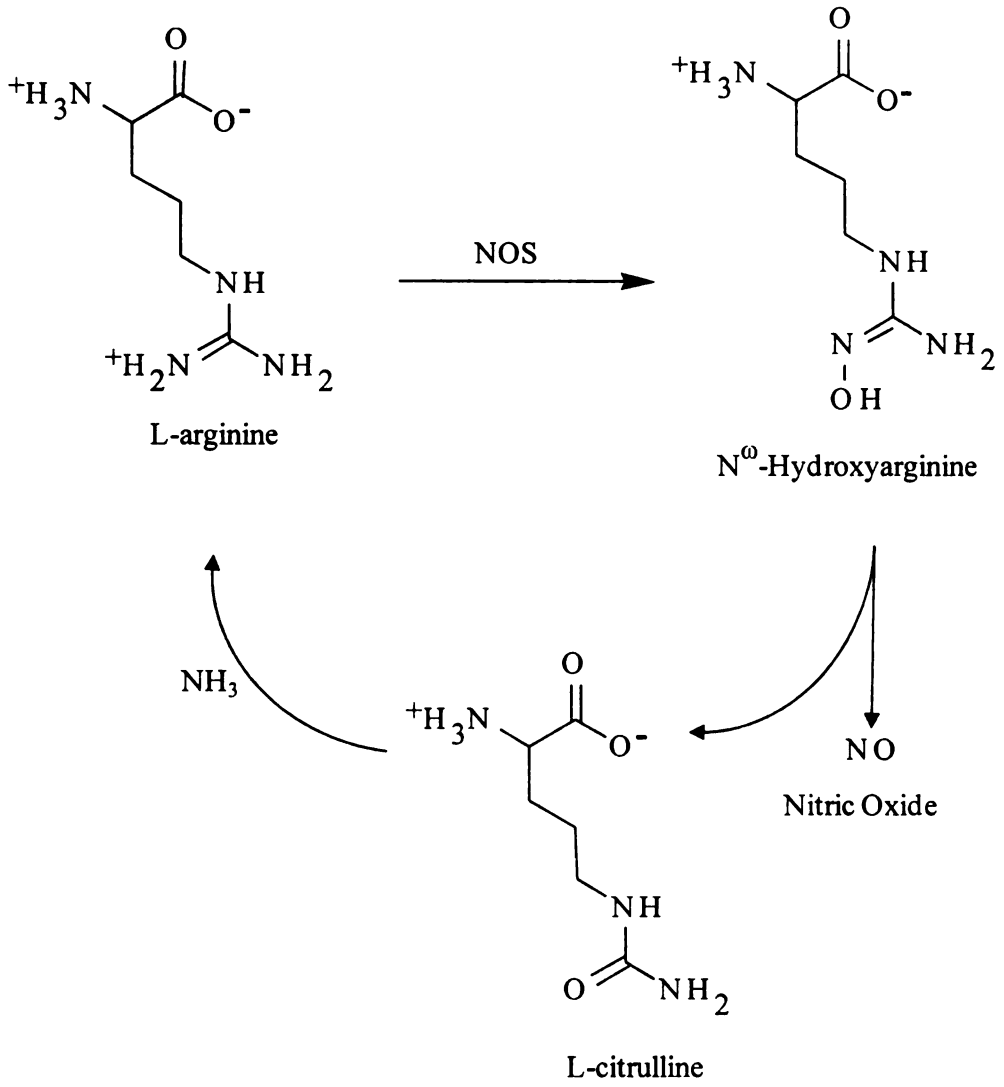


Figure 1.6 Biosynthetic pathway of nitric oxide from L-arginine

concentrations intracellularly.

The reaction is catalyzed by the NO synthases (NOS), which all utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 as cosubstrates (Figure 1.7). Full activity of NOS requires the presence of four co-factors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (H_4B) and a heme group.[107] Three NOS isoforms (Table 1.4) have evolved to function in animals, and each gene is located on a different chromosome.[108, 109] Two of three NOS isoforms are constitutively expressed in cells, and they synthesize NO in response to increased Ca^{2+} or in response to Ca^{2+}-independent stimuli such as shear stress.[110] These particular NOS function in signal transduction cascades by linking temporal changes in calcium level to NO production. NO then serves as an activator of soluble guanylate cyclase (sGC).[111]

Important in these two types of NO syntheses is the presence of the calmodulin (CaM) protein bound to the NOS enzyme. When increased levels of Ca^{2+} are present in the environment, CaM will bind to the Ca^{2+} resulting in the reduction of NOS (Figure 1.8). The constitutive enzymes are designated nNOS and eNOS (or NOS I and III, respectively), after the cell types in which they were originally discovered (rat neurons and bovine endothelial cells). An inducible NOS (iNOS or NOS II) is constitutively expressed only in select tissues, such as lung epithelium,[112] and is more typically synthesized in response to inflammatory or proinflammatory mediators.[113, 114]

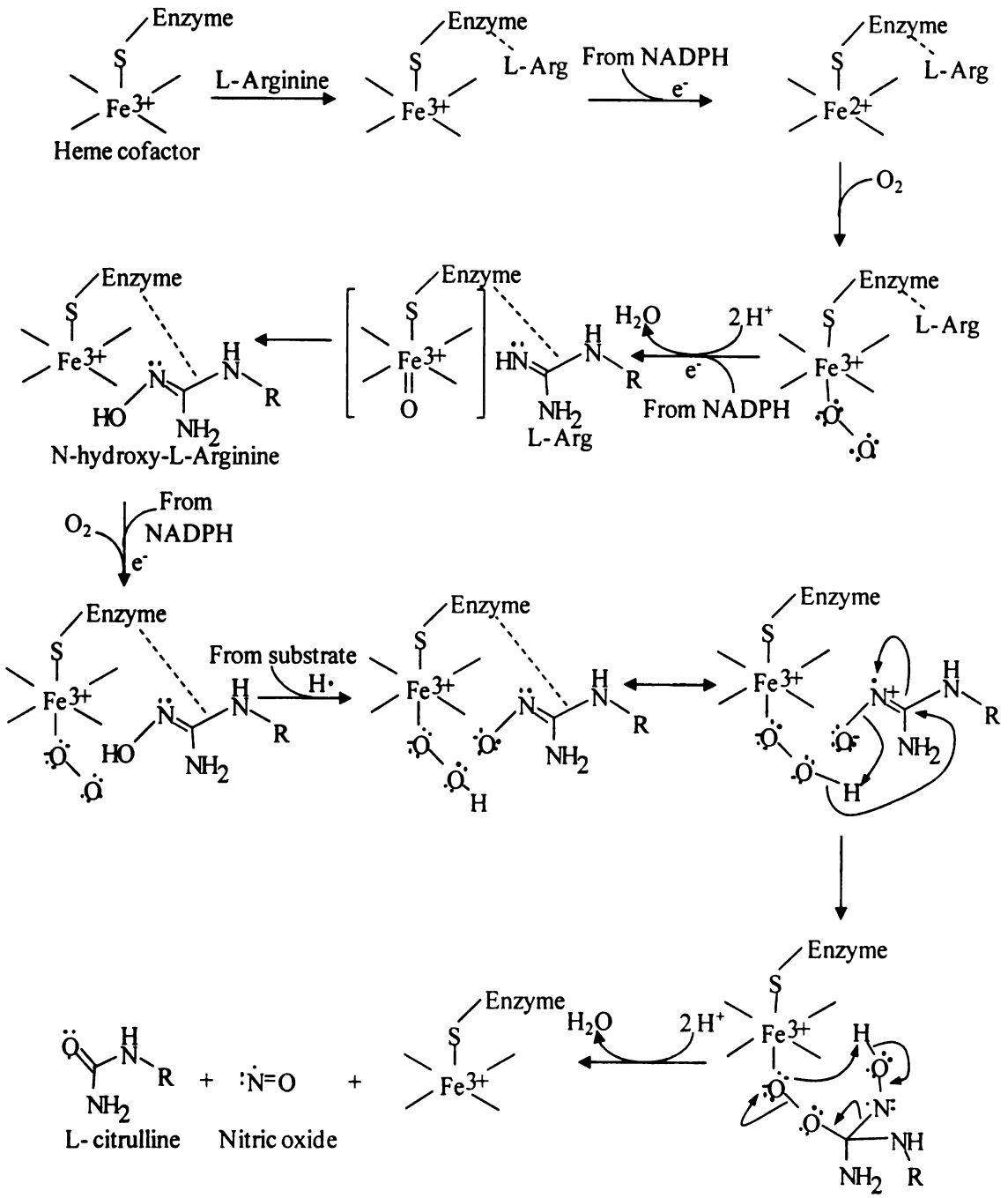


Figure 1.7 Generation of NO form L-arginine. NOS catalyses a multi-electron oxidation to form N-hydroxy-L-arginine (ArgOH), citrulline and NO. The first step involves the binding of L-arginine followed by the reduction of the ferric iron by an electron supplied by NADPH to form ArgOH. Incorporation of an additional oxygen molecule forms an iron-dioxy species, which abstracts a proton from ArgOH producing an iron-peroxy species and ArgOH radical. The final stage progresses through a tetrahedral intermediate between the iron-peroxy and ArgOH radical results in the production of citrulline, NO and the regeneration of the ferric iron

	cNOS (constitutive)		iNOS (inducible)
	nNOS (NOS III)	eNOS (NOS I)	NOS II
Source	Cardiovascular system	Central nervous system	Nonspecific immune system
Calcium dependency	Yes	Yes	NO
Function	Regulatory	Regulatory	Host defense
Examples	Relaxation of smooth muscle	Neurotransmitter	Kills bacteria and microorganisms
	Regulates blood flow and pressure		Seen in inflammatory conditions
	Inhibits platelet activation		

Table 1.4 Properties of NOS isoforms

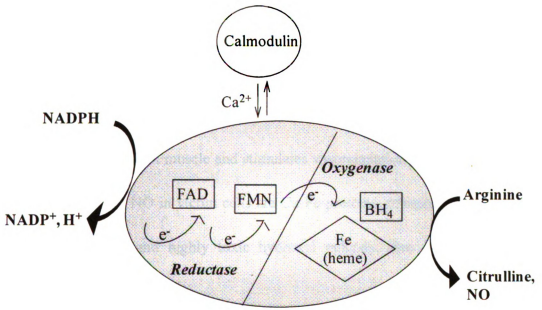


Figure 1.8 Schematic of NOS enzyme and participating co-factors. Increased Ca^{2+} levels enhances Ca^{2+}/CaM binding results in the reduction of NOS, passing electron to heme group and subsequent NO production

Although expression of iNOS is beneficial in host defense or in modulating the immune response, its expression is also linked to a number of inflammatory diseases.[113, 115, 116]

NO as a signaling molecule is completely different from classical mediators.[117] Figure 1.9 illustrates the constitutive and induced NO release and its signal transduction pathway. Unlike classical mediators, NO, a lipophilic, free radical gas, diffuses freely through the plasma membrane and does not need vesicles secretion from signaling cells or any cell surface receptors in the target cells in order to trigger a signal. It passes readily to the underlying smooth muscle and stimulates vasorelaxation (Figure 1.9a).[103, 104] The molecular targets of NO in victim cells are Cu-Fe proteins, releasing free Cu^{2+} and Fe^{2+} and generating O_2^- and highly toxic hydroxyl radicals, thus leading to large scale oxidative injury (Figure 1.9b).

1.3.2 ROLE OF NO IN THE CIRCULATION

Small arteries play an important role in the regulation of peripheral vascular resistance. The endothelium of resistance arteries regulates vascular function by way of its barrier role, through interaction with circulating cells such as platelets, which may release vasoactive or growth regulating agents, and by production of substances that modulate vascular tone and smooth muscle cell growth. However, NO serves as an important mediator in this regulation. The endothelium is an obvious target organ of

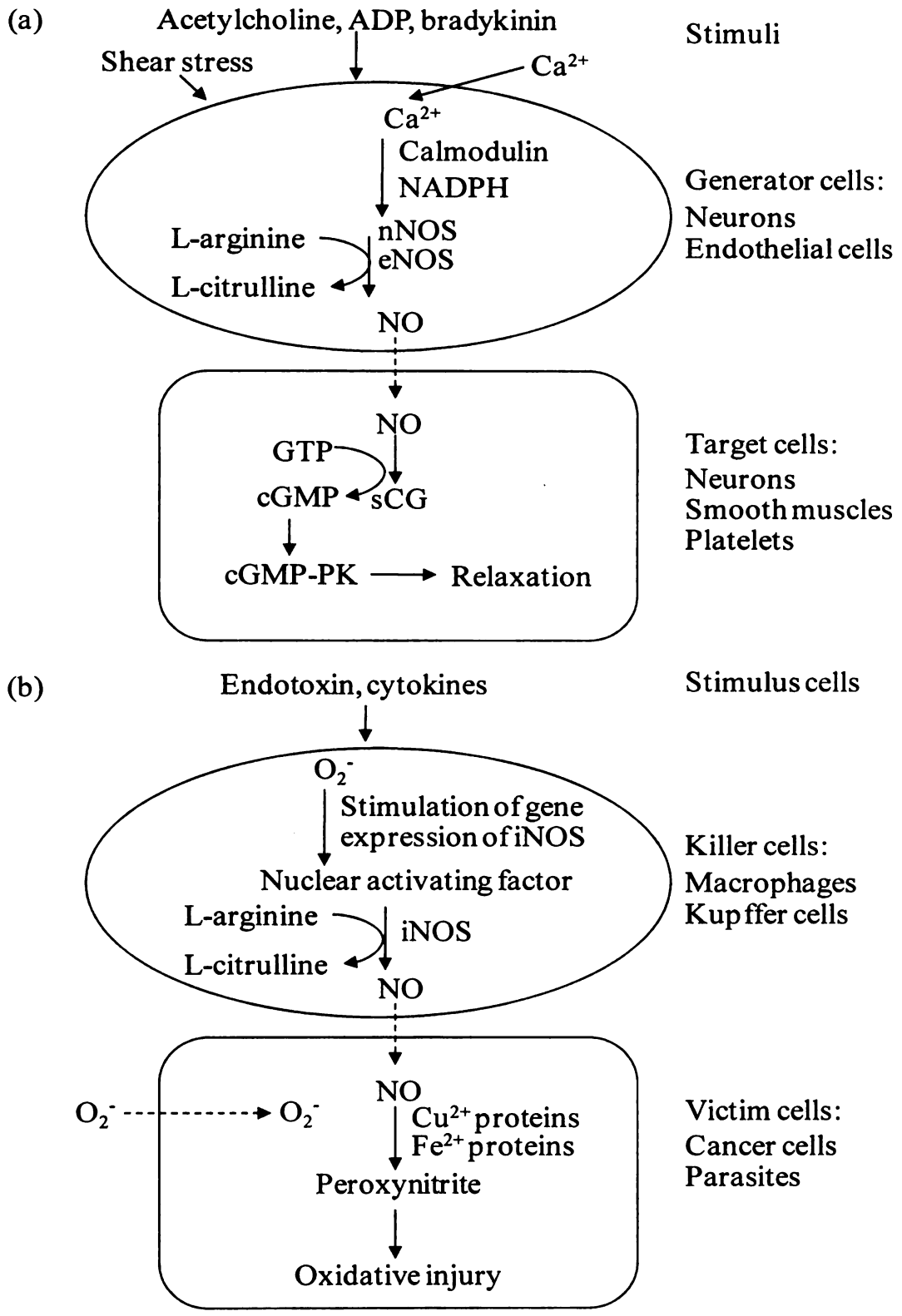


Figure 1.9 Schematic representation of (a) constitutive NO release and (b) induced NO release and their signal transudation pathways

cardiovascular risk factors. Accordingly, functional alterations do occur with aging, hypertension and hypercholesterolemia, all of which are associated with a decreased basal and stimulated release of endothelium-derived NO.

Relaxations in response to the abluminal release of endothelium-derived NO are associated with stimulation of sGC and in turn the formation of cyclic guanosine 3',5'-monophosphate (cGMP) in vascular smooth muscle cells (Figure 1.9a). Briefly, cGMP targets specific G-dependent protein kinases (PKG) that phosphorylate several key target proteins, including ion channels, ion pumps, receptors and enzymes. Once phosphorylated, these targets actively reduce the intracellular calcium concentration, which decreases myosin light chain kinase (MLCK) activity resulting in smooth muscle relaxation (Figure 1.10).[118-121] sGC, also present in platelets, is activated by the luminal release of endothelium-derived NO,[122] which limits adhesion and aggregation.[123] Therefore, endothelium-derived NO is a determinant in both vasodilation and platelet deactivation, and thereby represents an important antithrombotic feature of the endothelium.

NO also plays a crucial role in the regulation of blood pressure.[124] When infused intravenously, inhibitors of NOS such as L-N^G-monomethylarginine (L-NMMA) or N^{ω}-nitro-L-arginine methylester (L-NAME) have been shown to induce long-lasting increases in blood pressure and vascular resistance in the rabbit and human.[125, 126] This

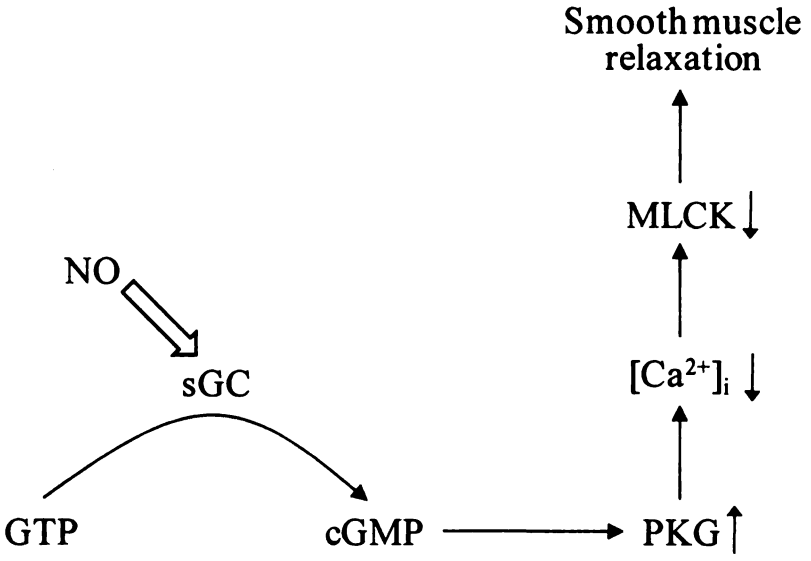


Figure 1.10 NO induced smooth muscle relaxation

demonstrates that the resistance circulation is in a constant state of vasodilation due to continuous basal release of picomolar quantities of NO by the vascular endothelium.

Furthermore, NO plays an important role in modulating vascular structure under physiological and pathophysiological conditions. In hypertension, resistance arteries adapt to the increased wall tension by changing their geometry. Accordingly, a reduced lumen diameter, an increased wall thickness, or both, can normalize the excessive tension applied on the vessel wall, which may protect the microcirculation against the blood pressure rise. The alterations in vascular wall structure and composition, induced by long-term changes in blood flow that lead to normalization of shear stress, are multifactorial in etiology, as the endothelium can regulate cell proliferation and extracellular matrix production through both NO-dependent and -independent mechanisms.[127-129]

Creation of an arteriovenous fistula (an artificial passageway between two vessels that are not normally connected) in the rabbit carotid circulation leads to an increase in carotid artery diameter and remodeling of the media that normalizes wall shear stress. These adaptive changes are partially attenuated by eNOS inhibition.[129] In marked contrast to wild-type mice, remodeling of the carotid artery is prevented in knockout mice with targeted disruption of eNOS.[130] Instead, eNOS mutants display a paradoxical hyperplastic increase in arterial wall thickness, suggesting that NO activity prevents

pathological changes in vessel wall morphology.[130]

1.3.3 NO AND PLATELETS

The appreciation of endogenous inhibitors for platelet activation was stimulated by the discovery of prostacyclin,[79] a major platelet-regulatory prostaglandin. However, it became apparent that the generation and release of this eicosanoid can only account in part for non-thrombogenic properties of vascular endothelium. Endothelial cells express the antiaggregatory activity even under conditions of complete inhibition of prostaglandin generation.[83]

Shortly after NO was recognized to be the EDRF with an important role in vasomotor control through its actions on vascular muscle,[96] it was also demonstrated that NO is an inhibitor of platelet function and plays a physiological role in the reduction of platelet activation.[123, 131] This deactivation by NO is achieved as platelets being the smallest of the blood cells, circulate closest to the endothelium, which is considered to be the most important source of NO in the vasculature. However, it was soon realized that platelets themselves are capable of biosynthesizing NO when they are activated.[106]

1.3.3.1 NOS IN PLATELETS

In contrast to megakaryocytes, which contain large amounts of RNA and DNA,

platelets contain trace amounts of DNA and small amounts of RNA. Therefore, the identification of DNA fragments coding NOS proteins requires application of polymerase chain reaction (PCR). A number of researchers extracted platelet RNA and amplified DNA fragments consistent with the expression of endothelial NOS but not inducible NOS or neuronal NOS in platelets.[132-134] Although the presence of eNOS in normal platelets appears to be beyond dispute, the identification of iNOS has proved to be controversial. However, it has been proven that both eNOS and iNOS are expressed in normal human and porcine platelets.[134-137] Importantly, platelet NOS, similar to endothelial NOS, is associated with a particular fraction of the platelet and undergoes intracellular translocation and activation during platelet activation.[106, 123, 136]

1.3.3.2 PHYSIOLOGICAL REGULATION OF PLATELET FUNCTION BY NO

NO available for platelet regulation is generated by both endothelium- and platelet- NOS. Stimulation of platelet and endothelial function plays an important role in the generation of NO. Tonic release of NO from the endothelial cells is mediated by shear stress,[138] while resting platelets generate small amounts of NO.[139, 140] Platelet adhesion and aggregation stimulate platelet NOS, leading to release of NO.[106, 139, 141, 142]

Both basal (shear stress dependent) and agonist stimulation release of NO have been implicated in platelet regulation. Experiments have shown the coronary and

pulmonary vasculatures generate NO to inhibit platelet adhesion under constant flow conditions.[143, 144] Similarly, bradykinin-stimulated endothelial cells release NO in quantities sufficient to inhibit platelet adhesion.[145-147]

Platelet aggregation induced by a variety of agonists, as well as by shear stress, is inhibited by NO released from endothelial cells.[84, 122, 146, 148-152] In addition to inhibition of adhesion and aggregation, NO disaggregates preformed platelet aggregates[84] and inhibits platelet recruitment to the aggregate.[153] Figure 1.11 summarizes the role of NO in regulating platelet function.

Similar to other cell types and tissue systems, the effects of NO on platelets are largely dependent on the stimulation of sGC and the resultant increase in the intraplatelet cGMP levels, hence activation of cGMP-dependent protein kinase (PKG). This in turn results in inhibition of platelet activation through various pathways. PKG promotes sarcoplasmic reticulum ATPase (SERCA)-dependent refilling of intraplatelet Ca^{2+} stores,[154] thereby inhibiting influx of Ca^{2+} and other cations and decreasing intracellular Ca^{2+} levels. PKG also phosphorylates the TXA_2 receptor, thereby inhibiting its function resulting in decreased platelet aggregation.[155]

In addition, two other mechanisms have been identified whereby cGMP prevents platelet activation. Firstly, cGMP indirectly increases intracellular cAMP through inhibition of phosphodiesterase;[156] cGMP and cAMP act synergistically to inhibit platelet

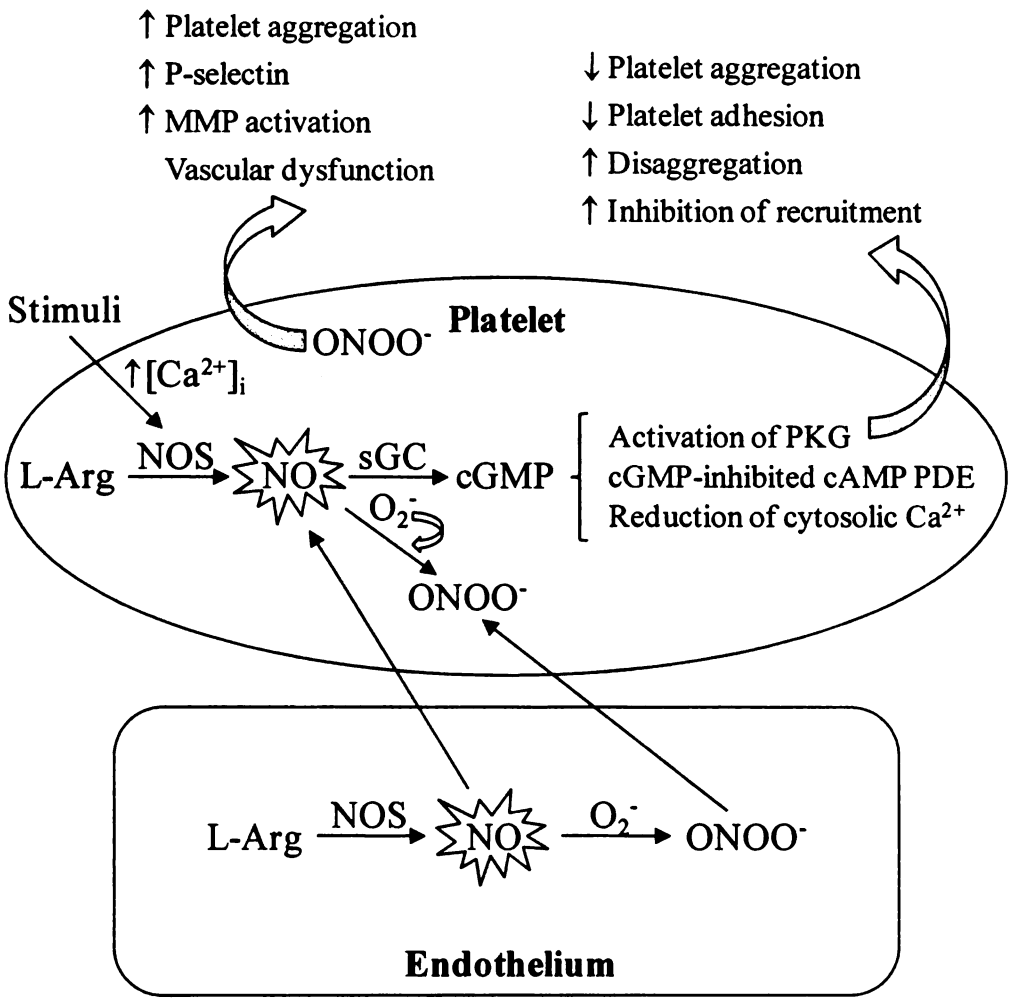


Figure 1.11 Overview of the role of NO in platelet function. NO generated from L-arginine (L-Arg) by the endothelial cells and platelets activates the soluble gunaylate cyclase (sGC) to increase the levels of cGMP that control the intracellular enzymes including protein kinase G (PKG), cGMP-inhibited cAMP phosphodiesterase (PDE), and the function of ion channel regulating calcium influx. NO can also react with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$)

aggregability.[146, 157] Secondly, cGMP indirectly inhibits the activation of GP IIb/IIIa fibrinogen receptors.[158]

Apart from the cGMP-dependent pathways described above, there is evidence that NO can also regulate platelet function independently of cGMP. NO has been shown to inhibit ATP-dependent Ca^{2+} uptake into platelet membrane vesicles in a manner which cannot be attributed to cGMP, because cGMP itself only has a weak effect on this uptake even at high concentrations.[159] However, the inhibitory effect of adenosine on platelet aggregation can be partially prevented by NOS inhibition,[160] suggesting that, while platelet-derived NO does not necessarily and consistently inhibit platelet aggregation in response to proaggregants, it enhances the antiplatelet effects of antiaggregatory mediators.

Platelet-derived NO inhibits recruitment of platelets to the growing thrombus.[161-163] This process is initiated by activated platelets at the site of vascular injury by secretion of ADP, serotonin, and TXA_2, and further promotes thrombin deposition and thrombus formation on the platelet surface. *In vitro*, platelet-derived NO inhibits aggregation between leukocytes and platelets, and in particular, between monocytes and platelets. This is considered an early and robust marker of platelet activation[164] implicated in the mechanism of atherogenesis and thrombosis.[165, 166]

Platelet-derived NO also modulates the rate of thrombus growth, through altering

platelet adhesion on to the surface, and is also sensitive to insulin or shear stress.[167, 168] Collectively, these data support a physiological role of platelet-derived NO in the modulation of platelet function and hence thrombus formation.

1.4 PATHOLOGICAL ROLE OF NO IN VASCULAR DISORDERS ASSOCIATED WITH PLATELET DYSFUNCTION

The vasodilator and platelet-regulatory functions of endothelium are impaired during the course of vascular disorders including atherosclerosis, coronary artery disease, essential hypertension and diabetes mellitus.[169, 170] A number of researchers correlated the changes in the endothelial function with the generation of NO. The endothelial dysfunction was ascribed to both decreased and enhanced generation of NO. To explain this discrepancy, it was proposed that these changes in NO generation are often accompanied by reduced bioactivity of NO.[171] The metabolism of NO and the interactions of NO with reactive oxygen species account for this reduced bioactivity of NO.[172]

A detrimental effect of superoxide ion generation on the NO-dependent cellular signaling was first demonstrated by Gryglewski *et al.*[173] In 1990, Beckman *et al* reported that the reaction of NO with superoxide could take place under physiological conditions and lead to the formation of peroxynitrite ($ONOO^-$),[174, 175] a highly reactive species that can oxidize various biomolecules in the cellular microenvironment. In 1994, it was found

that $ONOO^-$ can decrease the vasodilator and platelet-inhibitory activity of NO and prostacyclin.[176] However, thiols and glucose[176, 177] attenuated these detrimental effects of $ONOO^-$. The reaction of $ONOO^-$ with thiols in cell membranes and glucose in the extracellular fluid results in synthesis of NO donors that counteract the vasoconstrictor and platelet-aggregatory activities of the parent oxidant.[176, 178] Interestingly, there is now evidence that small amounts of $ONOO^-$ may be generated during aggregation of normal platelets.[179] Thus, $ONOO^-$ generated by platelets, is rapidly detoxified and converted to NO donors following reactions with platelet membrane thiols.[178] The oxidizing stress could decrease the efficiency of this regulating mechanism and precipitate platelet dysfunction and damage.

1.4.1 ATHEROSCLEROSIS, THBOMBOSIS AND HYPERTENSION

Thrombosis appears to be a major determinant of the progression of atherosclerosis. In early atherosclerosis, microthrombi present on the luminal surface of vessel[180, 181] can potentiate progression of atherosclerosis by exposing the vessel wall to clot-associated mitogens. In later stages of atherosclerosis, mural thrombosis is associated with the growth of atherosclerotic plaques and progressive luminal occlusion. Platelet activation and participation in thrombotic responses to ruptures of atherosclerotic plaques are critical determinants of the extent of thrombosis, increasing plaque growth, and the

development of occlusive thrombi.[182, 183] Increased adherence of platelets to vessel wall manifesting early atherosclerotic changes and the release of growth factors from α-granules can exacerbate the evolution of atherosclerosis.[184]

Atherogensis is associated with profound changes in the oxidative status of the vascular wall. Oxidative modifications of low-density lipoproteins (LDL) play a key role in atherogenesis, and a number of studies[171] have examined the effects of native and oxidized LDL on NO-mediated vascular functions. In most of these studies lipoproteins decreased the bioactivity of NO.[185, 186] The decreased bioactivity of NO in atherosclerosis could also result from changes in the metabolism and the generation of $ONOO^-$ from superoxide and inducible NO.[174] In addition, LDL inhibit L-arginine uptake into platelets and through this mechanism, decreases NOS activity and promotes thrombosis.[187] These effects are prevented by the administration of L-arginine in the diet.[188, 189] In contrast to LDL, high-density lipoproteins (HDL) decreased platelet activation and thrombosis by increasing NOS activity in platelet.[187] Moreover, human apolipopeotein E, which mediates hepatic clearance of lipoproteins, exerts a significant inhibitory effect on platelets through stimulation of platelet NOS.[190]

Lipid peroxidation also leads to free radical-catalyzed generation of prostaglandin isomers from peroxidation of arachidonic acid. Interestingly, some of these isoprostanes reduce the antiadhesive and antiaggregatory activity of NO on platelets.[191] Thus, lipid

peroxidation contributes to the pathomechanism of impaired bioactivity of NO in the cardiovascular system.

Ischemic heart disease and myocardial infarction are common manifestations of coronary atherosclerosis. NO inhibited microthromboembolism in the ischemic heart, protected myocardium against intracoronary thrombosis (Figure 1.12), and decreased platelet deposition owing to carotid endarterectomy.[192, 193] Moreover, decreased generation of NO by platelets is predictive of the presence of acute coronary syndromes in patients with coronary atherosclerosis.[162] In addition, acetylcholine-induced release of NO is impaired in patients with coronary artery disease, contributing to a reduction in the endothelial capacity to regulate platelet activation.[184] These observations clearly show that the alterations in the generation and action of NO are important for the pathogenesis of atherogenesis and its ischemic complications.

Interestingly, an impaired NO generation or action also underlies the pathomechanism of vasospastic and thrombotic changes of essential hypertension.[194, 195] Camilletti *et al* found that platelet NO production is reduced in hypertensive patients.[196] Platelet L-Arginine transport has also been reported to be reduced in hypertensives, attributable to downregulation of the membrane transport system.[197, 198] Asymmetric dimethylarginine plasma levels are greater in hypertensive patients compared with normotensive controls,[199] and this gives rise to enhanced inhibition of platelet NOS in

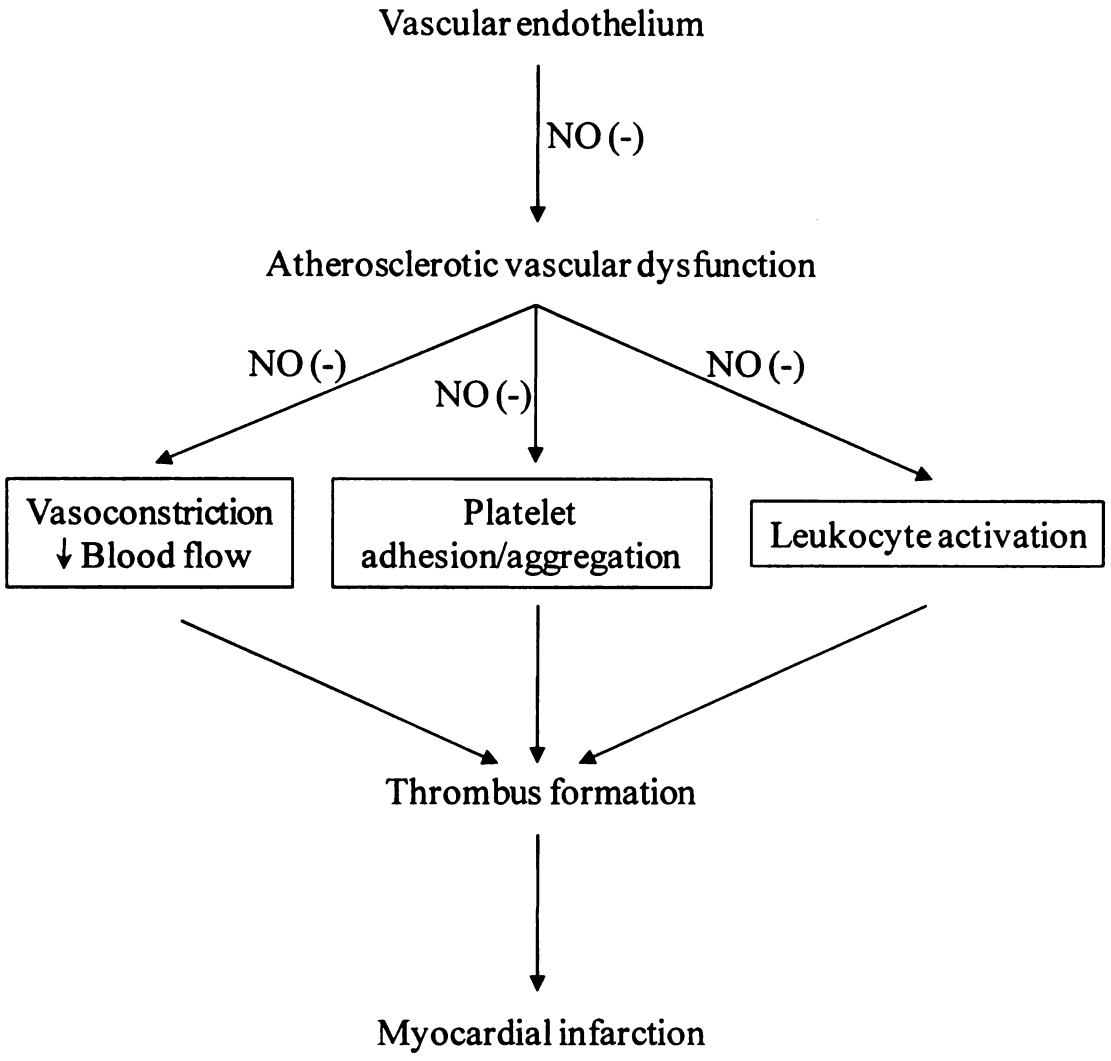


Figure 1.12 Overview actions of NO in myocardial infarction

hypertensives. Patients recently diagnosed, but not treated, with mild essential hypertension have been found to exhibit impairment in stimulated platelet NOS activity; in this study, although albuterol and collagen both increased platelet NOS activity in normotensive subjects, they failed to generate such an increase in hypertensives.[163] As these two agonists stimulate NOS through different pathways, it is likely that a generalized defect exists in the ability of platelet NOS to undergo stimulation in the context of hypertension.

1.4.2 DIABETES MELLITUS

There are indications that changes in the bioactivity and metabolism of NO are involved in the pathogenesis of vasculopathy in diabetes mellitus. Insulin, at physiological conditions, inhibits platelet activation via stimulation of platelet NOS.[200] Exposure of platelets to insulin decreases platelet aggregation in part by increasing synthesis of NO that, in turn, increases intraplatelet concentrations of cyclic nucleotides, cGMP and cAMP. Both of these cyclic nucleotides are known to inhibit activation of platelets. Thus, an insulin-dependent increase in NO production exerts antiaggregatory effects. In the context of diabetes, basal platelet NOS activity has been found to be decreased in both type I and type II diabetes mellitus as compared with healthy individuals.[201] This suggests that insulin deficiency of type I diabetes and in advanced

stages of type II diabetes contribute to platelet hyperactivity and diabetic angiopathy[202] by decreasing the inhibition of platelet reactivity induced by insulin.

1.4.3 CANCER

Platelets contribute to the cytotoxic cell effector system controlling neoplasia (tumor formation) and a part of this cytotoxic mechanism of platelets could be NO dependent.[203]

Platelets also play a role in the pathogenesis of tumor metastasis by increasing the formation of tumor cell-platelet aggregates, thus facilitating cancer cell arrest in the microvasculature. Tumor cell-induced platelet aggregation *in vivo* is modulated by the ability of tumor cell to generate NO, and this correlates with their propensity for metastasis.[204, 205] Indeed, human colon carcinoma cells isolated from metastases exhibited lower NO activity than cells isolated from primary tumor. Moreover, the expression of iNOS by murine melanoma cells inversely correlated with their ability to form metastases *in vivo*.[206] These data suggest that a differential synthesis of NO distinguishes between cells of low and high metastatic potential.

Another aspect of NO action on the metastatic cascade of events is its interactions with matrix metalloproteinases (MMPs). MMPs represent a family of matrix-degrading enzymes that play an important role in the growth, invasion, and metastasis of cancer

cells.[207] Sawicki *et al* have found that MMP-2 plays a crucial role in tumor cell-induced platelet aggregation.[208, 209] The release of MMP-2 was inhibited by NO donor agents, suggesting that NO interfere with cancer invasion and spread by reducing the release of MMPs.

1.5 PROJECT OBJECTIVE

Hyperactive platelets and associated thrombosis have been related to a number of cardiovascular diseases, and NO plays an important role in this physiology.[162, 210-212] When NO is released by the endothelium it prevents platelet adhesion to the vessel wall. However, when released by platelets, NO inhibits further recruitment of platelets to a growing thrombus.[153] Previous data from our research group showed that RBCs, upon deformation or under the influence of different agonists, release ATP[1, 213] that can further stimulate NO production from both the endothelial cells[214] and platelets[215, 216] resulting in vascular relaxation and inhibition of platelet activation.

In this work, it is hypothesized that these different cell types in the circulation are communicating through NO, mediated by the RBCs' ability to release ATP. Experiments have been performed not only to investigate platelet NO production release upon stimulation and activation using fluorescence spectroscopy, but also quantitatively measured the amount of NO employing a fluorescence probe with intracellular and

extracellular components. Moreover, this work also demonstrates the ability to measure NO production in platelets stimulated by RBC-derived ATP by employing a continuous flow analysis system as an *in vivo* system mimic. More specifically, an *in vitro* platform to immobilize endothelial cells in the channels of a microfluidic device to mimic *in vivo* microcirculation was used to monitor cell communication at the molecular level. This device is employed to monitor the physical interaction (adhesion) of platelets to an immobilized endothelium in the presence of platelet activator, inhibitor and RBCs. This approach is the first microfluidic device that allows multiple cell types to physically interact in the channels and this work further demonstrates the potential of these devices in the drug discovery process and drug efficacy studies.

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CHAPTER 2 FLUORESCENCE DETERMINATION OF NITRIC OXIDE PRODUCTION IN PLATELETS

2.1 KNOWLEDGE OF NITRIC OXIDE DETECTION

NO concentrations existing physiologically is essential for developing a quantitative understanding of NO signalling, for performing *in vitro* experiments with NO, and for measuring NO concentrations in disease states. Moreover, NO is involved in a wide range of biological systems in the body, such as the cardiovascular, nervous, reproductive, and immune systems.[1-4] Therefore, most research involving NO has primarily focused on the detection and quantitative determination of NO within these biological systems. Detection of NO *in situ* is often difficult due to its short half-life and low concentrations. However, a series of practical methods to detect NO (Table 2.1) has been developed using analytical methods including absorbance, chemiluminescence, amperometric, and fluorescence techniques.

Briefly, the first method utilizes horseradish peroxidase, a commercially available heme protein with ferric iron, to form a stable NO-ferric complex that induces large spectral changes at 396.5 and 420.0 nm in an absorbance spectrophotometer with a detection limit of 10 nmol/L.[5]

Chemiluminescence, more specifically the luminol/peroxide system, has also been used to detect NO in biological samples as low as 100 fmol/L.[6-9] The reaction of NO and

Method	Species detected	Detection scheme	Detection limit
Horseradish peroxide (HRP)	NO – HRP complex	Absorbance	10 nmol/L
Griess reaction	NO_2^-	Absorbance	0.1 μmol/L
Luminal reaction	$ONOO^-$	Chemiluminescence	100 fmol/L
Nafion coated carbon electrod	NO	Amperometry	10 μmol/L
DAF-FM (DA)	NO	Fluorescence	3 nmol/L

Table 2.1 Practical methods for the detection of NO in biological samples employing common analytical techniques

hydrogen peroxide generates peroxynitrite, a stronger oxidizing agent than hydrogen peroxide itself, which can then react with luminol to produce a detectable chemiluminescent product.

Another technique utilizes the Griess reaction, a method that measures the conversion of nitrite, an oxidation product of NO, to the diazonium ion that is then coupled to *N*-(1-naphtyl)ethylenediamine to form an azo derivative that is chromophoric. The limit of detection for the Griess reaction is about 0.1 μmol/L.[10]

Amperometry has also proven to be a useful tool in the detection of NO. By coating a carbon ink electrode with Nafion, a modification used to block nitrite from the electrode, NO was detected at concentrations as low as 10 μmol/L.[11] A decade ago, a value of about 1 μM seemed reasonable based on early electrode measurements[12-16] and a provisional estimate of the potency of NO for its guanylyl cyclase-coupled receptors, which mediate physiological NO signal transduction.[17-19] Since then, numerous efforts to measure NO concentrations directly using electrodes in cells and tissues have yielded an irreconcilably large spread of values.[14-16, 20-23] In compensation, data from several alternative approaches have now converged to provide a more coherent picture. These approaches include the quantitative analysis of NO-activated guanylyl cyclase, computer modeling based on the type, activity and amount of NO synthase enzyme contained in cells,[18, 24-26] the use of novel biosensors to monitor NO release from single endothelial

cells and neurones, and the use of guanylyl cyclase as an endogenous NO biosensor in tissue subjected to a variety of challenges.[27, 28]

The bulk of results reported here have focused on utilizing fluorescence based techniques for the detection of NO. Accordingly, of recent interest is the family of diaminofluorescein (DAF) fluorogenic indicators developed by Kojima et al,[29] but more specifically 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA). Membrane permeable, this probe is deacylated by intracellular esterases to 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM). The diacetate probe is essentially nonfluorescent until it reacts with byproducts of NO oxidation to form fluorescent heterocycles. Upon nitrosylation, the probe becomes trapped within the cytoplasm as shown in Figure 2.1. Importantly, the detection limit of NO with DAF-FM is approximately 3 nmol/L, which is 1.4 times lower than that of DAF-2, a probe similar to DAF-FM. DAF-FM is also known to be stable above pH 5.8 and because of the specificity for NO, DAF-FM will not react (in neutral solution) with nitrate, nitrite or any oxygen reactive species. DAF-FM is also not sensitive to ascorbic acid, a common species in medium and buffer system.

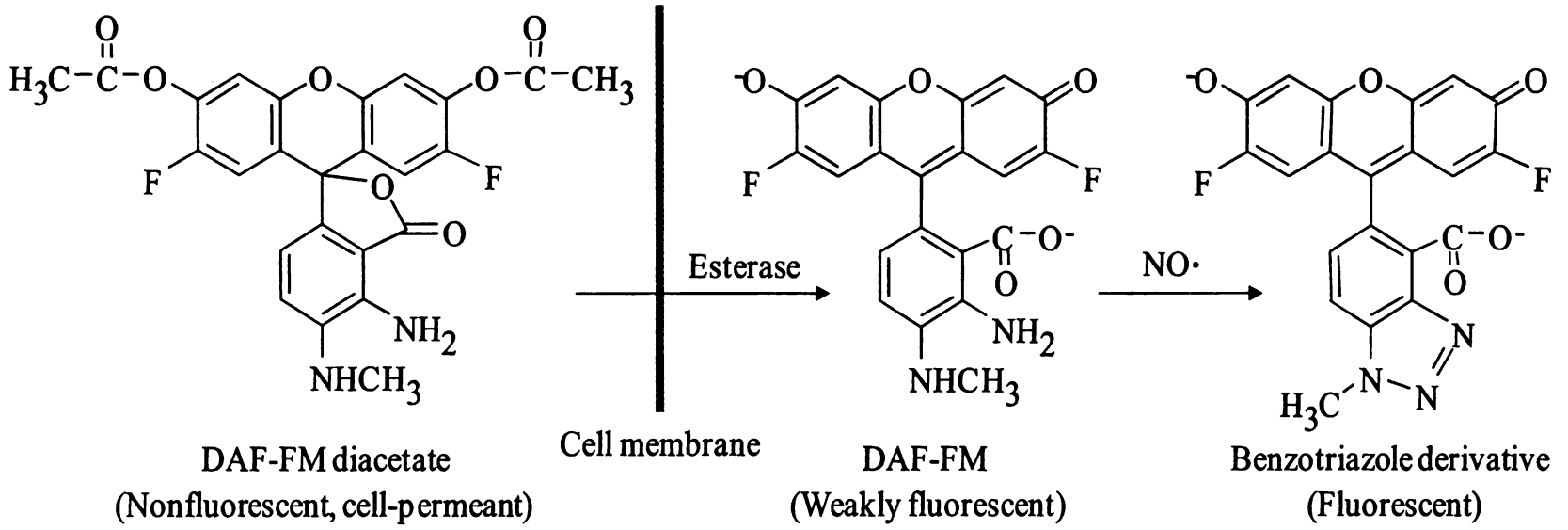

Figure 2.1 The reaction scheme of DAF-FM DA for the detection of intracellular NO production. DAF-FM forms a fluorescent benzotriazole derivative that has an excitation at 495 nm and emission at 515nm

2.2 NO PRODUCTION AND RELEASE FROM STIMULATED AND ACTIVATED PLATELETS

The circulation is a complex mixture that includes, but is not limited to, cells, macrophages, proteins, and metabolites. The white blood cells (leukocytes), red blood cells (erythrocytes) and platelets have well-defined roles in the bloodstream. For example, it is well-established that leukocytes play a major role in the immune system. Red blood cells (RBCs) are recognized as oxygen carriers *in vivo*, while platelets are generally considered major determinants in the blood clotting process. While each of the aforementioned roles of these cell types are understood and accepted, there also reports suggesting that the cells that flow through the circulation may actually participate in other processes in blood vessels.

RBCs have been shown to be determinants in the control of vascular caliber in the pulmonary and systemic circulation by releasing nanomolar to micromolar amounts of adenosine triphosphate (ATP) when these cells are deformed[30-35] or subjected to brief periods of hypoxia.[36, 37] Certain pharmacological agents are also capable of stimulating the release of ATP from RBCs.[38] The importance of this release of ATP *in vivo* is that ATP is a recognized stimulus of nitric oxide synthase (NOS) in endothelial cells.[39-42] NO is a multitasking molecule *in vivo*. It is a recognized dilator of blood vessels,[43-46] it is an inhibitor of platelet activation and aggregation,[47-50] and it is also a neurotransmitter.[51, 52] Although a relationship between ATP released from RBCs and endothelium-derived NO

has been established, there have been no reports of the synergy between ATP derived from RBCs and the ability of platelets to produce NO.

The relationship between ATP and platelet-derived NO may be profound. Sprague *et al* postulated that ATP was released from the RBC in response to mechanical deformation, as would occur in association with vasoconstriction or increased velocity of blood flow.[30] This RBC-derived ATP can then act on the endothelial cell to stimulate endogenous NO synthesis and enable the RBC to participate in local regulation of vascular caliber.[30, 34] In accordance with the availability of NO along the vascular wall, we have recently found that platelets have the ability to release ATP and produce their own NO (also has been shown to inhibit platelet adhesion to endothelial cells). These findings suggest that the RBC may be a determinant of platelet adhesion *in vivo*.

Platelets normally circulate without adhering to undisturbed vascular endothelium. Upon vascular insult, subendothelial collagen is exposed that acts as the primary stimulus for platelet activation. However, several other endogenous agonists also exist such as thrombin, ADP, thromboxane A_2, serotonin and epinephrine, which also promote platelet activation.[53] Platelet activation initiates a change in the platelet shape, thus promoting adhesion to the vascular walls and the subsequent recruitment of additional platelets. NO has been widely shown to mediate this process by activating soluble guanylate cyclase (sGC) which initiates a protein kinase G (PKG) dependant pathway.[54] In this construct,

NO becomes an important determinant in platelet adhesion *in vivo*.[50] If uncontrolled, these adhered platelets become a major constituent of thrombus formation and subsequent vessel blockage.

Recently, platelets have been reported to have unique properties in disease states other than cardiovascular problems. For example, a procedure used in multiple sclerosis, plasma replacement therapy, involves removing such formed elements as RBCs and leukocytes from the patient's plasma and then replenishing new plasma with the previously removed cells. The plasma contains the platelets so, in addition to obtaining new plasma, the patient has also receives new platelets. Interestingly, the platelets of people with multiple sclerosis have been shown to be more susceptible to hyperactivity, often aggregating more easily than the platelets of healthy people that do not have multiple sclerosis.[55] The importance of this platelet activity is that platelets are known to produce NO upon activation[56] and people with multiple sclerosis have been shown to have high levels of NO and NO metabolites in their cerebral spinal fluid and urine.[57, 58] This trait of platelets (prone to activation and aggregation) is also known to exist for patients that have cystic fibrosis.[59] Finally, patients with diabetes also have platelets that are known to be activated more readily than healthy, non-diabetic controls.[60] Exemplifying the importance and complexity of the relationship between platelets and NO is that NO has the ability to inhibit platelet activation.[50]

Interestingly, platelets have the ability to create bioavailable NO in the bloodstream in multiple ways other than by activation. For example, NO can be synthesized inside of the platelet by one of multiple isoforms of NOS[61, 62] or from the denitrosation of S-nitrosothiols (S-nitrosoglutathione or S-nitrosoalbumin) enzymatically[63] or by copper-containing proteins.[64] In each of these scenarios, the most common method that has been employed to date for measuring NO derived from cells is amperometry with either carbon or platinum electrodes.[11, 65] Advantages of such measurement schemes is the ability to measure the released NO without having to add any molecular probes to the system or performing any sort of derivatization chemistry to create a fluorophore or chromophore. Moreover, amperometric methods also enable the analyst to add some type of selectivity to their measurement when a modified electrode surface is employed during the measurement.[11, 65] However, the degree of selectivity depends on the number of coatings of the electrode surface and its integrity,[21, 23] and day-to-day electrode behavior can change dramatically.[22]

Here, we have employed diaminofluorofluorescein (DAF) probes[66] which, depending on the form, are rather specific for NO in the absence of certain interferences (such as ascorbate).[67-69] Specifically, we have used DAF-FM DA to perform intracellular measurements of NO when produced via platelet eNOS. However, to date there have been no reports of measuring platelet-derived NO using fluorescence spectrophotometry.

The DAF family of probes has been employed to determine NO production in, or NO release from, numerous cell types. However, none of the DAF probes have been employed to measure NO production in platelets. In the work reported here, the optimum conditions for measuring NO production in platelets, including probe concentration and incubation time, is determined. In addition, data is reported that demonstrates the ability to measure NO production in platelets using DAF-FM DA. When combined with an extracellular measurement (using DAF-FM) prior to the stimulation of NO production with either ATP or ADP, the ability to quantitatively determine the amount of NO released from the platelets is possible.

2.3 EXPERIMENTAL METHODS

Isolation and purification of platelets. Rabbits were anesthetized with ketamine (100 mg/mL, 0.12 ml/kg, i.m.) and xylazine (20mg/mL, 0.08 mL/kg, i.m.) followed by pentobarbital sodium (3 mg/mL, 3.2 mL/kg, i.v.). A cannula was placed in the trachea and the animals were ventilated with room air. A catheter was then placed into a carotid artery for administration of heparin (1000 units/mL, 0.5 mL/kg) and for phlebotomy. After heparin, animals were exsanguinated and the whole blood collected in a 50 mL centrifuge tube. Generally, 70 - 90 mL of blood was collected from the animal. Blood was centrifuged at 500 x g at 37°C for 10 minutes. The platelet rich plasma (PRP) was

decanted for the subsequent isolation of platelets. Platelets were isolated from the PRP by adding 1 mL of acid citrate dextrose (ACD, 41.6 mM citric acid anhydrous, 76.7 mM sodium citric acid anhydrous, 122.1 mM D-glucose) to 9 mL of the PRP and centrifuging at 1500 x g at 37°C for 10 minutes. The harvested platelets were then washed twice by centrifugation in a mixture containing Hank's Balanced Salt solution (HBSS, H1387 from Hanks' Balanced Salt, Sigma) and ACD (6:1 v/v). The washed platelets were then resuspended in HBSS as higher concentrated platelet stock solution.

In all experiments, platelet count was measured on the hemacytometer then adjusted to a platelet count of 3.0×10^8 platelets mL^{-1} as final concentration with HBSS. Platelets were generally harvested and prepared on the day of use. Our group has found that, if kept in the PRP, the platelets can be purified within 1-2 days after the surgical procedure and still produce reliable data. However, the platelets are generally consumed through experimental procedures on the day of harvesting them from the rabbit. All studies involving animal use were approved by the Animal Investigation Committee at Michigan State University.

Reagent preparation. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received without further purification unless otherwise noted. L-arginine (the substrate of NO production, pH was adjusted before experiments), N^{ω}-Nitro-L-arginine methylester hydrochloride (L-NAME, a selective eNOS/nNOS

inhibitor), and N^G-monomethyl-L-arginine (L-NMMA, a non-selective NOS inhibitor) were prepared as 10 mM stock solutions in HBSS. Adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) were prepared as 100 μM stock solutions in distilled and deionized water (DDW, 18.2 MΩ-cm). The DAF-FM/DAF-FM DA was prepared as a 5 mM stock solution in dimethyl sulfoxide (DMSO). In all studies, the total volume of platelets, DAF-FM/DAF-FM DA, inhibitor/agonist, and buffer were equal to 1.0 mL. Nitric oxide was prepared as a 38mM stock solution from spermine NONOate (Cayman Chemical, Ann Arbor, MI) by dissolving 10mg of the spermine NONOate solid in 1 mL of 0.01M sodium hydroxide (NaOH) solution. Working solutions were prepared by dilution of the alkaline NONOate solution in 0.1 M phosphate buffer (pH 7.4).

Fluorescence determination. Aliquots of DAF-FM/DAF-FM DA were added into each vial and incubated with the platelets for 30 minutes before each fluorescence measurement was taken. For those studies involving stimulation/activation with ATP/ADP, the agonist was added and equilibrated with platelets for another 30 minutes before measurements of NO production. In the contrast, studies involving inhibition with L-NAME/L-NMMA, the inhibitor was added with platelets for 30 minutes prior to fluorescent probe incubation. Fluorescence measurements were performed on an RF-5301PC spectrofluorometer (Shimadzu, Columbia, MD) at room temperature. Fluorescence emission spectra were obtained in a quartz cuvette with excitation

wavelength at 495 nm and emission wavelength at 515 nm. Both excitation and emission slit widths of 3 nm were used in all experiments. The sampling interval was 0.2 nm with a data acquisition rate of 50 Hz. All error bars in the figure represent standard error of the mean (SEM) for at least n = 4 different rabbits exsanguinated on different days and each bar was statistically significant at minimum $p < 0.05$.

2.4 RESULTS AND DISCUSSION

NO in platelets. Platelets are known to contain NOS, which upon stimulation, will produce and subsequent release NO.[61, 62] In order to determine if basal levels of NO could be measured using the DAF-FM DA probe, 100 μL of a suspension containing 3 x 10^9 platelets mL^{-1} were added to 100 μL of the DAF-FM DA probe (10 μM) and, after the solution was brought to 1.0 mL in total volume with HBSS, allowed to incubate for 30 minutes; all experiments were performed at room temperature. This time enabled the DAF-FM DA probe to cross the platelet membrane bilayer; once inside the platelet, the probe is able to detect NO after the diacetate groups are cleaved from the parent DAF-FM DA molecule. The incubation period enables this cleaving process to occur. A typical fluorescence spectrum from the platelets loaded with DAF-FM DA is shown in Figure 2.2a (middle trace). Also shown in this figure is a spectrum (top trace) obtained after DAF-FM DA loaded platelets were stimulated with 100 μL of 100 μM ATP, a

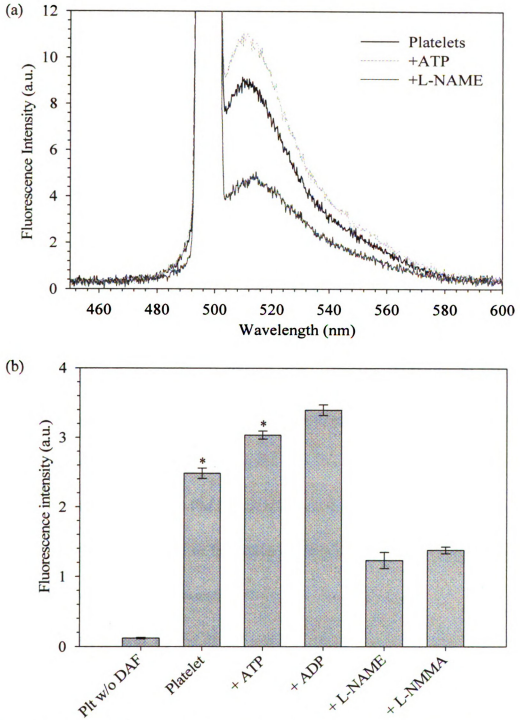

Figure 2.2 (a) Emission profiles for platelets in the presence of DAF-FM DA molecular probe for nitric oxide (middle trace), the platelets in the presence of the probe and 10 μM ATP (top trace), and the platelets in the presence of the probe after incubation in the NOS inhibitor L-NAME (bottom trace) (b) Quantitative data obtained from spectra, control consisted of platelets in the absence probe. Error bars represent SEM. $p < 0.05$ ($n = 5$)

recognized stimulus of NOS.[39] The increase in fluorescence emission is due to the increase in NO production inside of the platelets upon NOS activation by ATP. In order to provide evidence that this increase in the measured fluorescence emission was due to an increase of NO production (via NOS activation) in the presence of the ATP stimulant, platelets were incubated with L-NAME, a competitive inhibitor of NOS. As shown in Figure 2.2a, a 50.4% ± 2.3% decrease in fluorescence emission is measured from those platelets incubated with L-NAME (bottom trace). Figure 2.2b contains summarized data obtained from the spectra. The control consisted of platelets alone in the absence of DAF-FM DA, and demonstrated that no NO production could be measured unless DAF-FM DA is present. Upon stimulation or activation, the NO production in platelets was increased 22.3% ± 4.4% and 36.8% ± 5.1%, respectively compared to normal platelets incubated with DAF-FM DA. Since there is no nNOS in platelets, the inhibition of NOS activity by L-NAME or L-NMMA resulted in approximately equal decreases in NO production: 50.4% ± 6.0% and 44.5% ± 3.5%, respectively.

Optimization of DAF-FM DA concentration. Key to the success in measuring NO production in any cell type is the optimization of those variables affecting the signal intensity. An important variable in the studies reported here is the optimal concentration of the DAF-FM DA probe. Typically, most users of DAF-type probes employ concentrations of 10 μM. However, fluorescein-based probes are somewhat notorious for

resulting in large background signals, thus affecting the overall S/N ratio in a measurement scheme. Therefore, in order to optimize the resultant emission from NO production, various DAF-FM DA concentrations were loaded into a suspension of platelets (3×10^8 platelets mL^{-1} as final concentration), and allowed to incubate for 30 minutes before the fluorescence intensity was measured. Next, a mixture containing 100 μL of 10 mM L-arginine and 100 μL of 100 μM ATP was added to the platelet solution and, after 30 minutes, the emission resulting from ATP-induced NOS activation was measured. The data in Figure 2.3 show that platelets incubated with 0.1 and 0.5 μM DAF-FM DA displayed a change in fluorescence intensity upon the addition of DAF-FM DA to the platelet suspension. However, upon addition of the ATP stimulus, there was no statistically significant change in the fluorescence emission. There were significant changes in the fluorescence emission for concentrations of DAF-FM DA between 1 and 10 μM. In addition, results obtained from the studies involving the 1-10 μM concentrations indicate the background emission (DAF-FM DA alone represented by black bars in Figure 2.3) was lowest for the 1 μM trial. The signal from stimulated platelets (expressed as a percent change from the emission from platelets loaded with DAF-FM DA that were not stimulated with ATP) was highest for the 5 μM and 10 μM trials where an increase in emission intensity of ~40% was measured. Although a percent change of 37% was measured for the 1 μM DAF-FM DA concentration, the S/N ratio did

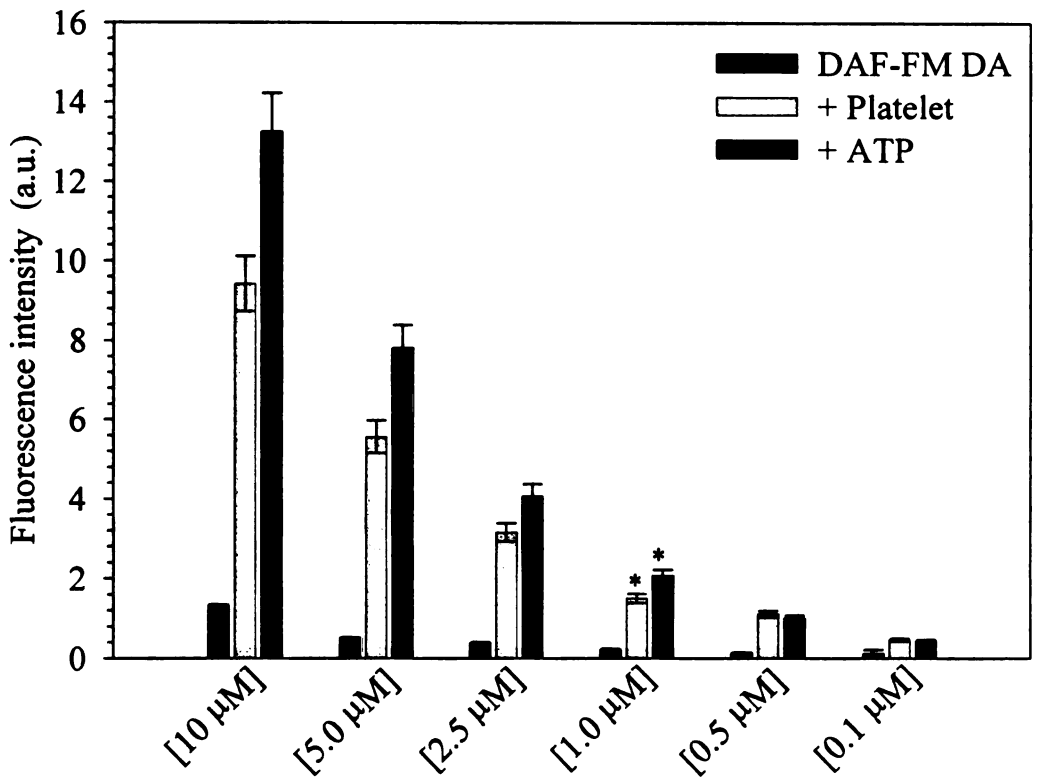

Figure 2.3 Fluorescence intensities using various concentrations of the DAF-FM DA probe. Emission intensities are shown for DAF-FM DA probe in the absence of platelets (black bar), the probe in the presence of the platelets (light gray bar), and in the presence of the probe, platelets, and ATP stimulus (dark gray bar). Error bars represent SEM. $p < 0.05$ ($n = 4$)

not statistically increase from 1 μM to 10 μM DAF-FM DA. Therefore, for the remaining studies, a 10 μM DAF-FM DA stock was diluted to a working concentration of 1 μM DAF-FM DA for all NO measurements.

Incubation time optimization. In addition to the concentration of the DAF-FM DA probe employed for monitoring the NO production in platelets, it is also important to investigate the time allotted for the DAF-FM DA to enter the cell and for the cleavage of diacetate from the parent probe molecule. Figure 2.4 contains data showing results from subjecting 100 μL of a 3 x 10^9 platelets mL^{-1} solution to 100 μL of a 10 μM DAF-FM DA solution (the final volume was adjusted to 1.0 mL with HBSS) for a specified period of time and monitoring the increase in fluorescence emission. Controls consisting of buffer alone with DAF-FM DA or buffer with L-arginine, ATP, and DAF-FM DA in the absence of platelets were measured to demonstrate that no significant increase in emission intensity is measured unless platelets are included in the reaction mixture. However, in the presence of platelets, a significant increase in fluorescence emission is measurable, even after 2.5 minutes of incubation time. This increase in fluorescence is even more pronounced in the presence of ATP. Interestingly, the highest ratio of fluorescence intensity in the presence of ATP to that when no ATP is added occurs within 2 - 3 minutes. In other words, although longer incubation periods for the DAF-FM DA will increase the overall emission intensity, the difference in fluorescence emission

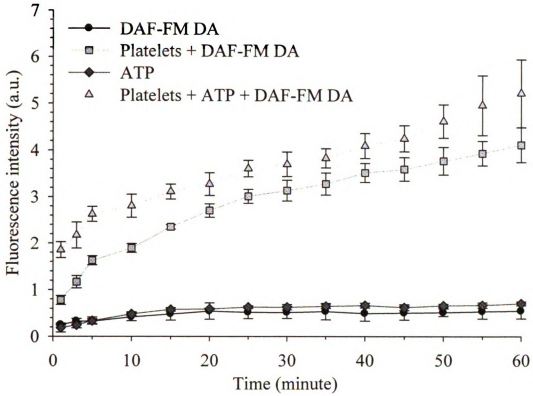

Figure 2.4 Measurement of the change in the fluorescence intensity as a function of time. The lower two traces are for DAF-FM DA (circles) and DAF-FM DA incubated with ATP (diamonds) in the absence of platelets. The top two traces are for platelets incubated with DAF-FM DA for the times specified on the time axis in the absence (squares) and presence (triangles) of ATP. For each data point shown in the top two traces, the fluorescence intensity is significantly higher for those platelets that were stimulated with ATP. Error bars represent SEM. (n = 4)

between basal levels of NO and those levels resulting from stimulation can be optimally measured within a minute or two of subjecting the platelets to the NO stimulus. The reason that the signal does not stabilize with time could be due to the ability of platelets to continue producing NO. Therefore, a fixed-time method is used to perform all experiments here.

NO production and platelet concentration. As mentioned above, the volume of platelets obtained from the whole blood of the rabbit is limited. Thus, in order to optimize the number of experiments that can be performed with a single harvesting of platelets from a mammalian subject, studies were performed to investigate the effect of platelet number on the measured emission signal. Volumes of a platelet solution containing 3 x 10^9 platelets mL^{-1} were incubated in 1.0 μM DAF-FM DA for 30 minutes prior to measuring the fluorescence emission from the probe-NO product. Data in Figure 2.5 indicate that the signal intensity increases as a function of the number of platelets in the reaction mixture. However, the overall ratio of the emission intensity between platelets with DAF-FM DA and those platelets incubated with the probe and ATP stimulus was statistically unchanged even up to 200 μL of platelets.

Discussion of NO in platelets. Figures 2.2 – 2.5 demonstrate that the DAF-FM DA probe can be employed for the intracellular determination of NO, not only in the presence of external agonists, ATP and ADP, but also after the inhibition by L-NAME

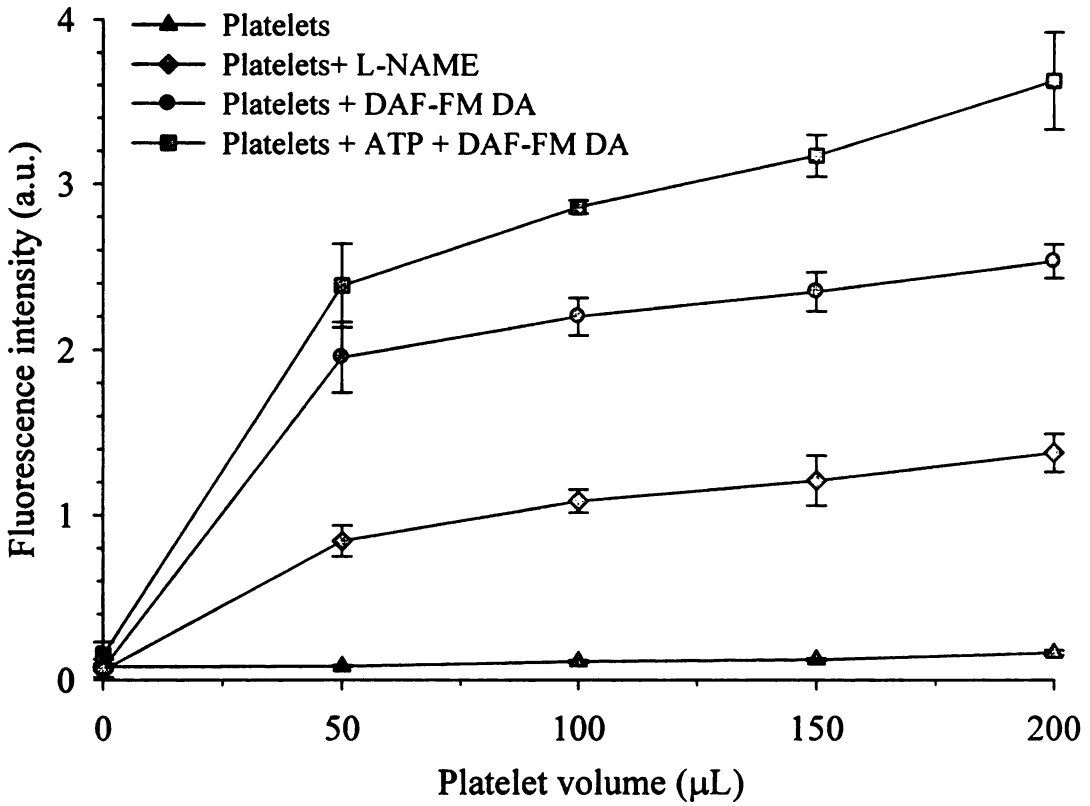

Figure 2.5 Signal intensities as a function of platelet number for platelets in the presence and absence of DAF-FM DA, in the presence of the probe with ATP, and in the presence of the probe and L-NAME. Error bars represent SEM. (n = 4)

and L-NMMA. The ATP stimulates the production of NO in platelets by binding to purinergic receptors on the platelet membrane. Therefore, although the ATP is able to stimulate NO production in the platelets, it does not result in the activation or aggregation of platelets. It is known that agonists of platelet activation and subsequent aggregation, such as ADP and thrombin, are able to stimulate NO production. However, this type of stimulation of NO (through platelet activation) is important because many features of platelet function are related to their ability to activate and aggregate. The data in Figure 2.6 show that the maximum NO produced in platelets upon activation with the ADP agonist increased by 52.3% ± 8.2% while ATP stimulation resulted in a maximum increase in fluorescence emission of 39.1% ± 6.2%. Thus, the use of the DAF-FM DA probe can be employed to measure NO production even in the presence of activated platelets. Importantly, these results also suggest that a greater amount of NO is produced in platelets that are activated by ADP as compared to stimulation with ATP.

NO released from platelets. A quantitative determination of the amount NO was also performed. The NO donor, spermine NONOate, was used as a standard in the method of multiple standard additions (Figure 2.7a). A 3.8 μM NONOate solution was incubated in HBSS at 37°C for 15 minutes, while at the same time, 100 μL of platelet solution (3×10^9 platelets mL^{-1}) and 100 μL of 10 μM DAF-FM DA were mixed and allowed to incubate for 15 minutes. Various amount of NONOate solution was added into

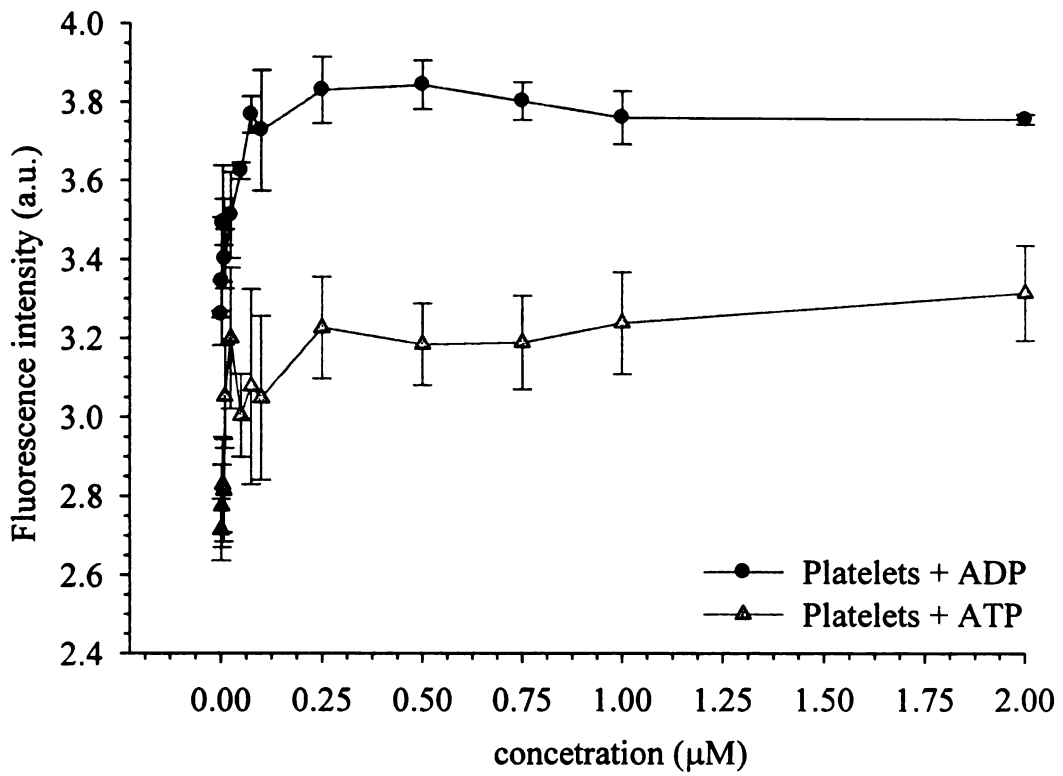

Figure 2.6 Monitoring the production of nitric oxide as a function of concentration of ATP and ADP. Relative to unstimulated platelets incubated with the DAF-FM DA probe, the increase in fluorescence emissions were 39.1% ± 6.2% and 52.3% ± 8.2% for platelets stimulated with ATP and activated with ADP, respectively. Error bars represent SEM. (n = 4)

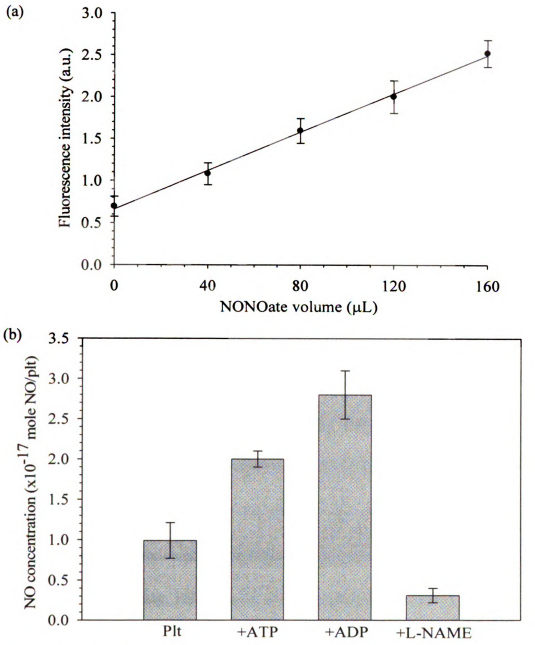

Figure 2.7 (a) A calibration curve was prepared using the method of multiple standard additions. The fluorescence intensity was measured after increments of NO were added to aliquots of platelets containing DAF-FM. (b) Quantitative determinations of NO released by the platelets are summarized in the accompanying bar graph. The concentration of extracellular NO in the presence of platelets alone (Plt) is $9.9 \pm 2.2 \times 10^{-18}$ moles NO/platelet. The extracellular NO levels increase to $2.0 \pm 0.1 \times 10^{-17}$ and $2.8 \pm 0.3 \times 10^{-17}$ moles NO/platelet in the presence of ATP (+ATP) and ADP (+ADP), respectively. In the presence of a NOS inhibitor (+L-NAME), the concentration of extracellular NO decreased to $3.1 \pm 0.9 \times 10^{-18}$ moles NO/platelet. Error bars represent SEM. (n = 4)

DAF-FM DA loaded platelet solution and allowed to incubate for another 15 minutes. The solution volume in each vial was adjusted to 1.0 mL with HBSS. The results showed that the amount of intracellular NO in platelets is $(2.7 \pm 0.3) \times 10^{-16}$ moles of NO/platelet (data not shown) as basal level of NO. Employing the method previously discussed with the addition of the DAF-FM probe (for extracellular NO determinations), Figure 2.7b indicates that a quantitative determination of NO released from platelets upon stimulation by ATP or activation by ADP is feasible. The data in Figure 2.7 revealed that the concentration of extracellular NO in the presence of platelets alone (i.e., no stimulus or activator) is $(9.9 \pm 2.2) \times 10^{-18}$ moles of NO/platelet. The extracellular NO levels increase to $(2.0 \pm 0.1) \times 10^{-17}$ and $(2.8 \pm 0.3) \times 10^{-17}$ moles of NO/platelet in the presence of ATP and ADP, respectively. To verify that the fluorescence signal was due to NO, the experiment was also performed in the presence of L-NAME. In the presence of the inhibitor, the concentration of extracellular NO decreased to $(3.1 \pm 0.9) \times 10^{-18}$ mole of NO/platelet. Importantly, Freedman *et al* demonstrated that, upon activation, platelets released about 5×10^{-17} moles of NO/platelet.[50] Here, we reported a value approximately 3×10^{-17} moles of NO/platelet,[70] which is less than that reported using amperometry, but still in good agreement with previous results.

2.5 CONCLUSIONS

The spectrofluorometric monitoring of NO production in platelets has been determined using DAF-FM DA as a molecular probe. The NO that was measured existed either as a basal level of NO found in the platelets, NO that was stimulated by ATP, or NO stimulated by activating the platelets with ADP. There have been some reports describing the use of DAF-2 DA, another member of the DAF family of molecular probes, for fluorescent monitoring of intracellular NO production. However, these reports were lacking since none of these previous studies investigated the parameters affecting the overall emission signal from the DAF-FM/NO product such as probe concentration, reaction time, and platelet density. Another important feature of the results presented here is that NO production in platelets can be determined upon activation with an agonist known to initiate platelet aggregation. That is, upon addition of ADP, platelets will activate and eventually aggregate. This activation has been shown to lead to the production and release of NO from platelets. The ability to monitor this NO production using a fluorescence probe may be advantageous over other forms of measuring NO production from platelets (such as amperometry) because, once activated, a platelet's ability to adhere to surfaces is dramatically increased. In fact, *in vivo*, it is the ability of the platelet to adhere to surfaces that leads to repair of lesions or clotting of blood. These same adhesion properties are also known to be determinants in the formation of a

thrombus on the inner walls of the vascular system, thus leading to thrombosis and, potentially, atherosclerosis and vessel blockage. The ability to measure NO produced in platelets using fluorescence spectrophotometry will enable those researchers studying the role of platelets in other biologically or medically related fields (diabetes, multiple sclerosis, cystic fibrosis) to monitor this important molecule using instrumentation found in most laboratories and microliter volumes of sample.

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CHAPTER 3 PLATELET-DERIVED NITRIC OXIDE IS AFFECTED BY RBC-DERIVED ATP

3.1 INTRODUCTION TO CIRCULATION

The main function of the circulatory system is to move nutrients, gases, and wastes to and from tissues and cells, as well as stabilize body temperature and pH to maintain homeostasis. Distinguished by types, it is considered that the circulatory system consists of the cardiovascular and lymphatic systems.

The main components of cardiovascular system are the heart and blood vessels. The blood vessels are comprised of arteries, arterioles, capillaries, veins and venules and the heart itself can be considered as a specialized type of blood vessel that has become adapted for pumping blood throughout the cardiovascular system.

Separate from the cardiovascular system is the lymphatic circulatory system, which contains capillaries and large vessels. This system is responsible for the transport of tissue fluid, or lymph, back to the circulatory system.

Distinguished by route, the circulation is composed of two systems, the pulmonary and systemic circulations. The vessels that supply blood to and from the lungs from the heart are referred to as the pulmonary circulation. Blood vessels serving the remainder of the body make up the systemic circulation. More specifically, the pulmonary circulation carries oxygen depleted blood away from the heart and to the

lungs where it is oxygenated; it then returns the oxygenated blood to the heart, while the systemic circulation carries oxygenated blood away from the heart and to the body returning deoxygenated blood back to the heart.

3.1.1 GENERAL INFORMATION ABOUT THE RED BLOOD CELL

Blood is responsible for a variety of processes in the body; it transports nutrients from the small intestine to the liver and from the liver and adipose tissue to other organs; it also transports waste products from the tissues to the kidneys. The bloodstream is responsible for carrying hormonal signals from one tissue to another and the transfer of oxygen from the lungs to tissues, as well as transport of carbon dioxide generated from tissue respiration to the lungs.

On average, most adults have 5 - 6 liters of blood circulating in the body. Over half of this volume is occupied by three types of cells: leukocytes, that are responsible for aiding the body in fighting infection; platelets, an essential component of the blood clotting process; and erythrocytes, or red blood cells (RBCs), that are responsible for carrying oxygen in the bloodstream. There are approximately 5×10^6 RBCs, $4 - 10 \times 10^3$ leukocytes, and $1.5 - 4.0 \times 10^5$ platelets in each microliter of blood in an average human. The remaining volume of blood is comprised of plasma that contains proteins,

lipoproteins, nutrients, waste, inorganic salts, and hormones; it is also responsible for carrying carbon dioxide back to the lungs.

Approximately 8 – 10 μm in diameter, RBCs are unlike other cell types because they are anuclueate when mature, which means they contain no nucleus and no DNA. RBCs are primarily composed of hemoglobin, a complex metalloprotein containing heme groups. The iron center of heme is responsible for the temporary linking of oxygen to RBCs in the circulation. The color associated with RBC is also due to the heme constituents on hemoglobin; oxyhemoglobin appears scarlet in color while deoxyhemoglobin is darker; it appears blueish in color in the vessels and through skin. RBCs are described as circular, biconcave disks. Importantly, this shape is optimal as it allows for the exchange of oxygen with surrounding tissues and organs.

In general, RBCs mature from stem cells in 7 days and circulate through the body for an average of 120 days. Human RBCs are produced in bone marrow by a process known as erythropoiesis. Once aged, an erythrocyte will undergo changes in its plasma membrane making it more susceptible to phagocytes and subsequent phagocytosis in the spleen. The majority of the RBC breakdown is recycled throughout the body including hemoglobin.

3.1.2 RBC-DERIVED ATP RELATED VASODILATION

Vasodilation is the widening of the blood vessels resulting from a relaxation of the smooth muscle wall of the vessels. This relaxation relies on removing the stimulus for contraction, which is primarily dependent on calcium and the phosphorlyation of myosin light chain. Therefore, vasodilation works by decreasing the intracellular concentration of calcium or a dephosphorylation of the myosin light chain.[1, 2] There are a number of substances that can dilate blood vessels including acetylcholine, bradykinin, as well as ATP.[3-5]

ATP, commonly known as a "universal energy carrier" or "molecular currency" is a multifunctional nucleotide generated and consumed within the body in a multitude of anabolic and catabolic reactions. ATP is mainly generated during respiration in non-photosynthetic organisms and during photosynthesis in photosynthetic organisms, and is readily consumed by many anabolic and catabolic enzymes.[6, 7] It is involved in signal transduction pathways and acts as a substrate for kinases, a group of enzymes that catalyze the transfer of a phosphate group from high energy donors, to specific target molecules.[8, 9]

Interestingly, ATP is known to be present in millimolar amounts within RBCs[10, 11] and upon being subjected to a stimuli such as mechanical deformation (as RBCs traverse through the microcirculation), O_2 tension (hypoxia) or low pH, these

RBCs release nanomolar to low micromolar amounts of ATP[12-15] into the lumen of the vessel.[12, 14, 16, 17] This is significant because it has previously been reported that endogenous NO production in the circulation is dependent on the presence of RBCs in the perfusate flow through an isolated rabbit lung.[18] It has also been determined that the production of NO was dependent on the RBCs' ability to release ATP in response to mechanical deformation.[19] The production of NO has been linked to changes in the endothelial lining of blood vessels as the result of stimulation from substances such as ATP[20] as well as shear stress.[21, 22] The binding of ATP to the P_{2y} receptor located on the endothelium[23-27] results in the synthesis of NO[28, 29] eventually lead to smooth muscle relaxation and an increase in blood flow to the target tissue.[14]

Collectively, these reports suggest a mechanism for control of vascular caliber in the circulation. In response to the RBC being continually deformed during flow through a vessel, it releases ATP thereby stimulating the synthesis of endothelial NO resulting in subsequent relaxation of the smooth muscle cells and this is illustrated in Figure 3.1. Accordingly, multiple groups have proposed a relationship between RBC-derived ATP release and vascular resistance in the pulmonary[18, 19, 29] and systemic circulation.[28, 30-32]

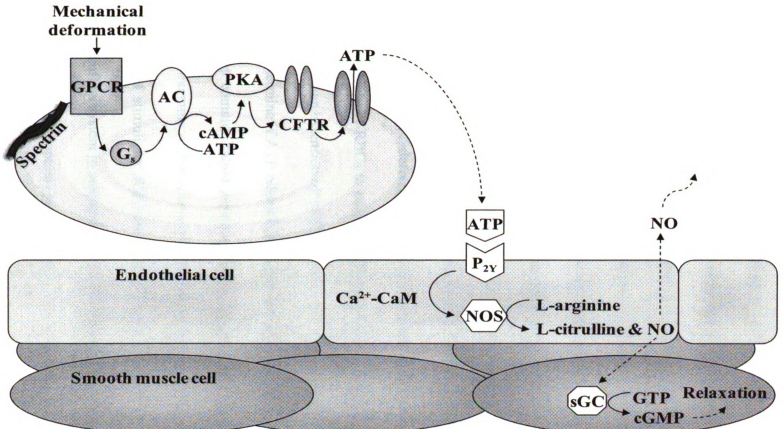

Figure 3.1 Illustration of the deformation-induced ATP release pathway. Activation of the G-protein (G_s) coupled receptor (GPCR) by mechanical deformation leads to the conversion of ATP to cyclic adenosine monophosphate (cAMP) by adenylyl cyclase (AC), which results in phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) by protein kinase A (PKA), upon which, stimulates ATP release from the cell. ATP further binds to P_{2y} receptor on the endothelium results in NO production eventually leads to vasodilation

3.2 ATP-MEDIATED NO PRODUCTION IN PLATELETS

Not only is RBC-derived ATP a stimulus of NO production in endothelial cell, resulting in relaxation of smooth muscle and subsequent vascular dilation, but also in platelets.[33-35] In question concerning the production and release of platelet-derived NO is the mechanism by which the NO production is stimulated. For example, ATP is a recognized stimulus of NOS and subsequent platelet NO production as previously discussed in Chapter 2; moreover, it is also known that ATP is released from platelets upon activation.[36] However, it has not been established if ATP released from activated platelets has the ability to stimulate the production of NO in platelets. Also, previous reports concerning RBC-derived ATP and ATP-stimulated NO production suggests that, in vivo, the ability of RBCs to release ATP may act as a stimulus for NO production in platelets. This is an extension of the RBCs role to stimulate endothelium NO.

To determine if ATP released from platelets affect platelet NO production and, to establish that communication between RBCs and platelets exists, experiments were performed to examine ATP-mediated platelet NO production and measure platelet NO in the presence of stimuli for RBC-derived ATP. These measurements were performed using a microflow system in conjunction with fluorescence detection.

Three stimuli (iloprost, pentoxifylline and metal-activated C-peptide) known to increase the release of RBC-derived ATP[37-39] were used in experiments. Iloprost has

already been shown to increase ATP release from RBCs due to the stimulation of IP receptor (prostacyclin receptor) and the increased RBC deformability.[40, 41]

Pentoxifylline (Trental), which has been reported to have beneficial effects in the diabetic kidney,[39, 42, 43] has also been recently investigated as a stimulus of ATP release by our group. Previous reports have shown that a pentoxifylline derivative may act as an anti-inflammatory agent by suppressing oxygen radical production or behave as a scavenger of reactive oxygen species. More recently, reports have suggested that adding pentoxyfilline to a diabetic patient's daily medicinal intake improved some of the complications associated with the disease, perhaps by reducing the amount of lipid peroxidation in the cell.[43] However, several reports have also demonstrated that pentoxyfilline acts specifically on improving RBC deformability, ultimately reducing fibrinogen concentration, platelet adhesion, and improving blood viscosity.

C-peptide, a byproduct of insulin synthesis, was initially believed to be a biologically inactive peptide.[44, 45] It is a 31 amino acid peptide that is released in equimolar amounts with insulin from the pancreatic beta cells. It is known that C-peptide facilitates the folding of insulin prior to cleavage from the proinsulin C-peptide hormone. However, our group has recently shown that metal (Fe^{2+}, Cr^{3+} and Zn^{2+}) –activated C-peptide can promote ATP release from RBCs by increasing glycolysis.[39, 46]

In this chapter, data are presented suggesting that pentoxyfilline and iloprost, two drugs known to improve blood flow in vivo, have the ability to indirectly increase NO production in platelets due to the ability of increasing RBC-derived ATP that are subjected to deformation while flowing through microbore tubing. Moreover, patients with type 1 and type 2 diabetes are known to have hyperactive platelets,[47, 48] which is defined as platelets that are more adhesive or are easier to active; and interestingly reports by separate groups have demonstrated that RBCs obtained from type 2 diabetic patients release less ATP than those obtained from healthy controls.[15, 39, 49] These results led us to speculate that increased platelet activity reported in diabetes is due to decreased stimulation of NO production secondary to reduced RBC-derived ATP. Here, data provide evidence suggesting that platelet NO is significantly increased when platelets were exposed to RBC supernatant that has been incubated with physiological concentrations of metal-activated C-peptide, not only in healthy control rats but also in type 1 and type 2 diabetic rats.

3.3 EXPERIMENTAL METHODS

Isolation and purification of RBCs. Whole blood was obtained from exsanguinated rabbits, and platelets were isolated and purified as previously described in Chapter 2. After the first centrifugation of whole blood and removing the platelet rich

plasma (PRP) and buffy coat, packed RBCs were resuspended and centrifuged three times in physiological salt solution (PSS; 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 140.5 mM NaCl, 21.0 mM tris(hydroxymethyl)aminomethane, and 11.1 mM dextrose with 5% bovine serum albumin in 18.2 MW-cm deionozed, distilled water (DDW), pH adjusted to 7.4) at 500 x g at 37°C for 10 min. Typically, 65 – 75% hematocrit RBC was obtained as stock solution.

In addition, for experiments involving in rats, whole blood was obtained from 4-month type 1 diabetic BB/Wor-rats, and age-matched control rats. Blood was obtained by cardiac puncture after anesthesia with isoflurane in a closed gas chamber. Typically, 6-8 ml of blood were collected into heparinized tubes from each rat. Platelets and RBCs were further washed in the same manner as mentioned in Chapter 2. All surgical procedures involving animals used in this study were performed under protocols approved by the Animal Investigation Committee.

Reagent preparation. All reagents were purchased from Sigma Chemical (St. Louis, MO) without further purification unless otherwise noted. The preparation of reagents (ATP, ADP, L-NAME, and DAF-FM DA) was described in Chapter 2. Thrombin (a platelet activator) was prepared at 1.2 units/mL in HBSS as stock solution. Platelets prepared for use with NF449 (an inhibitor of the P_{2x} receptor on platelets) (4′,4″,4‴- carbonylbis [imino-5,1,3-benzenetriyl bis(carbonyl-imino)] tetrakis(benzene-

1,3-disulfonic acid) octasodium salt) studies, were prepared by washing three times in an HBSS/ACD solution. The first two washes contained 1unit/mL apyrase (A6410, Apyrase Grade VI, Sigma, this grade has an exceptionally high ATPase/ADPase ratio to convert ATP in the solution to ADP/AMP). The third wash contained HBSS/ACD solution in the absent of apyrase.

For inhibitor studies of ATP release, glybenclamide (Sigma) was prepared by adding 49 mg to 2 mL of 0.1 M NaOH and 7.94 mL of a dextrose solution (1 g dextrose in 20 mL of DDW). The solution was carefully heated to 52°C until dissolved, resulting in a final stock concentration of 0.01 M. This stock was then diluted in the PSS to create a buffered solution of glybenclamide, to which washed RBCs were added (final concentration in 1% hematocrit RBCs was 100 μM).

For studies involving diamide, 0.0018 g of diamide was dissolved in 50 mL of DDW, creating a 200 μM stock solution. The stock solution was then diluted three times (1:10) in PSS, resulting a 0.2 μM diamide working solution (final concentration in 1% hematocrit RBCs was 40 nM). 500 μL of 54 μM solution of pentoxyfilline (0.0015 g dissolved in 100 mL of DDW) was prepared in PSS prior to the addition of 1.0 mL of 5% hematocrit RBCs (final concentration in 1% hematocrit RBCs was 5.4 μM). For studies involving iloprost, a procedure identical to that involving pentoxyfilline was followed. 0.5 g of iloprost was dissolved in 100 mL of DDW, creating a 13.8 mM stock solution.

50 μL of 100 μM solution of iloprost were prepared in PSS prior to adding 1.0 mL of 5% hematocrit RBCs (final concentration in 1% hematocrit RBCs was 1.0 μM).

Human C-peptide (American Peptide, Sunnyvale, CA, USA), 0.25 mg (MW 3020 g/mol) was dissolved in 10 ml of DDW to yield a concentration of 8.3 μM. The solution of Cr^{3+}/Zn^{2+} (derived from chromic chloride hexahydrate and zinc chloride) was prepared in DDW. The metal solution was then added in equimolar amounts to the C-peptide solution through a series of dilutions. Before adding to the RBCs, the metal–C-peptide mixture was diluted in PSS to avoid cell lysis that may occur upon direct contact of RBC with a non-buffered aqueous solution. All RBC solution in experiments had a final hematocrit of 7%.

Measurement of ATP from activated platelets. The experiment was performed using microbore tubing (Polymicro Technologies, Phoenix, AZ, USA) as a microcirculation mimic.[15, 50] A syringe pump (Harvard Apparatus, Holliston, MA, USA) was used to propel two solutions; one solution contained luciferase with 2 mg of luciferin added per 5 mL of solution to enhance the chemiluminescence sensitivity. The other syringe contained mixture of 300 μL of various concentrations of ADP and 200 μL aliquot of the platelets. After allowing 5 minutes for the ADP/platelet mixture to incubate, the two solutions were pushed through microbore tubing having an inside diameter of 50 μm and combined at a mixing tee at a flow rate of 6.7 μL min^{-1}. The resultant mixed

flow that eluted from this tee then traveled through a third section of tubing. This last section of tubing was placed over a photomultiplier tube (Hamamatsu, Iwata City, Japan) in order to measure the chemiluminescence at 540 nm resulting from the mixing of the luciferase solution with ATP that was released from activated platelets while flowing through the microbore tubing. The amount of ATP released was averaged from the activated platelets obtained from the whole blood of n = 5 exsanguinated rabbits. The system was calibrated each day with authentic ATP standards having concentrations from 0 to 1.5 μM. The correlation coefficient for this calibration is generally > 0.99, thus establishing a linear relationship between the emission and ATP concentration.

Fluorescence determination of platelet NO in a static system. Fluorescence emission spectra were obtained using 1 μM DAF-FM DA in a quartz cuvette with excitation wavelength at 495 nm and emission wavelength at 515 nm. This system for measuring platelet NO with DAF-FM DA is described in detail in Chapter 2. For studies involving the inhibition of the P_{2x} receptor, a 100 μM stock solution of NF449 was prepared by adding 1 mg of the NF449 to 6.6 mL of HBSS. NF449 is a selective P_{2x} receptor antagonist which inhibits ATP binding to the receptor. When using the inhibitor, 30 μL of the 100 μM stock solution was added to 3 mL of an apyrase-washed stock platelet suspension that contained 3×10^9 platelets/mL (DAF-FM DA loaded), the final concentration of NF449 used in all studies was 0.1μM. This solution was allowed to

incubate for 30 minutes prior to addition of a NOS stimulus (ATP) or a platelet activator (ADP or thrombin). After addition of these final reagents, the solution was allowed to incubate for an additional 30 minutes prior to obtaining the emission at 515 nm. For studies involving metal-activated C-peptide stimulated RBC-derived ATP, RBCs suspension was incubated with C-peptide in the manner as previously described for 5 hours prior to obtaining the supernatant. The supernatant from 7% hematocrit RBC suspension was obtained by centrifugation at 500 x g at 37°C for 10 minutes, then added and equilibrated with platelets for an additional 30 minutes prior to measuring the fluorescence.

Fluorescence determination of platelet NO in a flow system. Platelets were passed through microbore tubing in a manner similar to that described previously for measuring ATP from activated platelets. The system consists of 3 major components, namely a dual syringe pump, the sections of microbore tubing, and a flow-through fluorescence detector (Jasco) housed with a capillary flow cell. DAF-FM DA-loaded platelets and RBCs were mixed prior to being pumped through a section of microbore tubing using a syringe pump to propel the solution at a flow rate of 6.7 μL min^{-1}. The pump was a conventional syringe pump where the syringe was easily accessible. For studies involving stimulation with RBC-derived ATP in a flow system, 350 μL of 1% hematcroit RBC suspension was added and equilibrated with 150 μL of 3 x 10^8 platelets

mL^{-1} solution for 5 minutes prior to being pumped through for fluorescence detection. Previous experience with this type of syringe has proven that platelet sedimentation during the measurement portion of the analysis is not problematic because the platelets are usually in solution as a suspension. The fluorescent signal that is produced in the platelets due to NO production was determined using the aforementioned flow-through fluorescence detector and monitored with a program written in house with LabView (National Instruments).

3.4 RESULTS AND DISCUSSION

Self-stimulated NO production in platelets. Previous work by other groups provide evidence that platelets have the ability to release ATP upon activation with ADP and other agonists that result in activation of the platelets.[36] In addition, the data previously described in Chapter 2 shows that these platelets can also produce their own NO (intracellularly through NOS) via stimulation with ATP.[51] Such a scheme suggests that platelets can self-stimulate their own NO; that is, upon activation, the platelets release ATP which may, in turn, stimulate NOS and subsequent NO production. To date, there has been one example of self-stimulated production of NO in platelets.[52] This measurement was performed using a carbon electrode and showed a transient of NO

production upon activation with ADP. Moreover, that same measurement was not clear on whether the activation of the NOS was due to the ADP or the released ATP.

Substances such as collagen, thrombin, fibrinogen, and ADP all activate platelets and induce an ATP release. It was hypothesized that ATP release, and not the activators themselves, stimulate the NO production in platelets. To provide evidence for this theory, experiments were performed in which platelets were stimulated with various of ADP concentration to induce ATP release as shown in Figure 3.2 (black bars). Measurements of this ATP release, via the chemiluminescence assay for ATP described above, upon activation were verified. However, after 3 minutes of incubation with ADP, the platelet mixture was combined with 10 μL of a form of apyrase (50 units/mL) that breaks down ATP. After allowing the apyrase to react with this mixture for 2 additional minutes (which now contains ATP that was released from the activated platelets), the chemiluminescence from any remaining ATP was measured as shown in Figure 3.2 (gray bars). The ATP that was released from the activated platelets decreased in the presence of apyrase. Moreover, agents were shown to increase the production of NO by platelets as shown in Figure 3.3. However, when the ATP receptor on platelets (the P_{2x} receptor) was inhibited, the NO production decreased for every activator studied (gray bars in Figure 3.3). Collectively, the importance of this finding is that only ATP is able to stimulate NO production in platelets; the platelet activators (which results in a shape change of the

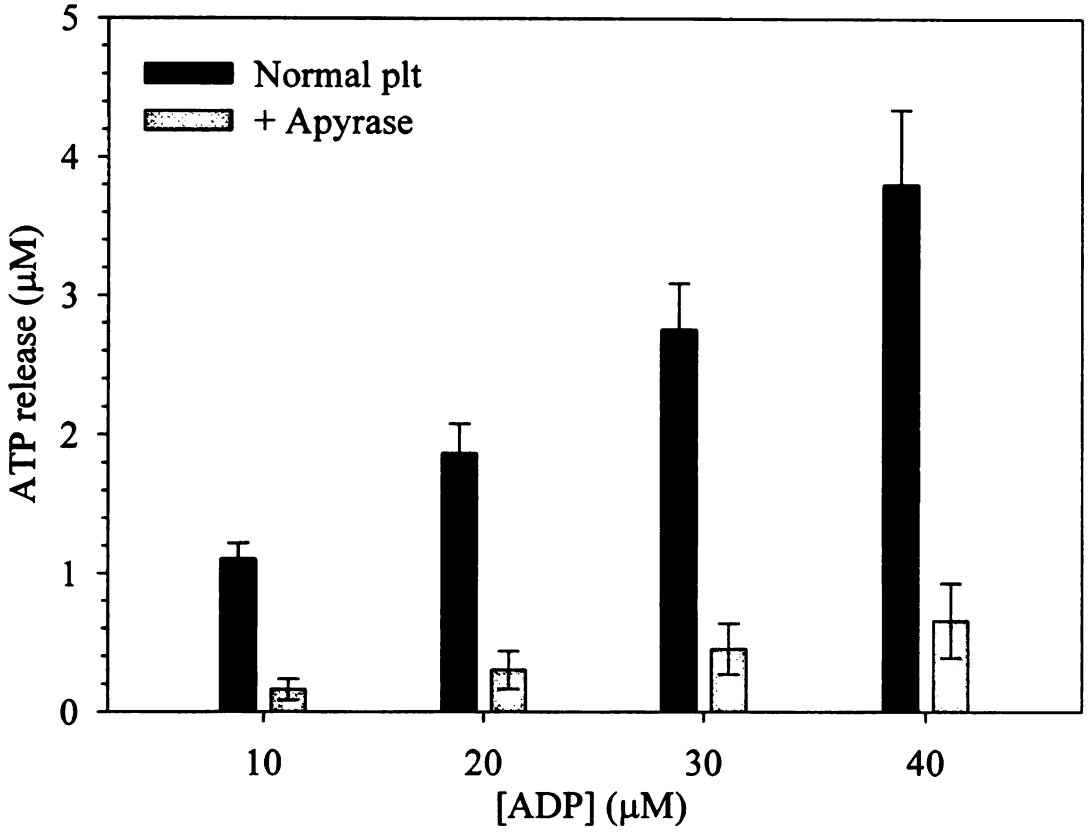

Figure 3.2 Chemiluminescence assays were performed as a function of ADP concentration in each sample to quantitatively detect ATP release from activated platelets. ATP that is released from the activated platelets decreased in the presence of apyrase (1.0 units/mL as final concentration). Error bars represent SEM. $p < 0.05$ ($n = 5$)

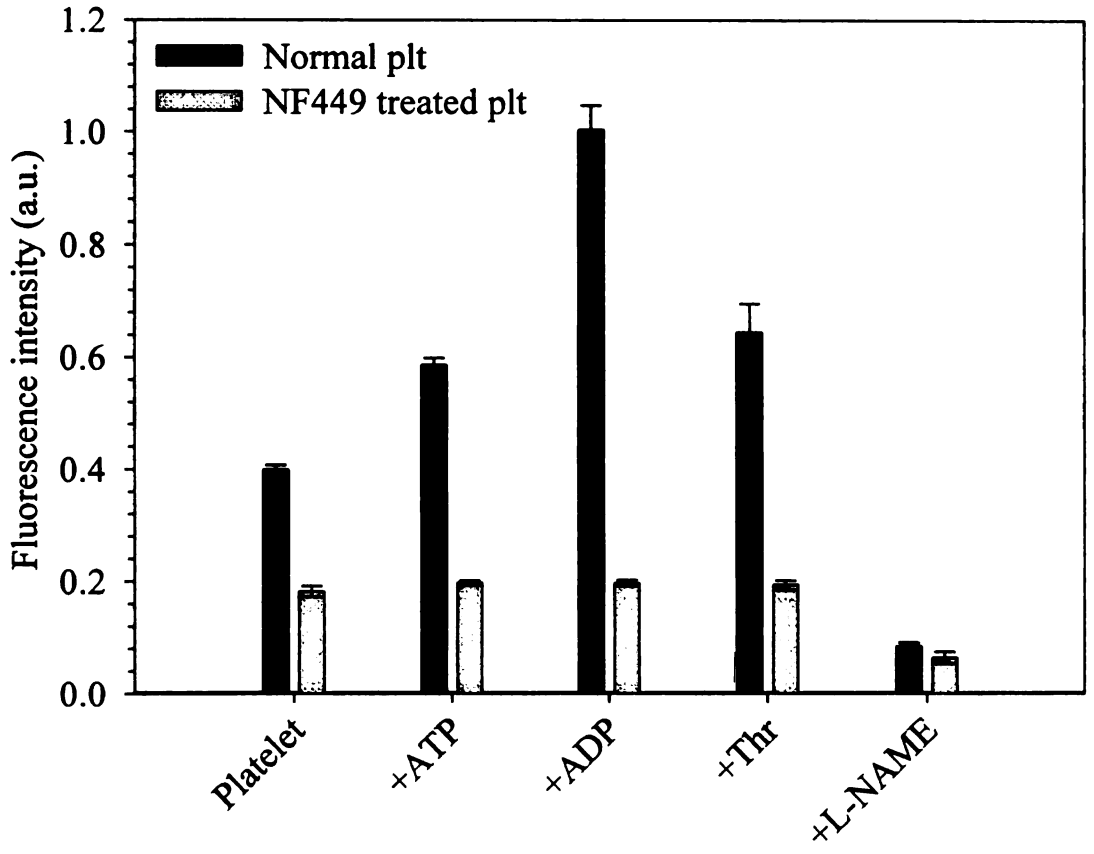

Figure 3.3 Evidence that NO production in platelets is due to ATP stimulation of the P_{2x} receptor. Platelets NO was measured in the absence and presence of a stimulus of NO (ATP) or activators of ATP release (ADP and thrombin) which lead to NO production. As shown, NO production increases in each case. However, identical measurements that were performed in the presence of NF449, a reagent that blocks the ATP receptor on platelets resulted in a decrease in NO production. This data suggested that NO production in platelets is largely dependent upon ATP binding to the platelet receptor. L-NAME, a NOS inhibitor decreased NO production with and without the P_{2x} blocker, verifying the measured signal in each case was due to NO. Error bars represent SEM. $p < 0.05$ (except L-NAME measurements) ($n = 4$)

platelets) do result in NO production, but perhaps through an ATP-mediated mechanism. This is potentially very important because it is known that some patient groups known to have low NO production or complications involving blood flow are also known to have RBCs that release lower than normal amounts of ATP upon pharmacological activation or physical deformation of the erythrocyte. Patients with primary pulmonary hypertension,[53] cystic fibrosis,[54] and diabetes[15, 49] are example patient groups with these lower ATP release values.

Measurement of platelet NO in a microflow system. In chapter 2, we demonstrated that platelet NO, in either extracellular or intracellular form, could be quantitatively determined using variations of the DAF family of probes.[51] However, those measurements were performed in a static system that did not involve flow. In vivo, platelets are part of the flowing blood stream, typically occupying the space closest to the vessel wall due to the non-Newtonian characteristics of whole blood (i.e., the platelets are displaced from the center of the flow by the larger RBCs). Therefore, to more closely mimic the physical conditions that the platelet would be subjected to in vivo, and to prepare for subsequent studies involving the RBC, it was imperative that the NO production by platelets be measured in a flowing stream.

The data shown in Figure 3.4 demonstrated the ability to measure both basal levels of NO in platelets and the increase in NO production when these platelets are

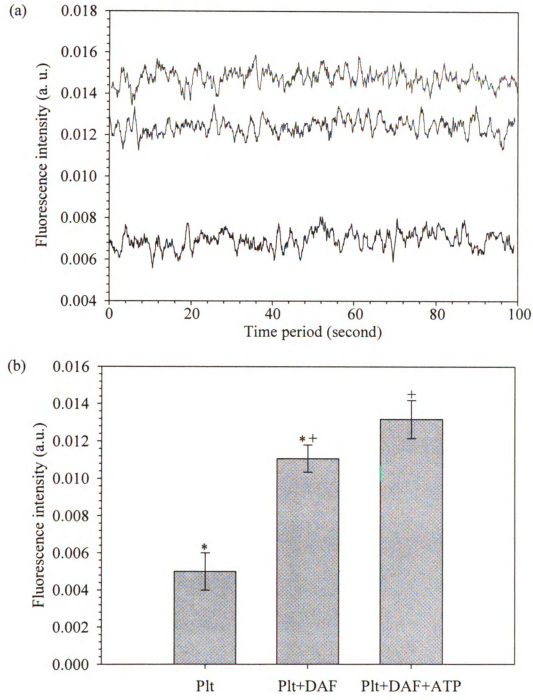

Figure 3.4 Increased NO production from platelets in the presence of 0.1 μM ATP. (a) The traces represent the measured fluorescence intensities from platelets only (lower trace), platelets in the presence of DAF-FM DA (middle trace), and platelets with DAF-FM DA in the presence of ATP (upper trace). (b) Bars represent the average of normalized results. Error bars represent SEM. $p < 0.05$ ($n = 3$)

stimulated with 0.1 μM ATP. The bottom trace in Figure 3.4a is essentially the background due to platelets flowing through the microflow system described above. The middle trace is the emission resulting from platelets incubated in 1 μM DAF-FM DA for 30 minutes prior to being pumped through the system. The upper trace is the fluorescence intensity of another aliquot of the DAF-FM DA-loaded platelets moving through the system; however, these platelets were stimulated with 0.1 μM ATP. As evident by the increase in the traces, the system enables the detection of basal and stimulated levels of NO production in platelets in a flowing stream.

RBC-stimulated NO production in platelets. Collectively, in Figures 3.2 and 3.3, the data suggested that the increase in NO production via platelet activation is due to an ATP stimulus alone. Moreover, the data shown in Figure 3.4 demonstrated that platelet NO can be measured via fluorescence spectrophotometry in tubing having a diameter that approximates an arteriole in vivo. These results suggested a possible unique relationship between RBCs and platelets in the circulation thus render the RBC a possible determinant of platelet physiology in vivo. Specifically, the RBC may be able to communicate with the platelet through RBC-derived ATP due to stimulus,[39] mechanical deformation[15, 49, 50] or pharmacologically,[19] resulting in NO production in platelets. Here, we determined that ATP derived from RBCs that had been incubated with zinc-activated C-peptide was able to increase the NO production by platelets by 67.5% ± 16.4% (Figure 3.5) compared

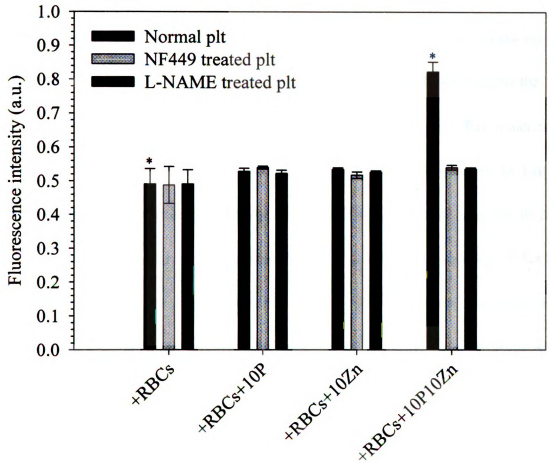

Figure 3.5 NO production of platelets incubated with supernatant from rabbit RBCs incubated with zinc-activated C-peptide (+RBCs+10P10Zn) and in the absence of zinc (+RBCs+10P) or absence of C-peptide (+RBCs+10Zn) at 5 hours (black bars). Platelets NO remain unchanged when platelets were pretreated with NF449 (light gray bars) and L-NAME (dark gray bars). Error bars represent SEM. $p < 0.05$ ($n = 3$)

to platelets alone. Importantly, when platelets were incubated without zinc or in the presence of only zinc (no C-peptide) the increase in NO production was insignificant. To demonstrate that the increase in NO production was due to the ATP from the RBCs, the platelets were incubated with NF449, an inhibitor of the P_{2X} ATP receptor on the surface of the platelet. As shown in Figure 3.5 (light gray bars), the NF449 reduced the NO production from platelets that had been incubated with supernatant from RBCs incubated with zinc-activated C-peptide to levels that were statistically equivalent to that of platelets alone. After establishing that the increase in NO production was due to ATP binding to the P_{2X} receptor on the platelet, the platelets were incubated with L-NAME prior to the addition of supernatant from RBCs that had been incubated with zinc-activated C-peptide. As expected, the fluorescence intensity was reduced to levels equivalent to platelets alone (dark gray bars in Figure 3.5) indicating that it was NO production that was being measured by the DAF-FM DA probe.

More specifically, data verifying the relationship between RBCs and platelets in the circulation was summarized in Figure 3.6. Here, the ability to measure platelet-derived NO upon stimulation with ATP secreted from deformed RBCs in a microflow system is demonstrated. The percent increase or decrease in platelet-derived NO (measured spectrofluorometrically with the DAF-FM DA probe) is presented for RBCs in the presence of either pentoxyfilline or iloprost. Pentoxyfilline is thought to

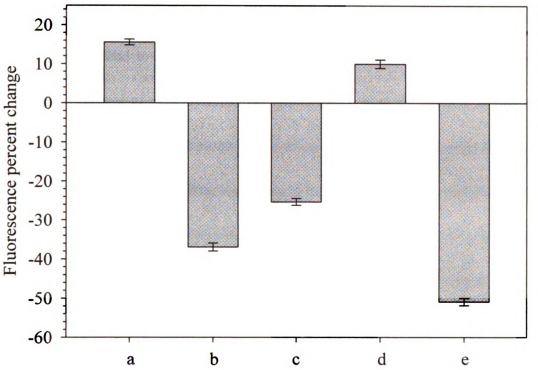

Figure 3.6 The percent change in fluorescence due to platelet NO production stimulated by RBCs in the presence and absence of stimulators and inhibitors of ATP release. The percent changes were reported relative to RBCs flowing with the DAF-FM DA-loaded platelets alone. In (a), RBCs incubated with pentoxyfilline prior to flowing with platelets in microbore tubing resulted in a 15.5% ± 0.8 increase in emission intensity; in (b), the RBCs were incubated with pentoxyfilline and diamide, resulting in a 36.9% ± 1.1% decrease in platelet NO; RBCs were treated with glybenclamide and pentoxyfilline in (c) and the platelet NO decreased by 25.3% ± 0.9%; in (d), RBCs were incubated with iloprost, resulting in an increase in platelet NO production of 10.0% ± 1.1%; the iloprost-induced increase in NO production was reduced in (e) where RBCs treated with glybenclamide and iloprost resulted in a decrease in platelet NO of 50.9% ± 0.9%. Error bars represent SEM. $p < 0.05$ ($n = 3$)

improve blood flow via its ability to make the RBC more deformable through a radical scavenging mechanism.[55, 56] Therefore, due to flow-induced shear, RBCs become more deformed in the presence of pentoxyfilline, and an increase in deformability would then be anticipated to lead to an increase in deformation-induced release of ATP from the RBC. In the presence of pentoxyfilline, the fluorescence from the platelet NO increased by 15.5% ± 0.8% due to the increased RBC-derived ATP release. However, this increase in platelet NO due to pentoxyfilline was reduced by 36.9% ± 1.0% and 25.3% ± 0.9% in the presence of diamide or glybenclamide, respectively. Diamide is a recognized oxidant that results in a stiffened membrane and reduced RBC deformability.[57] Glybenclamide is an anion transport inhibitor that will inhibit ATP release from RBCs. There have been reports of other pharmaceutical agents having the ability to stimulate ATP release from RBCs. Specifically, iloprost, a stable analogue of prostacyclin, has been reported to increase the release of RBC-derived ATP independent of flow.[13] In the presence of iloprost, a 10.0% ± 1.1% increase in platelet NO production, and a value that was reduced by 50.9% ± 0.9% in the presence of glybenclamide. Moreover, neither pentoxyfilline nor iloprost stimulated NO production in platelets in the absence of the RBC.

Increased RBC-stimulated platelet NO with diabetic rats. People with diabetes often suffer cardiovascular complications as a result of poor circulation of the blood.

Interestingly, RBCs from people with diabetes release less ATP than healthy indivuals.[15, 49] Because ATP is a primary stimulus for NO production in platelets, then recent reports involving ATP release from RBCs of patients with diabetes becomes important to platelet physiology. The significant decrease in concentrations of RBC-derived ATP from diabetic RBCs may contribute to decreased levels of NO production by platelets. Previously, it was shown that C-peptide had no direct effect on platelet behavior,[58] and studies performed in our laboratory have agreed with this prior report (even when the C-peptide was activated with zinc) (Figure 3.7). However, although there was no direct effect on the platelets due to treatment with C-peptide, there was an indirect effect due to C-peptide's ability to stimulate ATP release from RBC, which subsequently affected platelet behavior. As shown in Figure 3.8, the NO production from platelets obtained from type 2 rat models increased 26.1% ± 8.4%, while the NO production from control platelets was 43.8% suggesting a slight resistance to the effects of metal-activated C-peptide. Type 1 rat models, however, demonstrated an NO production increase of 27.8% ± 11.1% compared to platelets alone with the control increased similarly (31.1% ± 3.2%) as shown in Figure 3.9. This data suggested that ATP derived from zinc-activated C-peptide treated RBCs may play an important role in platelet hyperactivity and aggregation.

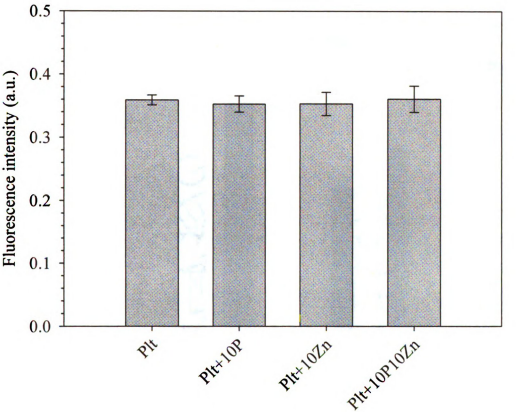

Figure 3.7 Control experiments of platelet NO production. Platelets were incubated with zinc-activated C-peptide (Plt+10P10Zn) and in the absence of zinc (Plt+10P) or absence of C-peptide (Plt+10Zn) at 5 hours. Each bar here was statistically insignificant, suggesting that C-peptide has no direct effect on platelet NO production. Error bars represent SEM. (n = 3)

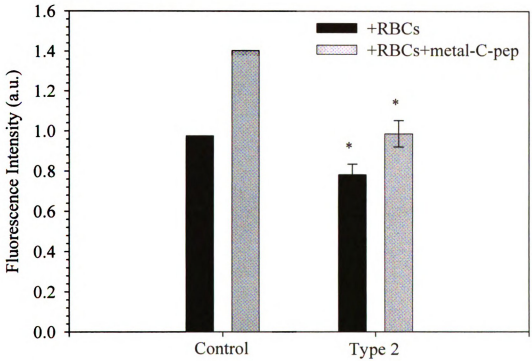

Figure 3.8 The effect of metal-activated C-peptide on the NO production by platelets from type 2 (BB/ZDB) and control rats. Black bars represent the fluorescence intensity from platelet NO production incubated with supernatant from rat RBCs and gray bars represent the fluorescence intensity from platelet NO production incubated with supernatant from rat RBCs incubated with metal-activated C-peptide at 5 hours. Platelet NO production from type 2 rats increased 26.1% ± 8.4% when RBCs were incubated with metal-activated C-peptide. Error bars represent SEM. $p < 0.05$ ($n = 3$)

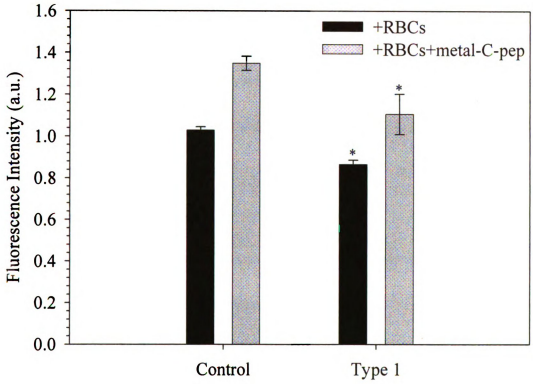

Figure 3.9 The effect of metal-activated C-peptide on the platelet NO production from type 1 and control rats. Black bars showed the platelet NO production incubated with supernatant from rat RBCs and gray bars showed the platelet NO production incubated with supernatant from rat RBCs incubated with metal-activated C-peptide at 5 hours. Error bars represent SEM. $p < 0.05$ ($n = 5$)

3.5 CONCLUSIONS

Once produced by the platelet, NO acts as a platelet inhibitor, reducing the activation and aggregation of platelets. The over-aggregation of platelets results in a thrombus formation, which if broken off, can become lodged in a small arteriole or capillary resulting in an embolism. As previously mentioned, ATP can stimulate platelet NO, here, the data provided evidence that RBC-derived ATP actually plays an important factor of platelet function in the circulation, especially considering the results from Figure 3.3 that suggest platelet NO is stimulated through ATP. Results shown here demonstrated that the ATP released by RBCs is able to stimulate NO production in platelets. The decreased NO production in platelets decreases substantially when RBCs are in the presence of a cell stiffening agent (diamide) or an inhibitor of ATP release (glybenclamide) or in the case of RBCs obtain from diabetic rat models. It should also be noted that neither of these agents have an effect on platelet NO production in the absence of RBCs. While in vivo studies will be necessary to truly verify any conclusions drawn here from in vitro studies, however, the data presented suggested that RBCs are able to stimulate NO production in platelets via their ability to release ATP and more in vivo circulation mimic experiments will be performed in next chapter to have a closer investigation of communications between these different cell types.

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CHAPTER 4 A CIRCULATORY MIMIC IN A MICROFLUIDIC DEVICE INCLUDING MUTLIPLE CELL TYPES

4.1 INTRODUCTION TO MICROFLUIDICS

The first microfluidic construct, a miniaturized gas chromatograph, was developed by Terry *et al* in 1979.[1] A few years later, small scale zone electrophoresis was performed in glass capillaries[2] followed by flow injection in microconduits.[3] Other early miniaturized devices included a coulometric acid-base titration system[4] and silicon-based micropump system.[5] Early on, there were concerns with these devices because peripheral pieces of equipment such as pumps, detectors, and chart recorders were still required to complete the analysis. It was not until the early 1990's that lithographic technology was combined with capillary electrophoresis (CE) to create a single device capable of sample injection, pretreatment, separation, and detection.[6]

As the popularity of genomics grew in the early 1990's, so did interest in developing technology for the analysis of complex molecules such as DNA and proteins. Microfluidic-based devices offered improvements in analytical performance with the ease of utilizing already developed technology. The leaders in microfluidics systems that analyzed multifaceted aqueous solutions were Manz,[7-11] Harrison,[12-17] Ramsey,[18-23] and Mathies.[24-27] Although early work in microfluidics ranged from biological sample purification[16] and amplification[10, 22] to high-throughput screening[28] and genomic

assays,[24, 27] other areas benefited from the potential of microfluidic applications. Point of care diagnostics based on immunological assays[29, 30] and cell counting[31] for clinical analysis was expanded, while devices for *in vivo* drug delivery and monitoring for disease conditions were developed.[32] The detection of single molecules on a microfluidic construct also became popular.[33-35] Although at the beginning the analytical performance of these devices was more desirable than their reduced size, miniaturization was the route to shorter analysis time and versatility. However, the central technology for a number of miniaturized systems was the microfluidic technology, where samples were manipulated in channels with dimensions on the order of 10 – 100 μm.

4.1.1 MATERIALS FOR MICROFLUIDIC DEVICES

Microsystems can be built on various substrates with a range of materials, from silicon,[5, 36] glass,[2] quartz,[37, 38] metals,[39] and organic polymers.[40] Each of these materials possesses certain advantages and disadvantages depending on the type of application.

Silicon processing was carried out by such conventional, planar fabrication techniques as photolithography and etching adapted from the microelectronics industry. Advantageous to silicon is its surface characteristics; it possesses a negative charge thereby supporting electroosmotic flow (EOF) for electrophoresis application and, because fabrication occurs by etching, the surface is cleaned as the channel is produced.

Moreover, silicon processing techniques are well developed, thereby enabling two and three dimensional shapes that can be reproduced with high precision.[41, 42] However, silicon is not suitable for optical detection because it is opaque in the visible/UV region of the spectrum and it conducts current, thereby limiting its usefulness for processes involving electric field compared to glass. Another shortcoming to silicon is the sealing process, which requires not only a clean room environment, but also high voltages or temperatures.

Since the advent of microfluidics, a significant amount of research has focused on introducing new types of materials into the field. Polymers[43] make a suitable alternative to silicon and glass because they are inexpensive, channels are easily formed by molding or embossing as opposed to extensive etching processes, and the devices have the ability to be sealed.[43-46] The surface chemistry of the polymer substrate is more easily modified than that of silicon and glass, thereby making polymers more applicable to the biomedical field.[47]

Two of the most actively developed polymers for microfluidics and other applications are poly(dimethylsiloxane) (PDMS) and poly(methylmethacrylate) (PMMA).[48-50] The polymer mainly discussed in this thesis is PDMS due to its transparency for optical observation and compatibility to biological samples. PDMS became a popular polymer substrate for soft lithography in the late 1990's when it was

applied in the fabrication of systems for biological and aqueous-based applications.[47] PDMS consists of a repeating (–Si–O–) backbone and each Si atom has two methyl ($-CH_3$) groups covalently attached to it.

There are several properties of PDMS that make it suitable for chemical and biological miniaturized systems. First, the elastomeric nature of PDMS allows it to conform to nonplanar surfaces and release easily from delicate or fragile structures without damaging the mold or itself. Second, PDMS is optically transparent down to about 300 nm[51] so it is amenable to optical detection schemes. Third, PDMS is chemically inert, non-toxic, and gas permeable providing a means to culture mammalian cells directly to the surface. Fourth, PDMS is very hydrophobic as the result of the methyl substituent groups attached to each silicon atom creating a polymer that is water impermeable. However, the surface properties of PDMS can be modified with plasma treatment. Finally, PDMS has the ability to seal reversibly to itself and other materials via van der Waals interactions or it can be irreversibly sealed after exposure to plasma.[52-54]

In contrast to the advantages of working with PDMS, its elastomeric properties are problematic when creating patterned stamps. Elastomers are subject to deformations such as pairing, sagging, and shrinking. Pairing occurs when either gravity or capillary forces stress the elastomeric features and result in collapse. Sagging is the result of the aspect ratio (length/height) of the relief structures becoming too small, compression will

occur between the stamp and the substrate to which it is adhered. Finally, the elastomer is subject to distortions that occur during the fabrication process as the result of the flexibility of the polymer.[55, 56] There are several other disadvantages that affect the performance of PDMS for certain applications. It is known that PDMS is incompatible with solvents such as toluene and hexane[57, 58] resulting in swelling of the elastomer. It has also been reported that PDMS shrinks approximately 1% upon curing.[56] The hydrophobicity of the polymer also creates some disadvantages, especially when dealing with biological applications. There exist several proteins and cell types that experience non-specific binding to PDMS that prevent the sample from flowing from one part of the device to another. Another disadvantage is that EOF is difficult to generate in PDMS due a lack of silanol groups (Si–OH) that are deprotonated at neutral and basic pH.

To date, a variety of polymeric materials have been reported for the fabrication of microdevices. Besides PDMS, PMMA and polycarbonate (PC) are the most popular polymer materials for microfabrication, either by embossing or injection molding, however other standard polymers include polyethylene (PE), polypropylene (PP), polystyrene (PS), and poly(etheretherketone) (PEEK).

4.1.2 PHOTOLITHOGRAPHY

Essential to the expansion of microelectronics, microfabrication - the generation of small structures, has become the standard for the creation of microprocessors and other microelectrical devices for the information and technology industry since its development in the late 1950's. The majority of the semiconductor circuitry was and continues to be made by this technology, although the escalating need for smaller features has strained the industry as smaller sizes of circuitry are increasingly difficult and expensive to produce. Microfabrication also gained popularity in areas outside of microelectronics, providing a means to miniaturize and construct devices that are more portable, produced at a lower cost, require less reagents and sample volumes, and have improved detection limits. The most popular method in microfabrication is photolithography,[59, 60] specifically, a transfer technique based on a projection-printing system in which an image is projected onto a thin film of photosensitive material (also known as photoresist), spin-coated on a wafer, and selectively expose to a radiation source. This exposed region will contain the pattern of the transferred image and unexposed material can be washed away with a developer solution. The portion of the negative photoresist unexposed to the radiation is soluble thus create positive relief on the wafer while the positive photoresist generates negative relief structures as illustrated in Figure 4.1.

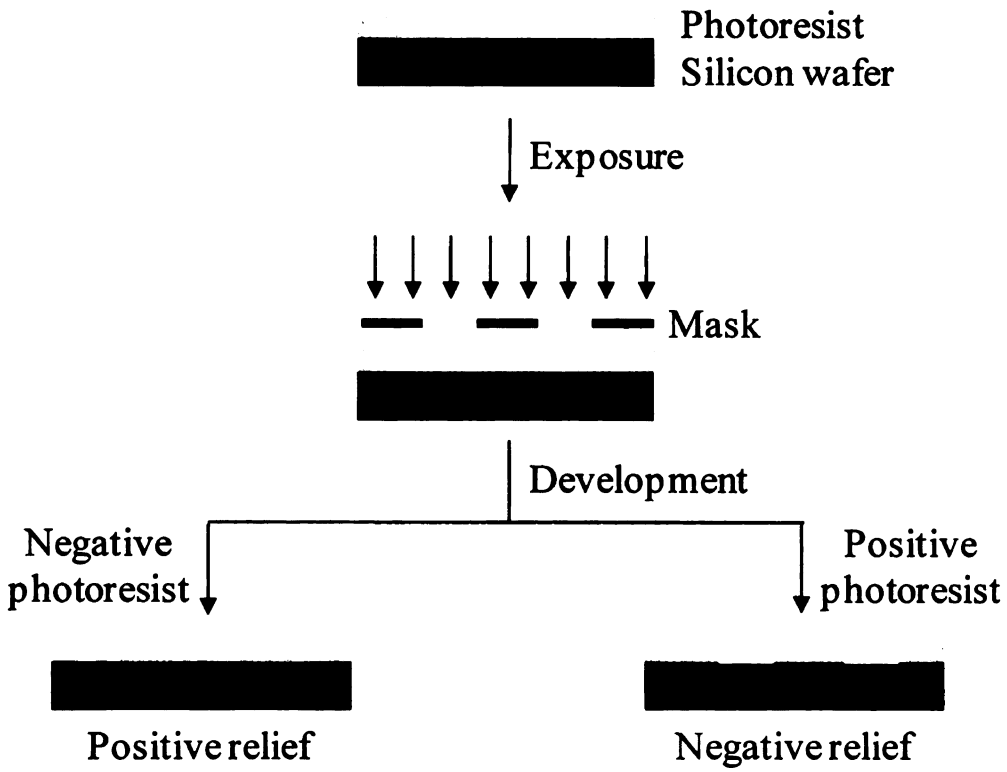

Figure 4.1 Two type of photoresist used result in different relief structure on wafer. The portion of the negative photosist exposed to the radiation is insoluble thereby create raised features corresponding to the mask, while positive photoresist generate negative relef structures after development

Though useful, photolithography has some disadvantages that limit its use in the preparation of devices. These constraints include the lack of technology to create features below 100 nm, expense, the complexity of patterning on non-planar surfaces, limited control over the chemistry of the patterned surface, and the lack of variation in materials used as photoresists. These limitations demonstrated a need for the development of non-photolithographic techniques to fabricate micro- and nano-structures.

4.1.3 SOFT LITHOGRAPHY

Soft lithography, named for its use of flexible organic materials as opposed to rigid, inorganic materials common with lithography fabrication, gained momentum in science and industry with increasing needs to generate structures smaller than 100 nm. Soft lithography is considered to be a non-photolithographic technique, and other non-photolithographic methods to create nanoscale structures include injection molding, embossing, and laser ablation. The strength of soft lithography is in replicating the master as opposed to fabricating a new device for each experiment performed. A few examples of soft lithography include microcontact printing (μCP), microtransfer molding (μTM), and replica molding (REM). These techniques have been reported to generate structures from 1 μM in μTM[61] to 30 nm in REM[46] laterally. Soft lithography requires a much smaller investment in materials as opposed to other lithographic methods and with the aid

of rapid prototyping and REM, it can take less than 24 hours to complete from design to stamp.[56] Moreover, preparation via soft lithography requires only an ambient laboratory setting as opposed to a required clean room environment for photolithography. Most importantly, soft lithography is not subject to limitations of optical diffraction and optical transparency as other types of lithography often are. This method also enables pattern transfer to curved materials.[48]

4.1.4 RAPID PROTOTYPING AND REPLICA MOLDING

Developed by the Whitesides group, rapid prototyping is a fabrication technique in which a design is patterned onto a silicon wafer. These designs often have features greater than 20 μm and their production is cost effective. The first step is to generate patterns in a design related software such as Freehand or AutoCAD and print them to polymer sheets. Photomasks contain the design pattern and are inexpensive to produce, approximately $1 per square inch as compared to chrome masks that can range from $200 to $500 per square inch. Although these masks are not durable for manufacturing microelectronic devices, they suffice for the rapid production of simple designs for microanalytical devices.

In general, soft lithography starts with a photomask that is placed on top of a silicon wafer coated with a thin film of photoresist; photolithography and developing

methods are used to transfer the design pattern to the wafer, thereby creating a master as illustrated in Figure 4.2 (more detail will be described in the experimental section). Once a master is complete, patterns can be formed in polymer substrates by a process known as replica molding. Specifically, REM duplicates the shape and structure present in a design by casting a polymer against a master. PDMS prepolymer, consisting of a particular ratio of base to curing agent, is mixed, degassed, and poured onto the silicon master. The polymer is cured in an oven in a period of time and the PDMS chip can be peeled away from the master.

4.2 CIRCULATORY MIMIC WITHIN A MICRODEVICE

RBCs are responsible for carrying oxygen from the lungs and delivering it to the body's tissues. Recent work has suggested that there are additional roles for the RBC in addition to supplying oxygen.[62, 63] It is known that while traversing the circulation, RBCs release nanomolar to micromolar amounts of ATP due to deformation and other stimuli such as hypoxia, pharmacological agents, or more recently, metal-activated C-peptide.[63-69] The importance of this *in vivo* release of ATP from the RBC is that ATP is a known stimulus of NOS, which catalyzes the production of NO in various cell types. NO is not only responsible for blood vessel dilation[70-73] but is also an inhibitor of platelet activation and subsequent aggregation.[74-77]

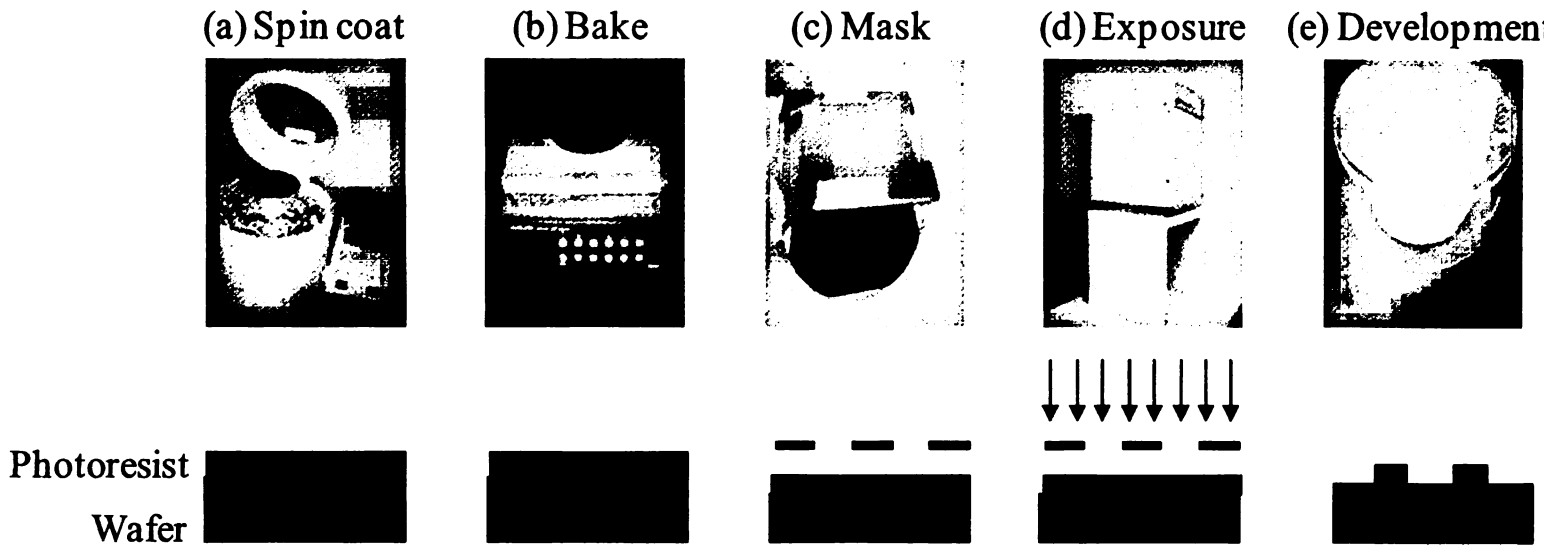

Figure 4.2 The process of photolithography for the master fabrication. (a) Spin-coat, results in approximately 100 μm thick photoresist on the 4" silicon wafer. (b) Bake, to evaporate solvent and compress the film. (c) Transparency mask alignment. (d)UV exposure, cross-link the exposed portion. (e) Development, remove the unpolymerized photoresist and obtain the master with desired raised features

Although platelets flow through the circulation and do not generally adhere to the endothelium, upon vascular injury, subendothelial collagen is exposed and stimulates platelet activation. Upon activation, the platelet shape changes allowing for adhesion to the vascular walls and even subsequent recruitment of additional platelets. However, left uncontrolled, the over-aggregation of platelets results in a thrombus formation, which if broken off, can become lodged in a small arteriole or capillary resulting in an embolism.

According to pieces of information described above and evidence presented in previous chapters, it is hypothesized that RBC-derived ATP plays an important role not only in controlling vascular caliber, but also in platelet function, especially for platelet adhesion to endothelial cells. Interestingly, it is known that hyperactive platelets are observerd in conditions[78-80] where circulatory RBC-derived ATP levels are either low (e.g., diabetes,[81, 82] cystic fibrosis,[83] pulmonary hypertension[83]) or high (e.g., sickle cell disease, multiple sclerosis), thus, people with these abnormal circulatory ATP levels may have higher rates of stroke and associated complications. However, to date, ATP-mediated platelet function has not been completed investigated.

There have been previous reports of microfluidic systems that enable certain features of the circulation to be investigated. Models of the blood brain barrier have been reported and successfully implemented for studies involving transport across an endothelium layer.[84] Other reports of a cell culture analogue, a device containing

different tissue types connected by a series of fluid channels, have also been reported.[85, 86] Many groups, including our own, have demonstrated the ability to culture or immobilize cells in the channels of various microfluidic devices. Previously, the ability to image NO production by endothelial cells immobilized in the channels of a microfluidic device,[87] as well as determine the concentrations of ATP released from RBCs flowing through microfluidic channels,[88] was demonstrated by our group.

In an extension of previous reports involving immobilized endothelial cells in microfluidic channels, the results in this chapter demonstrate the ability of platelets to adhere directly to endothelial cells in a microfluidic device. In this study, rather than immobilizing the cells directly onto the microfluidic channel, the microfluidic device was reversibly sealed to a petri dish. In this construct, the microfluidic device channels are simply used to direct reagent and fluid flow while the endothelial cells are immobilized to the surface of a petri dish. Upon immobilization, these cells then have platelets and RBCs directed over them through the channels of the microfluidic device under various conditions. Importantly, the channels are in parallel creating a high-throughput device capable of optimizing cell immobilization conditions. Therefore, the work presented here demonstrated the ability of multiple cell types (platelets, RBCs, and endothelial cells) to interact directly in the channels of a circulatory mimic device (Figure 4.3). Such a device should prove useful for investigating those pharmaceutical substances whose mechanism

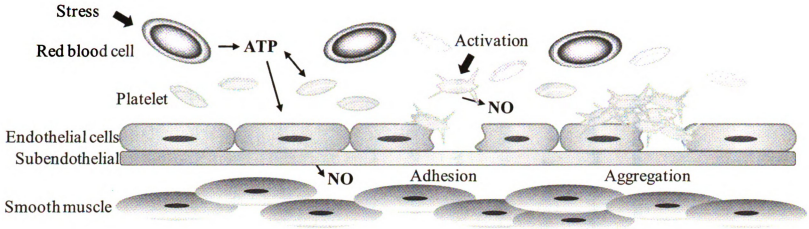

Figure 4.3 Illustration of processes going on in the blood stream. ATP released from RBCs results in platelet and endothelium NO production which leads to inhibition of platelet recruitment and vasodilation

of action is to prevent platelet activation *in vivo* or certain physiological interactions in the circulation.

4.3 EXPERIMANTAL METHODS

Preparation of a microfluidic device. Microfluidic devices were fabricated using standard soft lithographic technology. PDMS channel structures were produced following previously published methods.[48, 89] Briefly, masters for the production of PDMS microchannels were made by coating a 4 inches silicon wafer (Silicon, Inc., Boise, ID) with SU-8 10 negative photoresist (MicroChem Corp., Newton, MA) using a spin coater (Brewer Science, Rolla, MO) operating with a spin program of 2000 rpm for 20 seconds. The photoresist was prebaked at 95°C for 5 minutes prior to UV exposure with a near-UV flood source (Autoflood 1000, Optical Associates, San Jose, CA) through a negative film (2400 dpi, PageWorks, Cambridge, MA), which contained the desired channel structures drawn in Freehand (PC version 10.0, Macromedia, Inc. San Francisco, CA). Following this exposure, the wafer was postbaked at 95°C for 5 minutes and developed in Nano SU-8 developer (Microchem Corp.) (PGMEA, propylene glycol monomethyl ether acetate). The thickness of the photoresist was measured with a profilometer (Alpha Step-200, Tencor Instruments, Mountain View, CA), which corresponded to the channel depth of the PDMS structures. A 10:1 mixture of Sylgard

184 elastomer and curing agent (Ellsworth Adhesives, Germantown, WI) was used to increase the adhesiveness of the PDMS to aid in the reversible bonding procedure. This degassed mixture was poured onto the master and cured at 75°C for approximately 10 – 15 minutes. After this time, the PDMS layer was removed from the master and inlet holes were punctured using a 20 gauge luer stub adapter through the chip as well as 1/8" exit holes. A chip containing channels of 100 μm depth × 200 μm width × 2 cm length was used for all studies reported here. The channel depth corresponds to the height of the master, which was measured with the aforementioned profilometer.

Cell culture. Unless otherwise stated, all chemicals and reagents for cell isolation and culture were purchased from Lonza (Walkersville, MD), fluorescent dyes were from Invitrogen (Carlsbad, CA), and all other materials were purchased from Fisher Scientific (Pittsburgh, PA). Bovine pulmonary artery endothelial cells (bPAECs) were thawed and expanded in Endothelial Cell Basal Medium (EBM) supplemented with 0.1% gentamicin sulfate/amphotericin (GA- 1000), 0.1% human epidermal growth factor (rhEGF), 0.1% hydrocortisone, 0.4% bovine brain extract (BBE), and 5% fetal bovine serum (FBS). All reported experiments used bPAECs between passages 2 and 10. Once a confluent layer of cells has been obtained in a culture flask, the cells are ready to be subcultured or obtained. Cells were rinsed with 5 mL of HEPES for about 1 minute. The HEPES solution was then aspirated off, replaced with 5 mL of Trypsin/EDTA, and placed into a NUAIRE IR

Autoflow CO_2 water-jacked incubator (model NU-8500 at 5% CO_2, 37°C and humidified) for 1 minute. Trypsin is a protease that cleaves proteins that are used by the cells for adhesion to a substrate, however trypsin is not specific and will also cleave any other proteins it can find. As a result, the trypsin was only in contact with the cells for a minimal amount of time, approximately 1 - 2 minutes. As trypsin cleaves cellular proteins, the cell morphology is altered and the shape changes from an elongated cobblestone to a rounded sphere. Once approximately 90% of the cells have become dislodged (the solution will become cloudy as this process occurs), 10 mL of trypsin neutralizing solution (TNS) was added to stop the trypsin activity. This concentrated suspension was centrifuged at 1500 r.p.m. (revolutions per minute) for 5 minutes at 25°C in order to separate the cells from the solution. After centrifugation, the supernatant was aspirated out of the tube with care as not to disturb the cell pellet. Finally, 1 mL of equilibrated medium is added to the tube to resuspend the cells. This concentrated cell suspension is then equally pipetted into the previously prepared culture flasks (approximately 3 – 4 flasks). All handling and use of reagents were carried out under a NUAIRE Class II, type A-B3 biological safety cabinet with filtered laminar airflow to ensure safety and sterility of chemicals. All objects were sprayed with a 70% ethanol solution before entry into the hood to achieve and maintain a sterile environment.

Cell immobilization. The microfluidic device was placed with the array of

channels down into a 100 x 20 mm petri dish as shown in Figure 4.4. The petri dish is of the same nature to flasks used to culture bPAECs. Prior to bPAEC loading, the microchannels were coated with a cell adhesion protein, bovine plasma fibronectin (FN, Invitrogen). FN was prepared in 1 mL of DDW to a concentration of 1000 μg mL^{-1} and further diluted to a concetration of 100μg mL^{-1} in phosphate buffer solution (PBS) as working solution. To coat the microchannels, 100 μL of FN were added to the top of the inlet of the microchannel device and vacuum was applied at the exit hole to draw solution into each microchannel. An additional 10 μL of FN solution was added to the exit reservoir to keep the channel from drying out due to evaporation. The device was incubated at 37°C for 45 - 55 minutes, allowing for sufficient adsorption of the protein onto the surface. After removing excess FN solution from the inlets and reservoirs by pipet, the channel was dried with a stream of clean dry air (AirGas, East Lansing, MI), covered, and exposed to UV light for approximately 10 minutes. Cell pellet was obtained as described above and the pellet was suspended into 200 - 500 μL of equilibrated EBM. The concentrated cell solution was loaded into the microchannels in the same manner as FN and the device was incubated at 37°C and 5% CO_2 for 60 minutes. The channels were rinsed with equilibrated EBM 1 hour after initial seeding to remove any non-adherent cells. Additional cells were loaded into the channel and the above was repeated until the channels were >75%

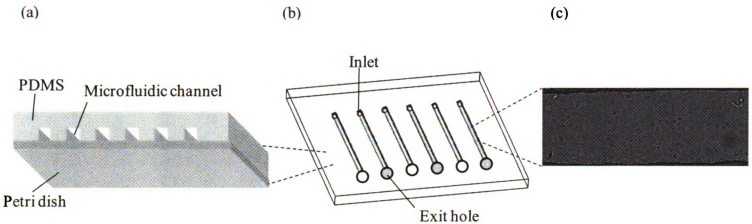

Figure 4.4 (a) Cross section of microfluidic array, each channel has dimensions of 100 μm width and depth. (b) PDMS array with inlet and exit holes for addressing flow to the system. (c) A confluent layer of bPAECs in a microfluidic device for mimicing of resistance vessels

confluent. On average, the time elapsed was approximately 4 - 8 hours from intital seeding of the channel with cells.

Isolation and purification of platelets and RBCs. All procedures followed previously described methods in Chapter 2.

Reagent preparation. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received without further purification unless otherwise noted. Reagents (ATP, ADP, L-NAME, C-peptide, and glybenclamide) were prepared following previously reported methods in Chapter 2 and 3.

Clopidogrel, (also as known as Plavix) was prepared as a 1.0 mM stock solution by dissolving a 35 mg tablet in 100 mL of diluted hydrochloric acid solution (pH ~4), while the working solution reported here was prepared by several dilutions of the stock into HBSS and the final concentration of clopidogrel with platelets was 40 μM.

Fluorescence labeling of platelets. Regarding the platelet adhesion studies, platelets were incubated with Cell Tracker Green CMFDA (1 μM as final concentration in HBSS) (Invitrogen) approximately 1 hour and extra fluorescence probe was removed by centrifugation. CMFDA can freely pass through cell membrane, but once inside the cell, is transformed into the cell-impermeant product due to reaction with thiols (e.g., glutathione) (Figure 4.5). Once the channels were confluent, platelets were hydrodynamically pumped over endothelium layer for 10 minutes at 37°C, 0.2 μL/min

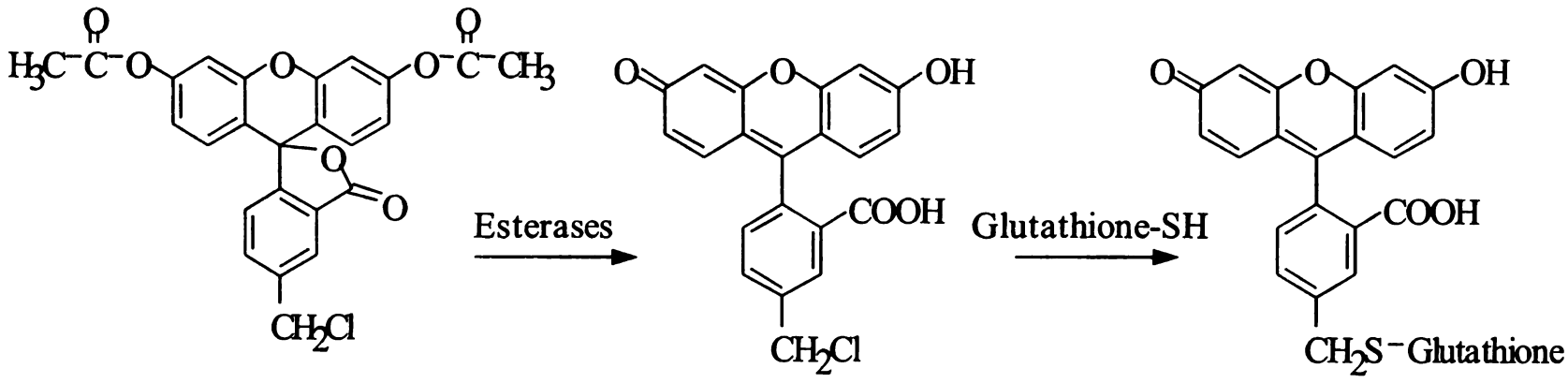

Figure 4.5 Reactions of CMFDA reagent. CMFDA is colorless and nonfluorescent until cytosolic easterases cleave off acetates, releasing a fluorescent product

and channels were rinsed with equilibrated EBM. Images were acquired using an Olympus IX71 microscope (Olympus America, Melville, NY) with an electrothermally cooled CCD (Orca, Hamamatsu) and Microsuite software (Olympus America). The microscope is fitted with a FITC filter cube (Chroma Technology Corp.) containing the excitation (460-500 nm) and emission (505-560 nm) filters.

4.4 RESULTS AND DISCUSSION

The effect of NO on platelet adhesion. As described in previous chapters, our quantitative research involving platelet activation was confined to cuvettes[90] or even in a flow system, was in the absence of endothelial cells.[91] These techniques focus on the platelet only and, while important in understanding platelet activity in the form of aggregation and molecules secreted by activated platelets, do not enable the monitoring of actual adhesion to an endothelium under the conditions of flow.

To demonstrate the ability of platelets to adhere to an immobilized endothelium upon activation, platelets were incubated in solutions containing various concentrations of ADP and hydrodynamically pumped through an endothelium-lined microchannel for 10 minutes as described above. Following this flow period, the channel was washed with equilibrated EBM and the resultant number of adhered platelets was determined by counting under fluorescent image conditions. A

representation of platelets adhering to the immobilized endothelial cells in the presence and absence of ADP is shown in Figure 4.6. In Figure 4.6, the images on the left are bright field images of the immobilized endothelium while the images on the right are the corresponding fluorescent images of the platelets adhering to the endothelium. In addition, the images were acquired for platelets alone and platelets in the presence of ADP. However, to demonstrate the importance of endothelium-derived NO on platelet adhesion, images were also obtained after the endothelial cells in the microfluidic channels had been incubated with L-NAME, a recognized inhibitor of eNOS and subsequent NO production. An examination of the fluorescent platelets in each image clearly shows the increased platelet adhesion due to ADP activation. Moreover, the data also show that a decrease in NO production by the endothelium increases the number of platelets adhering to the endothelial cells. The data in Figure 4.7 was the summary of the results represented in Figure 4.6 and additional concentrations of ADP that were investigated. The number of platelets adhering to the L-NAME treated immobilized endothelium in the absence of ADP was 35.2 ± 0.9, however, the number of adhered platelets was approximately the same to 35.4 ± 5.2 at ADP concentration of 1.0 μM but with untreated endothelium suggesting the importantance of endothelium-derived NO on inhibition of platelet adhesion to the endothelium

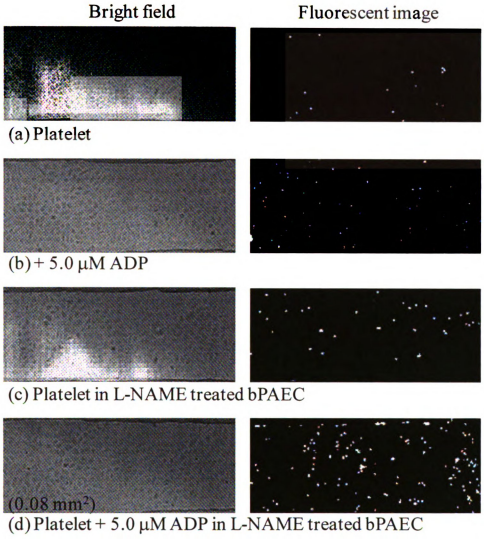

Figure 4.6 The effect of ADP activation and L-NAME on platelets pumped over a confluent channel of bPAECs. A stream of equilibrated medium was pumped over the endothelium to ensure any nonadherent platelets were removed before averaging the platelet count. Images in the left column represent bright field images, while those on the right represent the corresponding fluorescence images. (a) Untreated platelets, (b) platelets incubated in 5 μM ADP, (c) untreated platelet adhering to a 10 mM L-NAME treated endothelium, and (d) platelets activated with 5 μM ADP adhered to a 10 mM L-NAME treated endothelium

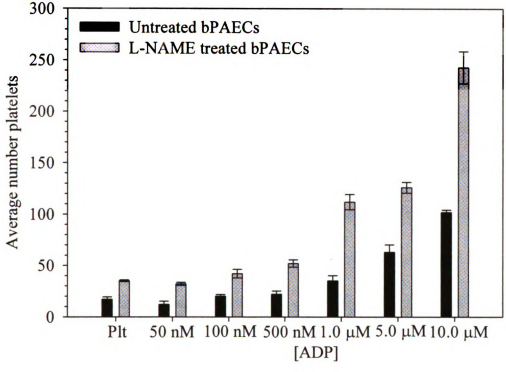

Figure 4.7 Varying concentrations of ADP from 50 nM to 10 □ M effects the number of platelets adhered to an untreated and L-NAME treated bPAEC monolayer. Non-activated platelets were also examined on similar untreated and treated surfaces. Error bars represent SEM. $p < 0.05$ ($n = 5$)

In contrast to endothelium NO, it has been hypothesized that platlet NO can inhibit platelet recruitment when adhere to the endothelium,[75] but this has never been proven. As shown in Figure 4.8, the number of platelets adhering to endothelium was 17.8 ± 0.5, however, the number increased approximately doule to 30.5 ± 0.6 when platelet NO was inhibited by L-NAME further proved the hypothesis by Freedman *et al* back to 1997. Collectively, no matter where NO is released from, it plays an important role in platelet adhesion. As shown dark gray bars in Figure 4.8, when platelets were pumped through the endothelium layer along with 40 nM exogenous NO, the number of platelets adhering to the endothelium decreased to 9.5 ± 0.6 and 23.5 ± 1.4 in the absence and presence of 5.0 μM ADP, respectively.

Applications for drug discovery. To demonstrate the utility of the microfluidic device for future studies involving drug discovery or drug efficacy, platelets were activated with increasing concentrations of ADP in the presence and absence of clopidogrel, a pharmaceutical often prescribed to prevent platelet aggregation. Clopidogrel is an inhibitor of P_{2y12} acceptor which is a ADP receptor on platelet membrane and is though to irreversibly block the ADP uptaking further inhibit platelet aggregation. As shown in Figure 4.9, platelet adhesion studies that were performed over the course of 3 days (using platelets from 3 different rabbits) resulted in an adhesion number of 15.3 ± 1.4. However, when this study was repeated using an aliquot of the

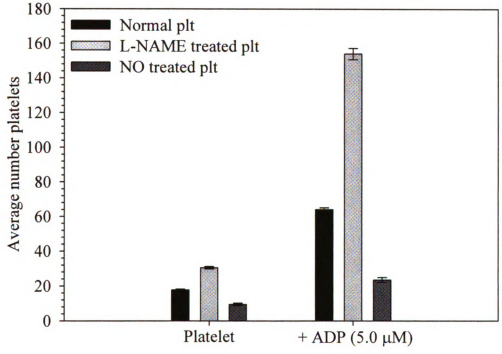

Figure 4.8 In contrast to inhibition of endothelium NO, platelets were incubated with 10 mM L-NAME prior to flowing through the bPAEC layer channel. The effect of exogenous NO was also examined shown as dark gray bars. Error bars represent SEM. $p < 0.05$ ($n = 4$)

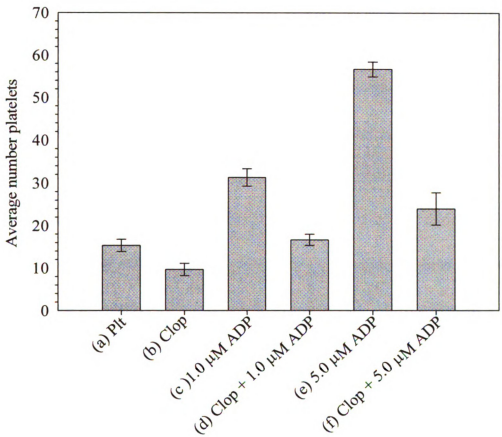

Figure 4.9 The effect of an anti-platelet drug, clopidogrel (Clop), on platelet adhesion to a bPAEC monolayer. Platelets were incubated with clop for 30 minutes before being pumped over the endothelium – (b), (d), and (f). In (d) and (f), after clop incubation and prior to being pumped over the endothelium, platelets were treated with 1.0 and 5.0 μM ADP. Error bars represent SEM. $p < 0.05$ ($n = 3$)

platelets that had been incubated in 40 μM clopidogrel for 30 minutes, the number of platelets adhering to the endothelium decreased to 9.7 ± 1.4. This decrease in platelet adhesion was even more pronounced in the presence of higher concentrations of platelet-activating ADP. For example, in the presence of 1.0 μM ADP, the number of platelets adhering to the endothelium was 31.3 ± 2.0. In the presence of clopidogreal and 1.0 μM ADP, the number of platelets adhering to the endothelium was decreased to a value (16.7 ± 1.3) that statistically overlaps with the number of platelets adhering to the endothelium in the absence of any ADP. Similar effects from the clopidogrel are also seen at higher concentrationso of ADP.

The effect of ATP on platelet adhesion in a multiple cell types system. People have abnormal circulatory ATP levels such as diabetes,[81, 82] cystic fibrosis, pulmonary hypertension[83] (decreased ATP amount in the circulation) or multiple sclerosis, sickle cell disease (increased ATP level), they also have been found having more readily active platelets.[78-80] Our group have hypothesized that ATP might have the dual effect on platelet adhesion and aggregation. Therefore, subsequent studies were performed to determine the role of ATP in platelet adhesion to an immobilized endothelium in the presence of flow. A microfluidic device was employed to investigate the effects of exogenous ATP, and ATP released from RBCs, on platelet adhesion to endothelial cells. As reported in Chapter 2 (Figure 2.6, also shown in Figure 4.10a), platelet NO production

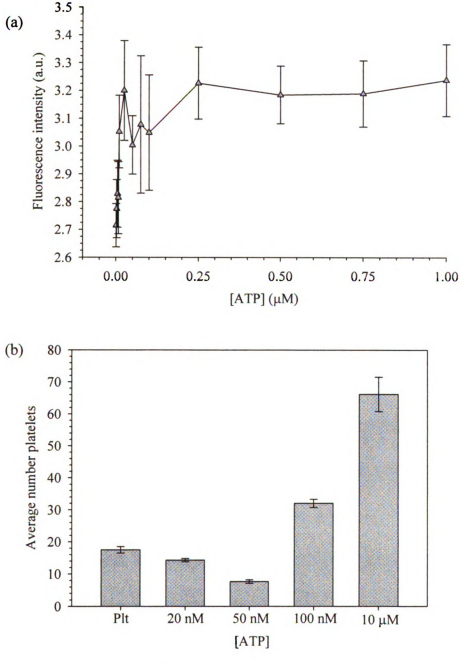

Figure 4.10 (a) Platelet NO production as a function of concentration of ATP. As ATP concentration was about 50 nM, platelet NO stopped increasing. Error bars represent SEM. (n = 4) (b) Platelets were treated with various concentrations of ATP (and were centrifuged to remove excess ATP) prior to flowing through a channel coated with a confluent layer of bPAECs. Error bars represent SEM. $p < 0.05$ (n = 5)

stopped increasing when ATP concentration was about 50 nM which perfectly fit in the platelet adhesion studies. Figure 4.10b summarizes the number of platelets adhering to the endothelium in the presence of various concentrations of exogenous ATP added to the platelets prior to pushing the platelets over the immobilized endothelium. The ATP that was added to the platelets was washed prior to adding to the microfluidic device, thus ensuring that the effect seen was from the ATP acting on the platelet as opposed to the ATP stimulating endothelium-derived NO that could also inhibit the platelet production. It was clearly evident that the adhesion trend as a function of ATP concentrations explained the curve in Figure 4.10a, when platelet NO stopped increasing, platelet adhesion started increasing. The data in Figure 4.11 also demonstrated the importance of considering sources of ATP in the bloodstream and possible effects on platelet behavior. Specifically, the platelet adhesion was monitored without the addition of any exogenous sources of ATP to the flowing stream. Rather, platelets were pumped over the endothelium along with RBCs in the solution. It is well-established that flowing RBCs subjected to shear-induced stress will release ATP;[68, 92] moreover, this ATP release can be inhibited or stimulated with glibenclamide and zinc-activated C-peptide, respectively. As shown in Figure 4.11, the platelet adhesion to the endothelial cells (15.7 ± 1.2) was decreased in the presence of RBCs (8.2 ± 1.4), but increases to a value of 18.6 ± 1.2when the RBCs were first incubated with an ATP release inhibitor prior to flowing with the

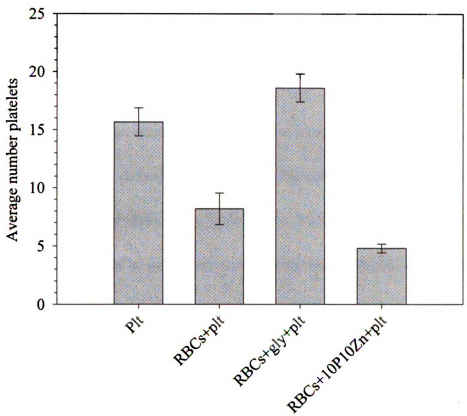

Figure 4.11 Platelets were incubated with RBCs in the absence and presence of glybenclamide (an ATP release inhibitor) or Zinc-activated C-peptide (an ATP release stimulus) prior to flowing over the confluent bPAECs layer. Error bars represent SEM. $p < 0.05$ ($n = 6$)

platelets through the endothelium layer. More specifically, platelets treated with RBCs incubated with zinc-activated C-peptide adhered less to an immobilized endothelium with only 4.8 ± 0.4, suggesting the importance of RBC-derived ATP for maintenance of normal platelet adhesion function.

4.5 CONCLUSIONS

Here, we report the creation of an *in vivo* process on an *in vitro* mimic and successfully used this device to monitor the physiological interaction (adhesion) of platelets to endothelial cells. The construction of the device is rather simplistic. Specifically, the use of a petri dish as the substrate to which endothelial cells are immobilized requires no pretreatment of the substrate (such as plasma oxidation). Moreover, the PDMS-chip was sealed to the substrate in a reversible manner, which also simplified construction of the microfluidic system. The device incorporates an important constituent of the bloodstream (platelets, RBCs, and endothelial cells) and enables the direct monitoring of their adhesion when activated. The importance of such a device is exemplified when considering work by Freedman *et al*[75] resulting in the conclusion that NO production from the endothelium prevents platelet adhesion to the endothelium. In turn, NO production by platelets may prevent platelets aggregating to each other, a process known as platelet recruitment. Moreover, the work presented here involving the

dual role of ATP as a platelet inhibitor and platelet activator is in concert with established findings involving hyperactive platelets, clinical outcomes, and certain types of disease as mentioned above. The work employed three different cell types in a single microfluidic channel, suggesting a possible method to investigate the communication between different cell types which such measurements would not be possible with current platelet aggregation or adhesion study technologies.

Therefore, such a device will enable future studies involving pharmaceutical candidates for reducing platelet activation to be investigated in a high-throughput manner using conditions that more closely approximate conditions in the bloodstream *in vivo*. Moremore, the changes in platelet behavior as a function of ATP concentrations monitored within the device, may serve as a starting point for explaining some of the clinical observations involving hyperactive platelets.

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CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

The discovery of NO as the endothelium-derived relaxing factor (EDRF) by Furchgott and Zadawaski has had a major impact,[1] not only on the cardiovascular system, but also in other field of scientific investigation.[2, 3] The fact that NO is synthesized by platelets, as well as endothelial cells, distinguished this vasoactive messenger from other factors such as prostacyclin or endothelin. However, platelet NO has been hypothesized to inhibit platelet recruitment[4] and sustain the suppression of aggregation[5] in a paracrine manner. On the other hand, it has been shown that RBCs are able to release high nanomolar amount of ATP subjected to mechanical deformation.[6-8] The importance of this release of ATP *in vivo* is that ATP is a recognized stimulus of nitric oxide synthase (NOS) in endothelial cells.[9] Although a relationship between ATP released from RBCs and endothelium-derived NO has been established, there have been no reports of the synergy between ATP derived from RBCs and the ability of platelets to produce NO.

In this thesis, a spectrofluorometric method to monitor NO production in platelets using a molecular probe was developed.[10] NO is quantitatively determined in platelets prior to, and after, stimulation with ATP or activation with ADP. Platelets obtained from the whole blood of rabbits were loaded with the fluorescence probe DAF-FM DA, and the subsequent NO production was measured as a fluorescent benzonitriazole.

Experiments were performed to determine the effect of probe concentration and probe incubation time in the platelets prior to measurement of the fluorescence. This information, combined with the method of multiple standard additions, was then employed to determine the moles of intracellular NO in the platelets ($2.7 \pm 0.3 \times 10^{-16}$ moles of NO/platelet) and the basal level of extracellular NO in the platelet sample ($9.9 \pm 2.2 \times 10^{-18}$ moles of NO/platelet). Moreover, this method was used to quantitatively determine the amount of NO released from platelets whose NO production was stimulated with ATP or ADP. The ability to monitor this NO production using a fluorescence probe may be advantageous over other forms of measuring NO production from platelets (such as amperometry) because, once activated, a platelet's ability to adhere to surfaces is dramatically increased.

Secondly, the communication between RBCs and platelets was successfully established using a flow through flourometer.[11] It has been shown that RBC-dervied ATP results in endothelium NO production and subsequent vasodilation suggesting that RBC may be a determinant of vascular caliber in the microcirculation. Moreover, studies have shown that patients with certain diseases such as pulmonary hypertension, cystic fibrosis[12] and diabetes,[8, 13] or complications arising from these diseases, have RBCs that release less ATP than RBCs obtained from healthy controls. To date, these reduced values of ATP release from the RBC have been discussed in terms of the endothelium and its inability to produce the proper levels of NO needed for vasodilatory purposes. In contrast

to endothelium NO, once produced by the platelet, NO acts as a platelet inhibitor, reducing the activation and aggregation of platelets. Here, we provided evidence that platelet NO is stimulated through RBC-derived ATP, suggesting an important role of ATP in platelet function. Interestingly, patients with diseases mentioned above also have hyperactive platelets,[14, 15] therefore, decreased levels of RBC-derived ATP and more active platelet may result in increased possibility of thrombus formation, further, stroke.

Finally, the creation of an endothelium mimic within a microdevice was successfully implemented to monitor multiple cell types in a single channel.[16] Here, the creation of a device to monitor an *in vivo* process on an *in vitro* mimic has been used to monitor the physiological interaction (adhesion) of platelets to endothelial cells. The device incorporated an important constituent of the bloodstream (platelets, RBCs, and endothelial cells) and enabled the direct monitoring of their adhesion when activated. This work also revealed the dual effect of ATP on platelet adhesion. In addition to the aforementioned diseases, there are other patient groups with hyperactive platelets who may have excessive extracellular ATP levels. For example, people with sickle cell disease whose cells are prone to hemolysis, are known to suffer from complications (e.g., stroke) associated with hyperactive platelets.[17] Moreover, our group has found that people with multiple sclerosis release excessive amounts of ATP from their RBCs in comparison to controls (unpublished results) and also have been reported to be more susceptible to deep vein thrombosis. Therefore, the changes in platelet behavior as a function of ATP

concentrations monitored with microfluidic technologies, may serve as a starting point for explaining some of the clinical observations involving hyperactive platelets.

5.2 FUTURE DIRECTIONS

The use of microfluidic devices will help improve our understanding of the microcirculation. Moreover, microfluidic devices have shown the capacity to be used in biomedical studies for diagnostic testing of certain disease states,[18] as well as in the pharmocokinetics field for drug efficacy.[19, 20] NO generated by endothelial cells and platelets has proven to be an important physiological and regulatory mediator modulating vessel wall hemostasis and preventing thrombosis. The changes in its generation or metabolism are likely to play role in the pathomechanism of vasospastic and thrombotic disorders. Flow induced shear is largely responsible for the tendency of blood elements to move towards the center of the flowing stream. If platelets were the only formed element in the blood, they would occupy the axial stream and unlikely interact with the vessel wall. However, in whole blood, numerous RBCs occupy the axial stream and force platelets to assume the position close to endothelial cells. Under physiologic conditions, platelets remain in a close contact with the endothelium and blood flow stimulates the endothelium NO, which regulates platelet activity.[21-23] The endothelium is an obvious target organ of cardiovascular risk factors. Accordingly, functional alterations do occur with aging, hypertension and hypercholesterolemia. All conditions are associated with a

decreased basal and stimulated release of endothelium NO. Clinical evidence substantiates the antiplatelets role of NO. Thrombotic complications associated with atherosclerosis, coronary artery disease, hypertension and diabetes mellitus[24-26] have been shown to involve impairment in NO synthesis and mechanism of action. Thrombi are complex structures that are composed not only of fibrin meshwork, but also contain blood-borne cellular elements such as platelets, leukocytes and RBCs and platelets play an essential role in the initial response they adhere to vessel wall components.

Platelet activity is known to enhance the procession of coronary atherosclerosis and to precipitate platelet-mediated coronary thrombosis and acute ischemic events (e.g., stroke).[27] Over the past 40 years, considerable research has been conducted to examine ways by which to inhibit abnormal platelet interaction with vessel wall in aforementioned patients. Owing to the important role of platelet adhesion, aggregation, and growth factor release on the damaged vessel surface (with the loss of functional endothelial cells and NO release), platelet inhibitors such as aspirin, ticlopidine and clopidogrel have been utilized to limit thrombosis. However, none of these inhibitors produced a completed satisfaction. Moreover, with more potent drug dose, the risk of excessive bleeding increased. The problem in finding a suitable platelet inhibitor is that a very high concentration is needed systemically to have a major effect locally at the site of damage or thrombus. Thus, there is a great interest in local delivery of substances with antiplatelet and anticoagulant properties. One of the substances that would be very beneficial to apply

locally is the targeting clot-dissolving therapeutics.

Improvement, have been made toward developing a fibrin-specific nanoparticle as a thrombolytic drug delivery system.[28] Briefly, once the nanoparticle targeted a thrombus, enzymes modified on particle surfaces can help dissolving the thrombus. However, more effective therapeutic approaches might be possible if an effective model can be developed. Thus, the device used in this work will be suitable for the study not only of the thrombus formation but also of providing an *in vitro* platform for the drug delivery. Figure 5.1 illustrated the creation of a thrombus in the confluent bPAEC layer channel. More specifically, the preparation of the microfluidic device was in the same manner as previously established except a layer of collagen was coated in the channel before fibronectin. Whole blood was pumped through while a 23 gauge needle poked through PDMS chip to create an "injury" in the channel. Whole blood was used in this method instead of RBCs suspension is because that species such as clot-factors facilitating coagulation processes are presented in whole blood. However, the problem with this method here is that it can't provide both precise spatial and temporal controls.

Methods such as laser-induced injury[29] or photothrombosis[30] have been well developed to generate a local disruption on the endothelium. The laser-induced method is the direct contact of the laser beam with the target vessel (here, it will be the endothelium layer), the prior fluorescently tagged platelets and proteins can then be pumped through the channel to generate both bright field and fluorescence images for thrombus

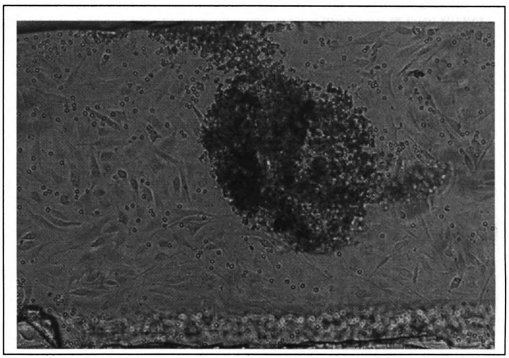

Figure 5.1 A thrombus formation mimic in the microfluidic channel

development. On the other hand, the photothrombosis procedure utilizes a photosensitive dye circulating in the channel. Once exposed to an external light beam, the dye turns into highly reactive oxygen radicals to disrupt the endothelium layer, resulting in subsequent endothelium injury. Thus, incorporation of any these methods with the device developed in this work will provide a more beneficial and precise model for *in vitro* studies.

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