INTRODUCTION

&

REVIEW OF LITERATURE
HEMOSTASIS

Vascular homeostasis is an important physiological event for the preservation of blood fluidity and vascular integrity. The fluid state of flowing blood must be carefully balanced against another important hemostatic property of blood that is meant to protect the vascular system from the loss of fluid due to an accidental breach of vascular integrity. These diverse yet coordinated reactions form the cornerstone of physiological hemostasis.

Stages of hemostasis

When a blood vessel is injured, the first discernible effect is the recruitment of the platelets to the site of injury forming primary hemostatic plug often referred as “white aggregate”. The reinforcement of this primary plug with fibrin which is the final product of blood coagulation cascade increases the effectiveness of the “primary plug”. The fibrin-rich “red-aggregate” is subsequently remodeled by the blood fibrinolytic system that repairs the vessel wall and finally returns to the physiological status. The complexity of hemostatic reactions requires flawless performance of the regulatory checkpoints that operate at various levels of hemostasis.

Blood Platelets

Morphology & Function

Platelets are small (~2µm in diameter), anucleated cell fragments first described by Italian researcher Bizozzerro and co-workers at the end of 19th century [Reed et al., 1985]. They are found in the bone marrow from megakaryocytes, which are extremely large cells of the hemapoietic series in the bone marrow that fragment into platelets either in the bone marrow or soon after entering the blood, especially as they try to squeeze through the pulmonary capillaries. The normal number of platelets in the blood is between 150,000-300,000/µl. Electron microscopy reveals a fuzzy coat (glycocalyx) which is thought to be composed of membrane glycoproteins, mucopolysaccharides and absorbed plasma proteins [White, 1993] (Figure 1).
Platelets move in an electric field because of their net negative surface charge; sialic acid residues attached to proteins and lipids are major contributors to this negative charge in platelets [Coller, 1984]. The electrostatic repulsion created by the negative surface charge help prevent resting platelets from attaching to each other.

**Ultra-structural features of platelets**

There are three major structural zones of the platelet, each related to a specific aspect of platelet function (Figure 2). The peripheral zone is involved primarily in adhesion, the sol-gel zone in contraction, and the organelle zone in secretion.

Adhesion of platelets to sites of vascular injury and to each other is a critical phase in formation of hemostatic plugs. Fundamental steps include conversion of non-sticky platelets to the adhesive state and release of endogenous chemical constituents essential for propagating platelet aggregation [Grette, 1962].

1. **Peripheral zone:** The peripheral zone of the platelet is critically involved in these events. It provides the template for chemical interactions generating the platelet response, the physical site for cell-cell adhesion and a trigger mechanism transferring the stimulus from outside the cells to the platelet interior. The peripheral zone of the platelet includes the exterior coat, the unit membrane and the submembrane area [White, 1972].
a) *Exterior coat:* The component of the peripheral zone in immediate contact with surrounding plasma is the exterior coat. Coat material is 150 to 200Å in thickness and covers the unit membranes of the cell surface and linings of the tortuous canalicular system penetrating the platelet surface.

b) *Unit membrane:* The middle layer of the platelet peripheral zone is a typical tri-lamellar membrane and is essential to the integrity of the cell. Surface-active agents, anti-histamines, local anesthetics, chelating agents, high and low salt concentration and lipid solvent injure the membrane and damage the cell. The changes are characterized by alterations in surface contour or by increased permeability with resultant swelling of the platelets.

c) *Submembrane area:* The area immediately under the unit membrane represents a transition between the peripheral zone and the sol-gel matrix of the hyaloplasm. Fine fragments are evident in the submembrane area of the peripheral circumferential band of microtubules.

2. **Sol-gel zone:** In studies with the light microscope, the interior of the platelets appears structure less except for a few granules and is thus called hyaloplasm. However, when examined by electron microscopy, the interior is found to be composed of masses of fibrous elements. An annular bundle of 250Å microtubule lying under the cell wall along with its greatest circumference is the most prominent fibrous system of the hyaloplasm. Microfilaments 50Å in diameter constitute a second system of fibers.

3. **Organelle zone:** A variety of formed organelles and particulate elements are embedded in the sol-gel matrix of the platelets.

   a) *Granules:* Granules are rich in phospholipids and contain hydrolytic enzymes including acid phosphatase, β-gluconuronidase and cathespin. Platelet fibrinogen, thrombosthenin, ATPase, ATP, ADP and serotonin have also been localized in granule fractions.
b) **Dense bodies**: The dense bodies of platelets are relatively few in number but play an important role in hemostatic function. Serotonin, ADP, catecholamines and platelet factor 4 have been associated with dense bodies.

c) **Mitochondria**: The mitochondria contribute significantly to the metabolic pool of ATP and as calcium repositories.

![Ultrastructural features observed in thin sections of resting platelets cut in the equatorial plane.](image)

Components include the exterior coat (EC), trilaminar unit membrane (CM), and submembrane area containing the specialized filaments of the membrane skeleton (SMF). The plasma membrane indentations from the walls of the channels of the surface connected open canalicular system (SCCS). The circumferential band of microtubules (MT) is seen as continuous band beneath the plasma membrane on the equatorial section. Glycogen granules (Gly) are prominent punctuate structures in the cytoplasm, and residual golgi zones (GZ) can also be identified. Organelles include mitochondria (M), dense granules (here termed as dense bodies (DB) and α-granules (G). The dense tubular system (DTS), the platelet equivalent of the sarcoplasmic reticulum, sequesters calcium.

**Composition of human platelets**

Platelets have many functional characteristics of whole cells, even though they do not have nuclei and cannot reproduce. In their cytoplasm are such active factors such as

1. Actin and myosin molecules, similar to those found in muscle cells as well as thrombosthenin that can cause the platelets to contract;
2. residuals of both the endoplasmic reticulum and the Golgi apparatus that synthesize various enzymes and especially store large quantities of calcium ions;

3. mitochondria and enzyme systems that are capable of forming ATP and ADP;

4. Enzyme systems that synthesize prostaglandins which are local hormones that cause many types of vascular and local tissue reactions;

5. An important protein called fibrin stabilizing factor;

6. a growth factor that causes vascular endothelial cells, vascular smooth muscle cells and fibroblast to multiply and grow, thus causing cellular growth that helps repair damaged vascular walls.

The cell membrane of the platelets is also important which is a coat of glycoproteins that repulses adherence to normal endothelium and yet cause adherence to injured areas of the vessel wall. In addition, the platelet membrane also contains large amounts of phospholipids that play multiple roles in blood coagulation process.

**Platelet Receptors**

The platelet receptors play the central role in the hemostatic function of platelets, allowing specific interactions and functional responses of vascular adhesive proteins and soluble platelet agonists. A wide variety of mobile transmembrane receptors are present [Rivera et al., 2009] in platelets of which some of the major platelet membrane receptors are (Figure 3):
Collagen receptors

Collagen receptors are believed to be most able to activate platelets and to cause adhesion and aggregation [Sixma et al., 1997]. Among these, glycoprotein receptors (GP) Ia/IIa and GP VI are believed to be responsible for collagen induced platelet activation. It has been suggested that activation via thrombin and ADP pathway may result in two different conformations of activated GP Ia/IIa with different ligand activity [Jung & Moroi, 2000]. Patients deficient in GP VI lack the ability to form thrombi on a collagen surface under flow conditions, with mild bleeding tendencies [Moroi & Jung, 2004].

Purinergic receptors

Purinergic receptors mediate activation by platelet derived ADP excreted from secretory granules of activated platelets. They constitute an autocrine mechanism of platelet aggregation [Gachet, 2005]. The purinergic-receptor family consists of P_{2}X ligand gated channels and G-protein coupled P_{2}Y receptors of which 7 and 8 subtypes respectively, has been described to date [Khakh et al., 2001; Gachet, 2005]. Platelets express mainly P_{2}Y_{1}, P_{2}X_{1} and P_{2}Y_{12}.

The P_{2}Y_{12} receptor is activated by ADP causing completion of aggregation of platelets in synergistic action with P_{2}Y_{1}, ADP-dependent amplification of aggregation induced by other
agonists, potentiation of the release reaction and stabilization of thrombi [Gachet, 2005]. Activation of both P$_2$Y$_{12}$ and P$_2$Y$_1$ receptors at the same time is required for normal ADP-induced aggregation, and antagonism of either one causes a dramatic inhibition of platelet aggregation [Jin & Kunapuli, 1998].

**Thrombin receptors**

Among the three thrombin activated protease activated receptor (PAR), PAR-1 and PAR-4 are expressed in human platelet [Kahn et al., 1999]. The PAR-1 is a high affinity receptor, activated by low concentrations of thrombin (<2nM) [Kahn et al., 1999] that causes GP Ibα as a cofactor for cleavage and also acts synergistically with ADP to amplify its responses [Gachet et al., 1997; De Candia et al., 2001; Adam et al., 2003]. When ADP is released from dense granules of activated platelets, ADP receptors, including P$_2$Y$_{12}$, become functional and take part in thrombin-induced aggregation. The PAR-4 is a low affinity receptor activated by higher concentrations of thrombin independent of ADP [Adam et al., 2003].

**TXA$_2$ receptor**

Thromboxane A$_2$ (TXA$_2$) is produced from arachidonic acid by cyclooxygenase (COX) enzymes. TXA$_2$ synthesis is caused by platelet activation by other agonists and is rapidly converted to thromboxane B$_2$ (TXB$_2$). The TXA$_2$ is a potent vasoconstrictor playing [Hamberg et al., 1975; Nakahata, 2008] an important role in the precipitation of ACS. The TXA$_2$ receptor officially called TP-receptor, belongs to the G-protein coupled receptor family (Figure 3).

**Adrenergic receptors**

The $\alpha_2$-adrenergic receptor mediated effect of catecholamines potentiates the effects of other agonists and in high concentrations inducing platelet aggregation and secretion [Anfossi & Trovati, 1996]. In physiological conditions, epinephrine mainly acts by potentiating platelet activation induced by other agonists [Lanza et al., 1988]. Interestingly, epinephrine significantly potentiates shear-induced platelet activation dependable on vWF and GPIb [Mustonen & Lassila, 1996]. Adrenergic receptors are linked, via G proteins, to the same intracellular pathways as P$_2$Y$_{12}$ [Jin & Kunapuli, 1998]. Epinephrine is therefore postulated to
synergize with ADP-induced responses mediated by P₂Y₁₂ receptors [Conley & Delaney, 2003].

**5HT₂-receptor**

Platelets have a serotonergic receptor 5HT₂. The activation of 5HT₂ receptor results in the increase of cytoplasmic Ca²⁺ and a shape change. Serotonin is a weak agonist of platelets and acts synergistically with other platelet agonists [Baumgartner et al., 1968; Pletsher, 1987].

**Overview of different platelet aggregating agents**

There are only few aggregating agonists currently available that can aggregate platelets as given below:

a) **Adenosine diphosphate:** Adenosine diphosphate (ADP), is a nucleotide. It is an ester of pyrophosphoric acid with the nucleoside adenosine consisting of pyrophosphate group, the pentose sugar ribose, and the nucleobase adenine (Figure 4). ADP is the product of ATP dephosphorylation by ATPases. ADP is stored in dense bodies inside blood platelets and is released upon platelet activation. ADP interacts with a family of ADP receptors found on platelets (P₂Y₁, P₂Y₁₂), leading to further platelet activation [Murugappa & Kunapuli, 2006].

![Figure 4: Ball and stick model of ADP](image)

b) **Collagen:** Collagen is the main structural protein of the various connective tissues in animals. As the main component of connective tissue, it is the most abundant protein in mammals, making up from 25% to 35% of the whole-body protein content. Collagen is the most thrombogenic component of the subendothelium [Baumgartner & Hardenschild, 1972]. Following vascular damage, collagen is exposed to
circulating platelets and both acts as a substrate for the adhesion of platelets [Cowan, 1981; Morton, 1989; Poole & Watson, 1995] and induces platelet activation [Poole & Watson, 1995]. The prevailing evidence proposes that two receptors are involved in the platelet response to collagen; integrin $\alpha_2\beta_1$ acts to adhere platelets to collagen, allowing platelets to interact with the lower affinity receptor GP VI, which is mainly responsible for platelet activation [Morton, 1989; Santoro, 1991] (Figure 5).

**Figure 5: Structure of collagen**

a) **Thrombin**: Thrombin is a serine protease that in humans is encoded by F$_2$ gene. Thrombin plays a central role in normal and abnormal hemostatic processes. It is assumed that $\alpha$-thrombin activates platelets by hydrolyzing the PAR-1, thereby exposing a new N-terminal sequence, a tethered ligand, which initiates a cascade of molecular reactions leading to thrombus formation. This process involves cross-linking of adjacent platelets mediated by the interaction of activated GP IIb/IIIa [Schwartz et al., 1995] (Figure 6).

**Figure 6: Structure of Thrombin**
b) **Epinephrine**: Catecholamines play an important role in platelet activation and aggregation, epinephrine being the most potent one. Catecholamines are substantially increased during stress, exercise or smoking and could result in clinically important platelet activation if their action was not rapidly regulated [Evangelou et al., 1998] (Figure 7).

![Figure 7: Structure of Epinephrine](image)

**c) Dermcidin isoform 2**: Dermcidin isoform 2 (DCN-2) has been reported to appear in the circulation in acute coronary syndrome (ACS)/acute myocardial infarction (AMI) was also found to be potent platelet aggregating agent [Ghosh et al., 2011]. It was also found that DCN-2 was a potent inhibitor of insulin induced NO synthesis in endothelial cells and also plays a role in the development of hypertension in animal model and in subjects suffering from systemic hypertension (Figure 8).

![Figure 8: Structure of DCN-2](image)

d) **N\textsuperscript{G}-nitro-L-arginine methyl ester**: N\textsuperscript{G}-nitro-L-arginine methyl ester (l-NAME), also referred to as, occurs naturally in living organisms (Figure 9), as it is a product of the degradation of
arginine-methylated proteins [Ji et al., 2009]. L-NAME is one of the first compounds which were intuitively employed to inhibit NOSes at the end of the eighties [Palmer et al., 1988; Sakuma et al., 1988; Aisaka et al., 1989]. It has been used widely as a general tool to decrease NO bioavailability or to establish the NO dependency of a physiological process. It is in this thesis the ability of L-NAME to aggregate platelets have been elucidated for the first time ever.

![Figure 9: Structure of L-NAME](image)

Platelet Activation

The inner surface of the blood vessel is lined with a single layer of endothelial cells where platelets do not adhere. Blood vessel injury is followed by the adhesion of platelets to the areas denuded of endothelium [Seiss, 1989]. When platelets adhere to the subendothelial matrix, the platelets change their shape from normal discoid to spherical (Figure 10) with the extrusion of long dendritic pseudopods [White, 1974] by the action of actin and myosin in platelet cytoplasm [George, 2000]. During the shape change secretory granules are organized into the center of the platelet (Figure 10). The secretory granules release their contents into plasma during platelet activation, which enhances further activation. The granule content also has procoagulant activities [Rendu & Brohard Bohn, 2001]. The most abundant surface protein, glycoprotein IIb-IIIa requires conformational change during platelet activation to express receptor function, mainly for fibrinogen [Shattil, 1998].
Platelet adhesion and aggregation

Normal spontaneous hemostatic plug formation to the sites of vascular injury depends on the immediate adhesion of circulating platelets with the lesion followed by rapid aggregation of platelets to each other. Fundamental steps include conversion of nonsticky platelets to the adhesive state and release of endogenous chemical constituents essential for propagating platelet aggregation [Grette, 1962]. The aggregation of platelets is initiated through the interaction of aggregating agents (agonists) to their specific receptors on platelet surface [Holmsen, 1979]. Although these agonists i.e. ADP, l-epinephrine, collagen and thrombin bind to their specific receptor molecules on the platelet surface, the agonist receptor interaction in each case results in the synthesis of prostaglandin G2 through cyclooxygenase 2 (COX 2), and in the release of ADP and other aggregating agents from platelets [Hamberg et al., 1974]. Interaction of ADP with its receptors on the platelet surface results in the platelet adhesion and thrombus formation [Jin et al., 2002].
The l-epinephrine, among all platelet aggregating agents is reported to be a weak aggregating agonist which aggregates platelets only at supraphysiologic concentration (> 2µM) through its interaction with α₂ adrenergic receptors on the platelet surface [Lalau Keraly et al., 1987; Grant et al., 1979]. While the sub optimal amount of ADP has been reported to fail to aggregate platelets, ADP nevertheless aggregates platelets at sub optimal concentrations in the presence of physiologic or sub-physiologic l-epinephrine in vivo might have an important role in the development of ACS. Emotional stress, which increases l-epinephrine “the stress hormone” in the system has been reported to be a major provocative factor for the ACS [Sheps et al., 2002], and as such the catecholamine might itself be responsible for the precipitation of the condition under emotional stress.

Initially plasma von willebrand factor (vWF) binds to collagen exposed from injured sub-endothelial layer. In conditions of low shear stress matrix proteins cause activation at the site of injury (Figure 11). In conditions of high shear stress, as in arteries, activation and adhesion depend largely on GP Ib-V-IX complex, which interacts with immobilized vWF. This interaction causes initial tethering of circulating platelets to the vessel wall. Thus, platelets slow down and roll over a vWF-coated surface. The rolling ends with firm attachment through GP Ia/IIa have become available via activation of rolling platelets. Firm attachment mediated by GP Ia/IIa also allows low-affinity GP VI to interact with collagen.
Recent studies have suggested that the differential roles of GP Ia/IIa and GP VI are modulated by changes at the extracellular matrix of the vessel wall induced by specific metalloproteinases, and both of these receptors participate in adhesion and aggregation alike [Ruggeri, 2002; Farndale et al., 2004]. The initial adhesion is followed by the recruitment of additional platelets into the growing platelet plug. Further activation occurs due to the release of agonists from the secretory granules of previously activated platelets. ADP is crucial for platelet activation, and plays the most important role in thrombus formation [Mills, 1996]. The activated GP IIb/IIIa-receptors in activated platelets
mediate platelet-platelet interaction, aggregation, by several ligands of which fibrinogen is most abundant [Bennett & Vilaire, 1979; Michelson, 2003]. After a platelet plug has been formed, it is then stabilized to prevent premature disaggregation. It has been suggested that outside-in signaling through cell surface integrins and tyrosine kinases of receptors have central roles in this phase of thrombus formation. The platelets also participate in localization, amplification and maintenance of the coagulant response at the injury site [Ilveskero et al., 2001; Michelson, 2003].

The platelets also regulate their own activation at the site of a platelet plug to prevent uncontrolled expansion of the thrombi. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) is an inhibitory receptor which mediates the inhibitory pathway. In addition, anti-thrombotic factors circulate in plasma and are secreted by platelets [Ruggeri, 2002]. Nitric oxide, prostacyclin and endothelial ecto-nucleotidase (NTPDase) are believed to be the most important endothelial regulators of the platelet activity. Nitric oxide and prostacyclin cause platelet inhibition and vasodilation and NTPDase neutralizes the prothrombotic release of platelets by metabolism of ADP [Marcus et al., 2005].

Coagulation

Recent advances in research have suggested a new model of coagulation as a cell-regulated overlapping process (Figure 12). Platelets support pro-coagulant reactions and vascular endothelial cells maintain anti-coagulant properties of the vasculature [Vine, 2009]. In healthy vessels the tissue factor pathway inhibitor (TFPI) inhibits coagulation factors. The complex formed by thrombin binding to thrombomodulin and protein C/protein S as well as another complex of endothelial surface heparinoids and anti-thrombin act as anti-coagulants at the site of injury, preventing excessive formation of thrombi [Monroe et al., 2005].
Figure 12: Coagulation Cascade
THROMBOSIS

Definition

Thrombosis is a pathological extension of hemostasis that occurs when the regulatory mechanisms of physiological hemostasis are inadequate [Radomski et al., 1999]. The interaction of platelets with the injured vessel wall in the formation of thrombus is of pivotal importance in the etiology and pathogenesis of coronary artery diseases and cerebrovascular or other vascular diseases (Figure 13) [Horne, 2005; Jennings, 2009; Falk, 1992].

Figure 13: Coronary artery disease (heart attack).

The main causes of thrombosis are given in Virchow's triad which lists hypercoagulability, endothelial cell injury, and disturbed blood flow.

a) Hypercoagulability

Hypercoagulability or thrombophilia, is caused by genetic deficiencies or autoimmune disorders. Recent studies indicate that neutrophils play a pivotal role in deep vein thrombosis, mediating numerous pro-thrombotic actions [Fuchs et al., 2010; Brill et al., 2011; Borissoff & Cate, 2011].
b) **Endothelial cell injury**

The causes of injury to the vessel's wall include trauma, surgery, infection or turbulent flow at bifurcations. The main mechanism is exposure of tissue factor to the blood coagulation system [Hypercoaguable disorders, 2007].

c) **Disturbed blood flow**

The causes of disturbed blood flow include stagnation of blood flow past the point of injury, or venous stasis which may occur in heart failure [Hypercoaguable disorders, 2007], in or after long periods of sedentary behavior, such as sitting on a long airplane flight. Also, atrial fibrillation, causes stagnant blood in the left atrium (LA) or left atrial appendage (LAA), and can lead to a thromboembolism [Hypercoaguable disorders, 2007]. Cancers or malignancies such as leukemia may cause increased risk of thrombosis by possible activation of the coagulation system by cancer cells or secretion of procoagulant substances (paraneoplastic syndrome), by external compression on a blood vessel when a solid tumor is present, or (more rarely) extension into the vasculature [Hypercoaguable disorders, 2007].

**Classification**

There are two distinct forms of thrombosis, venous thrombosis and arterial thrombosis, each of which can be presented by several subtypes.

1. **Venous thrombosis**

Venous thrombosis is the formation of a thrombus (blood clot) within a vein. There are several diseases which can be classified under this category which includes deep vein thrombosis, portal vein thrombosis, renal vein thrombosis, jugular vein thrombosis, budd-chiari syndrome, paget-schroetter disease, cerebral venous sinus thrombosis and cavernous sinus thrombosis.
2. Arterial thrombosis

Arterial thrombosis is the formation of a thrombus within an artery. In most cases, arterial thrombosis follows rupture of atheroma, and is therefore referred to as *atherothrombosis*.

   a) Stroke

A stroke is the rapid decline of brain function due to a disturbance in the supply of blood to the brain. This can be due to ischemia, thrombus, embolus (a lodged particle) or hemorrhage (a bleed). In thrombotic stroke, a thrombus (blood clot) usually forms around atherosclerotic plaques. Since blockage of the artery is gradual, onset of symptomatic thrombotic strokes is slower. Thrombotic stroke can be divided into two categories—large vessel disease and small vessel disease. The former affects vessels such as the internal carotids, vertebral and the circle of Willis. The latter can affect smaller vessels such as the branches of the circle of Willis.

   b) Acute coronary syndrome or acute myocardial infarction

Acute coronary syndrome (ACS) or acute myocardial infarction (AMI) or heart attack, is caused by ischemia, (restriction in the blood supply), often due to the obstruction of a coronary artery by a thrombus as detailed below.

   i) World-wide prevalence:

Each year, the American Heart Association (AHA), in conjunction with the Centers for Disease Control and Prevention, the National Institutes of Health, and other government agencies, brings together the most up-to-date statistics related to heart disease, stroke, and other cardiovascular and metabolic diseases and presents them in its Heart Disease and Stroke Statistical Update. Together, cardiovascular disease (CVD) and stroke produce immense health and economic burdens in the United States and globally (Figure 14). The 2011 overall rate of death attributable to CVD was 229.6 per 100 000 Americans or ≈1 of every 3 deaths in the United States. The death rates were 275.7 for males and 192.3 for females [American Heart Association Statistics Committee, 2015].
ii) National prevalence:

Cardiovascular diseases have been gaining importance in India recently because of increased incidence of the disease. It is the first among top 5 causes of deaths in Indian population (rural vs. urban, economically backward vs. developed states, men vs. women and at all stages vs. middle age) [Gupta et al., 2012]. In 2000, there were an estimated 29.8 million people with coronary heart disease (CHD) in India out of a total estimated population of 1.03 billion, or a nearly 3% overall prevalence [Gupta et al., 2008; Census of India, 2006]. According to World Bank estimates, CVD had a 31% share in the total burden of disease in 2015. In 2003, the prevalence was estimated to be 3-4% in rural areas and 8-10% in urban areas according to population based cross sectional surveys [Peters et al., 2001; Gupta, 2005; Gupta, 2004].

**Role of platelet aggregation on the precipitation of ACS and its pathophysiology**

The aggregation of platelets by various aggregating agonists like ADP, l-epinephrine, collagen or thrombin plays a critically important role in the events ranging from lifesaving normal blood
coagulation process [Willoughby et al., 2002] to the genesis of life threatening thrombosis leading to the development of ACS [Massberg et al., 2003; Furman et al., 1998]. Excess platelet aggregation at the site of injury, produced due to rupturing or fissuring of the atherosclerotic plaque present on the large or medium sized arteries serving the heart leads to the development of ACS [Furie & Furie, 2008; Fuster, 1996; Falk, 1992] due to the thrombus formation (Figure 15).

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**Figure 15: Thrombus formation**

Thrombus is the microaggregates of platelets embedded in fibrin mass [Furie & Furie, 2008; Furman et al., 1998; Massberg et al., 2003]. The formed thrombus not only physically blocks the normal circulation in the artery but the aggregation of platelets at the site also results in the formation of several proaggregatory prostaglandins including prostaglandin G₂ (PGG₂) and TXA₂.
INTRODUCTION & REVIEW OF LITERATURE

[Wong et al., 2010; Hamberg et al., 1974]. The last mentioned prostaglandin [Hamberg et al., 1975] is a potent vasoconstrictor and thereby causes further impairment of the blood circulation due to vasoconstriction [Wong et al., 2010; Hamberg et al., 1974; Hamberg et al., 1975].

Furthermore, during the platelet aggregation, ADP is also released from the platelets causing aggregation of more platelets [Remijn et al., 2002]. The blockade of normal blood circulation due to thrombus formation impedes the supply of oxygen, minerals and nutrients to the myocardial tissues that may ultimately cause series of symptoms including cardiac cell death associated with ACS and as such, the development of thrombus in the wall of the coronary artery is a critically important event in the precipitation of ACS [Furie & Furie, 2008; Falk, 1992; Fuster et al., 1996; Willoughby et al., 2002]. Similar atherosclerotic plaque rupture in the artery, in the brain resulted in subsequent formation of thrombus at the site of injury in the brain vessel wall leads to cerebrovascular disease commonly known as stroke [Elkind, 2006], and various thrombotic disorders predispose the system to pulmonary embolism and the post-phlebitic syndrome which are known to be a major cause of death and disability [Antman & Braunwald, 1998].

ACS more commonly known as heart attack is a common, sudden, and a major killer condition of all human race irrespective of their ethnic background [Klein, 2001; American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2015]. The major victims of heart attack are middle aged male people between the ages of 50-60. It has been estimated that 65% of all the population in the world will finally die of heart attack. According to official statistical data there is a significant difference between pre-menopausal women and age-matched men in morbidity and mortality from cardiac diseases and especially from myocardial infarction [Kameneva et al., 1999]. In women, the risk increases after 50 years of age. Although many factors can influence an individual’s risk for ACS, some factors are unique to women, including reproductive status. Menopause is associated with significant elevations in serum cholesterol levels and a 3-fold increase in the risk of ACS [Welty, 2001].
Atherosclerosis in the coronary artery commonly causes angina pectoris and myocardial infarction [Bergovec et al., 2009; Antman & Braunwald, 1998; Fuster et al., 1996]. Myocardial infarction generally occurs when there is an abrupt decrease in coronary artery blood flow due to thrombotic occlusion of a coronary artery previously narrowed by atherosclerosis resulting in the cardiac cell death (Figure 13) [Antman & Braunwald, 1998; Fuster et al., 1996]. During heart attack the interruption of normal blood circulation due to the atherosclerotic plaque rupture almost immediately causes extreme pain in the heart [Everts et al., 1996], nausea, vomiting [Goldberg et al., 1998] and dyspnea resulting in the development of ACS [Bergovec, et al. 2009; Milner et al., 1999; Zucker et al., 1997].

The rapid development of coronary artery thrombus at the site of vascular injury is facilitated by factors such as cigarette smoking, hypertension and lipid accumulation [Antman & Braunwald, 1998]. In most cases, infarction occurs when an atherosclerotic plaque fissures, ruptures, ulcerate, and when conditions favor thrombogenesis. In other words, mural thrombus forms at the site of rupture leading to coronary artery occlusion. Although the fatty streaks commonly proceed the development of more advanced atherosclerotic plaque, not all streaks progress to atheroma [Campbell & Campbell, 1994], while accumulation of lipid laden macrophages is the hallmark of fatty streaks, accumulation of fibrous tissues initiates the more advanced atherosclerotic lesion [Antman & Braunwald, 1998]. Although the mechanism of plaque rupture [Fuster et al., 1996] is not yet fully understood, but it may be due to the change in the pattern of the coronary blood flow (turbulence) resulting in the platelet aggregation occurring on the site initiated by a variety of agonists [Furman et al., 1998; Massberg et al., 2003]. Thrombin promotes platelet activation which is critically important for the development of thrombus in the coronary artery wall leading to thrombosis, ischemic heart diseases and other thrombotic diseases (Venus thromboembolism, deep vein thrombosis etc).

At the site of the ruptured plaque, the coagulation cascade is activated [Vine, 2009] on exposure to tissue factor [Morrissey et al., 1993; Eichinger et al., 1995] in the damaged endothelial cells. Factor VII and X are activated, ultimately leading to the conversion of prothrombin to thrombin.
[Papahadjopoulus & Hanahan, 1964; Nesheim et al., 1979] which then converts fibrinogen to fibrin. Fluid phase and clot bound thrombin precipitate in an auto-amplification reaction leading to further activation of coagulation cascade [Davie & Kulman, 2006]. The coronary artery eventually becomes occluded by a thrombus containing platelet aggregate and fibrin strand [Willoughby et al., 2002; Antman & Braunwald, 1998; Falk et al., 1992; Fuster et al., 1996]. The aggregation of platelets is mediated through the syntheses of PGG$_2$, prostaglandin H$_2$ (PGH$_2$) and TXA$_2$ due to the catalytic action of COX on arachidonic acid released from the platelet membrane due to the agonistic actions of the aggregating agents [Hamberg et al., 1974]. The following figure (Figure 16) shows the schematic representation of the mechanistic action taking place behind the aggregation of platelets.

![Figure 16: Summary of aggregation of platelets.](image)

1. ADP stimulates the release of Ca$^{2+}$ from the dense granules into the cytoplasm necessary for the activation of phospholipase A$_2$ (PLA$_2$), the mechanism of which remains obscure.
2. PLA$_2$ stimulates the release of arachidonic acid from the platelet membrane
3. Arachidonic acid through the activation of COX stimulates the release of prostaglandins and thromboxanes which ultimately leads to aggregation of platelets.
**Inhibition of aggregation of platelets**

Aggregation of platelets by aggregating agents is counteracted by several humoral factors that inhibit platelet aggregation to achieve homeostasis in the system. Furthermore, inhibition of platelet aggregation has been determined to play a critically important role in the prevention of ACS through the inhibition of thrombus formation [Fuster, 1996; Steering committee, 1989]. At present, the inhibition of platelet aggregation is thought to be mediated through three major pathways.

a) *Inhibition of platelet cyclooxygenase:* The inhibition of platelet COX results in the inhibition of synthesis of PGG₂, which in turn leads to the inhibition of platelet aggregation [Wong et al., 2010; Hamburg, 1975]. Various non-steroidal anti-inflammatory drugs including acetyl salicylic acid (aspirin), ibuprofen, indomethacin etc. are known to inhibit platelet aggregation [Catella-Lawson et al., 2001] through the inhibition of prostaglandin synthesis. Chronic ingestion of aspirin which induces inhibition of platelet aggregation *in vivo* has been reported to significantly reduce the incidences of ACS by numerous investigators [Steering Committee, 1989].

b) *Increase of cellular cyclic AMP level:* Increase of platelet cyclic adenosine monophosphate (cAMP) level either through the activation of membrane adenylate cyclase [Dutta-Roy & Sinha, 1987; Salzman & Levin, 1971; Haslam, 1971] or through the inhibition of cAMP phosphodiesterase results in the inhibition of platelet aggregation [Salzman & Levin, 1971]. Among these agents, prostacyclin, an arachidonate metabolite of endothelial cells, a potent inhibitor of platelet aggregation, that increases platelet cAMP level through the activation of membrane adenylate cyclase due to the binding of prostacyclin to its receptor sites on the enzyme has been most extensively studied [Tateson et al., 1977]. Prostaglandin E₁, a potent inhibitor of platelet aggregation, also activates adenylate cyclase through its binding to prostacyclin receptor linked adenylate cyclase has also been studied albeit to a lesser degree [Dutta-Roy & Sinha, 1987; Schafer et al., 1979].
c) **Nitric Oxide**: Mononitrogen monoxide, more commonly known as NO, believed to be the endothelial relaxing factor [Furchgott & Zawadzki, 1980], and is potent inhibitor of platelet aggregation [Yao et al., 1995; Coles et al., 2002; Sinha et al., 1998]. The compound is also a powerful vasodilator [Gruetter et al., 1979]. The vasodilatory property of NO could be important in the relaxation of vasoconstriction due to the release of TXA2 produced during platelet aggregation on the arterial wall in ACS. Currently, it is believed NO does not have any receptor on platelet surface and the compound is freely dissolved into the cytosol. NO has been reported to increase both cAMP and cyclic guanosine monophosphate (cGMP) levels [Coles et al., 2002; Sinha et al., 1998; Gruether et al., 1979].
NITRIC OXIDE

History of discovery of Endothelium Derived Relaxing Factor (EDRF)

Nitroglycerine has been used clinically for well over 100 years to treat angina pectoris but only recently has its mechanism of action been elucidated and attributed to NO and cGMP [Katsuki et al., 1977]. The unequivocal demonstration that nitrovasodilators elicit vascular smooth muscle relaxation via the actions of NO came in 1981, when organic nitrate and nitrite esters, inorganic nitroso compounds and nitrosoamines were shown to react with thiols to form intermediates-nitrosothiols, which were unstable and decomposed with the liberation of NO [Ignarro et al., 1980; Ignarro et al., 1981]. The liberated NO then activated guanylate cyclase and increased smooth cGMP levels, resulting in smooth muscle relaxation [Arnold et al., 1977]. A series of S-nitrosothiols were synthesized and found to be excellent NO donor molecules both in vivo and in vitro. The S-nitrosothiols, first described in 1980, represented the first known biologically active NO donor agents.

NO and its chemistry

NO is a colorless gas at room temperature and pressure (B.P.- 151.7°C). The maximum solubility of NO (at 1 atm partial pressure) in water at room temperature and pressure is approximately 2mM, which is slightly higher than the solubility of dioxygen (O₂) in water [Shaw et al., 1977]. It becomes immediately evident from a Lewis dot depiction that NO has one unpaired electron and thus exists as a free radical species.

The electronic structure of NO has been represented as:
At room temperature and pressure, NO has little propensity to react with itself in a radical-radical dimerization process.

**Free radical chemistry of NO**

NO reacts via simple radical-radical combination reactions with species processing unpaired electron such as \( \cdot\text{NO}_2, \ O_2 \). The ability of \( \cdot\text{NO} \) to “quench” other radical species also allows it to terminate radical chain reactions. A good example of this phenomenon is the effect of \( \cdot\text{NO} \) has on the \( O_2 \)- dependent oxidation of lipids [Wink et al., 1993; Hogg et al., 1983; Rubbo et al., 1996; Struck et al., 1995]. Due to the fact that many lipids contain “activated” allelic hydrogen atom of the unsaturated fatty acid (Lipid-H) by an initiating radical species (\( X' \)) to generate a lipid radical (lipid).

\[
\text{Lipid-H} + X' \rightarrow \text{Lipid'} + \cdot X - H
\]

The lipid radical then reacts with \( O_2 \) to generate an alkylperoxy radical (Lipid- \( \cdot\text{OO'} \)) which can further react with another lipid to form another lipid radical that can also react with \( O_2 \). These two reactions are chain propagating steps.

\[
\text{Lipid'} + O_2 \rightarrow \text{Lipid-} \cdot\text{OO'}
\]

\[
\text{Lipid-} \cdot\text{OO'} + \text{Lipid} - \cdot H \rightarrow \text{Lipid-} \cdot\text{OOH} + \text{Lipid'}
\]

NO is known to limit lipid peroxidation by acting as a chain terminating species.

\[
\text{Lipid-} \cdot\text{OO'} + \cdot\text{NO} \rightarrow \text{Lipid-OO-NO}
\]

Furthermore, \( \cdot\text{NO} \) has been reported to inhibit the generation of chain initiating species by altering the reactivity of metals known to serve as catalysts for their generation [Rubbo et al., 1996].
Chemical biology

NO is an endogenous mediator of numerous physiological processes that range from regulation of cardiovascular function to participation in memory [Dawson et al., 1992; Feldman et al., 1993; Ignarro & Gold, 1989; Moncada et al., 1991]. It has also been shown to be a promoter of the severity of different diseases including cancer and stroke [Gross & Wolin, 1995; Wink et al., 1998]. Unlike most other biological mediators, the in-vivo properties of NO are determined by its chemistry. The chemical reactions of NO falls into two distinct categories:

Direct: The direct reactions involve the reaction rate. The reactions in which NO combines with biological substrates at sufficiently rapid rates are to be of consequence involve either metals or radicals. Direct reactions between NO and thiols, are far too slow to occur to any considerable extent. The vast majority of reactions of NO in vivo are with metalloproteins containing iron. The most notable heme protein that forms a Fe-NO adduct in vivo is soluble guanylate cyclase [Murad, 1994]. On NO binding, the position of the iron within the porphyrin ring is shifted such that the distal histidine is decoupled in favor of the five-coordinate nitrosyl complex [Stone & Marletta, 1994; Yu at al., 1994].

In contrast to guanylate cyclase, binding of NO to monooxygenases such as cytochrome P-450 results in potent competitive inhibition of O₂ binding to the gene site [Khatsenko et al., 1993; Stadler et al., 1994]. NO mediated inhibition of cytochrome P-450 has some important pathophysiological implications. Binding of NO to the heme domain of cytochrome P-450 may serve as a protective mechanism against a variety of pathophysiological conditions by releasing free heme and activating hemeoxygenase in hepatocytes [Kim et al., 1995; Choi & Alam, 1996; Stocker, 1990]. Biological complexes containing metals other than iron are also affected by NO binding. As an example, nitrosylation of the aqueous form of the vitamin B₁₂ derivative cobalamine results in a diminished ability for this complex to serve a cofactor for methionine synthesis [Brouwer et al., 1996]. Scavenging of NO by cobalamine reduced the loss in mean arterial blood pressure induced by lipopolysaccharide (LPS) activated NOS [Greenberg et al., 1995]. The reactivity of NO with
metals is not limited simply to covalent interactions. The rapid reaction between NO and oxyhemoglobin to produce methemoglobin and nitrate \((k = 5 \times 10^7 \text{ M}^{-1} \text{ S}^{-1})\) [Eich et al., 1996] is the primary endogenous mechanism by which NO diffusion and concentration are controlled [Doyle & Hockstra, 1981; Feelisch, 1991; Lancaster, 1994].

\[
\text{Oxy Hb (Fe}^{2+} - \text{O}_2) + \text{NO} \rightarrow \text{met Hb (Fe}^{3+}) + \text{NO}_3^-
\]

It has also been reported that, consumption of \(\text{H}_2\text{O}_2\) by catalase is inhibited by cytokine stimulated hepatocytes and by synthetic NO donors and plays role in the tumoricidal activity of stimulated macrophages [Kim et al., 1995; Wink et al., 1996; Farias-Eisner et al., 1996]. The interaction of NO with radicals can also have protective effects. NO can react with oxyradicals formed during lipid peroxidation, which is an important component of the inflammatory process and cell death [Halliwell, 1991; Hogg et al., 1983; Rubbo et al., 1995].

**Indirect:** The indirect effects of NO are often associated with pathophysiological conditions and higher nitrogen oxides are thought to be the chemical species responsible for the etiology of numerous diseases. The biological properties of these species, principally \(\text{N}_2\text{O}_3\), \(\text{ONOO}^-\), NO• and NO\(_2\) are chemically driven much the same as NO itself. Aerobic NO solutions produce \(\text{N}_2\text{O}_3\) and when exposed to GSH, form the nitrosative product S-nitrosoglutathione (GSNO) [Wink et al., 1994]. Cobalamin, which forms a stable CoIII-NO complex under physiological conditions, has been shown to nitrosative thiols [Brouwer et al., 1996]. Under anaerobic conditions, ferrous nitrosyl hemes are quite stable. However, the corresponding ferric complexes require high NO concentrations for stability, as NO easily dissociates from the ferric iron. Physiological transport of NO and the formation of S-nitrosothiols may occur through non-heme iron sulfur nitrosyl complexes such as those observed in activated macrophages. Under high fluxes of NO, formation of these complexes has been indicated by electron paramagnetic resonanace (EPR) spectroscopy [Lancaster et al., 1990].
INTRODUCTION & REVIEW OF LITERATURE

The nitrosyl anion can be formed as a result of several different processes. A primary source of NO is the nucleophilic attack of reduced thiols by RNOS to form RSNO, which can subsequently form NO⁻ and disulfide.

\[
\begin{align*}
N_2O_3 + RSH & \rightarrow RSNO + HNO_2 \\
RSNO + RSH & \rightarrow RSSR + NO^- + H^-
\end{align*}
\]

Nitrosyl has also been suggested to be formed from the decomposition of iron dinitrosyl complexes similar to those observed in tumor cells exposed to activated macrophages [Pearsall & Bonner, 1982].

**Mixed direct and indirect effects:** A primary cellular target for the cytotoxic action of NO is the mitochondria [Moncada et al., 1991; Lancaster et al., 1990]. NO can repress oxidative phosphorylation in a reversible manner through regulation of intracellular calcium levels [Laffranchi et al., 1995]. Reversal of cytochrome c oxidase to active state occurs when the bound NO is reached to nitrogenous products by electrons from the respiratory chain [Barutaite & Brown, 1996]. The body may have several protective mechanisms to limit the indirect effects of RNOS on mitochondria. Inhibition of respiration was not observed in cells isolated from sites of experimentally induced inflammation in vivo [Fisch et al., 1996; Stadler et al., 1991]. This may suggest that oxyhemoglobin and diffusion of NO away from NOS containing cells play important roles in the extent of mitochondrial inhibition where RNOS formation is limited and reversible inhibition is only transient.

**Nitric oxide synthases: Historical introduction and functional aspects**

NO producing enzymes have been logically named nitric oxide synthases (NOS, Figure-17), but the derivation of the nitrogen of NO from the guanidino nitrogen of L-arginine, one of the amino acid building blocks of cellular proteins was unexpected. Before any of the enzymes were isolated and purified, first from rat brain cerebella [Bredt et al., 1990], the activities were often referred to as guanylate cyclase activating enzymes. In fact, the initial observations led to the proposal that NO
generated by nitroglycerine and other NO producing compounds acted on guanylate cyclase to cause increase in the tissue levels of cGMP [Arnold et al., 1977]. In 1980, Furchgott and Zawadzki showed that endothelial cells serve an obligatory role in acetylcholine mediated relaxation of arterial smooth muscle, which were seminal in further examination of the factors involved in signaling vasorelaxation [Furchgott et al., 1980]. Acetylcholine was postulated to interact with muscarinic receptors on the surface of endothelial cells to stimulate the release of an endothelium-derived relaxing factor (EDRF) that diffused into and interacted with the underlying vascular smooth muscle [Furchgott et al., 1980; Furchgott et al., 1981]. In 1986, Ignarro and others reported for the first time, after studying endothelium-independent NO-elicited and endothelium dependent relaxation of bovine pulmonary artery and vein, hypothesized that EDRF is either the same as NO or a labile nitroso precursor that spontaneously decomposes with the liberation of NO [Furchgott et al., 1981; Ignarro et al., 1986]. Endothelium-derived nitric oxide (EDNO) or NO is chemically unstable, with a half life of 3-5 seconds in aqueous solution under physiological conditions of concentration, temperature, pH and oxygen tension. In aqueous solution, NO spontaneously oxidizes primarily to NO$_2^-$, which is 5 or 6 orders of magnitude less potent than NO as a vasodilator [Ignarro et al., 1980; Gruetter et al., 1979]. In 1987, Palmer et al showed that NO, measured by chemiluminescence could account for biological action of EDRF released from cultured porcine aortic endothelial cells in response to bradykinin [Palmer et al., 1987].

Spectrophotometric monitoring of the formation of NO-hemoglobin from deoxyhemoglobin Ignarro et al., 1987] or the diazotization of sulfanilic acid [Ignarro et al., 1987] could account for the biological action of EDRF released from perfused artery, vein and freshly harvested bovine aortic endothelial cells. These later observations were subsequently confirmed by Schmidt et al [Schmidt et al., 1988].
Stuehr and Marletta had demonstrated that murine macrophages synthesize NO$_2^-$ and NO$_3^-$ in response to *Escherichia coli* lipopolysaccharide [Stuehr et al., 1985]. Macrophage cell culture experiments revealed that an L-arginine dependent biochemical pathway was involved in the biosynthesis of NO$_2^-$ and L-citrulline and that this pathway (Figure 18) was inhibited by N$^G$-methyl-L-arginine acetate ester (*l*-NAME), a close structural analog of L-arginine [Hibbs et al., 1987]. This arginine-dependent formation of EDNO was specific for L-arginine and was inhibited competitively by *l*-NAME [Palmer et al., 1988]. The clear demonstration of NO formation from L-arginine by vascular endothelial cells, together with the knowledge that NO undergoes rapid spontaneous oxidation to NO$_2^-$, prompted Hibbs et al. to reexamine the macrophage system, and they reported that NO was, after all, the immediate product of L-arginine and that NO$_2^-$ was, in turn, derived from NO [Hibbs et al., 1988]. The striking commonality of observations that close structural analogs of L-arginine antagonize endothelium dependent relaxation, that addition of L-arginine overrides such antagonism, and that arginine analogs cause endothelium dependent vascular smooth muscle contraction, together with the findings from experiments with macrophages and endothelial cells that NO is derived from the basic amino-nitrogen atom of the guanidine function of L-arginine. Furthermore, the NO is produced in such a manner that is inhibited by L-arginine analogs which suggests that a specific enzyme is involved in the catalytic conversion of L-arginine to NO. A novel citrulline forming enzyme activity present in the cytosolic fraction of vascular endothelial
cells was found, and as implicated its involvement in the formation of NO from L-arginine [Palmer et al., 1989]. The soluble fraction catalyzed the formation of L-arginine to citrulline in an NADPH-dependent manner, and the conversion was inhibited by l-NAME.

![Figure 18: Biosynthesis of nitric oxide](image)

Additional reports have surfaced that provide indirect evidence for the possibility that EDRF is entirely NO. Berkowitz and co-workers that the pharmacological properties of EDRF released from cultured arterial endothelial cells are not identical to those of authentic NO tested in the same bioassay [Shikano et al., 1987a; Shikano et al., 1987b; Long et al., 1987]. In another study, in which electron paramagnetic resonance spectroscopy was used, EDRF released from cultured arterial endothelial cells failed to generate a spectrum that was characteristic of that for authentic NO radical [Rubanyi et al., 1989].

In 1988, Garthwaite et al showed that NO could act as an intracellular messenger in the brain [Garthwaite et al., 1988] and subsequently Bredt and Synder reported the isolation and purification of neuronal nitric oxide synthase (nNOS) [Bredt & Synder, 1990]. In these studies, they showed the requirement of Ca\(^{2+}\)/calmodulin for the elicitation of L-arginine to L-citrulline conversion. It has also been reported that nNOS contains both Flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN) in 1:1 stoichiometry and the amino acid sequence of its 641-residue C-terminus was highly homologous to NADPH-cytochrome P-450 reductase [Bredt et al., 1991; Mayer et al., 1991].
Studies have shown the flavoprotein nature of inducible NOS (iNOS), having already demonstrated that iNOS from mouse macrophages absolutely requires tetrahydrobiopterin for activity [Mayer et al., 1991; Hevel et al., 1991; Tayeh & Marletta, 1989; Kwon et al., 1989; Mayer et al., 1990]. Crystal structures of both human eNOS and iNOS have been shown to contain ZnSO4 centers [Fischmann et al., 1999; Li et al., 1999]. All three isoforms have been found to contain zinc based on the biochemical studies of nNOS and eNOS [Miler, et al. 1999; Rodriguez-Crespo et al., 1999].

**Neuronal NOS** (nNOS, NOS 1 gene product), which produces NO on neuronal tissue in both the central and peripheral nervous system. Neuronal NOS also performs a role in cell communication and is associated with plasma membranes [Grozdanovic & Baumgarten, 1999].

**Inducible NOS** (iNOS, NOS 2 gene product), present in macrophages, smooth muscle cells, microvascular endothelial cells, fibroblast, cardiomyocytes, hepatocytes and megakaryocytes which can be found in the immune system but is also found in the cardiovascular system [Flesch et al., 1994].

**Endothelial NOS** (eNOS, NOS 3 gene product, Constitutive/cNOS), generates NO in blood vessels and is involved in regulating vascular function. A constitutive Ca^{2+} dependent NOS provide a basal release of NO [Awolesi et al., 1995]. eNOS is also associated with membranes of Golgi bodies within cells.

**Physiological actions of NO**

NO is implicated in a wide variety of physiological effects. The reaction of NO with the heme group of guanyl cyclase leading to the activation of guanyl cyclase is the principal effector reaction of NO in the cardiovascular system. Agonist-mediated increase in the endothelial cell calcium activates eNOS. Also, flow mediated vasodilation with exercise depends in part on the flow sensitive increase in the endothelial cell calcium, which leads to increased eNOS activity [Upchurch et al., 1997].
NO relaxes gastrointestinal smooth muscle and leads to reduced motility, relaxation of the sphincter of Oddi and relaxation of the lower esophageal sphincter.

Relaxation of the bronchial smooth muscle can be provoked by inhaled NO and endogenously produced NO may contribute to the maintenance of basal bronchial and basal pulmonary arterial tone [Loscalzo, 1992]. Some other effects of eNOS include maintenance of vascular integrity, impairment of leukocyte adhesion to the endothelium, inhibition of smooth muscle migration and proliferation. Endothelial NO plays a critical role in hemostasis wherein the basal production of NO by eNOS inhibits both adhesion and aggregation of platelets in vasculature.

Endothelium derived NO is an important determinant of cerebral blood flow. The nNOS in cerebral and glial cells contribute to the regulation of cerebrovascular tone, memory and learning through its involvement in long term potentiation in the CNS. NO is a likely transmitter of nor-adrenergic, non-cholinergic neurons and may thereby have a role in the regulation of myocardial contractility, heart rate, gastrointestinal motility, bronchial tone and penile erection.

The iNOS produced NO in macrophages, lymphocytes and neutrophils is an important determinant of immune and inflammatory responses. The bactericidal, fungicidal, viricidal, parasiticidal and tumoricidal activities of macrophages are partly determined by the robust elaboration of NO by iNOS [Loscalzo, 1995]. NO also limits lymphocyte proliferation and attenuates the allogenic immune response. The iNOS being expressed by a broad range of non-immune cell types, it is thought that NO produced by this isoform is involved in non-specific immunity, especially in the liver and lungs. By a similar mechanism, NO produced by iNOS may also be involved in apoptotic responses in a variety of cell types.

The effects of NO are modulated by both direct and indirect interactions that can be dose-dependent and cell-type specific. Understanding the regulatory mechanisms of NO in apoptosis and carcinogenesis provides important clues in diagnosis and treatment of tissue damage and cancer [Kim et al., 2001].
**Nitric oxide and ACS**

Platelet activation and recruitment are tightly regulated by products of the endothelium, including prostacyclin and NO [de Graff et al., 1992; Radomski et al., 1987]. NO inhibits platelet adhesion and aggregation [Stamler et al., 1989; Cooke et al., 1990], and prevents thrombosis in a model of endotoxin-induced glomerular damage [Shultz & Raij, 1992]. Endothelial production of bioactive NO is impaired in atherosclerosis [Bossaller et al., 1986] and in the presence of coronary risk factors including hypercholesterolemia, diabetes mellitus, cigarette smoking, and hypertension [Vita et al., 1996]. Furthermore, there is evidence that loss of endothelium-derived NO contributes to the pathogenesis of acute coronary syndromes [Okumura et al., 1992; Bogaty et al., 1994]. Both constitutive and inducible NOS have been identified in human platelets and megakaryocytoid cells [Mehta et al., 1995; Chen & Mehta, 1996; Pigazzi et al., 1995], and studies report NO release from aggregating platelets [Malinski et al., 1993; Radomski et al., 1990; Freedman et al., 1997]. Importantly, whereas platelet-derived NO appears to inhibit the primary aggregation response modestly, NO release from activated human platelets markedly inhibits platelet recruitment [Freedman et al., 1997] and thus may limit progression of intra-arterial thrombosis. However, the mechanism of release of arachidonic acid from the activated platelets in relation to NO synthesis still remains obscure.

**The second messenger role of nitric oxide**

It has been reported that NO as the second messenger of insulin for its diverse effects in various physiologic and pathologic events [Kahn et al., 2000]. Insulin plays an important role in carbohydrate metabolism, fat and protein metabolism [Czech, 1977]. The hormone also has its effects in atherosclerosis, thrombosis and neuropathological events. The effects of the hormone are the result of the binding of the protein molecules to the target tissue. The interaction of insulin with specific cell surface receptors results in the activation of the insulin receptor kinase [Weiss, 1993], which is thought to modify the biological activity of various proteins through the downstream phosphorylation by the activated enzyme. It has been recently shown that the administration of
physiologic concentrations of insulin in mice resulted in the decrease of blood glucose content with a simultaneous increase in plasma methemoglobin [Sinha et al., 1999]. The formation of methemoglobin suggested the formation of NO in the system. It has been demonstrated that physiologic concentrations of insulin specifically activates a membrane bound constitutive NOS. The NO formation catalysed by the NOS, produced insulin like effects on carbohydrate metabolism both \textit{in vivo} and \textit{in vitro}.  

ASPIRIN

Mechanism of action

The Hippocrates, who lived between 460 B.C. and 377 B.C. left historical records of the pain relief treatments, including the use of powder made from the bark and leaves of the willow tree to help heal headaches, pains and fevers. By 1829, scientists discovered that the compound called salicin in willow plants which gave the pain relief. In 1897, German chemist Felix Hoffman first synthesized aspirin and today it has become the most important antiplatelet agent. The anti-platelet effect of aspirin was first described by Morris in 1967. Aspirin exerts its effect through the inhibition of prostaglandin (PG) H-synthase 1 and 2 also known as COX 1 and 2. The COX 1 is known to predominate in platelet which utilizes arachidonic acid to produce PGH₂, which is further converted to TXA₂. The anti-platelet effect of aspirin is exerted by the irreversible inhibition of the COX-1 enzyme, inhibiting the production of PPG₂. The irreversibility is caused by the acetylation of strategic serine residues (Ser 529 in COX-1 and Ser 516 in COX-2) of COX- channel causing prevention of substrate access to the catalytic site of the enzyme. Anucleated platelets are unable to resynthesize the enzyme and thus depend on platelet turnover for its expression [Patrono et al., 2004].

Aspirin inhibits COX-1 in 1/100 to 1/50 concentrations compared to COX-2 [Cipollone et al., 1997]. Thus, smaller doses of aspirin are sufficient to inhibit COX-1, while inhibition of COX-2 is left incomplete. Platelet inhibition is not limited to COX-1 inhibition, enhancement of fibrinolysis, suppression of plasma coagulation and anti-inflammatory effects have been reported [Patrono et al., 2004]. Enhancement of fibrinolysis is caused by N-acetylation of lysyl residues of fibrinogen by aspirin (dose > 650mg twice daily) [Bjornsson et al., 1989]. Furthermore, aspirin has also been reported to inhibit platelet aggregation through the increase of NO in platelets resulting in the inhibition of thrombus formation in vivo [Karmohapatra et al., 2007 a]. Anti-inflammatory effects of aspirin are not only due to inhibition of COX-2 activity but aspirin also modifies the interaction between platelets and either neutrophils or erythrocytes, protects endothelial cells from oxidative
stress and improves endothelial dysfunction [Lopez-Farre et al., 1995; Podhaisky et al., 1997; Husain et al., 1998; Patrono et al., 2004].

**Uses of aspirin**

Aspirin has been evaluated in six primary prevention trials (US PHYSICIANS, Primary prevention project, Hypertension optimal treatment, UK Doctors, Thrombosis prevention trial and Swedish angina pectoris aspirin trial) including healthy, hypertensive, high-risk and stable angina patients. These trials showed that the level of cardiovascular risk is the major determinant of the absolute benefit of aspirin therapy, and aspirin use can be recommended only if a patient’s risk for coronary events is above 1.5% per year [Sanmuganathan et al., 2001; Patrono et al., 2004]. Risk reduction of 20-25% in adverse events has been shown with long term aspirin use in patients with previous atherothrombotic events or in other high risk categories (ATC, 2002). These results were obtained from a number of different anti-platelet agents combined, but aspirin was the drug most commonly studied and there was no clear evidence of differences between aspirin and other antiplatelet drugs (ATC, 2002).

The anti-thrombotic efficacy of a single dose of aspirin (162.5) at the time of acute coronary symptoms was proved by the ISIS-2 (International Study of Infarct Survival) which showed that aspirin was capable of preventing further atherothrombotic events and at the same time the treatment was shown to be safe. Proportional risk reduction of adverse events was 30%. The aspirin efficacy in acute stroke has also been established with a risk reduction of approximately 11% (ATC, 2002). In meta-analysis of total 40, 821 patients with acute stroke, there was a significant reduction of new strokes as well as cardiovascular deaths.

**Aspirin activated nitric oxide synthase (AANOS)**

The impressive effects of aspirin both for its preventive and curative properties in ACS are commonly believed to be consequence of the inhibition of platelet COX [Hamberg et al., 1974; Vane, 1971] through the acetylation of the enzyme [Roth & Majerus, 1975] resulting in the
inhibition of the synthesis of proaggregatory prostaglandins that inhibited platelet aggregation. This inhibition of platelet aggregation resulted in the inhibition of the thrombus formation which is reported to be critically important in the development of ACS [Kalgutkar et al., 1998]. However, there are several major drawbacks to explain the observed multifaceted antithrombotic effects of aspirin in ACS on the basis of inhibition of platelet COX. Furthermore, it has been reported that the treatment of platelet rich plasma (PRP) or washed platelet suspension in Tyrode’s buffer with aspirin resulted in the synthesis of NO [Chakraborty et al., 2003]. It has also been reported that the treatment of CaCl_2 induced clotted PRP, but not platelet free plasma, with aspirin in vitro resulted in the dissolution of the clot with the appearance of fibrin fibrinogen degraded products in the in vitro assay mixture [Karmohapatra et al., 2007a]. On the other hand, NO itself is reported to dissolve the formed PRP clot [Karmohapatra et al., 2007a]. The addition of l-NAME which inhibits NO production resulted in the inhibition of aspirin induced fibrinolysis.

Mechanistically, the inhibition of COX by aspirin would explain the inhibition of thrombus formation due to the inhibition of the enzyme would fail to explain the thrombolytic effect of the compound [Karmohapatra et al., 2007b]. In contrast, this thrombolytic effect of aspirin could be explained by the presence of an enzyme, NOS in platelet membrane. This enzyme has been purified to homogeneity, and was found to be a single polypeptide chain of Mr. 19kDa [Karmohapatra et al., 2007a], and could be activated by aspirin in the presence of Ca^{2+}. Direct acetylation of the enzyme by acetic anhydride also resulted in the activation of the NOS. The aspirin activated NOS was reported to be present not only in platelet but the enzyme was also reported to be present in erythrocytes, and endothelial cells [Chakraborty et al., 2003; Monobe et al., 2001]. However, it is not known whether the aspirin activated NOS in platelet was identical to the enzyme present in other cells.
ALPHA 1 ANTITRYPSIN

The protease inhibitor activity of human plasma was first discovered by Fermi and Pernossi in 1894 [Fermi et al., 1894]. However, the isolation and characterization of individual proteins have only emerged much later with the availability of new techniques. Ultimately, the so-called serum trypsin inhibitor responsible for antiprotease activity was first isolated in 1955 by Schultze and named α-1 antitrypsin (AAT) because of its occurrence in the α-1globulin fraction and its ability to inhibit trypsin [Schultze et al., 1955] (Figure 19). α-1 Antitrypsin (AAT), also referred to as alpha-proteinase inhibitor or SERPINA1, and is the prototypical member of the SERPIN (an acronym for serine proteinase inhibitor) family of protease inhibitors.

![Figure 19: Crystal structure of α 1-Antitrypsin complex with citrate](image)

AAT synthesis and regulation

AAT is mainly produced (70-80%) by liver cells, but it is also synthesized by monocytes, macrophages, pulmonary alveolar cells, and by intestinal and corneal epithelium. The normal plasma concentration of AAT ranging from 0.9 to 1.75 g/L and the protein is cleared with a half-life of 3-5 days [Kalsheker et al., 2002]. AAT is also present in saliva, tears, milk, semen, urine and bile [Chowandisai & Lonnerdal, 2002; Berman et al., 1973; Eden, 2010; Huang, 2004; Janciauskiene et al., 1996]. As an acute-phase reactant, plasma AAT levels increase rapidly (3-4 fold) in response to inflammation or infection. The concentration of AAT in plasma also increases during oral contraceptive therapy and pregnancy. During an inflammatory response, tissue
concentrations of AAT may increase as much as 11-fold as a result of local synthesis by resident or invading inflammatory cells [Janciauskiene, 2001; Boskovic & Twining, 1997].

**Protease inhibitory activity of AAT**

Until recently it was thought that inhibition of neutrophil elastase and proteinase 3 (PR3) is the primary function of AAT [Gooptu & Lomas, 2009]. Current studies demonstrate that AAT is an irreversible inhibitor for kallikreins 7 and 14 [Luo & Jiang, 2006], and that AAT also inhibits intracellular and cell-surface proteases. Matriptase, a cell surface serine protease involved in the activation of epithelial sodium channels, is one such protease. Findings that AAT inactivates the catalytic domain of matriptase *in vitro* and inhibits epithelial sodium transport both *in vitro* and *in vivo* [Tseng et al., 2008; Janciauskiene et al., 2008; Lazrak et al., 2009] support the notion that the inhibition of matriptase by AAT may offer a pharmacologic target for improving mucociliary clearance in both chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Several studies have shown that AAT inhibits the activity of caspase-3, an intra-cellular cysteine protease which plays an essential role in cell apoptosis [Petrache et al., 2006; Zhang et al., 2007]. A recent study by Bergin and collaborators, provides new evidence that AAT modulates neutrophil chemotaxis in response to soluble immune complexes by inhibiting ADAM-17 activity, also called TACE (tumor necrosis factor-a-converting enzyme) [Bergin et al., 2010].

**Other biological activities of AAT**

AAT has been reported to play an immunoregulatory role [Arora et al., 1978; Dabbagh et al., 2001], to inhibit neutrophil superoxide production, to enhance insulin-induced mitogenesis in various cell lines, and to induce a interleukin 1 (IL-1) receptor antagonist expression. *In vivo*, AAT has been shown to protect against tumor necrosis factor (TNFα) or endotoxin-induced lethality [Libert et al., 1996; Churg 2001]. Findings that AAT enhances the synthesis of both transferrin receptor and ferritin revealed a role of AAT in iron metabolism [Graziadei et al., 1997]. AAT has also been found to bind to the secreted enteropathogenic Escherichia coli proteins (EspB, EspD), thereby
reducing their hemolysis of red blood cells [Knappstein et al., 2004]. An interaction between AAT and Cryptosporidium parvum [Forney et al., 1996], a protozoan parasite, has been shown to inhibit Cryptosporidium parvum infection, suggesting a potential role for AAT in cryptosporidiosis. Aerosolized AAT also suppressed bacterial proliferation in a rat model of chronic Pseudomonas aeruginosa lung infection and in patients with cystic fibrosis [Griese et al., 2007]. It has recently been demonstrated that a specific 20-residue fragment of AAT (C-terminal peptide, residues 377-396, referred to as VIRIP) binds to the gp41 fusion peptide of HIV-1 and prevents the virus from entering target cells, thereby inhibiting HIV-1 infection [Munch et al., 2007]. These findings suggest that AAT may play a protective role in HIV-1-infected individuals [Forssmann et al., 2010].

In vitro, AAT was demonstrated to inhibit endotoxin stimulated TNFα and to enhance IL-10 expression in human monocytes, mediated by signaling through the cAMP dependent protein kinase A pathway which is targeted by number of anti-inflammatory drugs used and/or currently under development for COPD [Janciauskiene et al., 2007]. AAT also expresses a broad anti-inflammatory profile in gene expression studies on primary human lung microvascular endothelial cells, including the suppression of self-induced TNFα expression. Animal studies provide further evidence that AAT therapy prolongs islet graft survival in transplanted allogenic diabetic mice [Koulmanda et al., 2008; Lewis et al., 2008]. Current findings show that AAT stimulates insulin secretion and protects β-cells against cytokine induced apoptosis, and these effects of AAT also seem to be mediated through the cAMP pathway. In view of these novel findings, it is suggested that AAT may represent an anti-inflammatory compound to protect β-cells under immunological attack in type 1 diabetes and also therapeutic strategy to potentiate insulin secretion in type 2 diabetes [Kalis et al., 2010].

**Discovery of α1-antitrypsin deficiency (AATD)**

In 1952, C-B Laurell at Malmo University Hospital made outstanding contributions to protein research by introducing plasma protein electrophoresis as a tool for clinical investigations. By using electrophoresis, clinicians in respiratory medicine in Malmo unexpectedly noted the absence of the alpha1 band in two patients, both of whom suffered from severe respiratory insufficiency caused by
emphysema. This observation, published in 1963 by C-B Laurell and S Eriksson, established that AAT deficiency (AATD) is inherited and linked to emphysema (Figure 20).

**Figure 20:** α-1 Antitrypsin deficiency (AATD) is an inherited disorder affecting mainly the liver and lung.

Today it is well established that the Z, and also Siiyama (Ser53Phe) and Mmalton (52Phe del) variants of AAT form polymers and retain in the endoplasmic reticulum of the hepatocytes. The retention of AAT within hepatocytes results in protein overload that in turn is associated with juvenile hepatitis, cirrhosis, and hepatocellular carcinoma. Individuals with AATD have about 90% reduced levels of serum AAT and are at greatly increased risk for early-onset COPD, especially if they are cigarette smokers.

In the following years, several research groups in Europe and the USA demonstrated that AAT is an effective inhibitor of neutrophil elastase, thereby implicating elastase in the pathogenesis of
emphysema. Based on these developments, it was generally accepted that the protease-antiprotease balance is an essential requirement for respiratory health [Eriksson, 1996].

**Disease manifestations other than the lung and liver**

Diseases other than lung and liver diseases have been associated with AATD, but their association with AATD is less clear. Indeed, the multiple protective role(s) of AAT described above suggest that the deficiency of that protein may worsen the pathogenesis of other diseases rather than causing them.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Panniculitis</td>
<td>Valverde et al., 2008; Lyon, 2010</td>
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<tr>
<td>ANCA-positive vasculitis</td>
<td>Ranes &amp; Stoller, 2005; Esnault, 1997</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Teh et al., 1985; Breit et al., 1985</td>
</tr>
<tr>
<td>Chronic rhinosinusitis</td>
<td>Kitty &amp; Desrosiers, 2008; Maune et al., 1995</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>Needham &amp; Stockle, 2004; Witt et al., 2002; Rabassa et al., 1995</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Gambichler et al., 2006; Folwaczny et al., 1998</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>Pons et al., 1986; Klasen et al., 1980</td>
</tr>
<tr>
<td>Peptic ulcer disease</td>
<td>Elzouki et al., 2000; Andre et al., 1974</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Sandstrom et al., 2008; Lisowska-Myjak et al., 2006</td>
</tr>
<tr>
<td>Renal disease</td>
<td>Ting et al., 2008; Dieriks et al., 2007; Szonyi et al., 2004</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td>Ablin et al., 2009; Blanco et al., 2010</td>
</tr>
<tr>
<td>Idiopathic granulomatous mastitis</td>
<td>Schelsofot et al., 2001</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>Frederick et al., 1990</td>
</tr>
<tr>
<td>Aneurisms</td>
<td>Elzouki et al., 1999; Elzouki et al., 1994; Pezzini et al., 2004</td>
</tr>
<tr>
<td>Coronary atherosclerosis</td>
<td>Talmud et al., 2003</td>
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<tr>
<td>Cancer</td>
<td>Yang et al., 2009; Li et al., 2010; Topic et al., 2010; Lindor et al., 2010</td>
</tr>
</tbody>
</table>

*Table 1: Disease manifestations in AATD other than lung and liver.*