INTRODUCTION

Coronary Artery Disease.

Coronary artery disease (CAD) more commonly known as heart attack is a sudden, common and a major killer condition of human race [Klein. 2001. American Heart Association-Heart and Stroke facts, 2002]. It has been estimated that 65 % of all population in the world will finally die of heart attack (Fig.A). The major victims of heart attack are middle aged male people between the age of 50-60. The center of the circulatory system is the heart, which is the main pumping mechanism. Deoxygenated blood is collected by capillaries and through superior and inferior vena cava is supplied to the right chambers of the heart from where oxygenated blood in the lungs is finally pumped out by the left chambers to the coronary artery (the largest artery of the body), and from there the blood brings oxygen to all the cells of the body through the network of every small arteries and capillaries. The used blood from the body is returned to the heart through the network of veins. Through this circulation or supply of the blood to the heart walls the heart can contract and relax. Thrombus or platelet micro aggregates [Colman, Walsh, 1987. Furman, et al, 1998. Massberg, et al, 2003] in the fibrin mass formed on the site of injury on the coronary artery wall is mainly caused due to rupture or fissure of existing atherosclerotic plaques (Fig. B, Fig. C) [Falk, 1992. Fuster, et al, 1996] thereby causing the disruption of normal circulation in the vessel. The interruption of normal circulation almost immediately causes extreme pain in the heart [Everts, et al, 1996], nausea, vomiting [Goldberg, et al, 1998] and dyspnea that results in the development of CAD [Milner, et al, 1999. Zuker, et al, 1997] Cardiovascular disease mainly comprising of acute CAD leads to myocardial infarction, similar atherosclerotic plaque rupture in the artery, in the brain and the 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 (Fig.A). Antman, Braunwald, 1998].

Atherosclerosis of the coronary artery commonly causes angina pectoris and myocardial infarction [Antman, Braunwald, 1998. Fuster, et al, 1996]. Myocardial infarction generally occurs when there is an abrupt decrease in coronary artery blood flow following a thrombotic occlusion of a coronary artery previously narrowed by
atherosclerosis (Fig. C) [Antman, Baraunwald, 1998. Fuster, et al, 1996], slowly developing high grade. Coronary artery stenosis usually does not precipitate acute infarction because of the collateral network which develops over time. When a coronary artery thrombus develops rapidly at the site of vascular injury, this injury is produced or 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 condition favors thrombogenesis. In other words mural thrombus forms at the site of rupture and lead 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]. The complex atherosclerotic lesion, thus the arrival of smooth muscle cells and their collaboration of extracellular matrix is probably a critical transition, yielding a fibro-fatty lesion in place of a simple accumulation of macrophage derived foam cells. The histologic studies have demonstrated that the coronary plaque are prone to rupture, particularly those are rich lipid core with a thin fibrous cap. However the principal factor in the pathogenesis of coronary artery disease leading AIHD is the formation of atheromatous plaque in the coronary artery. The mechanism of the plaque rupture [Fuster, et al, 1996] is not yet fully understood, but may be due to the change in pattern of coronary blood flow (turbulence) resulting in the platelet aggregation (Fig. A, Fig. B, Fig. C) occurring on site initiated by a variety of agonists including [Colman, Walsh, 1987. Furman, et al, 1998. Massberg, et al, 2003] collagen, ADP, $\alpha$-epinephrine. Thrombin promotes platelet activation which is critically important for the development of thrombus in the coronary artery wall leading to thrombosis, AIHD disease, and other thrombotic diseases (Venus thrombo embolism, deep vein thrombosis).

Following the activation of platelets there is the production and release of thromboxane A2, a compound capable of inducing vasoconstriction [Roth, Majerus, 1975. Kalugutkar, et al, 1998], further platelet activation, and potential resistance to thrombolysis. In addition to generation of thromboxaneA2, activation of platelets by agonist promotes conformational changes in the glycoprotein IIb-IIa receptor [Furman, et al, 1998] once converted to its functional state, these receptor develop high affinity for the sequence of
glycine, aspartic acid on the fibrinogen α-chain and also for a dodecapeptide sequence on the fibrinogen γ-chain [Antman, Braunwald, 1998]. Since fibrinogen is a multivalent molecule, which can bind to two different platelets simultaneously, resulting in platelet cross-linking and aggregation [Falk, 1992. Fuster, et al, 1996].

![Heart Attack Image]

Figure – A: Coronary artery disease (heart attack)


Figure – B: Plaque buildup, formation of clot, coronary arterial blockage.
Blood platelets:

**Morphology and function:**

Platelets are small (~ 2 μm in diameter), anucleated cell fragments first described by Italian researcher Bizozzerro at the end of 19th century [Jurasz, et al, 2000]. They are formed in the bone marrow from megakaryocytes, which are extremely large cells of the hemopoitic 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 concentration of platelets in the blood is between 150,000-300,000/μl. Gross features of platelets can be observed using phase contrast microscopy ex vivo. Their development is controlled mainly by thrombopoietin, but also many cytokines and hormones [Kauhansky 1995]. Platelets circulate for approximately 10 days, before removal by macrophages [George 2000]. The main functions of platelets include normal hemostasis as well as vessel constriction and
repair. Platelets also participate in pathophysiological processes such as thrombosis, bleeding, inflammation, tumor growth and promotion of atherosclerosis [Ruggeri 2002]. There are three major structural zones of the platelet, each related to a specific aspect of platelet function. The peripheral zone is involved primarily in adhesion, the sol-gel zone in contraction, and the organelle zone in secretion.

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

Figure: D, activated and normal platelets.

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].

The components of the peripheral zone in immediate contact with surrounding plasma is the exterior coat. Chemical substances making up the coat could be easily extracted by
method formerly used to repair platelets for study in the electron microscope, but
improved fixation and cytochemical techniques have permitted visualization of the
platelet exterior coat. Coat material is 150 to 200 Å in thickness and covers the unit
membranes of the cell surface and linings of the tortuous canalicular system penetrating
the platelet substance.
The middle layer of the platelet peripheral zone is a typical trilaminar membrane and is
essential for the integrity of the cell. Surface-active agents, antihistamines, local
anesthetics, chelating agents, high and low salt concentration, and lipid solvent injure
the membrane and damage the cell. The changes are characterized by alteration in
surface contour or by increased permeability with resultant swelling of the platelets.
The area immediately under the unit membrane represents a transition between the
peripheral zone and the sol-gel matrix of the hyaloplasm. Because its structural
elements appear closely associated with the changes in the cell surface, the
submembrane area is considered to be a part of the peripheral zone. Fine fragments are
evident in the submembrane area of peripheral circumferential band of microtubules.

2. Sol-gel zone:
In studies with the light microscope, the interior of platelets appear structure less,
except for a few granules and is thus called the hyaloplasm. However, when examined
by electron microscopy, the interior is found to be composed of masses of fibrous
elements. An annular bundle of 250Å microtubules lying under the cell wall along 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. The granules, dense bodies, and mitochondria deserve special
comments. In addition, single glycogen particles are evident throughout the matrix, and
a compact mass of glycogen not bounded by a membrane is commonly observed.
Granules are usually oval or round, but variations in form are common. Each granule is
enclosed by a unit membrane. The granules are rich in phospholipids and contain
hydrolytic enzymes, including acid phosphatase, β-glucuronidase, and cathepsin.
Platelet fibrinogen, thrombosthenin, ATP ase, ATP, ADP, and serotonin have also been
1966].
The dense bodies of platelets are relatively few in number but appear to be very important in hemostatic function. Most, if not all, opaque organelles in human platelets originate from granules [White. 1968]. The transformation of granules to dense bodies is directly related to the uptake of serotonin. Dense bodies rapidly decrease in number during early viscous metamorphosis and are absent from platelets in late viscous metamorphosis and clot retraction. Serotonin, ADP, catecholamines, and platelet factor 4 have been associated with dense bodies [Holmsen, et al, 1969].

The mitochondria of platelets are simple in structure and few in number. They contribute significantly to the metabolic pool of ATP, for blockage of enzymatic glycolysis alone and do not affect the level of platelet ATP or energy requiring functions of the cell. In addition to their metabolic activity, platelet mitochondria may function as calcium repositories similar to mitochondria of smooth muscle.

**Composition of human platelets:**

*Human platelets (Fig. D) contain most of the common cellular constituents except DNA. Platelets are a heterogeneous population of cytoplasmic fragments ranging from > 5 to < 12 µm³ in volume, with an average volume of 5 to 7.5µm³. Glycogen, adenine nucleotides, protein, amino acids, and orthophosphate are all distributed heterogeneously among the platelets. This heterogeneity of volume and composition is probably a reflection of variations in platelet production as well as platelet age. The larger platelet is a heavier and relatively younger platelet, particularly under stress conditions; the smaller platelet is a lighter and probably older platelet. If a total (heterogenous) platelet population is employed, there is 119 mg protein or 0.78 X 10¹¹ platelets per gm wet weight or per milliliter packed platelets [Karpatkin. 1969]. Carbohydrates represent 1.9 % of the platelet wet weight, or 8.4 % of dry weight. There is no free glucose detectable in washed platelets [Karpatkin, Siskind. 1967]. Platelet hexosamine is distributed 75% in glycoprotein and 25 % in mucopolysaccharide. Glucosamine makes up the major hexosamine component of platelet glycoprotein, whereas galactosamine makes up 96 % of the amino sugar of mucopolysaccharides. At least 10 glycosidases have been identified and partially purified. The sialic acid of platelets consists predominantly of N-acetyl neuraminic acid. Proteins are the major constituents of platelet representing 12% wet weight and 52% dry weight. Starch -gel electrophoresis separates up to 15 protein fractions. Polyribosomes have been separated...*
on sucrose gradients and shown to be increased in heavy platelets. At least 15% of total platelet protein is composed of a contractile Ca\(^{2+}\)-Mg\(^{2+}\) dependent ATPase, thrombosthenin, which is similar in many respects to muscle actomyosin. The contractile protein seems to be composed of multiple polypeptide subunits, possibly polymeric in nature. Platelet myosin (thrombosthenin M) has been isolated as a dimer of molecular weight 550,000 with calcium ions ATPase activity, and as a monomer of molecular weight 200,000. A growth factor capable of stimulating the proliferation of arterial smooth muscle cells has been isolated from platelets (platelet derived growth factor). It is a high molecular weight protein, which is stable at 56°C and labile to pepsin treatment. The free amino acid pool in platelets has been analyzed and contains glutamic acid, aspartic acid, serine and glycine. Cystine, histidine, and methionine are present in trace amounts. Lipids make up 2.98% of the platelet wet weight and 14% of the dry weight. Phospholipid makes up 76% of the total lipid, neutral lipid 20% and lipoprotein 4%. The total phospholipid consists primarily of phosphatidyl choline, phosphatidyl ethanolamine, and sphingomyelin. Smaller amounts of phosphatidyl serine, phosphatidyl inositol, lyssolecithin, phosphatidic acid, and cardiolipin are also present [Karpatkin. 1972].

Platelets contain nucleotides of the purine bases adenine, guanine and hypoxanthine and of the pyrimidine bases uracil and cytosine. The ATP/ADP ratio of the storage pool is 0.8, with 80% of total ADP in the storage dense granules. Enzymes for the nucleotide “salvage pathway”, wherein extracellular hypoxanthine can be converted to AMP, are present. Platelets are capable of maintaining cationic gradients with surrounding plasma. Thus the intracellular Na\(^+\) and K\(^+\) contents of platelets are, respectively, 38.8 and 118 meq per liter of intracellular water. Intracellular K\(^+\) appears to be distributed in two compartments. The pumping of this cationic gradient requires energy (ATP), which is probably modulated via a Na\(^+\)-K\(^+\)-stimulated ATPase on the platelet membrane. This ATPase is inhibited by 10\(^{-4}\) to 10\(^{-5}\) M ouabain as well as by sulfhydryl inhibitors [Cooley, Cohen. 1967]. The powerful smooth muscle vasoconstrictor substance serotonin (5-hydroxy tryptamine) is normally present in platelets but absent in plasma. Following coagulation or platelet aggregation, 20-25% of total platelet serotonin is released into the serum [Zucker. 1959]. Platelets acquire serotonin from cells secreting this substance and concentrate it by an active transport mechanism requiring energy and extracellular Na\(^+\), K\(^+\), Cl\(^-\). Platelet membranes may be different in origin from plasma membrane of
other cells in that they are derived from endoplasmic vesicles, possibly endoplasmic reticulum of megakaryocytes [Yamada. 1957].

**Platelet activation**

As platelets are activated they change from their normal disc shape to a sphere with long dendritic extensions. The shape change is brought about by actin and myosin in platelet cytoplasm [George 2000]. During the shape change secretory granules are organized into the center of the platelet. Platelets have three types of secretory granules; dense granules, α-granules and lysosomes. The contents of secretory granules are either produced by platelets and megakaryocytes or acquired from plasma via endocytosis and pinocytosis facilitated by the canalicular system. Secretory granules release their contents into plasma during platelet activation, which enhances further activation. The granule contents also have procoagulant activities [Rendy & Brohard-Bohn 2001].

*Figure: E, platelet adhesion and activation.*
Platelet adhesion and aggregation

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, $\alpha$-epinephrine, collagen on thrombin binds 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 (Fig. E, Fig. F) [Jin, et al, 2002].

Among the platelet aggregating agonists, $\alpha$-epinephrine was found to be a weak aggregating agent when compared to other agonists. The catecholamine aggregates, platelets only at supraphysiologic concentration (> 2 µM) through its interaction with $\alpha_2$ adenergic receptors on the platelet surface [Lalau Keraly, et al, 1987.Grant & Scrutton, 1979]. Although physiologic and supraphysiologic amounts of $\alpha$-epinephrine fail to aggregate human blood platelets, the activation of $\alpha_2$ adenergic receptors on the platelet surface, by these amounts of $\alpha$-epinephrine has nevertheless, a profound effect on the aggregation of platelets induced by more potent aggregating agents. It has been noted that while the sub optimal amounts of ADP failed to aggregate platelets, ADP will nevertheless aggregate platelets at sub optimal concentrations in the presence of physiologic or sub - physiologic amounts of $\alpha$-epinephrine .The potentiation of platelet aggregation by $\alpha$-epinephrine at sub physiologic level in vivo might have important consequence in the development of AIHD. Emotional stress, which has been reported to be a major provocative factor for the AIHD [Sheps, et al, 2002], accompanies the increase of $\alpha$-epinephrine “the stress hormone” in the system and as such the catecholamine might itself be responsible for the precipitation of the condition under emotional stress.

Platelet activation (Fig. D, Fig. E, Fig. F) is induced at the site of vessel wall injury, which is represented by rupture of an atherosclerotic plaque. As the vessel wall is injured sub-endothelial collagen and other platelet activating factors such as von Willebrand factor (vWF) are exposed. Initially plasma vWF binds to exposed collagen. The first contact between platelet receptors and matrix components depends to a large extent on the shear stress at the site.
of injury. In conditions of low shear stress matrix proteins cause activation at the injury site. In conditions of high shear stress, as in arteries, activation and adhesion depend largely on glycoprotein (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, which has become available via activation of rolling platelets. Firm attachment mediated by GP Ia/IIa also allows low-affinity GP VI to interact with collagen.

Interactions of platelet GP VI with collagen induce further collagen-dependent activation of platelets. However, recent studies have suggested that the differential roles of GP Ia/IIa and GP VI are not this simple, but are in fact modulated by changes at the extracellular matrix of the vessel wall induced by specific metalloproteinases, and that both of these receptors participate in adhesion and aggregation alike [Ruggeri 2002, Farndale et al 2004]. The initial adhesion is followed by recruitment of additional platelets into the growing platelet plug. Platelets are activated by factors at the injury.
site, but more importantly further activation is mediated by agonists released from the secretory granules of previously activated platelets (Fig. D, Fig. E). Adenosine-diphosphate (ADP) and thromboxane (Tx) A2 are crucial secondary mediators of platelet activation. As platelets are activated GP IIb/IIIa-receptors undergo conformational changes to become active. Activated GP IIb/IIIa mediates 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 signalling through cell surface integrins and tyrosine kinases of receptors have central roles in this phase of thrombus formation. Platelets also participate in localization, amplification and maintenance of the coagulant response at the injury site [Ilveskero et al 2001. Michelson 2003].

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, antithrombotic 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 vasodilatation and NTPDase neutralizes the prothrombotic release of platelets by metabolism of ADP [Marcus et al 2005].

Coagulation

Coagulation of blood has classically been described as a cascade (Fig. G) dependent on adequate levels of coagulation proteins. However, recent advances in research have suggested a new model of coagulation as a cell-regulated overlapping process. Platelets support procoagulant reactions and vascular endothelial cells maintain anticoagulant properties of the vasculature. 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 antithrombin act as anticoagulants at the site of injury, reventing excessive formation of thrombi [Monroe & Hoffman 2005].
Platelet receptors

Collagen receptors
Several glycoprotein receptors are present at the platelet surface. A number of these have been proposed as collagen receptors, but at present GP Ia/IIa and GP VI are believed to be the main ones responsible for collagen induced platelet activation. Fibrillar collagen of types I and III are the major constituents of the blood vessel wall, although type IV is also present in basement membranes [Tiainen, 2008]. These collagen types are also the most able to activate platelets and to cause adhesion and aggregation [Sixma et al 1997].
Extrinsic pathway Intrinsic pathway

The GP Ia/IIa receptor, integrin α2β1, causes initial platelet adhesion to the subendothelial matrix. It requires activation by thrombin-, collagen- or ADP-pathways to become operational. 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]. While the activation with ADP has been suggested independent of Ca^{2+} the activation by collagen, especially collagen monomers, is dependent on physiological concentrations of Mg^{2+} and Ca^{2+} [Siljander & Lassila 1999. Jung & Moroi 2001]. The role of GP Ia/IIa in platelet adhesion on collagen is emphasized in environments of high shear. Monomeric collagen provides an important tool for studying the role of GP Ia/IIa as at physiological cation concentrations it is responsible for platelet adhesion [Siljander & Lassila 1999]. In comparison, the second collagen receptor GP VI is reported to adhere only to fibrillar collagen [Jung & Moroi 1998]. Indeed, modulation of the collagens revealed at an injury site is believed to mediate variable roles of GP Ia/IIa and GP VI in adhesion to collagen. Variation in GP Ia/IIa activity has been shown to have clinical importance, and has been associated with the C807T polymorphism of the GP Ia/IIa gene [Kunicki et al 1993, Kritzik et al 1998]. In addition, the C807T polymorphism has been associated with increased risk for atherothrombotic events [Carlsson et al 1999. Moshfegh et al 1999]. However, regarding the role of these polymorphisms as predictors of atherothrombotic disease the multifactorial nature of the disease needs to be taken into account. GP VI participates in initial platelet adhesion and activation in circulating blood in addition to the GP Ib-IX-V complex [Andrews & Berndt 2004]. In fact, it seems to be topographically associated with GP Ib-IX-V [Farndale et al 2004]. Patients deficient in GP VI lack the ability to form thrombi on a collagen surface under flow conditions, but clinically present with only mild bleeding tendencies [Moroi & Jung 2004]. However, the T13254C polymorphism of GP VI has been associated with myocardial infarction [Croft et al 2001]. The differential roles of GP Ia/IIa and GP VI in initial platelet adhesion and aggregation related to collagen have not yet been indisputably determined.

Adhesion receptors

In addition to collagen receptors, there are other adhesion receptors such as GP Ib-V-IX
complex, GP IIb/IIIa and receptors for fibronectin, vitronectin and thrombospondin. The GP Ib-V-IX complex causes tethering of platelets to perivascular vWF. This enables platelets to slow down at the point of vascular injury and to interact with collagen. GP Ib-V-IX is a complex of glycoproteins and the N-terminal globular domain of GP Ib is the major ligand-binding region. Patients lacking GP Ib or GP IX have Bernard-Soulier syndrome which causes bleeding diathesis [Andrews & Berndt 2004]. GP IIb/IIIa, integrin-α2β1, is the most abundant adhesion receptor. It requires a conformational change caused by platelet activation through adhesion receptors (GP Ib, GP VI, GP Ia/IIa), thrombin (protease activated receptors 1 and 4) or ADP (P2Y12, P2Y1, P2X1) receptors to become active. GP IIb/IIIa binds mainly fibrinogen, but also vWF, fibronectin, vitronectin and thrombospondin. As GP IIb/IIIa binds to vWF, especially in flow conditions, it offers a second mechanism of platelet adhesion to the vessel wall in addition to GP Ib-V-IX. The receptor mediates aggregation by binding fibrin and vWF. GP IIb/IIIa function is Ca2+-dependent. Patients with hereditary Glanzmann’s thrombasthenia have decreased or functionally abnormal GP IIb/IIIa which causes bleeding symptoms [Andrews & Berndt 2004].

Inhibition of platelet aggregation:
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 AIHD through the inhibition of thrombus formation [Fuster, et al, 1988. Steering Committee of Physicians Health study Research Group, 1989]. At present the inhibition of platelet aggregation is thought to be mediated through three major pathways
I. Inhibition of platelet cyclooxygenase- The inhibition of platelet cyclooxygenase results in the inhibition of synthesis of prostaglandin G2, which in turn leads to the inhibition of platelet aggregation [Hamberg, et al, 1975]. Various non-steroidal anti-inflammatory drugs including acetyl salicylic acid (aspirin), ibuprofen, indomethacin etc. are known to inhibit platelet aggregation 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 incidence of AIHD by numerous investigators [Steering Committee of Physicians Health study Research Group, 1989].

II. Increase of cellular cyclic AMP level. Increase of platelet cyclic AMP level either through the activation of membrane adenylate cyclase [Salzman & Levin, 1971. Haslam, 1971. Dutta-Roy & Sinha, 1987] or through the inhibition of cyclic AMP 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, increases platelet cyclic AMP 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, 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].

III. Nitric Oxide: Mononitrogen monoxide, more commonly known as nitric oxide (NO), believed to be the endothelial relaxing factor [Furchgott & Zwadzki, 1980], and is a potent inhibitor of platelet aggregation [Yao, et al, 1995. Coles, et al, 2002. Sinha, et al, 1998]. The compound is also a powerful vasodilator [Gruether, et al, 1979]. The vasodilatory property of nitric oxide could be important in the relaxation of vasoconstriction due to the release of thromboxane A2 produced during platelet aggregation on the arterial wall in AIHD. Currently it is believed that NO does not have any receptor on platelet surface and the compound is freely diffused into the cytosol.

Nitric oxide has been reported to increase both cAMP and cGMP level [Coles, et al, 2002. Sinha, et al, 1998. Gruether, et al, 1979]. Although the inhibition of platelet aggregation by NO was believed to be mediated through cellular cGMP level, the increase of cellular cAMP level by NO could be equally important in the inhibition of platelet aggregation [Gruether, et al, 1979]. The increase of platelet cGMP level is reported to be mediated through the inhibition of activation of cytosolic guanylate cyclase [Coles, et al, 2002]. Although NO does not activate membrane adenylate cyclase, it can increase platelet cyclic AMP level through specific inhibition of cytosolic low K_m cyclic AMP phosphodiesterase [Sinha, et al, 1998].

Acetyl salicylic acid, commonly known as aspirin, is known to inhibit platelet aggregation both in vivo and ex vivo conditions [Patrono, 1994] through the inhibition
of cyclooxygenase [Roth & Majerus, 1975] and by the stimulation of nitric oxide synthesis [Chakraborty, et al., 2003]. And as such, the compound has been reported to produce beneficial effects in the prevention of coronary artery disease [Steering Committee of Physicians Health study Research Group, 1989].

Aspirin

Mechanism of action

Aspirin was first synthesized in 1897 by German chemist Felix Hoffman and today it has an important position as an antiplatelet agent, among other indications. The antiplatelet effect of aspirin was first described by Morris in 1967. Aspirin exerts its effect by inhibiting the activity of prostaglandin (PG) H-synthase-1 and -2, also known as cyclooxygenase (COX)-1 and 2. COX-1 is known to predominate in platelets. Thus, in activated platelets COX-1 utilizes arachidonic acid (AA) to produce PGH2, which is further converted to TxA2, a potent vasoconstrictor activator, as well as other prostaglandins PGD2, PGE2, and PGF2α. The anti-platelet effect of aspirin is exerted by the irreversible inhibition of the COX-1 enzyme, inhibiting the production of PGG1. The irreversibility is caused by acetylation of strategic serine residues (Ser529 in COX-1 and Ser516 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 to1/50 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. COX-2, which is inducible, expressed mostly in leukocytes and connective tissue cells and constitutively in certain organs, is not markedly affected by small aspirin doses (50-325mg) administered once daily. Aspirin has been postulated to also have effects unrelated to TxA2 inhibition. Platelet inhibition not related 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) [Björnsson et al 1989]. Suppression of coagulation could be caused by vitamin K- counter effect of large doses of aspirin (>1500mg/d), inhibition of thrombin generation (dose >500mg) or acetylation of one or more clotting factors [Quick & Cleasceri 1960. Szczeklik et al 1996. Patrono et al
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 [López-Farré et al 1995. Podhaisky et al 1997. Husain et al 1998. Patrono et al 2004].

Pharmacokinetics
Aspirin is absorbed in the stomach and upper intestine. Normally peak plasma levels are reached within 30-40 min of aspirin uptake. However, when enterocoated aspirin is used the peak levels are reached between 3-4 h. Aspirin is rapidly cleared from the circulation; half-life of acetylsalicylic acid is 15-20 min. As the effects on Tx synthesis are irreversible they last for the life-span of platelets, gradually decaying if dosage is not repeated. As 10% of platelets are replaced every day, it has been estimated that after 5-6 days 50% of the platelets function normally.

Bleeding and other adverse effects
Bleeding complications are common in patients receiving aspirin therapy. The risk of major hemorrhage is approximately 1-2%. Aspirin-induced gastro-intestinal (GI) toxicity depends on the dose. GI toxicity is postulated to be caused by inhibition of PG synthesis and direct damage to gastric and intestinal mucosa, not by bleeding directly associated with TxA2 inhibition [Roth & Caverly 1994]. Thus, low-doses (100-300mg/d) used for inhibiting platelet activity are associated with a 2% risk of GI bleeding, which is similar to other antiplatelet agents.

Primary prevention
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].
Secondary prevention
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). In a large meta-analysis antiplatelet medication was associated with decreases in reinfarction, death and stroke in 18,788 patients with a history of myocardial infarction, 18,270 patients with a history of cerebrovascular events and 9,214 patients with peripheral arterial disease (ATC 2002). These results were obtained from a number of different antiplatelet 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).

Aspirin in acute atherothrombotic syndromes
The antithrombotic effect of aspirin has been well established. The ISIS-2 (International Study of Infarct Survival) study proved the efficacy of a single 162.5mg dose at the time of acute coronary symptoms in 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 altogether 40,821 patients with acute stroke there was a significant reduction of new strokes as well as cardiovascular deaths. Hemorrhagic and ischemic stroke were also separately assessed in almost all of these patients and antiplatelet medication seemed to increase hemorrhagic strokes by 1.9 per 1000 patients, but this was counter balanced with a reduction of ischemic strokes to 6.9 per 1000 patients [ATC 2002].

Aspirin in arterial fibrillation
Oral anticoagulation with warfarin is beneficial in reducing the risk of stroke in patients with atrial fibrillation [The Boston Area Anticoagulation Trial for Atrial Fibrillation Investigators 1990]. In comparison with warfarin, aspirin (75-325mg/d) was found to be significantly less effective in preventing stroke. However, aspirin caused a risk reduction of 25% and thus its use in patients unable to receive anticoagulation therapy is recommended [Patrono et al 2004].
Summary
Aspirin is beneficial in both acute atherothrombotic events and in the prevention of thrombosis in patients with stable atherothrombotic disease. The benefits of antithrombotic medication in these patients at the population level overcome the risks of adverse-effects. However, subgroups of patients who suffer from either thrombotic or bleeding complications exist.

Fibrinolysis and Thrombolysis.
Blood contains an enzymatic system called the fibrinolytic system (Fig. H), one of the main functions of which is the dissolution of fibrin clots in the blood vessels. The fibrinolitic system comprises a proenzyme, plasminogen, which can be converted to the active enzyme plasmin by different plasminogen activators (PAs).

**Human plasminogen** is single chain glycoprotein containing 791 amino acids residues and 2% carbohydrate. Its molecule of mass is about 92000 Da. The plasminogen molecule contain a total of 6 structural domains, each with different properties [Henkin et al, 1991. Ponting, 1992. Bachmann, In Hemostasis and Thrombosis; 1994]. The N terminal portion of the molecule consists of five kringle domains with the capacity to bind to fibrin. The kringle domain was first described by Magmussen et al, 1975 who compared the structure with Danish pastry. Together with the pre-activation peptide, the kringles control the ability of plasminogen to adopt different conformations. The protease domain resembles that of other serine proteases and contains the active site pocket His\textsuperscript{603}, Asp\textsuperscript{646} and Ser\textsuperscript{741}. These region also contains Ala\textsuperscript{601} which appears to be essential for the normal function of plasminogen, since mutation to Thr\textsuperscript{601} leads to an increased risk of thrombosis [Bachmann, In Hemostasis and Thrombosis; 1994].
Several forms of plasminogen in plasma are known and can be separated by affinity chromatography [Nieuwenhuizen, 1989]. The later form of plasminogen in plasma has glutamic acid at the N-terminal and its termed Glu-plasminogen. Others plasminogen forms generated by the catalytic cleavage by plasmin and containing mostly lysine at the N- terminal position, are termed Lys-plasminogen.

Glu-plasminogen exists in a close conformation that becomes extended when bound to lysine residues on a fibrin surface. A similar conformational change is believed to take place when Glu-plasminogen is converted to Lys-plasminogen. The physiological role of these conformational; changes is not well-known although the general effect is believed to be an increased plasminogen activation rate catalyzed by t-PA [Wu et al, 1990. Fredenburgh et al, 1992]. The opposite effect is observed in the presence of...
anions, in particular with Cl', which stabilises the closed form of Glu-plasminogen rendering plasminogen poorly activatable [Stack et al, 1991].

Plasmin: Activation of plasminogen by its natural activators, t-PA and u-PA, involved a bond cleavage at a specific site in the plasminogen molecule, which gave rise to a two chain molecule linked by two disulfide bonds. The plasminogen forms may degrade fibrinogen in a variety of ways resulting insoluble fibrin degradation products or fragments called X, Y, D and E. Plasmin is a relatively non-specific protease and can degrade not only fibrin but also many protein in both plasma and extra cellular spaces. In the coagulation pathway factor V, VIII and Von-Willebrand Factors are known target of plasmin. Plasmin activity is inhibited mainly by binding to the plasmin inhibitor, which forms a stable complex with plasmin devoid of proteolytic activity.

Two physiologic PA has been identified, initially based on their immunologic relationship with the PA found in tissues (tissue-type PA [t-PA]) or with PA found in urine (urokinase-type PA, [u-PA]). Inhibition of fibrinolysis occurs at the level of plasmin (mainly by α₂ antiplasmin). Physiologic fibrinolysis is highly fibrin-specific as a result of specific molecular interactions between PA, plasminogen, fibrin, plasmin, and α₂ antiplasmin [Collen, 1988]. Cardiovascular diseases, mainly comprising coronary artery disease leading to myocardial infarction, cerebrovascular disease causing strokes, and venus thrombosis predisposing to pulmonary embolism and post phlebitic syndrome, are major cause of death and disability. The triggering event in the clinical expression of the acute ischemic event is not the underlying atherosclerotic lesion, but a thrombotic obstruction of the artery. Thus the common cardiovascular diseases have, as their immediate underlying etiology, thrombosis of critically situated blood vessels with loss of blood flow to vital organs.

Approach to the treatment of thrombosis consist of the pharmacologic dissolution of the blood clot via the intravenous infusion of PAs. Currently, five thrombolytic agents are either approved for clinical use or under clinical investigation in patients with acute myocardial infarction. These agents are streptokinase (SK), two-chain u-PA (tcu-PA; urikinase), anisolated plasminogen streptokinase activator complex (APSAC), recombinant t-PA (rt-PA), and recombinant single-chain u-PA (rscu-PA; prourikinase), [Collen, 1988].
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 nitric oxide (NO) and cyclic GMP (cGMP) [Kaatsuki, et al, 1977]. Long before the first significant biological experiments with NO were conducted, the presence of cGMP in mammalian urine was reported, and this led to a widespread interest in the possible biological roles of this new cyclic nucleotide. In 1969, however, cGMP was detected in mammalian tissues and guanylate cyclase was first discovered and partially characterized. Guanylate cyclase catalyzes the conversion of guanosine triphosphate (GTP) to cGMP plus inorganic pyrophosphate in reaction that requires a divalent metal cation such as magnesium. Much of the early work involving cGMP suggested that it might function to mediate or signal cellular processes that are ultimately antagonistic or opposite in direction to those mediated by cAMP. In general, increase in cGMP production were associated with stimulation of cell function such as phagocytosis and discharge of lyposomal enzymes, whereas increases in cAMP production were associated with the inhibitions of these functions. Prior to the evidence that, cGMP might be involved in smooth muscle relaxation; cGMP was implicated in smooth muscle contraction [Linconn & Cornwell, 1991].
The unequivocal demonstration that nitrovasodilators elicit vascular smooth muscle relaxation via the actions of nitric oxide (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 muscle 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.

Nitric oxide and its chemistry:
Nitric oxide 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 & Vosper, 1977]. It becomes immediately evident from a Lewis dot depiction that -NO has one unpaired electron and thus is formally a free radical species.

\[
\begin{align*}
\text{:N=O:} & \quad \text{:N=O:} \\
1(\text{I}) & \quad 2(\text{II})
\end{align*}
\]

At room temperature and pressure, NO has little propensity to react with itself in a radical-radical dimerization process. At first glance, this may seem curious since dimerization would lead to a structure (ON-NO) whereby all atoms have a full compliment of eight valence electrons and therefore, would satisfy the octate rule. A partial rationale for the usually long and weak N-N bond in the -NO dimer may also be found in an examination of the nature of the unpaired electron in the -NO monomer.

**Free radical chemistry of NO:**

Nitric oxide reacts rapidly via simple radical-radical combination reactions with species processing unpaired electron such as -NO₂, O₂. The ability of -NO to “quench” other radical species also allows it to terminate radical chain reactions. A good example of this phenomenon is the effect of -NO has on the O₂ – dependent oxidation of lipids [Wink, et al, 1993. Hogg, et al, 1993. Rubbo, et al, 1994. Rubbo, et al, 1996. Struc, et al, 1995]. Due to the fact that many lipids contain “activated” allelic C-H bonds, they are susceptible to oxidative damage. Lipid peroxidation results from the net abstraction of an allelic hydrogen atom of the unsaturated fatty acid (Lipid-H) by an initiating radical species (X•) to generate a lipid radical (lipid)

\[
\text{Lipid-H + X•} \quad \rightarrow \quad \text{Lipid*-X-H}
\]

The lipid radical then reacts with O₂ to generate an alkylperoxy radical (Lipid - OO•) which can further react with another lipid to form another lipid radical that can also react with O₂. These two reactions are chain propagating steps.
Nitric oxide is known to limit lipid peroxidation by acting as a chain terminating species.

\[
\text{Lipid} - \cdot + \text{O}_2 \rightarrow \text{Lipid} - \text{OO} - \cdot \quad (3) \\
\text{Lipid} - \text{OO} - \cdot + \text{Lipid} - \text{H} \rightarrow \text{Lipid} - \text{OOH} + \text{Lipid} - \cdot \quad (4)
\]

Furthermore, \(-\text{NO}\) 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:**


a. **Direct:** The primary criterion for the significance of direct reactions in vivo is reaction rate. The reactions in which NO combines with biological substrates at sufficiently rapid rates to be of consequence involves either metals or radicals. Direct reactions between NO and thiols, are for 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 an 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, et 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 heme site [Khatsenko, et al, 1993. Stadler, et al, 1994]. Nitric oxide mediated inhibition of cytochrome P450 has some important pathophysiological squeal as well. Binding of NO to the heme domain of cytochrome P450 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 & Alan, 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 as 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=5X10⁷ M⁻¹ S⁻¹) [Eich, et al, 1996] is the primary endogenous mechanism by which NO diffusion and concentration are controlled [Doyle & Hoekstra, 1981. Feelisch, 1991. Lancaster Jr, 1994].

\[
\text{oxy Hb (Fe}^{2+} - \text{O}_2 \text{)} + \text{NO} \rightarrow \text{met Hb (Fe}^{3+} \text{)} + \text{NO}_3^\text{-} \tag{6}
\]


b. 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 N₂O₃, ONOO⁻, NO⁻ and NO₂ are chemically driven much the same as NO itself. Aerobic NO solutions
produce N₂O₃ and when exposed to GSH, form the nitrosative product S-nitrosoglutathione (GSNO) [Wink, et al, 1994]. Cobalamin, which forms a stable Co⁹⁺-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 nonheme 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 resonance (EPR) spectroscopy [Lancaster Jr & Hibbs Jr, 1990].

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

\[
\begin{align*}
N_2O_3 + RSH & \rightarrow RSNO + HNO_2 \\
RSNO + RSH & \rightarrow RSSR + NO^+ + H^+
\end{align*}
}\] (7) (8)

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 [Bonner & Pearsall, 1982].

c. **Mixed direct and indirect effects:** A primary cellular target for the cytotoxic action of NO is the mitochondria [Monacada, et al, 1991. Lancaster Jr & Hibbs Jr, 1990]. Nitric oxide 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 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, 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.**

Nitric oxide (NO•)-producing enzymes have been logically named nitric oxide synthases (NOS) [Fig. I], 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 [Braedt & Snyder, 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 nitric oxide 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, were seminal in further examination of the factors involved in signaling vasorelaxation [Furchgott & Zawadzki, 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 & Zawadzki, 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 NO₂⁻, which is 5 or 6 orders of magnitude less potent than NO as a vasodilator [Ignarro & Gruetter, 1980. Gruetter, et al, 1979]. In 1987, Palmer et al showed that NO, measured by chemiluniscence could account for the 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]. Many pharmacological properties of EDRF and authentic NO were similar, if not identical [Radomski, et al, 1987. Hutchinson, et al, 1987. Ignarro, et al, 1988]. On the basis of reactions in which NO reacts with oxyhemoglobin to form methemoglobin, EDRF released from cultured arterial endothelial cells was identified as NO [Kelm, et al, 1988]. The biological half life of EDNO under the conditions of bioassay is approximately 3-5 seconds, which is identical to that of authentic NO superfused over the vascular strips at concentrations (10-100nM) that elicit equivalent relaxant responses to those elicited by EDNO released from intact artery or vein and cultured aortic endothelial cells [Palmer, et al, 1987. Radomski, et al, 1987]. NO concentrations of 10-50nM have half-lives of 3-5 seconds whereas concentrations in excess of 300nM have biological half-lives of longer than 30 seconds. It has been reported that under
conditions when large amounts of EDNO are released from cultured endothelial cells, bovine pulmonary artery, or human umbilical vein, the half-life of EDNO is in excess of 30 seconds, which is similar for a pharmacologically equivalent concentration of authentic NO. It is also possible that the endothelium generates and releases both NO and a nitroso compound that is somewhat more chemically stable than NO itself. Stuehr and Marletta had demonstrated that murine macrophages synthesize NO\textsuperscript{3} and NO\textsuperscript{2} in response to *Escherichia coli* lipopolysaccharide [Stuehr & Maletta, 1985]. These observations were extended to include macrophages activated by *Mycobacterium bovis* BCG infection, lymphokines, or gamma interferon [Stuehr & Maletta, 1987].

Macrophage cell culture experiments revealed that an L-arginine-dependent biochemical pathway is involved in the biosynthesis of NO\textsuperscript{3} and L-citrulline and that this pathway (Fig. 1) was inhibited by N\textsuperscript{O}-monomethyl-L-arginine, 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 N\textsuperscript{O}-monomethyl-L-arginine [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\textsuperscript{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\textsuperscript{2} was, in turn, derived from NO [Hibbs, et al, 1988]. Moreover, NO was shown to be the actual cytotoxic activated macrophage effectors molecule. 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 in a manner that is inhibited by L-arginine analogs, 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 author as implicated its involvement in the formation of NO from L-arginine [Palmer & Moncada, 1989]. The soluble fraction catalyzed the conversion of L-arginine to
citrulline in an NADPH-dependent manner, and conversion was inhibited by N°-monomethyl-L-arginine. Thus this activity closely resembles the activity that is believed to convert L-arginine to NO. This similarity may be superficial, however, as Palmer and Moncada [Palmer & Moncada, 1989] was unable to demonstrate the concomitant formation of NO in the same reaction mixtures that formed citrulline from L-arginine. In cytotoxic activated macrophages the soluble fraction possessed enzymatic activity that catalyzed the conversion of L-arginine to NO plus citrulline [Maletta, et al, 1988]. This activity required NADPH and L-arginine; it was enhanced by Mg²⁺ and inhibited by N°-monomethyl L-arginine. Similar observations with activated macrophages were reported by Hibbs et al [Hibbs, et al, 1988]. Studies on activated macrophages, in that the concomitant formation of equal amounts of NO and citrulline from L-arginine in reactions involving the soluble fractions from aortic endothelial cells could not be demonstrated. Thus it is possible that the alleged NADPH-dependent monooxygenase in vascular endothelial cells is not present in the cytosolic or soluble fraction. It is possible that other citrulline-forming enzymes that do not generate NO as a second reaction product are present in the soluble fraction, whereas the monooxygenase that generates both NO and citrulline is present in the plasma membrane fraction. Studies on vascular endothelial cells should focus on a careful analysis of the sub cellular distribution of enzymatic activities capable of
concomitant generation of equal amounts of NO and citrulline from L-arginine. The distribution of this enzymatic activity may be different in vascular endothelium than in the macrophage. The high arginine concentration in endothelial cells is consistent with the findings that fresh vascular rings do not relax in response to L-arginine, whereas 24 hour incubated tolerant rings relax up to 100% [Ignarro & Gold, 1989]. Myers et al have used the chemiluminiscence procedure together with a bioassay to characterize the properties of EDRF released from bovine aortic endothelial cells [Myers, et al, 1989]. In contrast to the conclusions drawn by Palmer et al [Palmer, et al, 1987], these investigations argued that NO alone cannot account for the complete relaxant effects of EDRF. Additional reports have surfaced that provide indirect evidence for the possibility that EDRF is not entirely NO. Berkowitz and co-workers argue 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 & Berkowitz, 1987. Shikano, et al, 1987. 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 Snyder reported the isolation and purification of neuronal nitric oxide synthase (nNOS) [Bredt & Snyder, 1990]. In these studies they showed the requirement for 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]. These findings were to be portentous of mechanistic aspects of the NOS isoforms being similar to the interaction of NADPH-cytochrome P-450 reductase with a large number of heme-containing cytochromes P-450 in the endoplasmic reticulum of various mammalian organs.


**Insulin activated nitric oxide synthase:**

In 2000 for the first time Kahn et al demonstrated that insulin is capable of activating a membrane bound NOS in a wide variety of cells such as platelets, endothelial cells, liver, kidney, skeletal muscle cells. NO thus formed produces insulin-like effect in the system in the absence of the hormone itself [Kahn, et al, 2000]. This form of NOS could be activated by insulin in vivo and in vitro leading to the stimulation of NO synthesis. Although the presence of NOS in the soluble cytosolic fraction is well known, cytosolic NOS could not be stimulated by insulin, and the biochemical characteristics of the membrane bound insulin activated NOS is different from those of the cytosolic NOS, example for the synthesis of NO from L-arginine by the insulin-activated NOS, unlike the soluble cytosolic NOS, did not require the addition of ATP, NADPH in the reaction mixture although the addition of 1.5mM Ca²⁺ stimulated the formation of NO.

The IANOS, a constitutive membrane-bound enzyme was found to be a regulatory enzyme in the maintenance of homeostasis of plasma NO level. One of the interesting properties of IANOS was that the enzyme, could be activated by NO (the reaction product), representing a rather unusual case of feedback activation. The effect of NO on the IANOS however was biphasic in nature. At low concentration of NO (0.1-0.4 μM) IANOS was activated, but in the presence of higher concentrations of NO (>0.4 μM), the degree of stimulation was gradually decreased. The purified IANOS showed some properties similar to those of the insulin receptor itself [Bhattacharya, et al, 2001] in that not only could the insulin dependent activation of IANOS be blocked by anti-insulin receptor antibody but also the characteristics of the Scatchard plot of the hormone binding to the purified enzyme [Kahn & Sinha, 1990] and molecular weights of IANOS and its subunits were similar to those of the purified insulin receptor [Massague & Czech, 1980].