Prevention of Acute Ischemic Heart Disease by Using Estrogenic Metabolites

Thesis submitted for the degree of

Doctor of Philosophy (Science) in Biochemistry

Under the

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By

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DECLAIRATION

I hereby declare that the research work embodied in this dissertation entitled **"Prevention of acute ischemic heart disease by using estrogenic metabolites"** has been carried out by me in the Department of Biochemistry, Vidyasagar University, Midnapore, West Bengal, India under the supervision of **Dr. Smarajit Maiti**, Associate Professor, Head of the Department, Department of Biochemistry, Midnapore, West Bengal, India and **Prof. Asru K. Sinha**, Director, Sinha Institute of Medical Science and Technology, Kolkata, West Bengal, India. I also affirm that this work is a true reflection of my own research and has not been submitted partially or in full for any other degree or diploma to this or any other University or Institution.

Date: ……..

Place: …….

……..……

PRADIPTA JANA

Dedicated to My parents…

Acknowledgement

It is a great pleasure and satisfaction for me to embark on the movement and to thank the help from many persons, who in this path have walked along with me and touched my endeavours in their own way.

First and foremost, I would like to express my heartfelt gratitude to my research supervisor and mentor, **Prof. Asru K. Sinha** for shaping me scientifically what I am today. Sir, your extreme intellect and prodigious work ethic are unmatchable and above all perfection in anything that is done is admirable. I revere him as a scientist and take privilege and pride in being associated with such wonderful person during this phase of my career, from whom there is so much to learn, not only in science but also in general, about the way of life. Thanks for helping me at crucial times and keeping me in my ability. Thank you very much sir, for everything.

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Preface

This thesis is made as a completion for the degree of Doctor of Philosophy at the Vidyasagar University, Midnapore, West Bengal, India. The research work has been described herein was conducted under the supervision of Dr. Smarajit Maiti (Associate Professor and Head of the Department, Department of Biochemistry, Oriental Institute of Science & Technology, Vidyasagar University) and Prof. Asru K. Sinha (Director, Sinha Institute of Medical Science and Technology).

Cardiovascular diseases are common and the most vulnerable, which cause the highest number of death in the world. Due to heart attack the supply of blood in the circulation is obstructed and initiate an ischemic condition in the cardiac tissues which is followed by a severe pain. Demographic reports from all over the world have suggested that the occurrence of the acute ischemic heart disease (AIHD) matters on the age and sex of the human being. Women during their child bearing ages are protected from this disease but condition become defenseless after their menopause. These phenomenological reviews dragged me to ponder the possible role of estrogen, the female steroid hormone could have a preventive role. Researchers from all over the world have tried to reach to a conclusion but it remains speculative. The scientific development of recent trends is determined to find out the molecular mechanism and the biochemical pathway of the platelet aggregation and thrombus formation after atherosclerotic plaque rupture. Here, the role of estrogen on cardiovascular disease particularly on platelets which is responsible for thrombogenic condition have been investigated elaborately.

The chapters of this thesis are organized in a scientific order that was almost maintained throughout investigations. A total of four chapters were prepared including references for this thesis. In *chapter 1* a detailed literature survey was studied, including the molecular mechanism of the development of AIHD, role of various antiplatelet agents to inhibit aggregation of platelets, mechanism of fibrinolysis and thrombus dissolution, role of estrogen in AIHD. The mechanism of atherogenesis in the presence of different factors on the smooth muscle cell layer was elaborated in this section. Aggregation of platelets and thrombus formation was reviewed from previous researches from all over the world.

Chapter 2 designates the role of different estrogen metabolites on platelet aggregation. Activation of nitric oxide (NO) through non-genomic pathway was focused here as it was presumed that the inhibition of platelet aggregation related to NO synthesis.

Platelet aggregation is followed thrombus formation through an independent pathway. Whereas the dissolution of fibrin mass is different from platelet inhibition *Chapter 3* defines the thrombolytic activity of the estriol. Moreover, here the protein with NO activity was characterised. From available date, a conclusive role of NO in synthesising fibrin degradation products (FDPs) were determined.

In *Chapter 4,* some findings made the study so much interesting. Here, the study on the role of estriol on AIHD blood was carried out which went against the presumed postulation. Further research on this problem had shown the occurrence of a high amount of a protein named as dermcidin isoform-2 (DCN-2) in the AIHD circulation repress the inhibitory property of platelet aggregation by estriol.

Author is thankful to Sinha Institute of Medical Science and Technology, Oriental Institute of Science & Technology, Dept of Biochemistry, Vidyasagar University, IPGME&R and SSKM Hospital, Calcutta Medical College and Midnapore Medical College and Hospital for their support in providing instrumental as well as infrastructural facilities to fulfil this study.

This dissertation is ultimately based on various experimental and data obtained that were indispensable for successful completion of the research work. None of the text of the dissertation is taken directly from previously published articles. Several persons have contributed academically, practically and with support to complete this thesis and author is deeply indebted to all of them.

Pradipta Jana

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LIST OF ABBREVIATIONS

µLC/MS/MS=Liquid Chromatography tandem Mass Spectrometry

µM= Micro Molar

AA_1=Arachidonic Acid

ACS=Acute Coronary Syndrome

ADP= Adenosine Diphosphate

AHA=American Heart Association

AIHD =Acute Ischemic Heart Disease

AMI= Acute Myocardial Infarction

ASA=Acetyl Salicylic Acid

ATP=Adenosine Triphosphate

B_{max} =Maximum binding

$CaCl_2$=Calcium Chloride

cAMP=Cyclic Adenosine Monophosphate

cGMP= Cyclic Guanosine Monophosphate

COX=Cyclooxygenase

CRP= C Reactive Protein

DNA= Deoxyribonucleic Acid

DCN-2=Dermcidin Isoform 2

DHEA=Dehydroepiandrosterone

DTT=di-Thiothreitol

EDRF=Endothelium Derived Relaxing Factor

ELISA=Enzyme Linked Immunosorbent Assay

EPR=Electron Paramagnetic Resonance

ER=Estrogen Receptor

FDP=Fibrin Degradation Products

FSH=Follicle-Animating Hormone

GF/C=Glass Fibre Cation

GFP=Gel Filtered Platelets

GP=Glycoprotein

GSNO=S-nitrosoglutathione

HBSS =Hank's Balanced Salt Solution

HPLC=High Performance Liquid Chromatography

HRE=Hormone Receptor Element

HSC=Hematopoetic Stem Cells

HSD=Hydroxysteroid Dehydrogenase

ICAM=Intercellular Adhesion Molecule

IL=Interleukin

IFN-γ=Interferon γ

K_d =Dissociation Constant

l-NAME=NG-nitro-L-arginine methyl ester

LOX-1=Lectin-Like Oxidized Low Density Lipoprotein Receptor 1

LDL=Low Density Lipoprotein

M-CSF=Macrophage Colony Stimulating Factor

MCP-1=Monocyte Chemotactic Peptide-1

mg= Milligram

MIC=Minimum Inhibitory Concentration

MK=Megakaryocyte

MMP = Matrix Degrading Metalloproteinases

NaCl=Sodium Chloride

nM=nanoMolar

NO=Nitric Oxide

NOS=Nitric Oxide Synthase

NSAID=Non-Steroidal Anti-Inflammatory Drugs

NTPDase=Nucleoside Triphosphate Diphosphohydrolase

OxLDL=oxidised LDL

PAGE=Polyacralamide Gel Electrophoresis

PAF=Platelet-Activating Factor

PDGF=Platelet-Derived Growth Factor

PECAM-1=Platelet-Endothelial Cell Adhesion Molecule

PGE_1 =Prostaglandin E_1

PGG_2=Prostaglandin G_2

PGI_2 =Prostacyclin

PLG=Plasminogen

PSOX-Phosphotidylserine And Oxidised Lipoprotein

PRP =Platelet-rich Plasma

SD=Standard Deviation

SDS=Sodium Dodecyle Sulphate

SMC=Smooth Muscle Cell

STEMI=ST-elevated Myocardial Infarction

SR-A=Scavenger receptor A

TNF-α=Tumor Necrosis Factor α

tPA=tissue Plasminogen Activator

TPO=Thrombopoietin

$TxA_{2=}$Thromboxane A_2

UA=Unstable Angina

uPA=urokinase Plasminogen Activator

V_{max}=Maximum Velocity

vWf =von Willebrand Factor

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INTRODUCTION

INTRODUCTION

Acute ischemic heart disease (AIHD) is a condition that affects the blood supply in the blood vessels which are narrowed or blocked due to atherosclerotic plaque formation [Bhatia S, 2010] or due to thrombus formation after atherosclerotic plaque rupture. This disease is the number one killer disease in the world. In the beginning of this decade in the USA, statistical reports from American Heart Association (AHA) has shown AIHD causes one death out seven and more than six million Americans have a new AIHD [Mozaffarian et al, Heart Disease and Stroke Statistics, 2015]. India is experiencing a fast health transition with the rising weight of AIHD [Srinath Reddy et al, 2005]. After the age of 20yrs, the assessed predominance of AIHD is around 3–4 per cent in rural areas and 8–10 per cent in urban zones, speaking to a twofold ascend in rural areas and a sixfold ascend in urban regions between the years 1960 and 2000 [Gupta et al, 2008].

Inflammatory processes play an important role in the development of AIHD. Then again, both atherosclerosis and inflammation bring about the narrowing of the coronary artery, and along these lines diminishing the nutrients, O_2 and minerals in the bloodstream [Fuster V et al, 2005]. Increase of inflammatory markers, i.e. TNF-α [Boesten LS, 2005] and IL-6 [Haverkate F, et al, 1997] in the atherosclerotic lesion has been found to be elevated and so present in the increased level in AIHD [Moliterno DJ et al, 2013]. However, platelet aggregation has an important role in the life saving blood coagulation process [Colman RW, et al, 1987 for comprehensive literatures] but the sudden rupture of these plaques or fissuring on the wall of artery is followed by the excessive occurrence of platelet aggregation by aggregating agents like ADP, *l*-epinephrine, collagen or thrombin, which may result in the formation of thrombus (actually a micro aggregate of platelets embedded in fibrin mass) [Furman MI et al, 1997; Falk E, 1992; Fuster V et al, 1996]. The thrombus thus formed could physically block the normal circulation of the blood in the heart muscles which may lead to the development of acute ischemic heart disease (AIHD) [Furman MI et al, 1997]. The humoral factors like prostacyclin (PGI_2), prostaglandin E_1 (PGE_1) [Whittle BJ, et al, 1978] have been reported to inhibit platelet aggregation through the cellular increase of cyclic AMP [Acharya K et al, 2001] and cyclic GMP level [Kanowitz PJ, 1981]. In other way, the

inhibition of platelet aggregation affected by various platelet anti-aggregatory humoral factors which includes insulin [Trovati M, et al, 1997], interferon-α [Bhattacharyya, M et al, 2009] as well as pharmacological agent like acetyl salicylic acid (aspirin) [Karmohapatra SK, 2007] in particular, has been reported to reduce the incidences of AIHD [Bhattacharyya, M et al, 2009] by inhibiting platelet aggregation not only through the stimulation of nitric oxide synthase (NOS) but also due to the inhibition of cyclooxygenase [Smith JB et al, 1971]. It should be mentioned here that the thrombolytic agents are all fibrinolytic agents that are capable of converting plasminogen (Mr.89kDa) to plasmin (a serine protease, which is responsible for the fibrinolytic effect of these agents, Mr.75kDa) [Karmohapatra SK et al, 2007].

Extensive demographic studies have demonstrated that women in their childbearing ages (i.e. before menopause) are markedly resistant to developing AIHD, when compared to that in the male counterpart [Nelson HD et al, 2002; Gouva L, 2004]. These studies suggested a cardio protective role of estrogens and it could be expected that the humoral estrogens in women before the onset of menopause were involved in the inhibition of platelet aggregation in the prevention of the condition. However, no reports on the effect of estrogens on the inhibition of platelet aggregation in vitro are available. The basic issue related to the inhibition of platelet aggregation by estrogens was further complicated due to the fact that for the expression of the estrogen effect, the presence of DNA in the target cell is needed [Levin ER, 2005]. As human blood platelets do not contain DNA, no alternative mechanism for the estrogen induced inhibition of platelet aggregation independent of DNA in these cells is currently available.

In the present study, it is reported that the inhibition of platelet aggregation in platelet-rich plasma (PRP) by estradiol, the most potent estrogenic steroid hormone, as well as by estriol, which is a less potent estrogenic hormone than estradiol itself in women [O'Neil MJ, 2006], stimulated platelets to synthesize NO. We further report that estriol is one of the most powerful inhibitors of platelet aggregation currently known, and the inhibition of platelet aggregation was mediated by the stimulation of NO synthesis because of the activation of a membrane-bound NOS in platelets independent of DNA.

In the context of estriol as a potent inhibitor of platelet aggregation, the obvious question was, if estriol was such a potent inhibitor of platelet aggregation why did the steroid fail to inhibit platelet aggregation in the development of ACS or AMI in man?

It was found that, the mechanism of the resistance of the platelet aggregation from the AIHD subjects was due to the systemic appearance of dermcidin isoform 2 (DCN-2), a stress induced protein. Presence of DCN-2 conferred the resistance of platelets to estriol from the AMI subjects due to the "cross talk" between the receptors of DCN-2 and estrogen on the platelet surface. Synthesis of cytokines also reduced up to a level due to the presence of estrogen. We have also reported that the pre-existence of sufficient amount of estrogen can induce nitric oxide production that acts as a good vaso-dilating agent.

CHAPTER 1

REVIEW OF LITERATURE

1.1. Molecular Mechanism for the development of Acute Ischemic Heart Disease
1.2. Role of antiplatelet agents to inhibit aggregation of platelets
1.3. Mechanism of fibrinolysis and thrombus dissolution
1.4. Role of estrogen in AIHD

1. *REVIEW OF LITERATURE*

1.1. Molecular Mechanism for the development of AIHD:

AIHD is an assemblage of sicknesses that comprised; stable angina, unstable angina, myocardial infarction, and sudden coronary death [Wong ND, 2014]. Confinement of blood stream to the heart causes ischemia (cell starvation optional to an absence of oxygen) of the myocardial cells [Fuster V et al, 2005]. Myocardial cells may die due to the absence of oxygen and this is known as a myocardial dead tissue (usually called a heart attack). It prompts the heart muscle following its death and later, myocardial scarring without heart muscle re-growth.

1.1.1. Development of atherosclerosis:

Normally, coronary artery ailment happens when a piece of the smooth, versatile coating inside a coronary artery (the courses that supply blood to the heart muscle) creates atherosclerosis which was first described by the Italian anatomist Giovanni Morgagni in 1761. Atherosclerosis (ath-er-o-skler-O-sis) is a disease in which plaque (plak) builds up inside arteries. Plaque is made up of fat, cholesterol, calcium salt, and other substances found in the blood. With atherosclerosis, plaque hardens and the supply route's coating gets to be solidified, and swollen with a wide range of "gunge" that limits the flow of oxygen-rich blood to organs and other parts of the body. Atherosclerotic plaque formation involves 5 major steps:

A. Low density lipoprotein (LDL) accumulation in the intima

Atherosclerosis is portrayed by aggregation of low density lipoprotein (LDL)-determined cholesterol in the intima, the internal layer of the blood vessel wall. The starting extracellular lipid statement in the sub endothelial layer of the blood vessel intima is taken after both by intracellular lipid affidavit creating foam cell arrangement and by the advancement of an extracellular lipid center somewhere down in the intima (Figure 2.1). The explanation behind LDL accumulation in the blood vessel intima is not clear, but rather seems, by all accounts, to be because of development of altered LDL, which, as opposed to

local LDL, has a tendency to collect both intra-and extracellularly [Schwenke, 1989] found that the living arrangement time of LDL in a typical blood vessel intima at destinations inclined to create atherosclerotic injuries was significantly expanded. All the more as of late, utilizing a more modern dynamic methodology [Tozer, 1997] discovered no distinction at LDL home time between sore safe and sore inclined blood vessel destinations without indications of atherosclerosis, yet watched a sensational increment at LDL living arrangement time after the improvement of greasy streak injuries. An LDL adjustment in the blood vessel intima, thus, seems to require tying of the LDL particles to the extracellular lattice, where they are presented to toxic cell-inferred chemicals and operators for prolonged time frames.

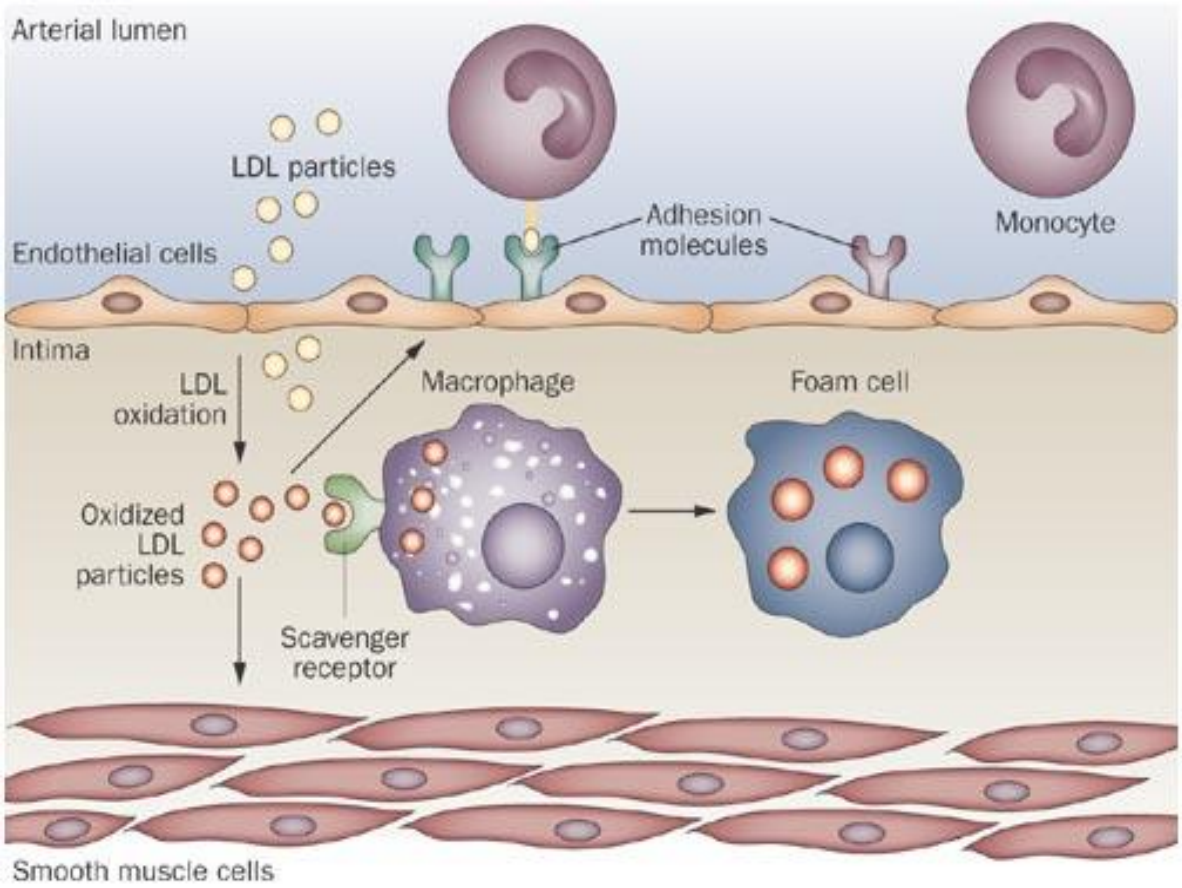

Figure-1.1: Effects of LDL particles on the vessel wall.
Circulating LDL particles invade the arterial wall and accumulate in the intima, where they undergo chemical modifications, such as oxidation. Modified LDL can induce endothelial cell activation and expression of adhesion molecules. Furthermore, intimal macrophages can internalize modified LDL particles through scavenger receptors and become foam cells—a key process in the development of atherosclerotic plaque. Oxidized lipids probably modulate smooth muscle cell functions, for example increasing their adhesion to macrophages and foam cells in the plaque. [Figure courtesy: Rocha VZ et al, 2009]

Decorin, the small proteoglycan was found to tie apoB-100 of LDL by its glycosaminoglycan chain thus connect LDL to collagen. Lipoprotein lipase, by binding to both the glycosaminoglycan chain of decorin [Riessen et al 1994] and to the lipids of LDL, could essentially reinforce the coupling of LDL to decorin.

B. Oxidation of LDL:

The exact location and procedure of oxidation of LDL remains obscure. However, it seems to happen in a special microenvironment where LDL molecules are no longer protected by antioxidants like vitamin E or butylated hydroxyl-toluene [Steinbrecher et al, 1984] that so effectively protect it in whole plasma or in extracellular fluid. When macrophages (and maybe other cell sorts too) hold fast to a substratum, they carry on somewhat like the limbs of an octopus, i.e. micro-domains of the cell film append themselves to the dish in a round example making pockets. These pockets or microenvironments sharply decrease antioxidant level and accelerate the formation of the oxidised LDL (OxLDL) by oxidation. NADPH oxidase, 15-lipoxygenase, myeloperoxidase, the mitochondrial electron transport framework, and others on a basic level assume a part in the oxidation of LDL. Which of these add to LDL oxidation in vivo and to what degree is still questionable, however investigation of items confined from atherosclerotic sores emphatically underpins the association of lipoxygenases [Folcik et al, 1995; Kuhn et al, 1994] and of myeloperoxidase [Leeuwenburgh et al, 1997].

C. Recruitment of monocytes-macrophages:

While oxidation of LDL in the vessel wall got the most consideration, it appears to be likely that oxidation of LDL happens at numerous different destinations, maybe at all locales of inflammation. In view of the penetration by neutrophils and monocyte/macrophages the conditions for LDL oxidation at inflammatory destinations would be favourable.

D. Oxidized LDL uptake and foam cell formation

A group of receptors located on the macrophage membrane has the ability to recognize a versatile number of macromolecules, particularly help to engulf by phagocytic cells, and known as scavenger (cleaner) receptors.

Scavenger receptor A (SR-A) are encoded from chromosome no. 8 and expressed in macrophages, endothelial cells and vascular smooth muscle cells. Oxidative stress, OxLDL, macrophage colony stimulating factor (M-CSF) and Phorbol esters play important role in increasing SR-A. SR-A I and SR-A II affect in the formation of atherosclerotic lesion and an increased susceptibility to bacteria and virus.

Class G scavenger receptor, binds to phosphatidylserine and oxidised lipoprotein (SR-PSOX), usually show elevated expression by TNF-α, IFN-γ and OxLDL in macrophages or monocytes and dendritic cells.

LDL and monocytes move through the endothelium, where OxLDL is formed, stimulates production of monocyte chemotactic peptide-1 (MCP-1), which is released from smooth muscle cells [Nelken, N et al, 1991] and is taken up to generate macrophage-derived foam cells. Formation of foam cells, by scavenger receptors specifically CD36 (class B scavenger receptor) and lectin-like oxidized low density lipoprotein receptor 1 (LOX-1), a class E scavenger receptor occurs due to transformation of macrophages. Basically, these receptors expressed on macrophages, endothelial cells and platelets as pro-inflammatory stimuli, including OxLDL, inflammatory cytokines, for example, TNF-α, shear stress, oxidative stress, phorbol ester, endothelin-1, and angiotensin II make microenvironment for atherogenesis.

E. Formation of a fibrous cap:

Endothelial actuation and penetration of monocyte macrophages are vital essentials for fibrous cap formation, which involves multiplication and the relocation of smooth muscle cells and net network statement [Björkerud et al, 1996]. Macrophage foam cells and endothelium go about as a wellspring of development elements and chemo-attractant for smooth muscle cells. On the other hand, development calculates alone don't animate smooth muscle cell multiplication or migration. Fibrous cap formation appears to be a relatively late event in atherosclerosis, occurring over and, by implication, as an evolution from macrophage-rich fatty streak lesions [Stary H et al, 1995]. Macrophage, endothelial and platelet-derived growth factor (PDGF) promote further replacement and expansion of smooth muscle cell (SMC) to collagen-rich fibrous tissues. These tissues form a fibrous cap in between a necrotic core and towards a luminal surface with high content of type I collagen.

The collagen, elastin, and proteoglycans of the fibrous matrix are mainly produced by SMCs, and this secretory function of lesional SMCs are reflected by their ultrastructural phenotype. Plaque SMCs are characterized by an abundant rough endoplasmic reticulum and Golgi complex and only sparse myofilaments [Stary, 1999].

1.1.2. Atherosclerotic plaque rupture

After Giovanni, William Heberden, an English doctor described a painful "disorder of the breast, marked with strong and peculiar symptoms and sense of strangling and anxiety" [Jay V, 2004]. Heberden coined the name *angina pectoris* for this syndrome; it was British physician Edward Jenner and Caleb Parry, who linked this excruciating "disorder of the breast" to the "hardening" of arteries that had been described by Morgagni [Ashley et al, 2004].

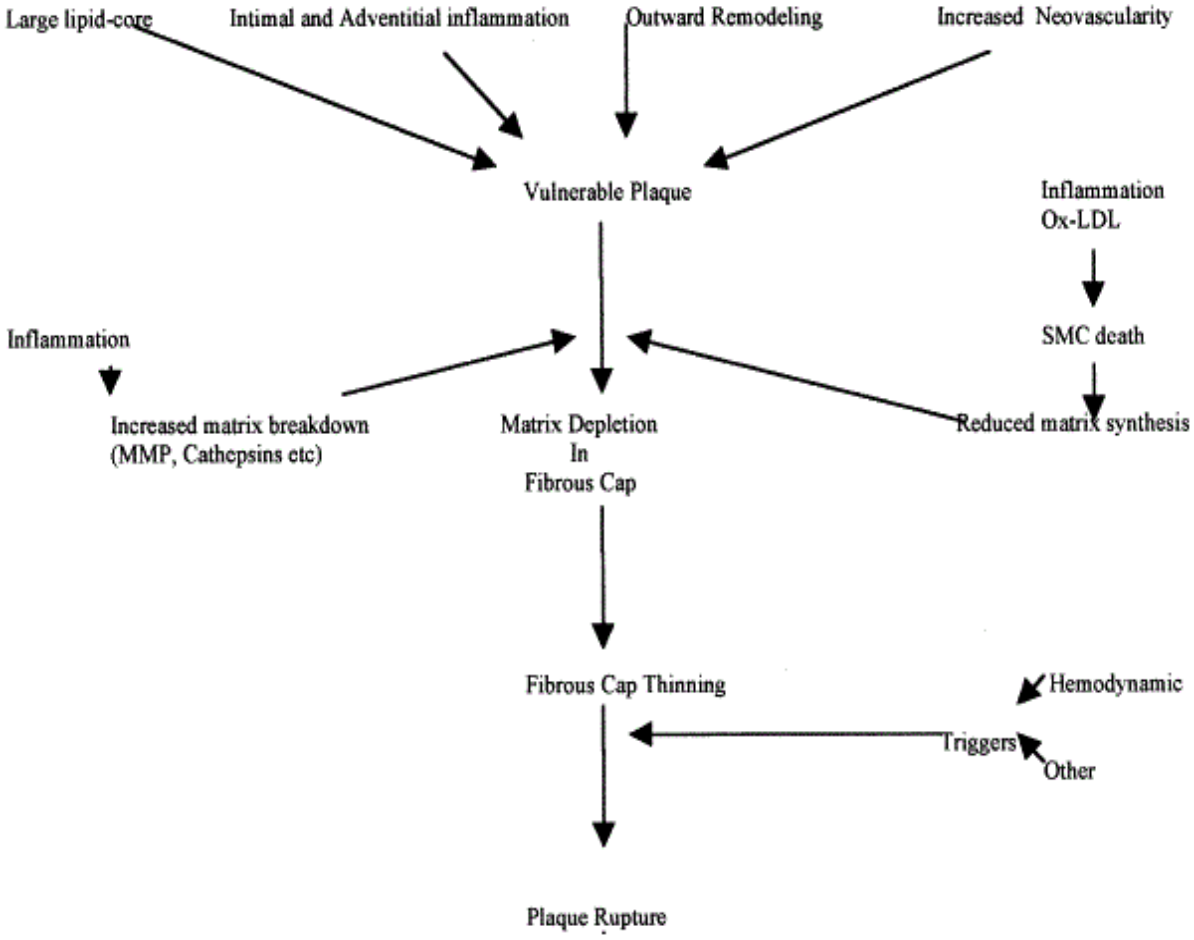

Figure 1.2: Conceptual model depicting the potential pathophysiologic mechanisms of plaque rupture.

Atherosclerotic plaque rupture connected with fractional or complete thrombotic vessel impediment is principal to the proceeding of ischemic coronary disorders [Montoro-García, 2014]. Plaques that create just mellow to-direct angiographic luminal stenosis are regularly those that experience sudden interruption, prompting unstable angina or acute myocardial infarction (Figure 2.2). Plaques with expanded lipid substance seem more inclined to rupture, especially when the lipid mass is stored capriciously inside of the intima. Particularly cap margin or shoulder region [Falk et al, 1995], and thinner fibrous cap with increased level of lipoprotein onto the lesions are the primary site for atherosclerotic plaque rupture [Virmani et al, 2000]. Uptake and metabolism of lipoproteins, discharge of growth factors, and synthesis of catalysts and lethal metabolites that may encourage plaque rupture. What's more, the specific synthesis or setup of a plaque and the hemodynamic movements to which it is uncovered may decide its susceptibility to disturbance.

The sudden rupture of a defenceless plaque may happen suddenly without clear triggers. By contrast, it may take after a specific occasion, for example, intense physical movement (particularly in somebody unaccustomed to general activity), extreme passionate injury, sexual action, presentation to unlawful medications (cocaine, weed, amphetamines), introduction to frosty, or intense disease [Muller et al, 1989; Willich et al, 1992; Peters et al, 2001; Mittleman et al,2001; Muller, 2000].

An increase in the number of inflammatory components in the atherosclerotic lesion leads to the active inflammation [Van der Wal AC et al, 1994]. At each step of this process, inflammatory cytokines are implicated making the atherosclerotic process a chronic inflammatory disease.

Endless high-review stenosis of the coronary arteries can instigate transient ischemia that prompts a ventricular arrhythmia, which may end into ventricular fibrillation prompting demise. Conversely, an unreasonable event of platelet aggregation, especially by ADP in human [Mills DC, 1996], on the site of the atherosclerotic plaque burst or fissuring on the mass of conduit may bring about the development of thrombus. Injection of ADP into the circulation resulted in the thrombus formation on the “normal” endothelial layer of the

coronary artery wall in the absence of atherosclerotic plaque rupture in the animal model using mice [Bhattacharyya et al, 2009].

1.1.3. Inflammatory response

Over the last few decades, a plausible model linking lipids and inflammation to atherogenesis has emerged. The tunica adventitia is included in the inflammatory procedure of atherosclerosis. This data, got primarily in the aorta, recommends a dynamic part of an adventitial lesion in generating an immune response [Kohchi et al, 1985; Wilcox et al, 1996; Houtkamp et al, 2001]. Houtkamp et al. exhibited the vicinity of follicular aggregates made out of B and T cells, reticulo-dendritic cells ($CD21^{+}$), and macrophages in the aortic adventitia [Houtcamp et al,2001]. These infiltrates look like mucosa-related lymphoid tissue and could assume a dynamic part in the humoral safe reaction of cutting edge atherosclerosis.

Few studies have been directed for the coronary corridors. Kohchi et al., 1985 and Stratford et al., 1986; watched a significant increment in the rate of adventitial inflammation in patients with lethal AMI. Neither one of the groups associated the adventitial infiltrate with the plaque sort. All the more as of late, Higuchi et al. had shown significantly more lymphocytes and micro-vessels in coronary guilty party injuries than in stable sores in patients with deadly AMI [Higuchi et al, 2002]. Maseri et al, 1978; estimated a part of an adventitial inflammatory infiltrate in coronary vasospasm. In the external layer of the adventitia of infarct-related coronary supply routes in patients with myocardial localized necrosis, other than lymphocytes and macrophages, numerous mast cells were found in contact with tangible nerve fibers [Laine et al, 2000].

According to the oxidation hypothesis, low-density lipoprotein (LDL) retained in the intima, in part by binding to proteoglycan, undergoes oxidative modification [Berliner et al, 1997; Williams et al 1998]. Elevated values of circulating inflammatory markers such as CRP, TNF-α, serum amyloid A, IL-6, and IL-1 receptor antagonist commonly accompanies ACS. Such elevations correlate with in hospital and short-term adverse prognosis [Berk et al, 1990]. It may reflect not only a high prevalence of myocardial necrosis, ischemia-reperfusion damage, or severe coronary atherosclerosis but also a primary inflammatory

instigator of coronary instability. An increase in the number of inflammatory components in the atherosclerotic lesion leads to the active inflammation [Van der Wal AC et al, 1994]. In particular, prospective epidemiological studies have found increased vascular risk in association with increased basal levels of cytokines such as IL-6 and TNF-α [Ridker et al, 2000; Harris et al, 1999] cell adhesion molecules such as soluble ICAM-1, P selectin, and E selectin [Hwang et al, 1997; Ridkar et al, 2001] and downstream acute-phase reactants such as CRP, fibrinogen, and serum amyloid A.48,49,65–70a Several traditional cardiovascular risk factors track with these inflammatory biomarkers, in particular central obesity and body mass index.

1.1.4. Platelet aggregation and coagulation

A. Platelet morphology and composition

Platelets are tiny cell-lets (1.5-3μM or about 20% of the diameter of red blood cells) in the blood, which play a major role to stop bleeding by forming aggregates and initiating the coagulation process in blood vessel injuries. Dr. Marlene Williams, Assistant Professor of Medicine and CICU Director for Johns Hopkins Bay View Medical Center says “Platelets are the cells that circulate within our blood and bind together when they recognize damaged blood vessels. There’s an evolutionary reason why they’re there. It’s to stop us from bleeding.”

In the year 1841, George Gulliver, an anatomist and physiologist at St. Bartholomew’s Hospital, London sketched a picture of platelet for the first time by using a twin lensed compound microscope that was made by Joseph Jackson Lister. By improving resolution of this microscope the platelets were seen for the first time, Lionel Beale in 1864 and Max Schultze in 1865, first published articles on platelets and described as "spherules". Richard Hill Norsis, a physician from Queen's College, Birmingham was the first to announce the action of platelets in the year 1880. William Osler, in the year 1886, designated this ‘colorless protoplasmic disc’ as the third blood corpuscle. James Wright observed the structural characteristics by finding them with stain and termed as ‘plates’ [Wright JH, 1906] and later it became the universally accepted term as “platelets” [Wright JH, 1910]. Platelets are also denoted as thrombocytes due to having its ability to clot, but this is usually specified

for non-mammalian vertebrates which are mononuclear cells [Michelson AD, 2013] but have the thrombogenic activities like platelets.

Platelets are anucleated cells and derived from megakaryocytes by hematopoesis. Basically self-renewing hematopoetic stem cells (HSC) produce multipotent progenitors (MPP) [Morrison et al, 1994] (Figure 2.3). This procedure includes the dedication of multipotent hematopoietic stem cells toward megakaryocyte (MK) progenitors. The multiplication and differentiation of MK progenitors, the polyploidization of MK precursors and the development of MK. Mature MK produce platelets by cytoplasmic fragmentation happening through a dynamic and managed process, called pro-platelet development, and comprising of long pseudopodial extensions that break in the blood flow. Recent experiences have shown that the MK and erythroid heredities are tightly related at both the cell and molecular levels, particularly in the transcription factors that manage their differentiation programs [Vodyanik et al, 2006]. Megakaryocytopoiesis is managed by two sets of

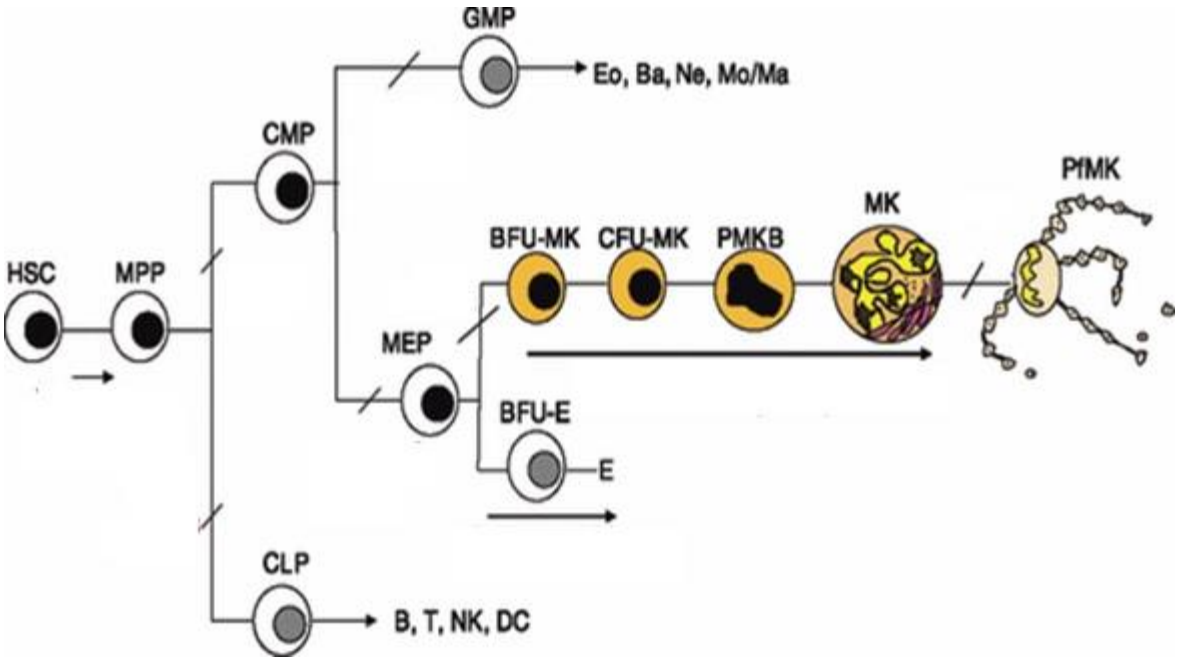

Figure 1.3: Scheme of transcription factors involved in the development of megakaryocytic lineage.
HSC, hematopoietic stem cell; MPP, multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; Eo, eosinophil; Ba, basophil; Ne, neutrophil; Mo/Ma, monocyte/macrophage; MEP, megakaryocyte/erythrocyte progenitor; BFU-MK, burst-forming unit megakaryocyte; CFU-MK, colony-forming unit megakaryocyte; PMKB, promegakaryoblast; MK, megakaryocyte; PfMK, pro-platelet forming megakaryocyte. (Figure Courtesy: Chang et al, 2007)

transcription factors, those controlling the differentiation procedure, for example, GATA-1, and those directing pro-platelet development, for example, NF-E2. The humoral variable thrombopoietin (TPO) is the essential controller of MK differentiation and platelet creation through the incitement of its receptor MPL. Various acquired or inborn pathologies of the MK ancestry are currently clarified by molecular variations from the norm in the movement of the transcription factors included in megakaryocytopoiesis, in the Tpo or c-mpl genes, and additionally in signalling molecules connected with MPL [Pang et al,2006].

Peripheral Zone

Peripheral zone plays main role in the platelet activation. Chemical interaction in platelet response is provided by this site and the physical location for adhesion between platelets, as also as the signal mechanism for transferring the stimulus from outside to platelet interior is also performed in this zone. Peripheral zone includes exterior coat, the unit membrane, and the sub-membrane area [White, 1972]. The components of peripheral zone in immediate contact with surrounding plasma are the exterior coat.

The middle layer of the platelet peripheral zone is a typical tri-laminar membrane and is essential for the integrity of the cell. Surface-active agents, antihistamines, local anaesthetics, chelating agents, high and low salt concentration and lipid solvent injure the membrane and damage the cell. The changes are characterised 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 sol-gel matrix of the hyaloplasm. Because its structural elements appear closely associated with the changes in the cell surface, the sub-membrane area is considered to be a part of a peripheral circumferential band of microtubules.

Sol-gel Zone

However, when examined by electron microscope, 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 fibres.

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.

In studies with the light microscope, the interior of the platelets appear structurally less, except for a few granules and is thus called the hyaloplasm. 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-gluconuronidase and cathepsin. Platelet fibrinogen, thrombosthenin, ATPase, ATP, ADP and serotonin have also been localized in granule fractions [White et al, 1965; Nachman et al, 1967; Msrcus, 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 of dense bodies is directly related to the uptake of serotonin. Dense bodies rapidly decrease in number during early 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 the blockade of enzymatic glycolysis alone and do not affect the level of platelet ATP or energy requiring functions of the cells. In addition to their metabolic activity, platelet mitochondria may function as repositories similar to mitochondrion of the SMC.

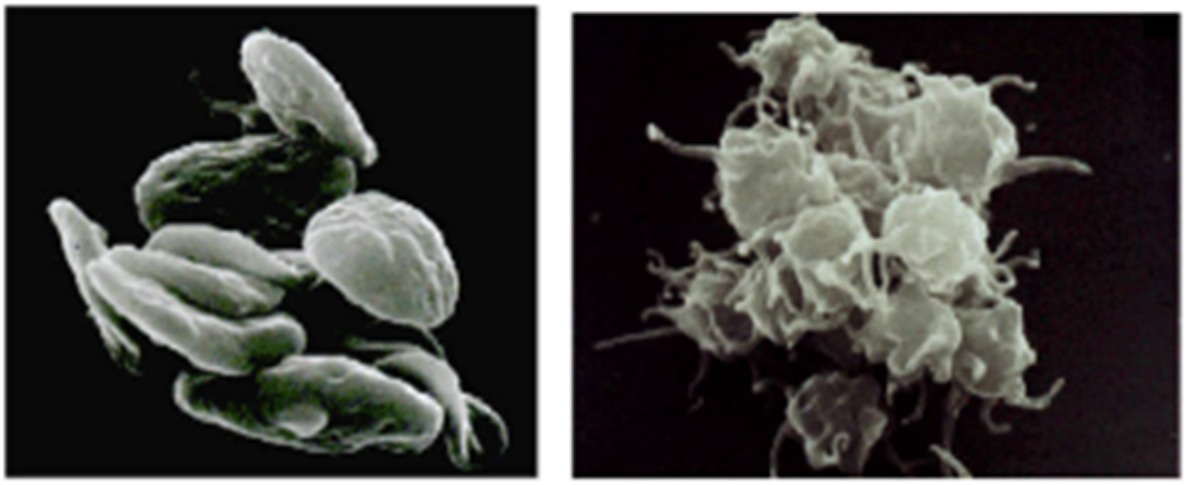

Figure 1.4: Change in shape of the resting platelets is representing above, shows the formation of pseudopods in activated platelets (Figure Courtesy: Cremer et al, 2015***)***

Chemical composition of Human platelets

Human platelets (Figure-2.4) contain most of the common cellular constituents except DNA. Platelets are a heterogeneous population of cytoplasmic fragments ranging from >5 to <12μm^3 in volume, with an average volume of 5-7.5μm^3. Glycogen adenine nucleotides, protein amino acids and orthophosphate are all distributed heterogeneously among the platelets. The 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 younger platelet particularly under stress conditions is a lighter and probably old platelet. If a total (heterogeneous) platelet population is employed, there is 119mg protein or 0.78 X10^{11} platelets/gm wet weight or per millilitre packed platelets [Karpatkin, 1969]. Carbohydrate represents 1.9% of the platelet wet weight or 8.4% of dry weight. There is no free glucose detectable in washed platelets [Karpatkin et al, 1967]. Platelet hexosamine is distributed 75% in glycoprotein and 25% in mucopolysaccaride. 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 the platelets consists predominantly of N-acetyl nuraminic 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 MW 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 analysed 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 phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Smaller amounts of phosphatidylserine and phosphatidylinositol, lysolecithine, phosphatidic acid and cardiolipin are also present [Karpatkin, 1972].

ATP/ADP ratio of the storage pool is 0.8, with 80% of the 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^+-K^+-stimulated ATPase on the platelet membrane. This ATPase is inhibited by 10^{-4} to 10^{-5}M ouabin as well as by sulphydril inhibitors [Cooley et al, 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 the total serotonin are 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^+ and Cl^-. Platelet membranes may be different in origin from the plasma membrane of other cells in that they are from endoplasmic vesicles, possibly endoplasmic reticulum of megakaryocytes [Yamada, 1957].

B. Platelet activation

Physiologic activators that can activate platelets both *in vivo* and *in vitro* are diverse in nature. Some are particulate, such as collagen. Others are proteolytic enzymes, such as thrombin or trypsin, and still others are low molecular weight compounds such as ADP, serotonin, or epinephrine (Figure 2.4). Each of these agents has specific receptors on the outer surface of the plasma membrane, discussion of which is beyond the scope of this review.

Other stimuli arise under pathologic conditions. Among these are antigen-antibody complexes or aggregated gamma globulin that reacts with an Fc receptor on human platelets and a complement receptor on rabbit platelets [Zucker, 1974, Nachman et al, 1980]. Platelet-activating factor (PAF)-acether (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is produced by activated leukocytes and stimulates platelets at low concentrations [Marcus et al, 1981; Chesney et al, 1982].

Platelet activation is complementary, mutually dependent process in haemostasis. Platelets have a normal disc shape before activation but changed into a sphere with long dendritic extensions. Most stimuli cause a change in shape (Figure 2.4). This change involves first the formation of very fine (0.1µ*m* diameter) pseudopodia (i.e., filopodia) from the rim of the disc, followed by a general "rounding up" of the platelet so that it becomes a spiny sphere, often with much broader pseudopodia [Nachlmas, 1983]. The shape change is brought about by actin and myosin in the platelet cytoplasm [George, 2000]. It has been reported before that the change in phosphatidylserine (PS) concentration in platelet membrane causes a change in shape of the platelet. During the shape change secretory granules are organized into the center of the platelet. The cytoskeletal structure becomes dis-balanced, round off balloon like pseudopods and form membrane bleb [Bevers et al, 1991, Heemskerk, 2000]. The contents of secretory granules are either generated from platelets and megakaryocytes or acquired from plasma via endocytosis and pinocytosis facilitated by the canalicular system.

It may be suggested that the pro-coagulant activity and subsequent production of thrombin increases on the platelet surface due to this platelet shape change [Ehrman et al, 1978].

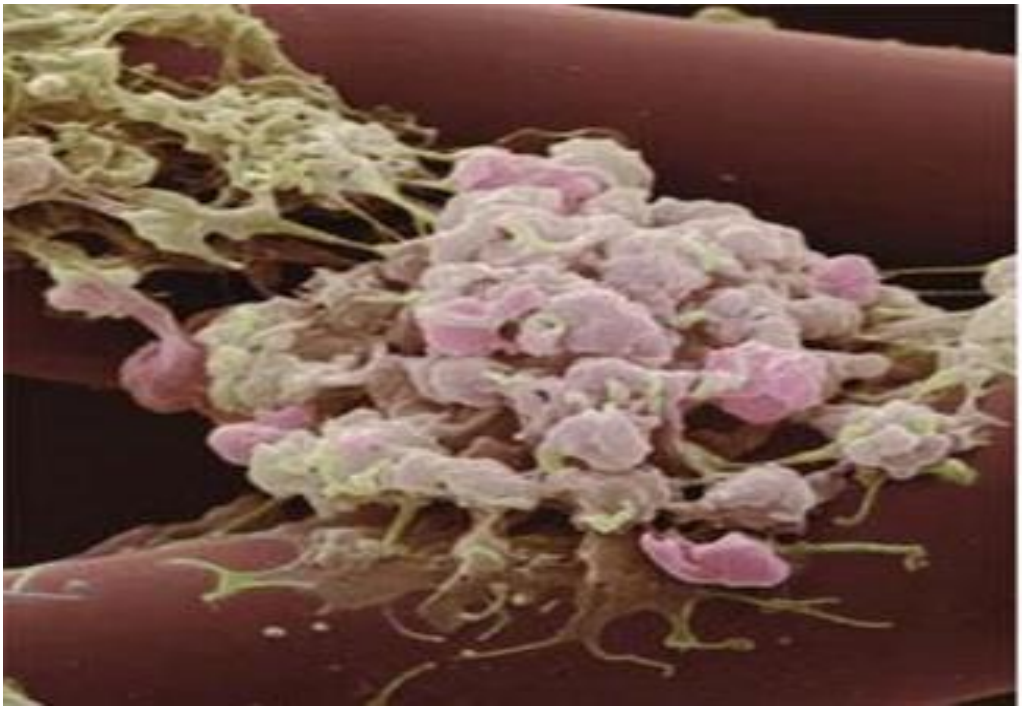

Figure 1.5: ***Activated platelets aggregating, a process regulated by aggregating agents*****.**
An essential part in the platelet activation process is the interaction of adenosine di-phosphate (ADP) with the platelet receptor (Figure Courtesy: Steve Gschmeissner).

C. Platelet adhesion and aggregation

The aggregation of platelets is initiated after plaque rupture through the interaction of aggregating agents (agonists) to their specific receptors on the platelet surface [Holmsen 1979] (Figure 2.5).

Role of adenosine di-phosphate (ADP)

Although these agonists i.e. ADP, *l*-epinephrine, collagen and thrombin binds to their specific modules on the platelet surface, they all effects on the activation of platelets resulting in the synthesis of prostaglandin G_2 through cyclooxygenase 2 (COX 2) and help to release ADP and other aggregating agents from platelets [Hamberg et al, 1974]. Interaction of ADP with its receptors on platelet surface results in the adhesion and thrombus formation [Jin et al, 2002]. It is also reported before that the injection of either ADP, which is a cyclooxygenase activator [Mills DC, 1996], into the circulation, resulted in the thrombus formation on the "normal" endothelial layer of the coronary artery wall in the absence of atherosclerotic plaque rupture in the mouse model. ADP and thromboxane A_2 (TxA_2) are

crucial secondary mediators of platelet activation. As platelets are activated GP IIb/IIIa-receptors undergo conformational changes to become active.

Role of fibrinogen

Activated GP IIb/IIIa mediated interaction between platelets and aggregation by several ligands of which fibrinogen is most abundant [Bennett et al, 1979; Michelson, 2003]. After the platelet plug has been formed, it is stabilized to prevent premature disaggregation then. It has been suggested that outside-in signaling through cell surface integrin and tyrosine kinase receptors have the main role in this phase of thrombus formation. Platelets also participate in localization, amplification and maintenance of coagulant response at the injury site [Ilveskero et al, 2001; Michelson, 2003]

Role of l-epinephrine

Among the platelet aggregating agonists, *l*-epinephrine was found to be weak aggregating agonists. The catecholamine aggregates at supra-physiologic level (>2µM) *in vivo* through its interaction with α_2 adrenergic receptors on the platelet surface [Lalau et al, 1987; Grant et al, 1979]. Although *l*-epinephrine at physiologic and supra-physiologic level fails to aggregate platelets, it can activate α_2 adrenergic receptor that helps to propagate aggregation by using other aggregating agents even at sub optimal level. It has been noted that while the ADP at sub micro molar range failed to aggregate platelets, it will nevertheless aggregate platelets in the presence of physiologic or sub-physiologic range of *l*-epinephrine. The potentiation of platelet aggregation by *l*-epinephrine at sub-physiologic level might have important consequences 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 *l*-epinephrine "the stress hormone" in the system and as such catecholamine might itself be responsible for the precipitation of the condition under emotional stress.

Role of collagen

At the site of vessel wall injury platelets get activated by the expose of sub endothelial collagen and other platelet activating factor, von Willebrand factor (vWf) become exposed and plays a crucial role in high shear induced aggregation [Moake et al, 1986; 1988; Peterson et al, 1987]. 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. Indeed, vWf binding of the GP Ib-V-IX complex is shown to be an essential step in the process, whereas the nature of the adhesive protein, or proteins, engaged in the interaction with GP IIb/IIIa, absolutely required for aggregation [Ikeda et al, 1991]. 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 a firm attachment through GP Ia/IIa also allow low affinity GP VI to interact with collagen.

Interaction of GP VI with collagen induces 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]. The initial adhesion is followed by recruitment of additional platelets into the growing platelet plaque. 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.

Several glycoprotein receptors are present at the platelet surface. A number of these have been proposed as collagen receptor, out of them GPIa/IIa and GP VI are believed to play the main role in collagen induced platelet activation. Fibrillar collagen for type I and type III are major constituents for blood vessel wall, although type IV is present in the basement membranes [Tiainen, 2008]. These collagen receptors are mostly able to activate platelets, which are followed by initiation of adhesion and aggregation of platelets [Sixma et al, 1997].

Role of Dermcidin isoform-2 (DCN-2)

Dermcidin is an 11kDa protein secreted from the sweat gland. A glycosylated N-terminal peptide of this protein is involved in cachexia in cancer patients [Stewart et al, 2008].

In the year 2010, Ghosh et al reported the presence of a dermcidin isoform-2 (DCN-2) protein (Figure 2.6) occurs in high amount in AMI patients. It demonstrated that DCN-2 could have a significant role in the activation of platelets with the corresponding decrease in nitric oxide (NO). Inhibitory property of DCN-2 to decrease NO insists to form micro-aggregates of platelets and propagate a condition of pro-thrombotic condition.

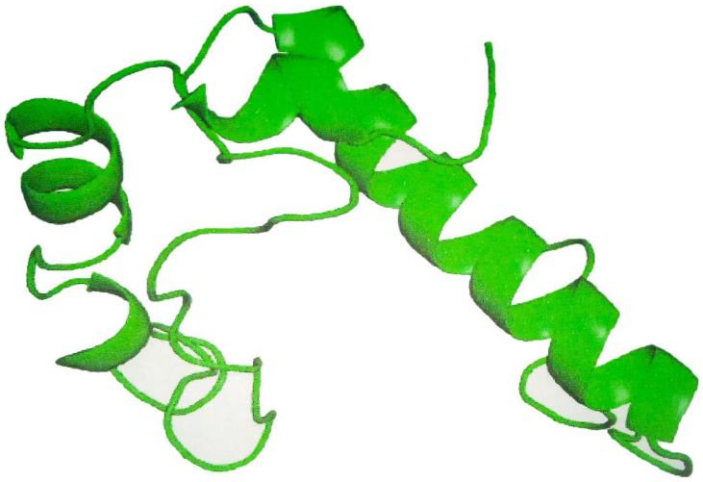

Figure 1.6: Three dimensional structure of Dermcidin isoform-2 (Ghosh et al, 2010)

1.1.5. Blood Coagulation

Coagulation of blood has classically been described as a cascade (Figure-2.7) dependent on adequate levels of coagulation proteins. Platelets support pro-coagulant reactions and vascular endothelial cells maintain anticoagulant properties of the vasculature. In healthy vessels the tissue factor pathway inhibitor (TFPI) inhibits coagulation factor. A new model of coagulation by thrombin binding to thrombomodulin and protein C/protein S as well as other complex of endothelial surface heparinoids and antithrombin act as anticoagulants at the site of injury, revenging excessive formation of thrombi [Monroe et al, 2005; Hoffman et al, 2005].

A. Extrinsic pathway and Intrinsic pathway

The GP Ia/IIa receptor, integrin α2β1, causes initial platelet adhesion to the sub-endothelial matrix. It requires activation by thrombin, collagen or ADP pathways to become operational. It has been suggested that the activation of thrombin and ADP pathway may result in two different conformations of activated GP Ia/IIa with different ligand activity [Jung et al, 2001]. While the activation with ADP has been suggested independent of Ca^{2+}, the activation by collagen, specifically collagen monomers, is dependent on the physiological concentration of Ca^{2+} and Mg^{2+} [Siljander et al, 1999] The role GP Ia/IIa in platelet adhesion on collagen is emphasized in environments of higher 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 et al, 1999]. In comparison, the second collagen receptor GP VI is reported to adhere only to fibrillar collagen [Jung et al, 1998]. Indeed, modulation of the collagen revealed at an injury site is believed to mediate variable roles of GP Ia/IIa and GP VI in adhesion of 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 1a/IIa gene [Kunicki et al, 1993; Kritjik et al, 1998]. In addition, the C807T polymorphism has been associated with increased risk for athero-thrombotic risk [Carlsson et al, 1999; Moshfegh et al, 1999]. However, regarding the role of these polymorphisms as predictors of athero-thrombotic diseases the multi-factorial 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 [Farndale et al, 2004]. Patients deficient in GP VI lacks the ability to form thrombi on a collagen surface under flow conditions, but clinically present with only mild bleeding tendencies

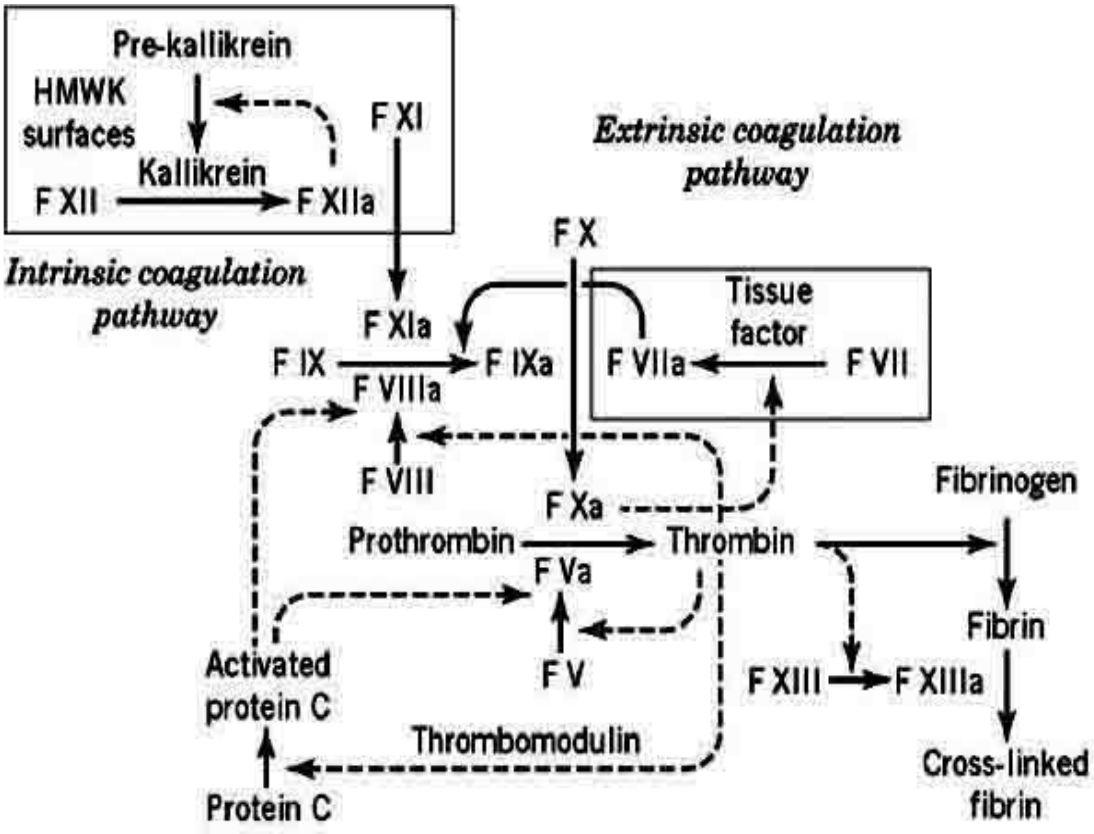

Figure-1.7: ***Blood coagulation; Extrinsic and Intrinsic pathway***

[Moroi et al, 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.

1.1.6. Ischemic condition and stress due to hypoxia

Thrombus formation after atherosclerotic plaque rupture results in the ischemic condition-an imbalance between the supply (perfusion) and demand of the heart for oxygenated blood. Ischemia comprises not only insufficiency of oxygen, but also reduced the availability of nutrient substrates and inadequate removal of metabolites.

More than 90% of patients with AIHD have atherosclerosis of one or more of the coronary arteries. The clinical manifestations of coronary atherosclerosis are generally due to the progressive encroachment of the lumen leading to stenosis (chronic, "fixed" obstructions) or to acute plaque disruption with thrombosis (generally both sudden and dynamic), which compromises blood flow. A fixed obstructive lesion of 75% or greater (i.e., only 25% or less lumen remaining) generally causes symptomatic ischemia induced by exercise; with this degree of obstruction, the augmented coronary flow provided by compensatory vasodilation is no longer sufficient to meet even moderate increases in myocardial demand. A 90% stenosis can lead to inadequate coronary blood flow even at rest.

1.2. Role of antiplatelet agents to inhibit aggregation of platelets:

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 (PECAM-1) is an inhibitory receptor which mediates the inhibitory pathway. In addition, athero-thrombotic factors circulate in plasma and are secreted by Platelets [Ruggeri, 2002]. Nitric oxide (NO), 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 the metabolism of ADP [Marcus et al, 1966].

Those components which play a role in platelet activity are termed as anti-platelet agents. These compounds are classified into three groups.

1.2.1. Cyclooxygenase (COX) Inhibitors:

COX is an enzyme that is in charge of the synthesis of prostanoids including prostaglandins, prostacyclin and thromboxane. Prostaglandins, potent bioactive lipid messengers derived from arachidonic acid (AA_1), were first extracted from semen, prostate, and seminal vesicles by Goldblatt and von Euler in the 1930s and demonstrated to lower blood pressure and cause smooth muscle contraction [Goldblatt MW, 1933]. Bergström and colleagues purified the first prostaglandin isomers during the 1950s and 60s [Bergstrom S, et al, 1964], and in 1964, van Dorp et al. and Bergstrom et al. independently identified AA, a 20-carbon tetraenoic fatty acid (C20:4ω6) as the precursor to prostaglandins [Bergstrom S, et al, 1964; van Dorp DA et al, 1964]. The cyclooxygenase response through which AA is enzymatically cyclized and is oxygenated to yield endoperoxide-containing prostaglandin G2 (PGG_2) was later distinguished by Samuelsson and associates [Hamberg M et al, 1973; Hamberg M et al, 1974]. COX_1 and COX_2 are almost

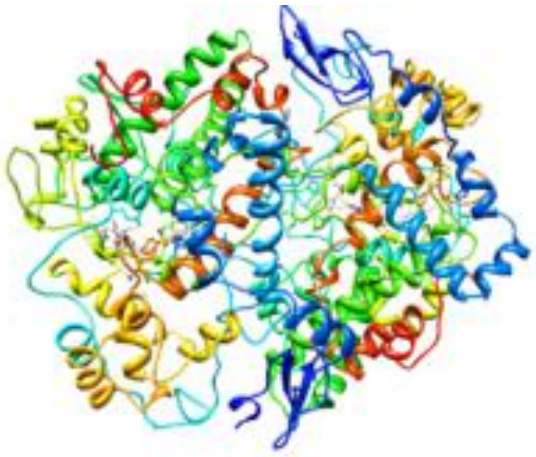

Figure 1.8: Three dimensional structure of Cyclooxygenase-2 (PDB id: 6COX***)***

similar molecular weight proteins with 70kDa and 72kDa respectively, with 65% homology between them. A change in Ile_{523} in COX_1 to Val_{523} in COX_2 (Figure 2.8) makes them selective for their inhibitors as Val_{523}in COX_2 is smaller in size and allows access to hydrophobic side pocket (which Ile_{523} sterically hinders).

There are so many compounds inhibit COX, which are commonly known as non-steroidal anti-inflammatory drugs (NSAID) [J. R. Vane, 1976]. These drugs have analgesic (pain-killing), antipyretic and anti-inflammatory effects. The term non-steroidal is used to denote that the sources of these drugs are not steroid compounds. Among various NSAIDs pharmacologic agents like acetyl salicylic acid (ASA) or aspirin, ibuprofen and naproxen are particular.

Aspirin is non-particular and irreversibly represses both structures [Sharma et al, 1997] (yet is feebly more specific for COX-1). Typically, COX produces prostaglandins, the majority of which are genius incendiary, and thromboxane, which advance clotting. Aspirin adjusted COX-2 produces lipoxins, the majority of which are mitigating. More current NSAID drugs called COX-2 specific inhibitors have been produced that hinder just COX-2, with the expectation for diminishment of gastrointestinal side-effects [Warner et al, 2002]. It inhibits platelet aggregation through the COX pathway.

1.2.2. Increase in cellular cyclic AMP level

Increase of platelet cyclic AMP level either through the activation of membrane adenylate [Salzman et al, 1971; Haslam, 1971; Dutta-Roy et al, 1987] or through the inhibition of cyclic AMP phosphodiesterase results in the inhibition of platelet aggregation [Salzman et al, 1971]. Among these agents, prostacyclin and arachidonate metabolite are potent inhibitor of platelet aggregation, increases platelet cyclic AMP level through the activation of the platelet membrane adenylate cyclase as prostacyclin binds to its receptor site on the enzyme that has been most extensively studied [Tatrson, 1977]. Prostaglandin E_1, a potent inhibitor of platelet aggregation, also activates adenylate cyclase through its binding to prostacyclin receptor. As adenylate cyclase is the part of prostacyclin receptor the activation of prostacyclin receptor causes the simultaneous activation of adenylate cyclase [Dutta-Roy et al, 1987; Schafer et al, 1979].

1.2.3. Production of NO in the system

A. Chemistry of NO

Mono-nitrogen monoxide, more commonly known as nitric oxide (NO), is a colorless gas at room temperature and pressure. The maximum solubility of NO (at 1atm partial pressure) in water at room temperature and pressure is approximately 2mM, which is slightly higher than the solubility of di-oxygen (O_2) in water [Shaw et al, 1977]. It becomes immediately evident from Lewis dot depiction that ·NO has one unpaired electron and thus is formally a free radical species. At room temperature and pressure, NO has less propensity

$$\overset{\cdot}{:}N{=}O: \quad :N = O\overset{\cdot\cdot}{:} \qquad (1)$$

1(I) 2(II)

to react with itself in a radical-radical dimerization process. At first glance it may seem curious since dimerization would lead to a structure (ON-NO) whereby all atoms have a full complement of eight valence electrons and therefore, would satisfy the octate rule.

A partial rational for the usually long and weak N-N bond in the NO dimmer may also be found in an examination of the nature of the unpaired electron in the NO monomer.

B. Free radical chemistry of NO

NO reacts rapidly via simple, radical-radical combination reactions with species processing unpaired electron such as ·NO_2, O_2. 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 O_2 –dependent oxidation of lipids [Wink et al, 1993; Hogg et al, 1993; Rubbo et al, 1994; Rubbo et al, 1996; Struck 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).

$$\text{Lipid} - H + X\cdot \longrightarrow \text{Lipid}\cdot + X\text{-}H \qquad (2)$$

The lipid radical then reacts with O_2 to generate an alkyl peroxy radical (Lipid-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.

$$\text{Lipid}\cdot + O_2 \longrightarrow \text{Lipid-OO}\cdot \qquad (3)$$

$$\text{Lipid-OO}\cdot \longrightarrow \text{Lipid-OOH} + \text{Lipid}\cdot \qquad (4)$$

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

$$\text{Lipid-OO}\cdot + \cdot\text{NO} \longrightarrow \text{Lipid-OO-NO} \qquad (5)$$

Furthermore, ·NO has been reported to inhibit the generation chain initiating species by altering the relativity of metals known to serve as catalysts for their generation [Rubbo et al, 1996].

C. **Role of NO in Biology**

NO plays its role as an endogenous mediator of numerous physiological processes that range from regulation of cardiovascular function of participation in memory [Dawson et al, 1992; Feldman et al, 1992; Ignarrow, 1989; Monkada et al, 1991]. The *in vivo* properties of NO can be determined by its chemistry.

Direct role of NO

The Direct reaction 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 metalloprotein containing iron. The most notable heme protein that forms an Fe-NO adduct *in vivo* is soluble guanylate cyclase [Murad, 1994].

In contrast to guanylate cyclase, binding of NO to monooxygenases such as cytochrome P450 has some important patho-physiological squeal as well. Binding of NO to heme domain of the cytochrome P450 could how a protective mechanism against a variety of pathophysiological conditions by releasing free heme and activating hemeooxygenase in hepatocytes [Kim et al, 1995; Choi et al, 1996; Stocker, 1990]. Nitrosylation of the vitamin B_{12} (cobalamine) in aqueous condition results in a reduced sensitivity to serve as a cofactor for methionine synthesis [Brouwer et al, 1996]. The rapid reaction with oxyhemoglobin to produce methohemoglobin and nitrate ($k=5 \times 10^7\ M^{-1}\ S^{-1}$) [Eich et al, 1996] is the primary

endogenous mechanism by which NO diffusion and concentration are controlled [Doyle et al, 1981; Feelisch, 1991; Lancaster Jr, 1994].

$$\text{OxyHb } (Fe^{2+}\text{-}O_2) + NO \longrightarrow \text{metHb } (Fe^{3+}) + NO_3^- \qquad (6)$$

It is also reported that consumption of H_2O_2 by catalase is inhibited by cytokine stimulated by hepatocytes and by hepatocytes and by synthetic NO donors and plays a role in the tumoricidal activity of activated macrophages [Kim et al, 1995; Wink et al; 1993; Farias-Eisner et al, 1996].

Indirect role of NO

Pathophysiological conditions and higher nitrogen oxides associated with the indirect effect of NO and are thought to be the chemical species which are responsible for the etiology of the disease. S-nitrosoglutathione (GSNO), a nitrosative compound is formed from GSH in the presence of aerobic NO solution [Wink et al, 1994]. Physiological transport of NO and the formation of S-nitrosothiols may occur through the non-heme iron sulfur nitrosyl complexes. Under high fluxes of the NO, formation of these complexes has been indicated by electron paramagnetic resonance (EPR) spectroscopy [Lancaster Jr et al, 1990].

Mixed direct and indirect effect

A primary cellular target for the cytotoxic action of NO is the mitochondria [Moncada et al, 1991; Lacaster et al, 1990]. The body may have several protective mechanisms to limit indirect effects of NO on mitochondria. Inhibition of respiration is occurred in those cells which were 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 NO formation is limited and reversible inhibition is only transient.

Biological Production of NO

Nitric oxide synthase (NOS) is responsible for the synthesis of NO in biological system [Figure 2.9]. Guanidino nitrogen from *l*-arginine, an amino acid which plays an important role as the building block in the protein synthesis, serves the nitrogen derivation of NO. Guanylate cyclase, in the rat brain cerebella, was often referred to, show the activity [Bredt

et al, 1990]. As also as the initial observation led to propose that the guanylate cyclase the tissue level of cGMP and help to produce NO [Arnold et al, 1977]. In late days' research, it has been shown that the endothelial cells relax arterial smooth muscle through acetylcholine, were seminal in further examination of the factors involved in vaso-relaxation [Furchgott et

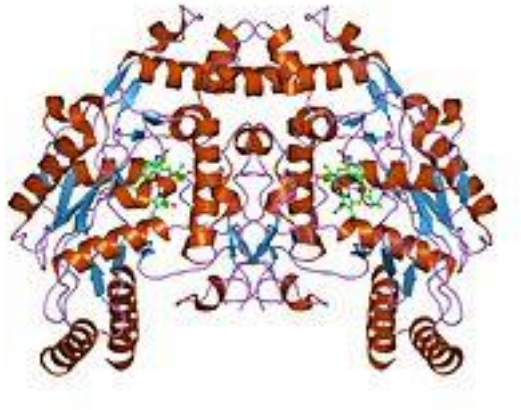

Figure 1.9: A three dimensional structure of endothelial nitric oxide synthase (eNOS). PDB id: IM9J

al, 1980]. Acetylcholine was postulated to interact with muscarinic receptors on the surface of the endothelial cells to stimulate the release of an endothelium derived relaxing factor (EDRF) that interacts with the underneath smooth muscle line by diffusing into the layer [Furgott et al, 1980]. Ignarrow, in the year 1986 described that EDRF is either same as NO or a labile nitroso precursor that spontaneously decomposes with the liberation of NO [Furchgott et al, 1984; Ignarro et al, 1986]. NO assayed by the chemiluminescence method could account for the biological action of EDRF in cultured endothelial cells in response to bradykinin [Palmer et al, 1987].

Spectrophotometric assay of NO-hemoglobin from deoxyhemoglobin [Ignarro et al 1987] or diazotization of sulfanilic acid [Ignarro et al, 1987] could account for the biological activity of EDRF released from perfused artery, vein and freshly cultured bovine endothelial cells. The reaction of EDRF from cultured endothelial cells, with oxyhemoglobin resulted in

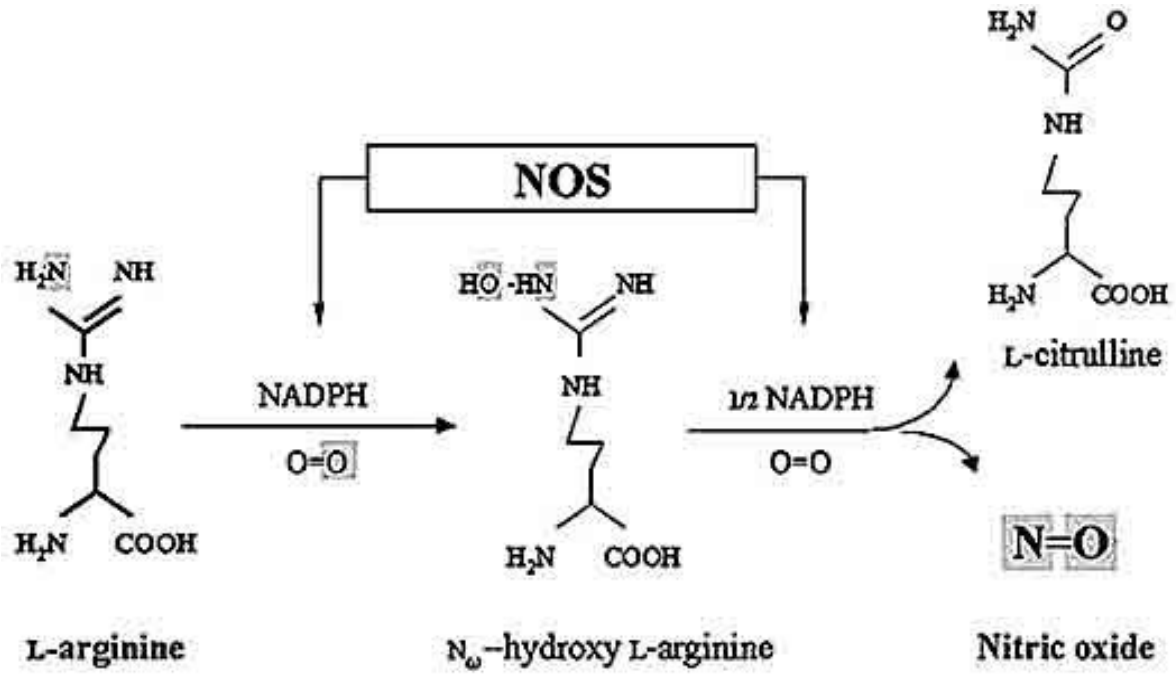

Figure 1.10: Biochemical synthesis of nitric oxide (NO) from l-arginine by nitric oxide synthase enzyme

the formation of methemoglobin confirmed that the released product is NO [Kelm et al, 1988].

Macrophage cell culture experiments revealed that an *l*-arginine dependent pathway is involved in the biochemical synthesis of NO and *l*-citrulline (Figure2.10) and that this pathway was inhibited by N^G-monomethyle *l*-arginine, a close structural analog of *l*-arginine [Hibbs et al, 1988]. The soluble fraction catalyzed the conversion of *l*-arginine to *l*-citrulline in an NADPH dependent manner. An enzyme present in activated macrophages solution has shown the conversion of *l*-arginine to NO and *l*-citrulline [Marletta et al, 1988].

Garthwaite et al demonstrated that NO could act as an intracellular messenger in the brain [Garthwaite et al, 1988]. In this study it was found that there is a requirement of Ca^{2+}/calmodulin for the elicitation of *l*-arginine to *l*-citrulline conversion by NOS.

Studies have shown the flavoprotein nature of inducible NOS (iNOS), requires tetrahydrobiopterin for activity [Mayer et al, 1991; Hevel et al, 1991].

NO 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 A_2 (TxA_2) produced during platelet aggregation on the arterial wall in AIHD. Currently it is believed that NO does not have any receptor on the platelet surface and compound is freely diffused into the cytosol.

While the humoral factors like prostacyclin (PGI_2), prostaglandin E_1 (PGE_1) [Whittle BJ, et al, 1978] have been reported to inhibit platelet aggregation through the cellular increase of cyclic AMP, other humoral inhibitors of platelet aggregation including insulin [Trovati M, 1997], interferon-α [Bhattacharyya M, 2009]and the pharmacologic agent, aspirin has also been reported [Karmohapatra SK, 2007] to inhibit platelet aggregation through the stimulation of nitric oxide synthase (NOS). NO 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 the cGMP level, the increase cellular cAMP level by NO could be equally important in the platelet aggregation [Gruether et al, 1979]. The increase of platelet cGMP level is reported to the inhibition of guanylate cyclase activation. [Coles et al, 2002]. Although NO does activate membrane adenylate cyclase, it can increase platelet cAMP level through specific inhibition of cytosolic low K_m cAMP phosphodiesterase [Sinha et al, 1998].

Recent studies demonstrate that the inhibition of platelet aggregation mediated through the direct activation of the plasminogen in the plasma to plasmin which is independent of cGMP or cAMP pathway but through NO pathway. The inhibition of platelet aggregation through the lysis of the interlinking peptide bridges of fibrinogen molecules among platelets.

1.3. Mechanism of fibrinolysis and thrombus dissolution:

Under physiological conditions, both coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, inhibitors, cofactors and receptors. Molecular links between these systems permit localized, timely removal of ongoing or acutely induced fibrin deposits. These co-ordinated molecular events insure blood fluidity while preventing blood loss (Esmon et al, 1999; Degen, 2001; Kolev et al, 2003).

1.3.1. Activation of plasminogen

Plasminogen (PLG) primarily in the liver (Raum et al, 1980), having 791 amino acids forms five homologous triple loop structures called 'kringles' (Forsgren et al, 1987). Plasminogen has two noteworthy glycoforms available in human - type I plasminogen contains two glycosylation moieties (N-linked to N289 and O-linked to T346), while type II plasminogen contains just a solitary O-connected sugar (O-connected to T346), type II plasminogen is specially enrolled to the cell surface over the type I glycoform. On the other hand, type I plasminogen seems all the more promptly enlisted to blood clusters.

Available for use, plasminogen receives a shut, actuation safe compliance. After tying to clumps, or to the cell surface, plasminogen receives an open frame that can be changed over into dynamic plasmin by an assortment of enzymes and NO.

In fibrinolysis, fibrin coagulation, the result of coagulation, is broken down [Cesarman-Maus et al, 2005]. Activation of PLG, by cleavage of a single Arg-Val peptide bond at position 560–561 (Holvoet et al, 1985), gives rise to the active protease, plasmin.

A. Role of urokinase plasminogen activator (uPA)

uPA is a kind of S1 family serine protease, was isolated from human urine by McFarlane in the year 1947. Later, it has been obtained from blood and extracellular matrix.

uPA basically cleaves inactive PLG to form plasmin to proceed into the mechanism of fibrinolysis.

uPA consists of 411 residues of amino acids with three domains: the serine protease domain; the kringle domain and the growth factor domain. A proteolytic cleavage at Lys_{158}-Ile_{159} in pro-urokinase leads to form activated uPA.

B. Role of tissue plasminogen activator (tPA)

tPA is also a kind of S1 family serine proteinase, released from various mammalian tissues especially from endothelial cell. The activation of tPA is enhanced by the presence of fibrin, which itself is lysed by activating tPA that formed plasmin from plasminogen. Recombinant tPA (r-tPA) used for the treatment of ST-elevated myocardial infarction (STEMI), ischemic stroke and pulmonary embolism.

A significant difference between uPA and tPA is that tPA maintain the particular cleavage site.

C. Role of NO in plasminogen activation

NO plays a typical role in the proteolytic activity [Sinha et al, 2002]. In the year 2007, Karmahapatra et al described that in plasmin synthesis, NO itself, can cleave the PLG through a proteolytic pathway [Karmahapatra et al, 2007].

1.3.2. Formation of fibrin degradation products (FDPs)

The *in vivo* formation of fibrin and its subsequent secondary fibrinolytic digestion yield a variety of crosslinked FDPs. D-dimer is a fibrin degradation product (or FDP), a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis. It is so named because it contains two crosslinked D fragments of the fibrin protein [Adams et al, 2009], known as FDP_1 and FDP_2.

The dissolution of the thrombus on the coronary artery was reported to help the restoration of the normal blood circulation in the heart and may consequently resolve the ensuing condition [Hoylaerts M et al, 1982].

1.4. <u>Role of estrogen in AIHD</u>:

Extensive demographic studies have demonstrated that women in their childbearing ages (i.e. before menopause) are markedly resistant to developing AIHD, when compared to that in the male counterpart [Nelson HD et al, 2002; Gouva Let al, 2004]. These studies suggested a cardio protective role of estrogens.

1.4.1. Estrogen and its types

Estrogens [Greek *οἶστρος* (oistros)= "verve or inspiration" and the suffix *-gen*= "producer of"] are hormones that are imperative for sexual and conceptive improvement, mostly in women. They are likewise alluded to as female sex hormones. The expression "estrogen" alludes to the majority of the artificially comparable hormones in this class, which are estrone, estradiol (essential in women of reproductive age) and estriol.

A. Biological synthesis of estrogen

Estrogens, in females, are delivered fundamentally by the ovaries, and amid pregnancy, the placenta. Follicle-animating hormone (FSH) invigorates the ovarian generation of estrogens by the granulosa cells of the ovarian follicles and corpora lutea. A few estrogens are additionally delivered in smaller amounts by different tissues, for example, the liver, adrenal glands, and the breast. These optional sources of estrogens are particularly important in postmenopausal ladies. Fat cells produce estrogen too [Nelson et al, 2001].

In females, biosynthesis of estrogens occurs in theca interna cells in the ovary, by the formation of androstenedione from cholesterol. Androstenedione is a substance of the feeble androgenic movement which serves transcendently as a precursor for more powerful androgens, for example, testosterone and estrogen. This compound crosses the basal membrane into the encompassing granulosa cells, where it is changed over either instantly into estrone, or into testosterone and afterward estradiol in an extra step. The transformation of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD), though the change of androstenedione and testosterone into estrone and estradiol, separately is catalyzed by aromatase, chemicals which are both communicated in granulosa cell. Interestingly, granulosa cells lack 17α-hydroxylase and 17,20-lyase, while theca cells express these enzymes and 17β-HSD yet need aromatase. Henceforth, both granulosa and theca cells are crucial for the generation of estrogen in the ovaries.

Estriol is just produced in significant amounts amid pregnancy as it is synthesized by the placenta from 16-hydroxydehydroepiandrosterone sulfate [Raju et al, 1990] an androgen steroid made in the fetal liver and adrenal organs.

The human placenta produces pregnenolone and progesterone from coursing cholesterol. Pregnenolone is changed over in the fetal adrenal organ into dehydroepiandrosterone (DHEA), a C19 steroid, then along these lines sulfonated to dehydroepiandrosterone sulfate (DHEAS). DHEAS is changed over to 16-OH DHEAS in the fetal liver. The placenta changes over 16-OH DHEAS to estriol, and is the prevalent site of estriol union.

Estrogen levels shift through the menstrual cycle, with levels most noteworthy close to the end of the follicular stage just before ovulation.

Out of three major naturally occurring estrogens (estrone [10-20%], estradiol [10-30%], and estriol [60-80%]) in women, estriol is the most abundant but with least estrogenic effect and can be used as safe estrogen.

B. Estrone

Out of three natural estrogens estrone is the least abundant metabolite usually secreted from ovary as well as adipose tissues. Adolf Butenandt, in the year 1929 discovered and isolated estrone from urine. This compound is white, odorless and solid crystalline powder when purified. It was enlisted as the carcinogen in the Fourth Annual Report on Carcinogens in National Toxicology Program, USA in 1985.

C. Estradiol

Estradiol is the main estrogen hormone which plays the major role in maintaining the reproductive health in female [Ryan, 1982]. Growth in the lining of vagina, the endometrium, the cervical glands, the lining of Fallopian tubes are maintained by estradiol in females. In case of male, it plays a role in the development of sartoli cells as synthesized in a little amount from Leydig cells of the mammalian testis. Estradiol also have a role in preventing the apoptosis of the sperm cells [Pentikäinen et al, 2000]. It has also been reported to have an effect on other tissues particularly bone. Estradiol effect on blood cells has also been reported before [Strzemienski et al, 1987]. Ability of estradiol to activate neutrophils to synthesise NO has been informed by Durán et al in the year 2000 [Durán et al, 2000].

D. Estriol

Estriol is the most abundant estrogen metabolite and in high level during pregnancy of the women. Generally, occurs in postmenopausal women at low level and in case of men its

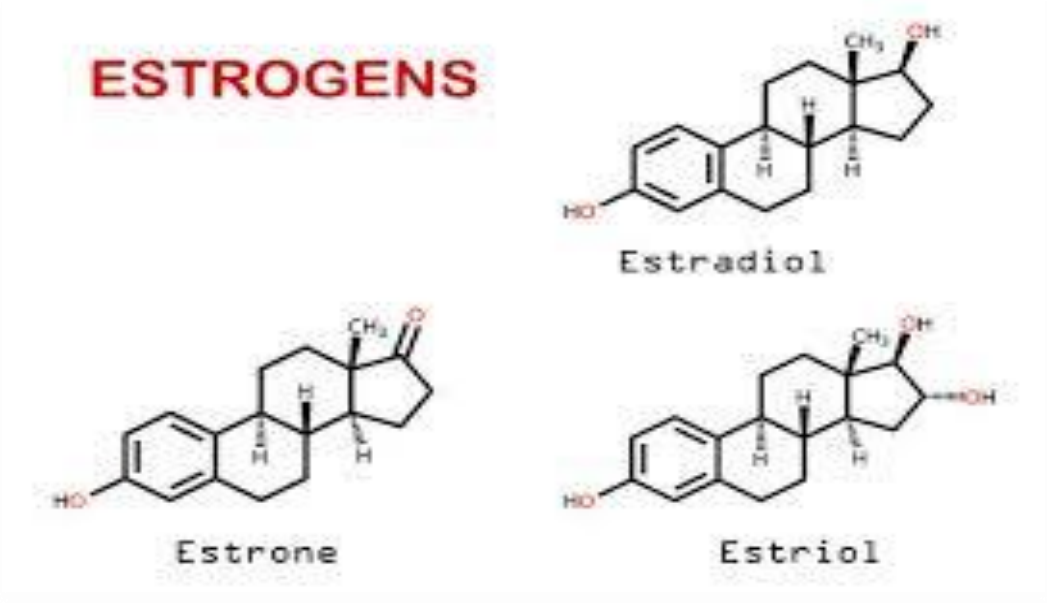

Figure 1.11: Chemical structure of natural estrogen metabolites

level not changes significantly. Both estradiol and estrone has the fate to be converted to estriol ultimately. This estrogen metabolite has the least estrogenic effect and termed as 'weak estrogen'. As the other two estrogens are blamed to have the pro-cancer or carcinogenic activity this molecule is thought to have antagonistic activity to breast cancer [Lappano et al, 2010].

1.4.2. Previous studies on AIHD using estrogen

The use of postmenopausal estrogens first gathered momentum during the sixties under the banner of "feminine forever." The promise to women that estrogens would hold back the ravages of time went unfulfilled, and instead they learned that they were being put at increased risk of endometrial cancer [Smith DC et al, 1975]. Disturbing news emerged: estrogens increased the risk of AIHD in men, and oral contraceptives increased the risk of AIHD and stroke in middle-aged women [Coronary Drug Project Research Group, 1970; Royal College of General Practitioners' Oral Contraception Study, 1981]. Result: from the

mid-seventies onward, estrogen use declined precipitously [Hemminki E et al, 1988]. Better news started emerging in the eighties: estrogen users appeared to be at lower risk of AIHD [Stampfer MJ et al, 1985].

Fallaciously, a study conducted with estradiol (0.625mg X 4times per day) to post-menopausal women increased the incidences of AIHD by 50% instead of leading to the reduction of the occurrence of the condition in the participants [Nelson HD et al, 2002]. And, as such, the role of estrogen in the prevention of AIHD remains obscure.

On the other hand, the non-genomic effects of estrogen have also been studied [Falkenstein E et al, 2000]. So the role of estrogen is hypothesized on the cells which are not reported as a target tissue of estrogen. The binding of an agonist to its own receptors may down-regulate or upregulate the numbers of receptors of another agonist on the platelet membrane [Kahn NN et al, 1990]. This down-regulation or the up-regulation of the receptor numbers on the platelet surface by its ligand was affected by the binding of a different agonist to its own receptors generally called “cross talk” between receptors [Kahn NN et al, 1990].

AIMS AND OBJECTIVES

Since previous reports have suggested the possible cardio-protective role of estrogen therefore studies will be conducted as followed-

I. To determine the role of the hormone to activate any cardio-protective protein(s) in platelet.
II. To study the role of protein(s) that is activated by estriol in the regulation of blood circulation.
III. To characterize estrogen binding protein.
IV. To find out possible therapeutic for the prevention of AIHD from the estrogenic metabolites.
V. To determine the differences in binding affinity of estriol (E_3) or other estrogenic metabolites with the protein.
VI. Long term goal is to validate and optimize the efficacy of the screened therapeutic agent (if any) for major clinical use.

STUDY DESIGN

- The nitric oxide assay was carried out in platelet-rich plasma (PRP) to determine the role of estriol in producing NO.
- Preparation of platelet-rich plasma (PRP) and gel filtered platelets (GFP) for *in vitro* studies.
- The role of estriol to inhibit ADP induced platelet aggregation was carried out.
- *Ex vivo* Clot (fibrin mass) was prepared by using $CaCl_2$ and role of estriol on fibrinolysis was analysed.
- Estriol binding to enzyme-protein present in platelets was purified and was characterized by determining the enzymatic activity (K_m and V_{max}).
  At the same time estradiol binding to the proposed protein was also compared.
- Analysis of estriol binding to the protein by determining dissociation constant (K_d) and number of ligand molecules bound/molecule of protein (n).
- Determination of the cross-talk effect of DCN-2 protein with the estriol in the inhibition of pathogenic effect of DCN-2 in acute myocardial infarction (AMI) patients.

CHAPTER 2

ROLE OF ESTROGEN METABOLITES IN THE INHIBITION OF PLATELET AGGREGATION

2. *ROLE OF ESTROGEN METABOLITES IN THE INHIBITION OF PATELET AGGREGATION*

2.1. Introduction:

The aggregation of platelets by aggregating agents such as ADP, L-epinephrine, collagen, or thrombin is an essential physiologic event in the life-saving blood coagulation process [Colman RW, et al, 1987 for comprehensive literatures]. In contrast, excessive platelet aggregation at the site of atherosclerotic plaque rupture on the coronary artery, particularly by ADP in humans, has been reported to result in the development of acute ischemic heart disease (AIHD), commonly known as 'heart attack'. The formation of thrombus (a micro-aggregate of platelets embedded in fibrin mass) on the wall of the coronary artery blocks the normal blood circulation in the heart muscle and may lead to the development of a life threatening condition [Furman et al, 1998; Falk, 1992; Fuster et al; 1996].

As the above-mentioned platelet-aggregating agents may lead to the formation of thrombus on the site of atherosclerotic plaque rupture, the inhibition of the platelet aggregation by several factors present in the circulation is known to counteract the excessive platelet aggregation that might be crucial for the prevention of AIHD. Among these humoral factors capable of inhibiting platelet aggregation are prostacyclin [Whittle et al, 1978], insulin [Trovati et al, 1997], and interferon-α [Bhattacharyya M, 2009]. These factors, including prostacyclin, insulin, and interferon-α, have been reported to be some of the most potent inhibitors of platelet aggregation in that these compounds are capable of inhibiting platelet aggregation at nmol/l concentrations. Although prostacyclin has been reported to inhibit platelet aggregation by increasing the platelet cyclic AMP level, interferon-α and insulin are reported to inhibit platelet aggregation by the stimulation of nitric oxide (NO) synthesis induced by these compounds in platelets [Bhattacharyya et al, 2009]. NO, thus formed, is not only reported to increase both cyclic AMP [Acharya et al, 2001] and cyclic GMP [Kanowitz, 1981] in platelets for the inhibition of platelet aggregation. The oxide has also been reported to activate plasminogen to plasmin, which results in the dissolution of intra-platelet fibrinogen bridges that is essential for the aggregation of platelets [Bhattacharyya et al, 2009]. The plasmin-induced lysis of the fibrinogen bonds resulted in

the inhibition of platelet aggregation in the absence of an increase in the cyclic nucleotide level in platelets.

It is well known that women, before menopause, are resistant to the development of AIHD [Nelson et al, 2002; Gouva et al, 2004].

In the above context, where the inhibition of the platelet aggregation might lead to the prevention of AIHD, it could be expected that the humoral estrogens in women before the onset of menopause were involved in the inhibition of platelet aggregation in the prevention of the condition [Gouva et al, 2004]. However, no reports on the effect of estrogens on the inhibition of platelet aggregation in vitro are available.

The basic issue related to the inhibition of platelet aggregation by estrogens was further complicated by the fact that for the expression of the effect of estrogen, the presence of DNA in the target cell is needed [Levin, 2005]. As human blood platelets do not contain DNA, no alternative mechanism for the estrogen-induced inhibition of platelet aggregation independent of DNA in these cells is currently available.

Here, we report that the inhibition of platelet aggregation in platelet-rich plasma (PRP) by estradiol, the most potent estrogenic steroid, as well as by estriol, which is reported to be one of the more abundant albeit considerably less potent estrogenic hormones than estradiol itself in women [O'Neil et al, 2006], stimulated platelets to synthesize NO. We further report that estriol is one of the most powerful inhibitors of platelet aggregation currently known, and the inhibition of platelet aggregation was mediated by the stimulation of NO synthesis because of the activation of a membrane-bound nitric oxide synthase (NOS) in platelets independent of DNA.

2.2. Materials and Methods:

2.2.1. Ethical clearance:

The study, which used blood samples from normal volunteers, was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology (Calcutta).

All participating volunteers were asked to sign an informed consent form before they were requested to donate blood.

2.2.2. Chemicals

Estriol, estradiol, ADP, and Triton X-100 were obtained from Sigma Aldrich (St Louis, Missouri, USA). The estrogens were dissolved in 0.9% NaCl. ADP used in the study was dissolved in distilled water and the pH was adjusted to 7.4 at 0°C just before use and discarded after use.

The solvents used for preparing stocks were freshly prepared.

2.2.3. Selection of volunteers

The volunteers selected (women=20, men=20; n=40) were between 25 and 40years of age. None of the volunteers had a history of neither diabetes mellitus nor systemic hypertension and never had any cardiovascular nor cerebrovascular disease. The participants had no life threatening infections at the time of the investigation and had not been hospitalized for any condition at least 6 months before their blood samples were collected. All volunteers were asked to stop using any medications including acetyl salicylic acid (aspirin) for at least 4 weeks before they consented to donate blood.

Female volunteers donated their blood when they were in the mid-follicular phase of their menstrual cycle. The estradiol and estriol concentration ranged between 37–53 and 24–30pmol/l, respectively. None of the female volunteers had ever received any contraceptive medication.

2.2.4. Collection of blood from the volunteers and the preparation of platelet-rich plasma and gel-filtered platelet

Peripheral blood sample (30ml) was obtained by venipuncture using 19-G siliconized needles, collected in plastic tubes, and anticoagulated by mixing one volume of sodium citrate with 10 volumes of the blood by gentle inversion. The final concentration of the anticoagulant was 13mmol/l. The blood samples were centrifuged at 200g for 15min at room

temperature (23°C). The supernatant fraction, PRP, was collected and used for further studies. The cell-free blood plasma was prepared by centrifuging the anticoagulated blood sample at 10,000g for 30min at 0°C. The gel-filtered platelet (GFP) were prepared from the blood of a single donor (200ml) using the method described previously [Dutta-Roy et al, 1987]. Both estradiol and estriol present in the plasma were removed from platelets by sepharose gel filtration.

2.2.5. Platelet aggregation

The aggregation of platelets was studied using 2mmol/l ADP as the aggregating agent with an aggregometer as described before [Chakraborty et al, 2003]. To determine the effect of estriol or estradiol as the inhibitor of platelet aggregation, different concentrations of the estrogens were added to PRP and were incubated for 45min at 37°C before the aggregation of platelets was initiated by using ADP. The minimum inhibitory concentration (MIC) of either estriol or estradiol was defined as the amount of the compound that completely inhibited platelet aggregation induced by 2.0mmol/l ADP as described above.

2.2.6. Determination of nitric oxide synthesis

The synthesis of NO was determined by the conversion of oxyhemoglobin into methemoglobin as described previously [Bhattacharyya, 2009]. The formation of NO in the reaction mixture was verified independently using the chemiluminescence method [Cox et al, 1982].

2.2.7. Assay of nitric oxide synthase

Typically, a reaction mixture containing 0.5–0.8mg of the protein preparation as the source of NOS was incubated with 10mmol/l *l*-arginine in the presence of 2mmol/l $CaCl_2$ in a total mixture of 1.0ml in Tyrode's buffer, pH 7.4, as described previously [Chakraborty et al, 2003]. The formation of NO in the supernatant was quantified using the method described above.

2.2.8. Treatment of gel-filtered platelet with Triton X-100

In a certain phase of the study, it was necessary to release platelet membrane proteins as the source of NOS. For this purpose, GFP was prepared from 200ml of blood from a single donor. The GFP was treated with 0.05% Triton X-100 (v/v) for 30min at 0°C with occasional gentle shaking. We have reported previously that the treatment of GFP with a low

concentration of the detergent resulted in the release of platelet membrane proteins without dissolving the whole platelets [Dutta-Roy et al, 1987]. The supernatant thus obtained was centrifuged at 30000g for 60min at 0°C. The clarified supernatant was concentrated to 1.0ml [Dutta-Roy et al, 1987] and dialyzed against 0.9% NaCl overnight. The dialyzed supernatant was used as the source of NOS.

2.3. Results:

2.3.1. Prevention and therapeutic role of estriol against ADP induced platelet aggregation in PRP

The treatment of PRP from normal volunteers with 2.0μM ADP resulted in the complete aggregation of platelets (Figure-2.1A). When the same PRP was pre-treated with different amounts of estriol as indicated, and incubated for 45 min at 37°C, and subsequently treated with 2.0μM ADP, it was found that the minimal inhibitory concentration (M.I.C.) of estriol

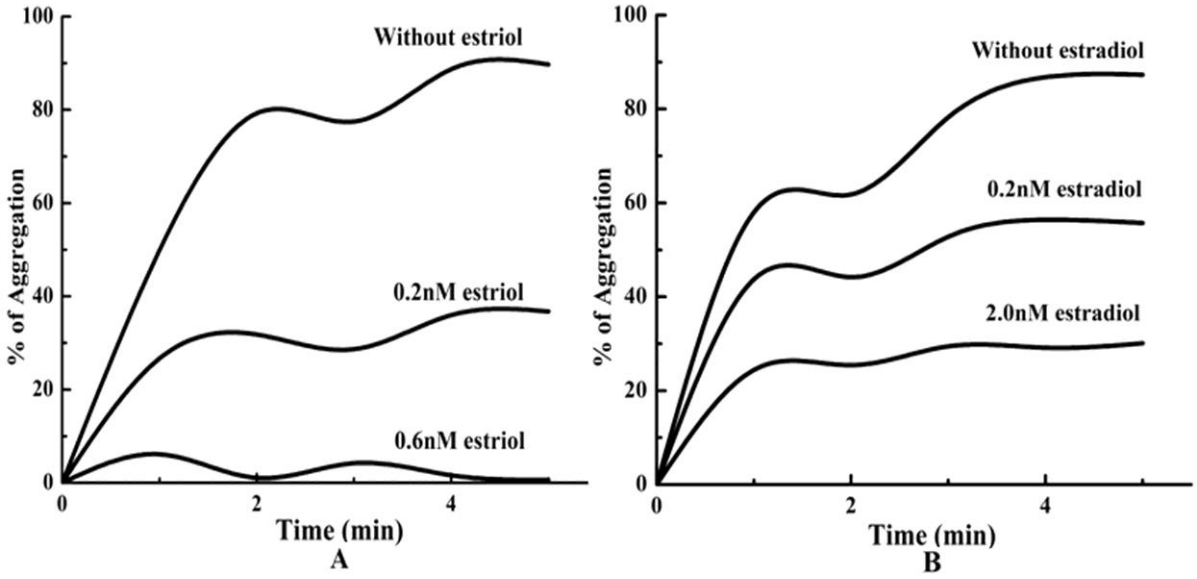

Figure-2.1: ***ADP-induced platelet aggregation inhibition by estriol (A) or by estradiol (B) in platelet-rich plasma.***
Before the initiation of platelet aggregation induced by 2.0mmol/L ADP, the platelet-rich plasma was incubated with or without different concentrations of estriol or estradiol as indicated for 45 min at 37°C. The profiles of platelet aggregation are typical from more than 40 different experiments using 40 different individuals.

that completely inhibited platelet aggregation induced by ADP was 0.6nM (Figure-2.1A). In contrast, when the same PRP preparation was incubated with different amounts of estradiol under identical conditions, it was found that the MIC of estradiol for the ADP induced platelet aggregation was 2.0nM ($p<0.005$, $n=40$) (Figure-2.1B) indicating that estriol was >2-fold powerful inhibitor of platelet aggregation when compared to estradiol under identical conditions.

2.3.2. Synthesis of NO in PRP induced by estriol or by estradiol

To ascertain the role of NO in the inhibition of platelet aggregation by the estrogens, the production of NO induced by both of these steroids in PRP was determined (Figure-2.2). Although both estriol and estradiol were able to stimulate NO production, the former steroid was a more potent inducer of NO synthesis in platelets than the latter.

While the maximal synthesis of NO in the presence of estriol in PRP was achieved at 0.6nM, the maximal production of NO in PRP occurred at 2.0nM estradiol (Figure-2.2). The amount of NO produced at 2.0nM estradiol in PRP was found to be 0.179nmol/10^8cells/h and was 0.55nmol/10^8cells/h when treated with 0.6nM estriol. These results indicated that estriol was >3-fold powerful stimulator of NO synthesis than that produced by estradiol in the same platelet preparation for the maximal inhibition of platelet aggregation which was induced by ADP.

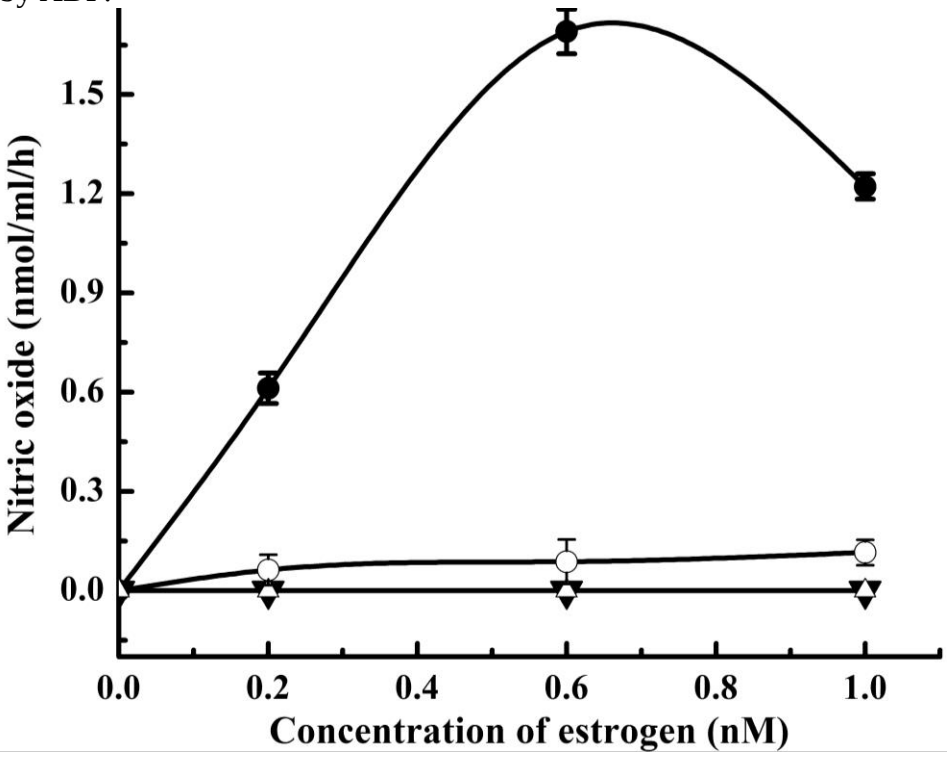

Figure-2.2: ***Stimulation of nitric oxide (NO) synthesis by estriol or estradiol in platelet-rich plasma (PRP)***. PRP from five different volunteers was incubated with different concentrations of estrogens as indicated for 45 min at 37°C. After incubation, NO synthesis in the PRP was determined as described in the Materials and methods section.
Whereas the solid circles (—●—) represent estriol, the open circle (—○—) show estradiol. The formation of NO is indicated by the hollow triangle (—Δ—) in PRP treated without estriol or estradiol and in PRP in the presence of estriol or estradiol with 10 mmol/L NG-nitro-L-arginine methyl ester. The solid inverted triangles (—▼—) show the formation of NO in PFP without estriol or estradiol.

2.3.3. The mechanism of the estrogen induced NO synthesis in platelets

As human blood platelets do not contain DNA and as it has been reported that the expression of estrogen effect (in the present case, the synthesis of NO by the steroids in platelets) through the synthesis of *de novo* NOS in GFP through the stimulation requires a non-genomic mechanism for the estrogens' effects in the inhibition of platelet aggregation due to NO production as reported before or in the fibrinolysis of the PRP clot.

As such, it was thought that the blood plasma might contain NOS that catalysed formation of NO from *l*-arginine (the substrate of NOS). However, no NOS activity in the presence or absence of estradiol or estriol could demonstrate in the human blood plasma (Figure-2.2).

Efforts were next made to find out whether platelet membrane might contain basal NOS activity that was activated by the estrogens as shown in the figure-2.2. To test the possibility of increase in NO in the platelet membrane, GFP was treated with 0.05% Triton X-100 as

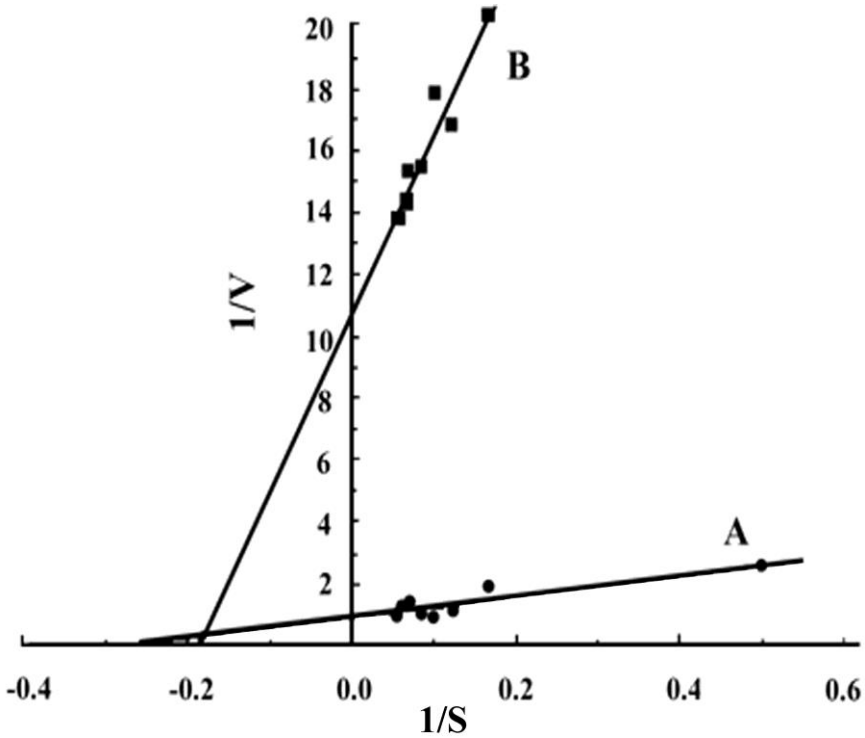

Figure-2.3: ***Lineweaver–Burk plots of the supernatant of Triton X-100-treated gel filtered platelet in the presence and absence of estriol.***
The gel-filtered platelets were treated with 0.05% Triton X-100 and the supernatants from the incubation mixture were dialyzed as described in the Materials and Methods section. The dialyzed supernatant was used as the source of nitric oxide synthase. Line (A) represents the Lineweaver–Burk plot of the dialyzed supernatant in the presence of 0.6nmol/L estriol; line (B) represents the Lineweaver–Burk plot of same dialyzed supernatant in the absence of the added estriol.

described in the Materials and Methods. Experiments were carried out to find membrane proteins in the supernatant without dissolving the whole platelets that might show the presence of basal NO releasing ability in the released proteins.

We have reported before that the treatment of intact platelets with low concentration of Triton X-100 (0.05%vol/vol) released membrane proteins in the supernatant without dissolving the whole platelet as described below.

When the dialyzed supernatant was tested for the presence of NOS, the basal activity of the protein in the dialyzed preparation was found to be present. Lineweaver Burk plot of the NOS in the dialyzed supernatant was constructed in the presence and absence of 0.6nM estriol.

Lineweaver-Burk plots of the NOS activity demonstrated that in the absence of the added estriol, the K_m was 5.28mM with V_{max} 0.029nmol NO/mg/h (Figure-2.3, Line-B). When the same platelet supernatant was treated with 0.6nM estriol K_m was found to be reduced to 3.42mM with concomitant increased V_{max} to 0.337nmol NO/mg/h (Figure-2.3, Line-A).

In other word, the basal NOS activity of platelet membrane protein was stimulated ≈10 fold in the presence of 0.6nM estriol.

2.4. **Discussion**

It is generally believed that all steroids, including estrogens, mediated their effects through the synthesis or inhibition of protein in the target cells, that is, those cells that are capable of expressing the effects of the steroid [Levin, 2005].

As such, for the expression of the steroids' effect, the presence of both DNA and the nuclear steroid receptors is needed [Olefsky, 2001]. However, we have reported that the effects of estriol [Ganguly Bhattacharjee K, 2012] and progesterone [Ganguly, 2012], as well as NO itself, led to the synthesis of maspin, an anti-breast cancer protein in neutrophils, which suggests that NO was 'acting' like the second messenger in these cases.

In some cases, it has been described that estriol at 100nmol/l can synergize the increased platelet aggregation [Akarasereenont, 2006], but the amount of estriol (100nmol/l) was much higher than the concentrations that showed the highest NO synthesis by estriol (0.6nmol/l) in our study. It was also found that the synthesis of NO was decreased when the concentration of estradiol or estriol was increased beyond the optimum level of estrogens for the maximal synthesis of NO in platelets.

Our results, as described above, showed that NOS activity induced by estradiol or estriol did not involve the participation of DNA but probable non-genomic activity of the estrogen β receptor [Khetawat et al, 2000] (Figure 2.3). Our results also showed that not estradiol, but estriol was the more powerful inhibitor of platelet aggregation in PRP.

Indeed, it was found that estriol was not only a more powerful inhibitor of platelet aggregation than estradiol (MICE2.0nmol/l) but is one of the most powerful inhibitors of platelet aggregation currently known (MIC 0.6nmol/l), in that no other inhibitors of platelet aggregation were reported to completely inhibit (100%) platelet aggregation at sub-nanomolar concentrations as a result of stimulation of NO synthesis in platelets. It was found that 0.55nmol NO/10^8 cells/h was produced by 0.6nmol/l estriol and 2.0nmol/l estradiol synthesized 0.179nmol NO/10^8 cells/h (Fig. 2A and 2B).

In this context, it should be mentioned that extensive trials using estradiol to prevent AIHD in menopausal women failed to reduce the occurrence of the deadly condition in the participants [Nelson et al, 2002]. The basis of this trial, however, was an extension of estrogen replacement therapy for the prevention of AIHD, which itself is only phenomenological in nature. In some other cases, investigators claimed that endogenous estradiol may play a role in increased platelet aggregation by ADP or adrenaline [Akarasereenont, 2006]. However, inhibition of platelet aggregation in GFP that is free of endogenous estradiol occurred using a minimal concentration of estradiol (2nmol/l) as in the case of PRP. The investigation erroneously presumed that the effect of estrogens on the inhibition of AIHD was mediated by estradiol. We found that not estradiol but estriol was the major inhibitor of platelet aggregation that might prevent AIHD in women. Recent studies further showed that estradiol may actually be a thrombogenic agent in vivo by increasing platelet aggregation [Manson et al, 2003].

However, the beneficial effect of estradiol could be related to its effect as an antihypertensive [Xue et al, 2009] and an anti-diabetogenic [Louet et al, 2004] agent that could result in the control of atherosclerosis but was not necessarily related to the inhibition of platelet aggregation in vivo. Our study also showed that the estrogenic effects of estradiol or estriol were not necessarily related to their ability to inhibit platelet aggregation, which has a major effect in the prevention of AIHD as supported by numerous studies using acetyl salicylic acid [Steering Committee of the Physicians Health Study Research Group, 1989]. Perhaps, the use of estriol might have produced a better outcome in the prevention of AIHD compared with that obtained using estradiol [Nelson et al, 2002].

CHAPTER 3

ESTRIOL-INDUCED FIBRINOLYSIS DUE TO THE ACTIVATION OF PLASMINOGEN TO PLASMIN

3. *ESTRIOL-INDUCED FIBRINOLYSIS DUE TO THE ACTIVATION OF PLASMINOGEN TO PLASMIN*

3.1. Introduction

The aggregation of platelets is a life-saving physiologic event that plays an essential role in blood coagulation process [Colman RW et al, 1987 for comprehensive literatures].

In contrast, excessive platelet aggregation particularly at the site of atherosclerotic plaque rupture on the coronary artery is reported to result in the AIHD due to the formation of thrombus (a micro-aggregate of platelets embedded in fibrin mass) on the plaque rupture site. Thrombus thus formed obstructs the normal blood circulation that is essential for the physiologic activity of the musculature of the heart, and the blockade might precipitate AIHD due to the thrombosis [Falk, 1992; Fuster et al, 1996].

The dissolution of the thrombus on the coronary artery was reported to help the restoration of the normal blood circulation in the heart and may consequently resolve the ensuing condition [Hoylaerts et al, 1982]. For this purpose, various thrombolytic agents are therapeutically used [Sherry, 1977; Fletcher et al, 1976]. It should be mentioned here that the thrombolytic agents are all fibrinolytic agents that are capable of converting plasminogen to plasmin (a serine protease, which is responsible for the fibrinolytic effect of these agents) [Summaria et al, 1967]. Although nitric oxide is not an enzyme, and known to be a potent inhibitor of platelet aggregation [Bhattacharyya et al, 2009], the inorganic compound has been reported to convert plasminogen to plasmin in the absence of any cofactors or cells and was found to cause fibrinolysis [Bhattacharyya et al, 2009; Yao et al, 1995; Sinha et al, 1998; Karmahapatra et al, 2007].

Various compounds and agents including acetylsalicylic acid (aspirin) [Karmahapatra et al, 2007], interferon-α [Bhattacharyya et al, 2009], insulin [Chakraborty et al, 2004] which are reported to stimulate nitric oxide synthesis in platelets, and nitric oxide itself, are all reported to be efficacious fibrinolytic agents through the activation of plasminogen (Mr. 89kDa) to plasmin (Mr. 75kDa) due to excision of a peptide chain ‘excision peptide’, (Mr. 14 kDa) from plasminogen molecule probably due to the nitric oxide induced breakage of cross-strand disulfide (S–S) bridges [Karmahapatra et al, 2007].

It has also been reported that the efficacy of aspirin in the prevention of AIHD was not only related to the inhibition of platelet aggregation due to the inhibition of cyclooxygenase alone but was also due to the aspirin-induced stimulation of nitric oxide synthesis in platelets that resulted in the activation of plasminogen to plasmin in the plasma leading to the dissolution of the formed thrombus on the arterial wall [Karmahapatra et al, 2007].

As estriol was found to be one of the most potent inhibitor of platelet aggregation at 0.6nmol/l through synthesis of the nitric oxide in platelets, studies were carried out to determine the role of estriol, if any, as a fibrinolytic agent on ex-vivo clotted platelet-rich plasma (PRP).

3.2. Materials and methods

3.2.1. Ethical clearance

The protocol used in the study involved normal humans and was carried out in accordance with the Helsinki agreement, which was approved by the Internal Review Board, Sinha Institute of Medical Science & Technology, Calcutta, West Bengal. All volunteers signed informed consent form before they were included in the study.

3.2.2. Chemicals and antibody

Estriol (purity 98%), goat anti-rabbit immunoglobulin G-alkaline phosphatase and fibrinogen were obtained from Sigma-Aldrich. Polyclonal antibody against estriol was obtained from Thermo Scientific. Maxisorp plates for ELISA were obtained from Nunc, Roskilde, Denmark.

All other chemicals used were of analytical grade.

3.2.3. Selection of volunteers

The study was conducted by involving both male and female volunteers (n=40, men 30, women 10) between the ages of 20 and 40years. They had no history of systemic hypertension or diabetes mellitus. They were also clarified for any life-threatening infectious diseases if they had. The volunteers did not suffer from cardiovascular or cerebrovascular diseases and had not been hospitalized for any reason at least for 6 months before they participated in the study. Women volunteers, who had never received any contraceptive medications, were asked to donate their blood in the mid-follicular phase of their menstrual cycle. No medications including acetyl salicylic acid (aspirin) was not taken by the participants at least for 4 weeks before they donated blood.

3.2.4. Preparation of platelet-rich plasma and platelet-free plasma from blood samples

Blood samples (20ml) were obtained by venepuncture by using 19-gauge siliconized needles, collected in plastic vials, anti-coagulated by using sodium citrate (13mmol/l final concentration) as described before [Chakraborty et al, 2003]. The PRP and platelet-free plasma (PFP) were prepared from the anti-coagulated blood samples as described [Chakraborty et al, 2003].

3.2.5. Synthesis of nitric oxide in platelets in the presence or absence of estriol

Incubation mixtures containing PRP were incubated with 10mmol/l of *l*-arginine in the presence or absence of estriol in different times. In control experiments nitric oxide synthase (NOS) activity in the PFP was similarly treated with the steroids under identical conditions. In our preliminary experiments, it was found that estriol was able to synthesize maximal nitric oxide when PRP was incubated at 37°C. After incubation of either PRP or PFP with 0.6nmol/l estriol at 37°C, the synthesis of nitric oxide was determined by the conversion of oxyhaemoglobin to methaemoglobin by the spectralchanges of the absorption maxima at 525 and 630nm as described earlier [Bhattacharyya et al, 2009]. Appropriate control experiments were carried out by incubating PRP with 10mmol/l of *l*-arginine in the absence of the added estrogen.

The amount of nitric oxide formed in the presence of estriol was calculated by subtracting nitric oxide formation in the control experiment. The quantitation of nitric oxide was independently verified by chemiluminescence method [Cox et al, 1982].

3.2.6. Preparation of gel-filtered platelets

Gel-filtered platelets (GFPs) were prepared by gel filtration of PRP on Sepharose CL-2B and suspended in Tyrode's buffer without Ca^{2+}, pH 7.4 as described before [Dutta-Roy, 1987].

3.2.7. The lysis of the clotted platelet-rich plasma in the presence of added estriol

The PRP or PFP was clotted after incubation with estriol in the presence of $CaCl_2$ (10mmol/l) in silicon-coated glass tubes as described earlier [Karmahapatra et al, 2007]. The clot lytic activity of the added estriol (0.6nmol/l) was recorded by non-weighted photography and by the determination of the accumulated fibrin degradation products in the lysate as described below at different times. Experiments were also carried out by adding 0.1mmol/l *l*-NAME (NG-nitro-L-arginine methyl ester) to PRP to inhibit estriol-induced nitric oxide synthesis in platelets [Dutta-Roy et al 1987] before the PRP or PFP was treated with estriol and subsequently clotted by $CaCl_2$.

3.2.8. Determination of fibrin degradation products, FDP_1 and FDP_2 by the estriol induced fibrinolysis of the clotted PRP

The fibrinolysis of the clotted PRP or PFP was determined by determining the amounts of fibrin degradation product-1 (FDP_1) and fibrin degradation product-2 (FDP_2) accumulated in the clot lysate by SDS-polyacralamide gel electrophoresis of the lysate [Carr et al, 1995]. Both FDP_1 and FDP_2 bands were excised out of the gel after the electrophoresis, and amounts of the proteins were determined as described by Lowry et al [Lowry et al, 1951]. Appropriate control experiments were run by using similar Vol of only 0.9% NaCl without estriol to determine the accumulation of FDP_1 and FDP_2 in the clot that might be formed due to clot retraction and not by fibrinolysis.

3.2.9. Isolation of protein from the intact platelet membranes by using Triton X-100

Triton X-100 (0.05% v/v) was added to the GFP suspension in Tyrodes' buffer, pH 7.4, prepared from 200ml of blood obtained from a single donor, and incubated at 0°C with occasional shaking for 30min as described before [Dutta-Roy et al, 1987]. The supernatant was subsequently collected and was dialyzed against 0.9% NaCl overnight to remove the detergent at 4°C. Release of NO was catalysed by the dialyzed protein was determined as described below.

Typically, ≈0.6mg of protein in the supernatant was incubated with 10μM of *l*-arginine containing 2.0mM $CaCl_2$ in 2.5ml reaction mixture with 0.6nM estriol for 45 min at 37°C and the release of NO was determined as described above.

Lineweaver-Burk Plot of the electrophoretically purified protein with NOS like activity (described below) was constructed in the presence and absence of estriol.

3.2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the released proteins in the supernatant from the Triton X-100 treated GFP

The dialyzed supernatant from the Triton X-100 treated platelets was electrophorosed on SDS-polyacrylamide gel and the protein bands were stained by Coomassie brilliant blue [Fazekas et al, 1963]. The protein bands in an identical gel, but not stained, were excised out from the gel. Each protein band was separately excised, triturated in 0.9% NaCl, and clarified by centrifugation and dialyzed as described above. The NOS activity of each of the eluted and dialysed protein bands was subsequently determined.

The 69kDa protein band in the gel that released NO was reduced by using di-thiothreitol (DTT) and electrophorosed on SDS polyacrylamide to determine the subunit composition of the protein [Cleland, 1964].

3.2.11. The amino acid sequence analysis of the protein with NO releasing ability

The amino acid sequence analysis of the electrophoretically purified protein, first in the presence and then in the absence of SDS, (as described above) that showed stimulated release of NO in the presence of 0.6nM estriol was performed at Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer. The protein sample was processed as instructed by the service provider.

3.2.12. Binding of estriol to the electrophoretically purified protein (Mr.69kDa) with NO producing ability

The details of the binding of estriol to a platelet membrane protein that had the ability to release NO as described above was carried out by the methods that we have described recently [Jana et al, 2013]. Briefly, the binding of estriol to the electrophoretically purified protein was carried out by separating the bound estrogen from the unbound estrogen by using micro glass fibre cation (GF/C) membrane filters by using Millipore filtration [Kahn et al, 1990]. The amount of the estriol was quantitated by ELISA using estriol antibody [Bhattacharjee et al, 2012]. The specific binding was determined by subtracting nonspecific binding from the total binding.

3.2.13. Scatchard plot analysis

Scatchard plot [Scatchard, 1949] of the equilibrium binding of estriol to the electrophoretically purified protein with NO producing activity was determined by the ELISA of the steroid as described before [Bhattacharjee et al, 2012].

3.2.14. Statistical analyses

The results shown are mean±standard deviation (SD) of at least 5 different experiments using the blood samples from 5 different donors each in triplicate.

The significance “p” of the results was determined by student’s t test, and $p<0.001$ was considered to be significant (n=15). The coefficient of correlation “r” was determined by Pearson test by using GraphPad Prism software.

3.3. Results

3.3.1. The estriol induced fibrinolysis of the clotted PRP due to the stimulation of NO synthesis in platelets

Experiments were carried out to determine the role of estriol, if any, on fibrinolysis of the clotted PRP induced by NO released in platelets through plasmin formation [Karmahapatra et al, 2007].

Incubation of clotted PRP with 0.6nM estriol was found to result in the gradual

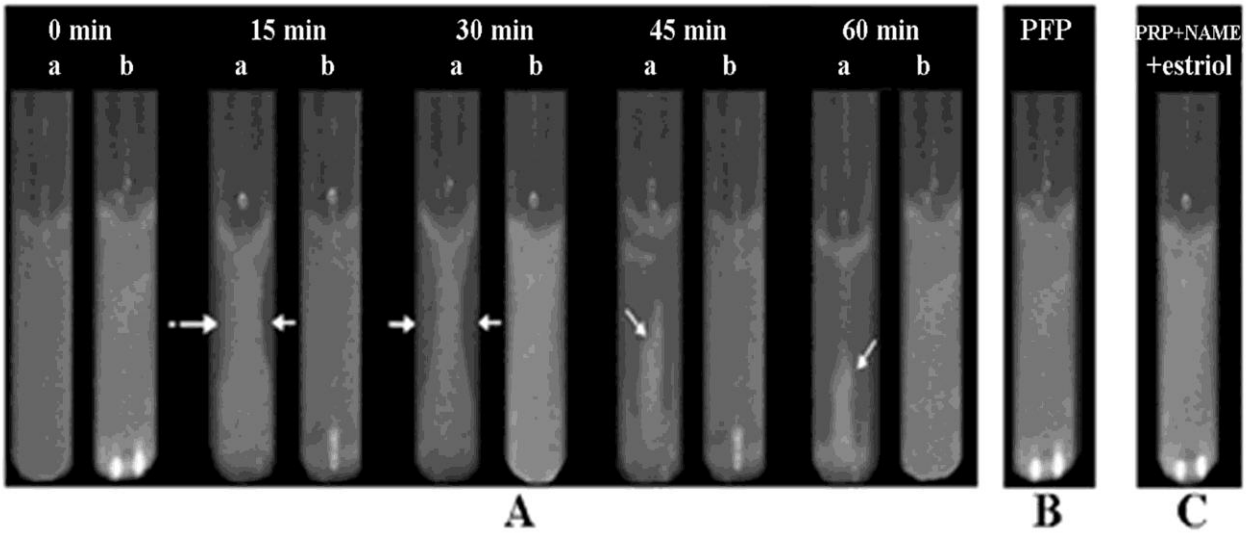

Figure-3.1*: The lysis of the clotted platelet-rich plasma in the presence of 0.6nmol/l estriol at different times.*
(A) The lysis of clot at different times is shown by the white arrow (→) in the tube marked 'a'. The tubes marked 'b' show no clot lysis at different times in the absence of the oestrogen. In these tubes, only vehicle (0.9% NaCl) was used. The tubes were incubated for different time as indicated and the lysis was recorded by photography as described in Materials and Methods section. (B) The absence of clot lysis in the presence of 0.6nmol/L estriol in the case of the clotted platelet-free plasma (PFP) under identical condition.
(C) The effect of addition of *l*-NAME to the PRP on the clot lysis in the presence of 0.6nmol/L estriol. The

increase of the clot lysis from 0min to 60min at 37°C as shown in the tubes marked "a" at different times (Figure 3.1A), and it was found that at 60min the lysis of the PRP clot was maximally achieved. In control experiments where the PRP was clotted only in the presence of 0.9% NaCl (vehicle for estriol) no clot lysis could be seen in the tubes marked "b" at different times (Figure-3.1A).

When platelet free plasma was clotted in the presence of 0.6nM estriol no clot lysis could be found (Figure 3.1B). The addition of 0.1mM *l*-NAME that inhibited NO synthesis

in the platelets incubated with 0.6nM estriol, resulted in the complete failure of the estrogen to lyse the clotted PRP (Figure-3.1C).

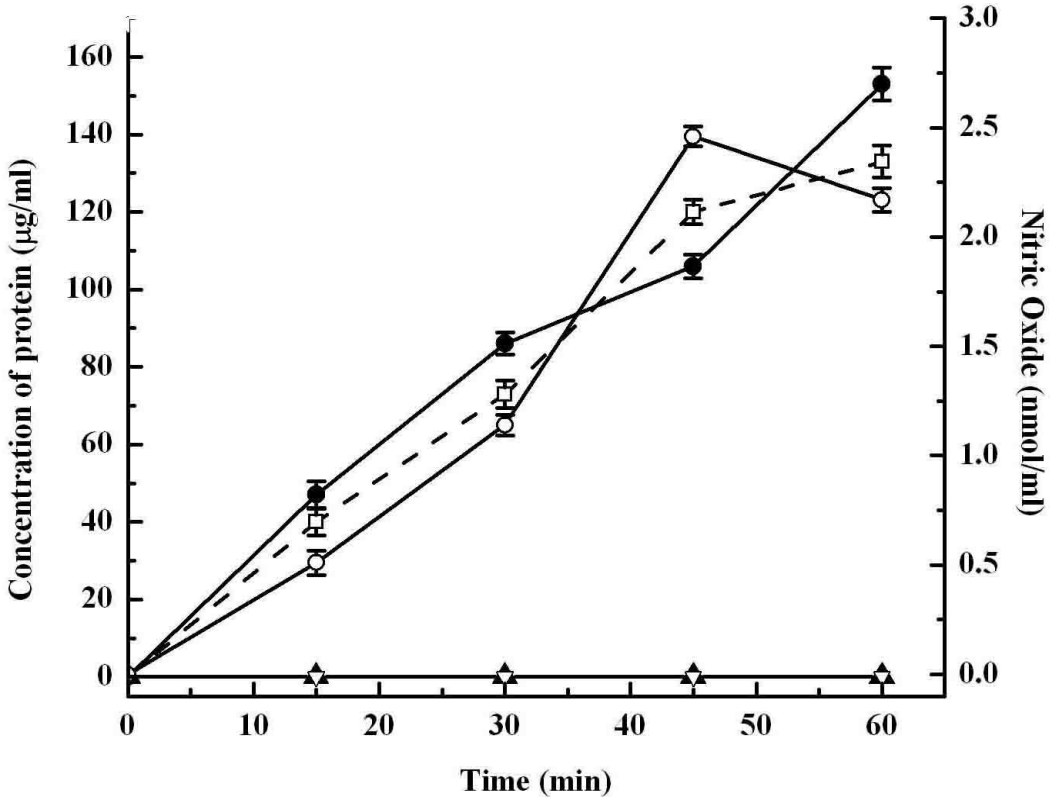

Figure-3.2: ***Accumulation of fibrin degradation products (FDP1 and FDP2) in the lysate and synthesis of nitric oxide in the presence 0.6nmol/l estriol at different times***.

The appearance of hollow circles (-○-) describes the production of nitric oxide at different times as indicated.

The solid circles (-●-) or the continuous line represents FDP1 and hollow squares (-□-) or the discontinuous line shows the FDP2, respectively, at different times as indicated.

Solid triangles (-▲-) show the absence of FDP_1 and FDP_2 in the lysate in the presence of both estriol and 0.1mmol/l *l*-NAME at different times.

The hollow inverted triangles (-▽-) demonstrate that the accumulation of FDP_1 and FDP_2 in the lysate in the absence of estriol wherein only equal Vol of only 0.9% NaCl was used and incubated under identical conditions.

Mean±SD of at least five experiments each in triplicate using blood samples from five different volunteers. After 60 min incubation of estriol, the level of FDP_1 and FDP_2 was significantly different ($P<0.001$, n=15) from the level FDP1 and FDP2 in the absence of estriol after 60min.

When the lysates were obtained from the above experiment after different times of incubation, and the accumulation of fibrin degradation product-1 (FDP_1) and fibrin degradation product-2 (FDP_2) in the lysate were determined (Figure-3.2), the amounts of both FDP_1 and FDP_2 in the lysate were found to be increased with the increase of the incubation time, and at 60min the maximal amounts of both FDP_1 and FDP_2 were found to be accumulated in the lysate (Figure-3.2). In control experiments where an equal Vol of 0.9% NaCl was used instead of estriol and the PRP was clotted by $CaCl_2$, no such increase

in the amounts of FDP_1 and FDP_2 due to fibrinolysis could be found. Thus excluding the possibility that the observed PRP clot lysis was due to the clot retraction where no accumulation of either FDP_1 or FDP_2 in the lytsate takes place. The correlation of coefficient "r" between the NO synthesis and the appearance of FDP_1 and FDP_2 were determined to be 0.9774 and 0.9087.

3.3.2. The mechanism of estriol induced stimulation of NO release in platelets

As human blood platelets do not contain DNA, the role of estriol in the NO production in PRP or in GFP through the stimulation of NOS requires a non-genomic mechanism (i.e. DNA independent) for the estrogens' effects in the inhibition of platelet aggregation due to NO production as reported before [Jana et al, 2013] or in the fibrinolysis of the PRP clot through estriol induced NO production as described above.

And, as such, efforts were made to determine whether platelet membrane might contain a protein that could be activated in the presence of the estrogens to release NO in PRP or in GFP as reported before [Jana et al, 2013]. To test the possibility of increase in NO in the platelet membrane, GFP that could be stimulated by estriol was treated with 0.05% Triton X-100 as described in the Materials and Methods. Experiments were carried out to find whether the treatment of intact platelets with the low amount of Triton X-100 that has been reported before to result in the release of membrane proteins in the supernatant without dissolving the whole platelets [Dutta-Roy et al, 1987] that might show the presence of basal NO releasing ability in the released proteins.

3.3.3. *Characterization of the platelet membrane proteins with NO releasing activity that was stimulated by estriol in the supernatant released by the treatment of platelets by Triton X-100*

The supernatant obtained from the Triton X-100 treated platelets was electrophorosed on polyacrylamide gel with SDS which showed the presence of several major protein bands when stained with Coomassie blue (Figure 3.3A). These protein bands were separately excised out from another identical gel that was not stained. The eluted protein from each band in the gel was triturated in 0.9% NaCl, clarified and dialyzed as described in the Materials and Methods. Only the 69kDa protein band showed enhanced NOS activity when treated with 0.6nM estriol. The eluted protein of 69kDa from the gel was next concentrated and re-electrophorosed on polyacrylamide gel in the absence of SDS that again demonstrated the presence of a single protein (Figure-3.3B). The reduction of protein did not show the presence of any subunits suggesting that the 69kDa platelet protein with NOS activity was probably a single polypeptide chain (Figure-3.3C).

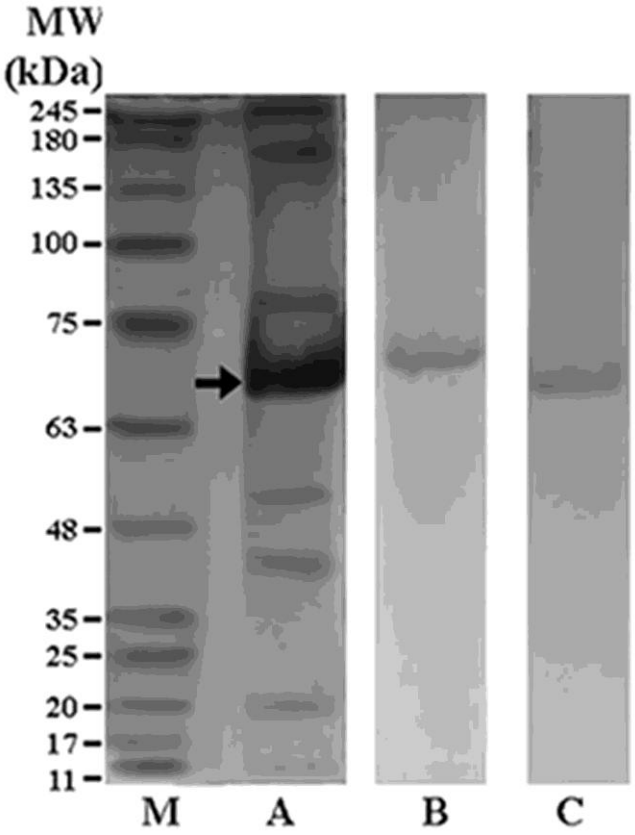

Figure-3.3: ***PAGE of the supernatant from the Triton X-100 treated gel filtered platelet suspension.***
Lane A: SDS-PAGE of the supernatant from GFP treated with Triton X-100 stained with Coomassie blue. The arrow shows the protein band of 69kDa.
Lane B: PAGE of the protein band in the Lane A (black arrow) that was excised out and reelectrophoresed in the absence of SDS.
Lane C: The protein from Lane B was isolated, treated with dithiothreitol and electrophoresed in SDS-polyacrylamide gel.
Lane M: Marker proteins in SDS polyacrylamide gel. Protein bands in all cases were stained by coommassie brilliant blue.

When the electrophoretically purified protein was treated with either estriol or estradiol, the synthesis of NO from *l*-arginine in the presence of Ca^{++} in the

reaction mixture could be demonstrated. However, it was found that the amounts of NO produced in the presence of 0.6nM estriol were >3 fold (1.77nmol NO formed/mg protein/h) when compared to that produced in the presence of similar amounts of estradiol (0.49nmol of NO formed/mg of protein/h) under identical conditions indicating estriol was more potent stimulator of the platelet membrane NOS than estradiol.

Lineweaver Burk plot of the NOS activity of the protein purified by the repeated electrophoresis (Figure-3.3B) as described above in the presence and absence of estriol demonstrated that K_m of *l*-arginine in the absence of added estrogen was 8.637mM with V_{max}=16.33nmol NO formed/mg protein/h (Figure-3.4, Line-B). In contrast, when the same protein was treated with 0.6nM estriol the K_m was found to be reduced to 6.093mM with

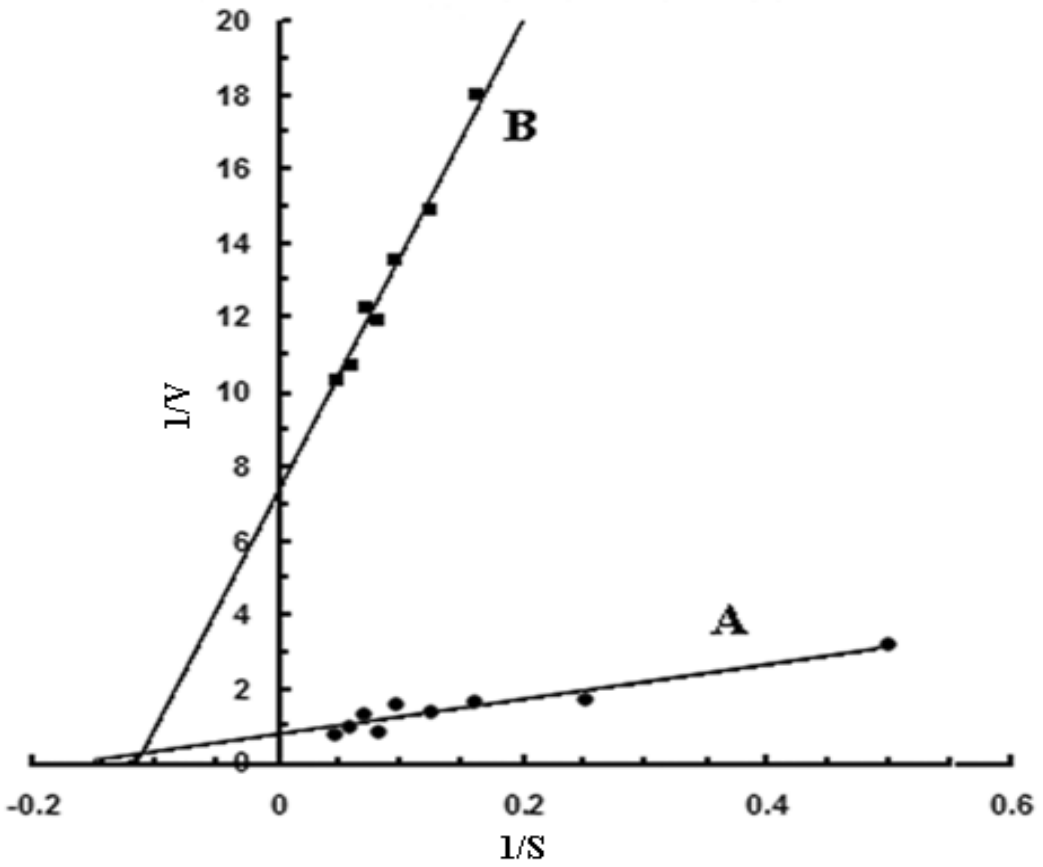

Figure-3.4: ***Lineweaver–Burk plots of the nitric oxide synthase activity of the electrophoretically purified 69kDa protein in the presence and absence of estriol.***
The 69kDa (Mr.) protein was purified by PAGE first in the presence and then in the absence of SDS. The purified protein was studied for its nitric oxide synthase activity by Lineweaver–Burk plots in the presence and absence of 0.6nmol/L estriol added to their action. Line A: Lineweaver–Burk plot of the 69-kDa protein in the presence of 0.6nmol/l estriol in the reaction. Line A and Line B represents Lineweaver-Burk plot of the 69kDa protein in the presence and absence of 0.6nmol/L estriol respectively.

corresponding increase of Vmax to 154.2nmol NO formed/mg protein/h indicates that the rate of NO release was stimulated by ≈ 10fold in the presence of 0.6nM estriol over the basal NO releasing ability (Figure-3.4, line-A).

The Uniprot data bank matching of the amino acid sequence of the purified platelet protein with the NO releasing ability (MW. 69kDa) identified to be Human serum albumin precursor (Uniprot ID: **P02768**) as shown in the Figure-3.5.

MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAF
AQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTV
ATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTA
FHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAAC
LLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAE
FAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKEC
CEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
MFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFK
PLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRN
LGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCT
ESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALV
ELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

Figure 3.5: The amino acid sequence of the 69kDa protein with NOS activity
The electrophoretically purified protein with 69kDa Mr. was used for the determination of the amino acid sequence. The details of the procedures and the Uniprot data bank of the protein by matching the amino acid sequence

3.3.4. *The equilibrium binding of estriol to the electrophoretically purified protein and Scatchard plot analysis of the binding characteristics*

The binding profile of estriol to the binding profile of estriol to the electrophoretically purified protein (Figure 3.3B) from platelets showed a saturable binding characteristic and the presence of specific estriol binding sites in the protein as determined by the subtraction of the nonspecific binding from the total binding (Figure-3.6, Panel-A).

Scatchard plot of equilibrium estriol binding characteristics to the purified platelet protein

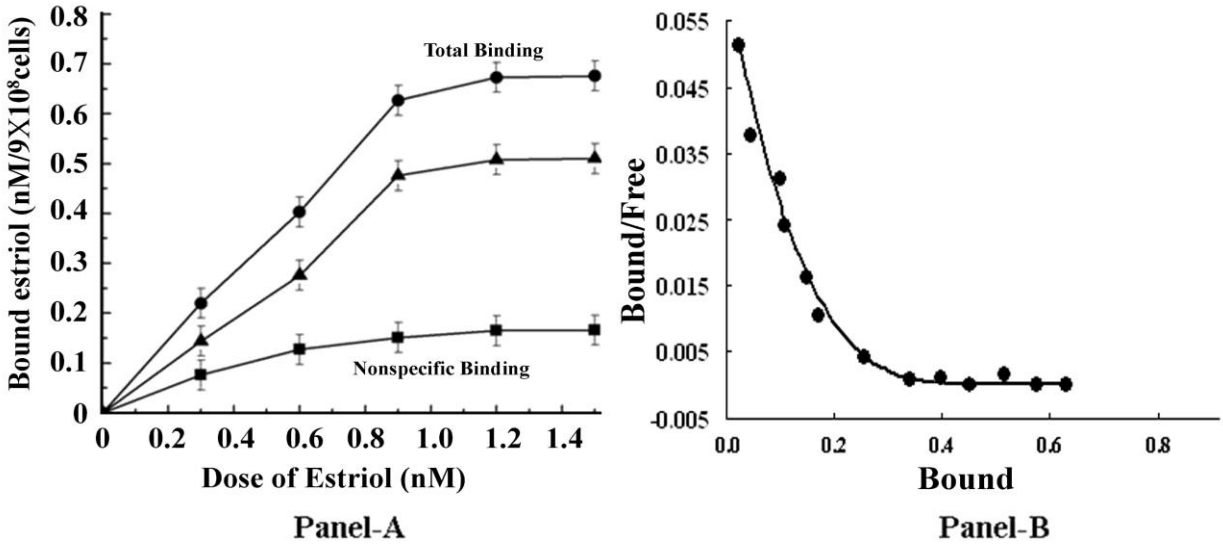

Figure-3.6: ***The equilibrium binding of estriol to the 69-kDa protein with nitric oxide synthase activity that was stimulated by estriol.***

(A) The nonspecific and specific binding of estriol to the 69-kDa protein was determined. Values obtained from total binding and nonspecific binding were significantly different when $P<0.001$, $n=15$.

(B) Scatchard plot of the equilibrium binding to the 69-kDa protein was determined by separating the bound estriol from the unbound by using GF/C filters and the quantity of the estriol was determined by

produced a curvilinear profile of estriol binding to the purified 69kDa protein, suggesting the presence of heterogeneous binding sites of the estrogen in the protein molecule with one high affinity (Kd_1=6.002nM) low capacity($n_{1=}$39±2 molecules estriol/molecule of protein) and one low affinity (Kd_2=243nM) high capacity ($n_2=1.4X10^3$ molecules estriol/molecule of protein) binding sites of estriol in the Mr.69kDa protein (Figure-3.6, Panel-B).

3.4. Discussion

These results demonstrated that estriol; one of the most abundant ovarian hormones was a potent stimulator of NO production by a platelet membrane associated protein of Mr. 69kDa which resembles NOS like activity. As described in the results, the binding of estriol to this 69kDa protein resulted in the ≈ 10-fold stimulation of the basal NO production ability of the protein. And, as such, it can be inferred that the 69kDa protein, identified to be human serum albumin precursor (Figure-3.5) might not only bind to estriol with high affinity (Kd_1=6,002nM), but the binding of the estrogen to this protein resulted in the stimulation of the basal NO production by ≈ 10fold. In this context it was also noted that the Kd_1 of estriol binding to 69kDa protein was in ranges similar to the optimal concentration of the estrogen for the maximal inhibition of platelet aggregation as reported earlier [Jana et al, 2013]. The low affinity (Kd_2=243nM) with high capacity binding sites (n_2) probably indicated non-specific binding of fatty acids like molecules by the human serum albumin precursor.

ERα or ERβ binding to estrogen resulting in upregulation of NOS gene expression and NO production is reported [You, 2003], which may be ruled out in our present investigation because platelet is an anucleated cell. The 69kDa protein was apparently acting like a 'receptor protein' for the production of the NO induced by estriol in the absence of DNA in human blood platelets. These results suggested a non-genomic expression of the estrogen effect in anucleated platelets apparently in the absence of nuclear receptors or DNA which are currently believed to be involved in the expression of the steroid effect at least in the nucleated cells [You et al, 2003]. There is a possibility of nitrosothiol formation with NO and the 69kDa protein as predicted to be human serum albumin precursor that is highly *cys* rich [Kharitonov et al, 1995]. Estrogen binding to albumin followed by structural modifications may also promote the sustained release of NO, which is able to perform further physical functions [Chakrabarti et al, 2010].

In the effects of the steroids that mediate their effects through the expression or inhibition of specific proteins through hormone receptor element (HRE) in the DNA [Levin, 2005]. We have reported before that both estriol and progesterone were capable of

inducing “maspin” synthesis, an anti-breast cancer protein in the nucleated cells, through the synthesis of NO in the nucleated cells [Bhattcharjee et al, 2012]. Interestingly, as we have reported here, that estriol was also mediates its effect through NO synthesis in the inhibition of platelet (anucleated cells) aggregation either in PRP or in GFP [Bhattacharyya et al, 2009 which might suggest NO was acting like the “messenger” of these steroids in these cases apparently without the synthesis of proteins. In the above context, it should also be mentioned that the effect of aldosterone that has an essential role in the electrolyte balance in the kidney function apparently was capable of mediating its effect in a DNA independent way [Logvinenko, 2007].

Estriol at 0.6nM was found to be a potent inhibitor of platelet aggregation as described before [Jana et al, 2013], and it was also a thrombolytic agent in the post platelet aggregation event of thrombus formation as shown in the Figure-6.1 and Figure-6.2 above.

As described above, the NOS like activity of the platelet membrane protein by estriol was 3 times greater than estradiol which is known to be a more potent estrogenic metabolite than estriol proves the estrogenic potency of estrogen was not necessarily related to any physiologic activities [Soladan, 2003] and our result suggested that the antithrombotic property of estriol could have a more important role in the prevention of AIHD than that of the estradiol in women before the onset of menopause.

In the above context, the role of estriol induced NO release that only reported to result in the inhibition of platelet aggregation, but as described here NO was also found to be a potent thrombolytic agent through the activation of plasminogen to plasmin (a serine protease) through the excision of 14kDa Mr. peptide from plasminogen. Then the formation of plasmin was independent of Hageman factor dependent slow pathway for the systemic production of plasmin [Bhattacharyya et al, 2009]. And, as such, the estriol effect as both the inhibitor of platelet aggregation and a thrombolytic agent that is similar to those of acetyl salicylic acid, currently known to be one of the most beneficial compounds against the AIHD. Perhaps, estriol could have similar beneficial effects against AIHD due to thrombus formation on the coronary artery wall.

Finally, it should also be mentioned here that NO is only known compound currently known that inhibits platelet aggregation when it increases. The decrease of the basal NO

level actually led to the aggregation of platelets even in the absence of ADP [Banerjee et al, 2013].

CHAPTER 4

"CROSS-TALK" BETWEEN RECEPTORS OF DERMCIDIN ISOFORM-2 AND ESTRIOL ON PLATELET MEMBRANE

4. *"CROSS-TALK" BETWEEN RECEPTORS OF DERMCIDIN ISOFORM-2 AND ESTRIOL ON PLATELET MEMBRANE*

4.1. Introduction:

Platelet aggregation by different platelet aggregating agents, including ADP, *l*-epinephrine, collagen or thrombin has a critically important role in physiologic events in the lifesaving blood coagulation process [reference Colman et al, 1987 for comprehensive literatures]. In contrast, an excessive platelet aggregation on the atherosclerotic plaque rupture site in the coronary artery is reported to develop into thrombus formation that has been demonstrated to obstruct the normal circulation of the blood in the musculature of the heart, leading to acute coronary syndrome (ACS) as well as acute myocardial infarction (AMI), known to be the major killers of the human race (Fustar, 1996). The inhibition of platelet aggregation by several humoral factors, including insulin (Chakraborty et al, 2004), prostacyclin (Whittle et al, 1978), estriol (Jana et al, 2013) and pharmacological compound like acetyl salicylic acid (aspirin) through their ability to inhibit platelet aggregation are considered to produce a counter veiling effect on the excess platelet aggregation leading to homeostasis and consequently might help to prevent the occurrence of ACS (Karmahapatra et al, 2007).

It is generally accepted that the expression of the steroid effects, including estrogens (of which estriol is one) in the hormone responsive cells is mediated through the binding of the steroid to one of the nuclear receptors (Levin, 2005). Binding of the agonist changed the conformation of the nuclear receptor that in consequence binds to a specific sequence in the DNA known as hormone responsive element (HRE) that results either in the expression or in the inhibition of the gene that results in the synthesis or in the inhibition of protein(s) that mediates the steroid effect in the cells (Levin, 2005). The validity of this assertion in the effect of estriol (one of the estrogens) in the stimulation of nitric oxide (NO) synthesis as a potent inhibitor of platelet aggregation in platelets may actually pose a special problem in that we have recently reported that, estriol, at 0.6nM probably acted, to the best of our knowledge, as the most potent inhibitor of human platelet aggregation currently known through the production of NO in platelets (Jana et al, 2013). As human blood platelets do not contain DNA, the effect of estriol in the inhibition of platelet aggregation through the NO

synthesis catalyzed by nitric oxide synthase (NOS) cannot be mediated through the DNA dependent expression of the NOS gene in the platelets.

In the context of estriol as a potent inhibitor of platelet aggregation, the obvious question was, if estriol was such a potent inhibitor of platelet aggregation why did the steroid fail to inhibit platelet aggregation in the development of ACS or AMI in man?

We report herein the mechanism of the resistance of the platelet aggregation from the AMI subjects was due to the systemic appearance of DCN-2 (Ghosh et al, 2011) in the circulation that conferred the resistance of platelets to estriol from the AMI subjects due to the “cross talk” between the receptors of DCN-2 and estrogen on the platelet surface.

4.2. Materials and Methods:

4.2.1. Ethical clearance for human subjects

Platelet rich plasma (PRP) both from a control and from the subjects with AMI was obtained. Appropriate protocol according to Helsinki agreement was approved by the Internal Review Board, Sinha Institute of Medical Science & Technology, Calcutta, West Bengal. All volunteers signed informed consent forms before they were included in the study. Informed consent forms were also signed by the control subjects.

As the subjects affected with AMI were not always physically fit to sign an informed consent form, the collection of blood was carried out by attending physician of the Department of Cardiology, Calcutta Medical College, Kolkata with the consent from next of kin of the patient and in the presence of legal councillor to the patients. The blood (5.0ml) was withdrawn only after the attending physician obtained consent from next of kin of the patient and decided that the withdrawing of the blood sample was safe and permissible, both for the confirmation of the occurrence of AMI by plasma Troponin-I level and of platelet aggregation studies.

4.2.2. *Chemicals and antibody*

Estriol (98% purity) and goat anti-rabbit immunoglobulin G-alkaline phosphatase were obtained from Sigma-Aldrich. Polyclonal antibody against estriol was obtained from Thermo Scientific. Maxisorp plates for enzyme linked immunosorbent assay (ELISA) were obtained from Nunc, Denmark. All other chemicals used were of analytical grade.

4.2.3. Selection of volunteers

Subjects who participated in the study did not have any history of systemic hypertension or diabetes mellitus or were suffering from any life threatening infection. The participants did not suffer from cardiovascular or cerebrovascular diseases and no record had been found to be hospitalized for any reason at least for 6 months before they participated in the study. The volunteers were asked not to take any medications including acetyl salicylic acid (aspirin) for at least 4wks before they donated blood. None of the female volunteers had ever received any contraceptive medications.

4.2.4. The selection of AMI patients

The blood sample was withdrawn from both ST elevated AMI (STEMI) and non ST elevated AMI (nSTEMI)-UA patients. However, when such differentiation was not possible, the subject was not included in the study. The presence of Q wave in the EKG was routinely followed for the patient selection. At presentation all AMI patients had characteristics of chest pain for AMI for more than 120min. The occurrence of AMI was confirmed by the determination of Troponin-I by collecting blood samples within 6h of hospitalization before the initiation of any cardiac therapy. The blood sample was drawn from 30 AMI Patients (M=15, F=15) with an equal number of age and sex matched control volunteers.

4.2.5. Preparation of platelet-rich plasma (PRP) and platelet free plasma (PFP) from blood samples

Blood samples (5.0ml) were obtained by venepuncture by using 19 gauge siliconized needles, collected in plastic vials, anti-coagulated by using sodium citrate (13mM final concentration). The PRP and PFP were prepared from the anti-coagulated blood samples as described before (Chakraborty et al, 2003).

4.2.6. *Production of NO in platelets in the presence or absence of estriol*

Incubation mixtures containing PRP were incubated with 10μM of *1*-arginine in the presence or absence of different concentrations of estriol with triplicate experiments for each sample. To determine NO production in the PFP in the control experiment, the platelet free plasma was similarly treated with the steroids under identical conditions. In our preliminary experiment, it was found that the estrogens were able to maximally produce NO after 45 min incubation of the platelet preparations at 37°C. After incubation of either PRP or PFP for 45 min at 37°C in the presence of the estrogens, the production of NO was determined by the conversion of oxyhemoglobin to methemoglobin by the spectral changes of the absorption maxima at 525 and 630nm as described before (Karmohapatra et al, 2007). Production of NO was confirmed by chemiluminescence method (Cox et al, 1982).

4.2.7. Aggregation of platelets

Platelet aggregation both in control and AMI platelets were determined by using 2.0μM ADP as the aggregating agent with an aggregometer as described before (Chakraborty et al, 2003). Aggregationof AMI platelets and in control platelets was determined in equal number

of age and sex matched volunteers (n=30, M=15, F=15) with triplicate experiments in each . To find out if the estriol has any role to inhibit both control and AMI platelets, with 0.6nM estriol was added to PRP as described before (Jana et al, 2013).

4.2.8. Isolation of estriol binding protein from the intact platelet membranes by using Triton X-100

Gel filtered platelets (GFP) from PRP were prepared by using a Sepharose 6B column as described before (Jana et al, 2013). The GFP suspension in Tyrodes' buffer, pH 7.4, was prepared from 200ml of blood obtained from a single donor. Triton X-100 (0.05% v/v) was added to the GFP and incubated at 0°C with occasional shaking for 30min as described before (Dutta-Roy et al, 1987). Synthesis of NO catalysed by the dialyzed protein from the GFP suspension supernatant was determined as described below. The platelet supernatant thus obtained was electrophoresed first in the presence of sodium dodecyl sulphate (SDS) in polyacralamide gel and the protein bands were stained by coommassie brilliant blue (Fazekas et al, 1963). In an identical experiment where the same supernatant was similarly subjected to SDS gel electrophoresis, but was not stained by coommassie blue, the protein bands were excised from the gel and separately triturated in 0.9% NaCl, dialyzed overnight against 0.9% NaCl and was concentrated by using polyethylene glycol (Howe et al, 1964) the NOS activity of the preparation was determined as described before (Jana et al, 2015).

Typically, ≈27μg/ml protein in the supernatant was incubated with 10μM of *l*-arginine containing 2.0mM $CaCl_2$ in 2.5ml reaction mixture with 0.6nM estriol for 45 min at 37°C and the production of NO was determined as described above.

4.2.9. Characterization of the estriol binding protein

The platelet membrane protein, thus obtained, was re-electrophoresed in polyacrylamide gel in the absence of SDS was dialyzed, concentrated as described above. NOS activity with 0.6 nM estriol was determined in crude and in each step of purification of the protein.

The isolated and dialyzed protein from the gel was also subjected for its purity by micro-capillary reverse-phase HPLC Nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer at Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology.

4.2.10. Determination of allosteric modulation of the platelet membrane protein for NO production

The platelet membrane proteinpurified by repeated electrophoresis as described above was treated with 0.6nM estriol, and the activity of NOS was determined by using different concentrations of *l*-arginine (the substrate of NOS) to determine V_{max} for the assessment of the effect of estriol, as a possible heterotropic allosteric activator (Nelson et al, 2010).

4.2.11. Determination of the effect of estriol on tamoxifen treated NO production in protein

The production of NO by the protein was studied by using tamoxifen; a well-known antagonist of estrogen receptor (Shiau et al, 1998) to determine whether the compound can nullify the effect of estriol on NO production. Typically, the purified NOS from the platelet membrane preparation, as described above was treated with different concentrations of the estrogen receptor antagonist and after incubation for 45 min at 37˚C, the production of NO in the reaction mixture was determined as described.

4.2.12. The Scatchard plot analysis

The binding characteristics of estriol to platelets in the presence or absence of Tamoxifen or DCN-2 were carried out by the Scatchard plot analysis (Scatchard, 1949) of the equilibrium binding of the agonist.

4.2.13. Statistical Analysis

The results shown are mean±standard deviation (SD); the significance (p) of the results was determined by the student t-test. The coefficient of correlation (‘‘r’’) was determined by Pearson test. The dissociation constant (K_d) and the number of the DCN-2 binding sites (B_{max}) in the Scatchard plot analyses were determined by Microsoft Office Excel.

4.3. Results

4.3.1. Purification of estriol binding protein in platelet membrane

To find out the purity of the protein that produce NO due to binding with the estriol the assay for NO production was done at each step of the protein purification as described in the Methods and Materials. It was found that in PRP (crude) the amount of NO formed 0.025nmol NO/mg protein and final product from the re-electrophoresis of the protein was found to be purified about 22,814 fold when compared with the PRP (Table-4.1).

Table 4.1: ***Summary of the protein purification steps*****:**

Steps of the purification	Total Protein (mg)	Specific activity (nmol NO produced/mg protein)	Fold Purifiction	Yield (%)
1. Platelet rich plasma (PRP)	**61.3**	**0.025**		
2. Gel filtered platelets (GFP)	**12.23**	**0.125**	**5**	**19.95**
3. Dialyzed protein from GFP suspension (Triton X-100 treated)	**0.038**	**40.52**	**1621**	**0.061**
4. Electrophoresed protein	**0.0027**	**570.37**	**22814.8**	**0.004**

4.3.2. Determination of allosteric modulation of the NOS activity of the protein in the presence of estriol

To find out whether the steroid could activate the enzyme as a heterotrophic allosteric activator when the basal level synthesis of NO was determined in the absence of the added estriol, it was found that the synthesis of NO production showed V_{max}=0.42nmol NO/ml with K_m=1.14µM (Figure-4.1). When the same preparation was incubated with 0.6nMestriol the NO production in the estriol treated NOS demonstrated V_{max}=3.16nmol NO/ml with K_m=6.32µM (Figure-4.1). In other word the rate of synthesis of the NO was increased by 8 folds in the presence of the steroid in the absence of new protein synthesis.

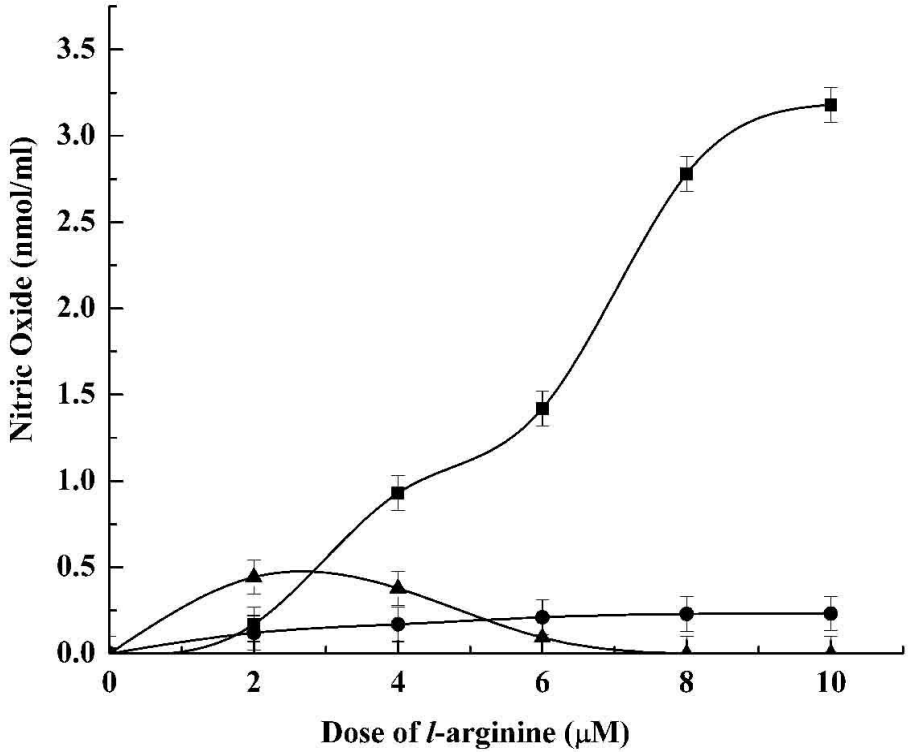

Figure-4.1: ***Allosteric modification of the platelet membrane (NOS) prepared in the presence and absence of estriol and tamoxifen***.
Platelet NOS was specified to homogeneity as described in the Materials and Methods. The purified membrane protein was treated with 0.6nM estriol and incubated for 45 min at 37°C. The enzyme preparation was next incubated with different concentrations of substrate (*l*-arginine) and synthesis of NO was determined.
The solid squares (-■-) = NOS treated with estriol (0.6nM) in the absence of tamoxifen.
Solid circles (-●-) = Control experiment in the absence of both estriol and tamoxifen.
Solid triangles (-▲-) represent the effect of tamoxifen (1.0nM) in the presence of estriol (0.6nM).
The result shown are mean±S.D. of at least 10 different experiments by using blood samples from different donors.

To determine whether the role of estrogen on the platelet surface NOS through the interaction of the steroid with the platelet surface receptors, pre-incubation of platelet with 1nM tamoxifen, an estriol receptor antagonist (Shiau et al, 1998) for 15min before the addition with the estrogen for 45 min resulted the inhibition of NO synthesis with V_{max}=0.44nmol/ml with K_m=0.76 µM (Figure-4.1).

4.3.3. Inhibition of estriol induced NO synthesis in the presence of estrogen receptor inhibitors

Different concentrations of estriol were incubated to find out the ability to stimulate the NOS and tamoxifen (0.1nM, 1nM, 10nM and 100nM) in another set were pre-incubated to find out its ability to inhibit estriol binding to the receptor and subsequent synthesis of NO. It was found that tamoxifen at 1nM concentration is able to inhibit NO synthesis maximally. The profile of the NO synthesis by estriol in the absence and presence of tamoxifen shows a difference in the production of NO from 1.54±0.043nmol/3 X 10^8 platelets to 0.42±0.011nmol/3 X 10^8platelets respectively (Figure-4.2).

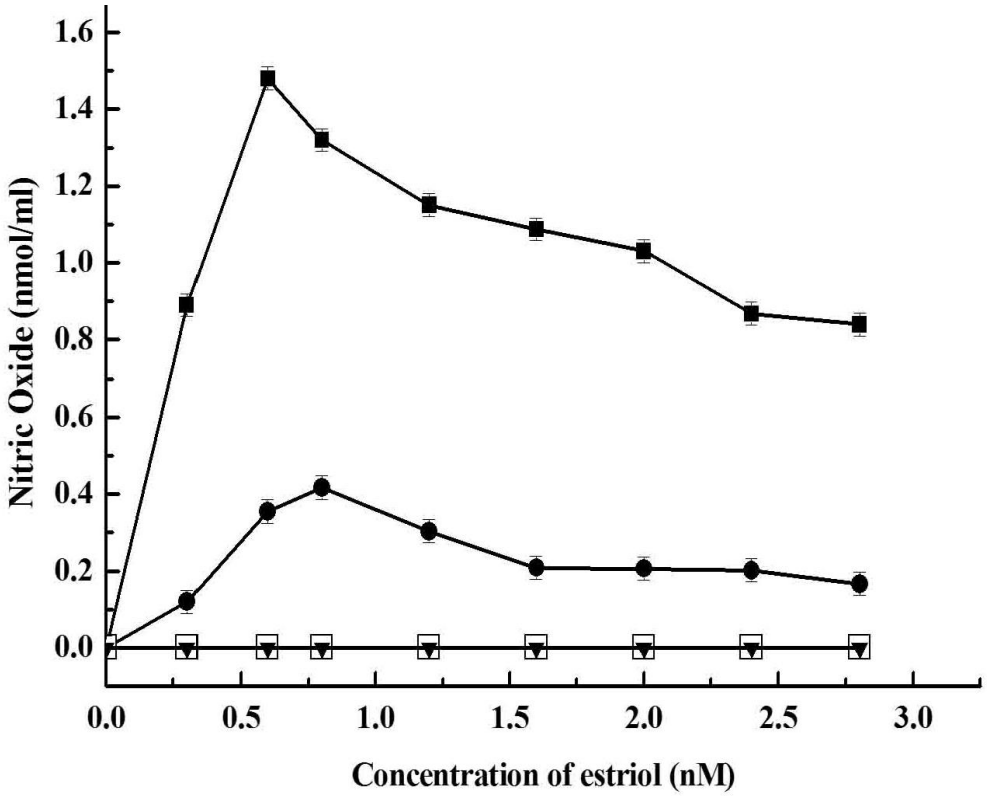

Figure-4.2: ***Effect of tamoxifen on estriol induced NO production in platelet membrane.***
The purified NOS from the platelet membrane was incubated with different concentrations of estriol as indicated. After incubation for 45 min at 37°C the synthesis of NO was determined. The synthesis of NO by the NOS preparation was also carried out in the presence of *l*-NAME.

As DCN-2, an inhibitor of NOS (Ghosh et al, 2011) has been reported to be present in the circulation in ACS and AMI when the estriol induced NO synthesis was determined in platelets, in AMI subjects, it was found that the synthesis of NO was decreased to 0.067±0.006nmol NO/3 X 10^8 platelets that contrasted the synthesis of 1.49±0.032nmol NO/3 X 10^8 platelets in the presence of 0.6nM estriol (Figure-4.3).

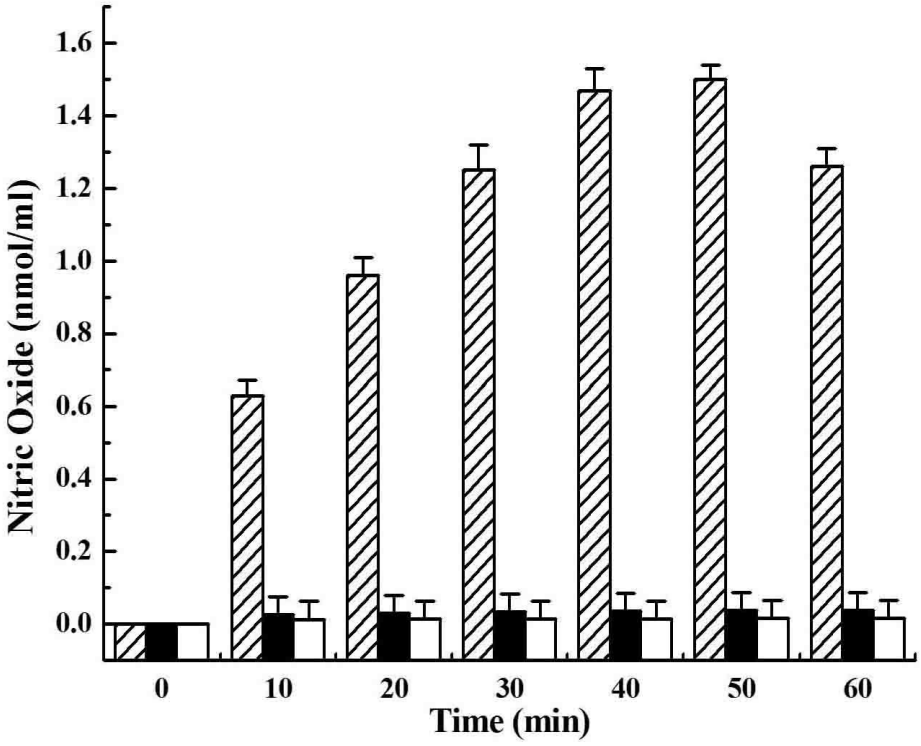

Figure 4.3: ***Stimulation of NO in platelets from the subjects with acute myocardial infarction (AMI) compared to normal platelet rich plasma.***

Platelet-rich plasma from the subjects with AMI was prepared (n=30, M=15, F=15). Platelet rich plasma was also prepared from age and sex matched male and female normal subjects. The platelet-rich plasma preparations were treated with 0.6nM estriol for 45 min at 37°C.

Stripped bar (▨) = The production of nitric oxide in different times in normal platelet rich plasma induced with 0.6nM estriol.

Solid bar (■) = The production of NO in AMI platelet rich plasma in the presence of 0.6nM estriol.

Hollow bar (□) = The production of NO at different times in the absence of estriol with added equal of vehicle (0.9% NaCl).

4.3.4. The impairment of estriol induced NO synthesis and platelet aggregation inhibition in AMI

When the PRP from AMI subjects (n=30, M=15, F=15) was treated with 0.6nM estriol and the aggregation of platelets was determined in the presence of 2.0µM ADP, it was found that the AMI platelet was not inhibited that contrasted the inhibition of platelet aggregation by 100% induced by the same amount of estriol in the presence of equimolar ADP under identical conditions in control PRP (Figure-4.4).

Aggregation of platelets in AMI subjects (n=30, M=15, F=15) and their age sex matched control was performed (Table-2). It was found that the mean value of aggregation in AMI platelelets

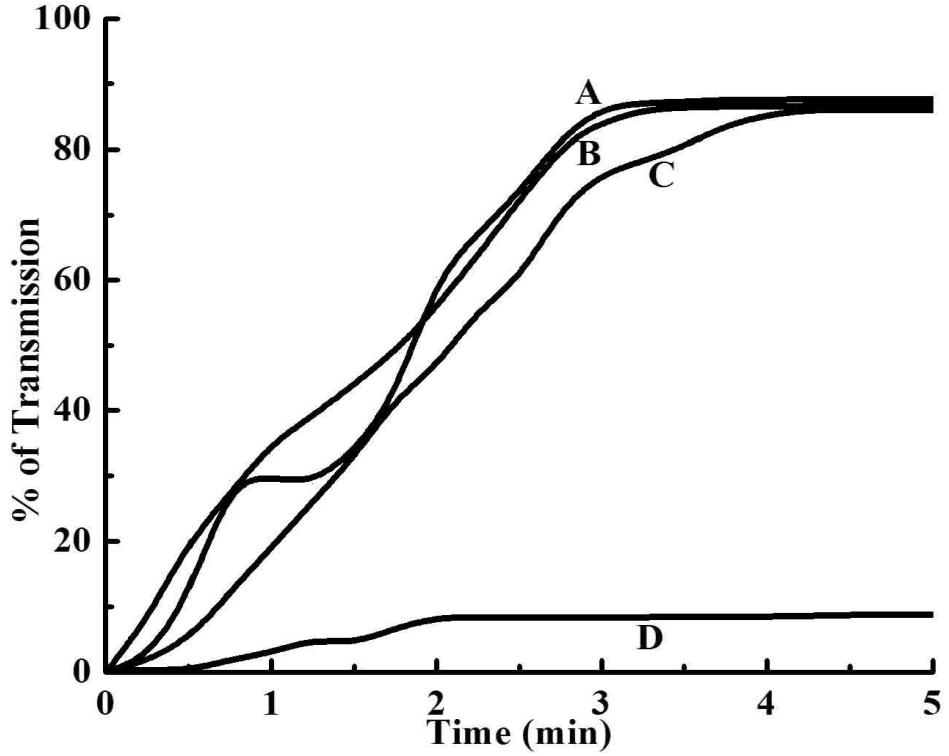

Figure-4.4: ***The effect of estriol on the ADP induced platelet aggregation in PRP from normal volunteers and from the subjects affected with AMI***
Platelet-rich plasma (PRP) was prepared from both AMI subjects (n=30, M=15, F=15) and from age and sex matched normal volunteers. The aggregation of platelets was initiated by treating the PRP with 2.0µM ADP and the aggregation was followed up to 5 min as shown.
A= the aggregation of AMI platelets without estriol,
B= aggregation of normal PRP without estriol ,
C= aggregation of AMI platelets incubated with estriol,
D= aggregation of normal PRP incubated with estriol.

(87.1±4.4% of transmis sion) did not change much (83.29±3.81% of transmission) due to incubation with 0.6nM estriol. Whereas, in control platelets (age and sex matched with AMI samples), the mean aggregation of platelets (82.64±8.29% of transmission) has been changed to 12.86±4.21% of transmission.

Correlation of coefficient "r" between platelet aggregation (% of transmission) in the presence of 0.6nMestriol and DCN-2 level have shown +0.974565 and +0.994845 respectively (Table-4.2).

Table 4.2: Aggregation profile (% of transmission) of platelets from AMI and control platelets

Parameters	AMI Samples			Normal Samples		
	Aggregation profile of platelets (% of transmission)		DCN-2 level (pmol/ml)	Aggregation profile of platelets (% of transmission)		DCN-2 level (pmol/ml)
	Platelets without estriol (A)	Platelets with estriol (C)		Platelets without estriol (B)	Platelets with estriol (D)	
Range	**82.7-91.5**	**79.48-87.1**	**78-194**	**74.35-90.93**	**8.68-17.1**	**4-39**
Median	**87.12**	**81.28**	**114**	**85.37**	**11.96**	**18**

"Pearson *r*" (correlation coefficient) = +0.974565 between DCN-2 level and platelet aggregation profile of AMI platelets in the presence of 0.6nM estriol.

"Pearson*r*" (correlation coefficient) = +0.994845 between DCN-2 level and platelet aggregation profile of control platelets in the presence of 0.6nM estriol.

In both cases the significance (*P* value) was P<0.001 between DCN-2 level and platelet aggregation profile in the AMI and control platelets as determined by the MannWhitneyU test with the medians significantly different.

Blood samples were collected from both AMI subjects and age and sex matched control (n = 30 in each group) by venipuncture as described in Methods and Materials. The plasma DCN-2 level was determined by ELISA. Aggregation of platelets was determined by platelet aggregometer.

4.3.5. Changes in binding characteristics in the Scatchard plot analysis of estriol to the protein in the presence and absence of tamoxifen

The Scatchard plot analysis of estriol to the protein showed the shifting of the curvilinear plot towards the left side of the X axis (Figure-4.5). This shifting profile signified the change in estriol binding to the protein was due to the treatment of the protein (NOS) with tamoxifen. The heterogeneous binding sites on curvilinear binding profile of the 69kDa protein to the estriol in the absence of tamoxifen showed one high affinity (Kd_1=8.77nM) and one low capacity (n_1=51±2 molecules estriol/molecule of protein) and one low affinity (Kd_2=243nM) high capacity (n_2=1.4X10^3molecules estriol/molecule of protein) receptor population.

In the presence of tamoxifen, the binding of estriol to the protein with a curvilinear binding profile showed heterogeneous binding sites with a high affinity (Kd_1=15.19nM) and a low capacity (n_1=27±2 molecules estriol/molecule of protein) and one low affinity (Kd_2=8.77nM) high capacity (n_2=104 molecules of estriol/molecule of protein) binding sites.

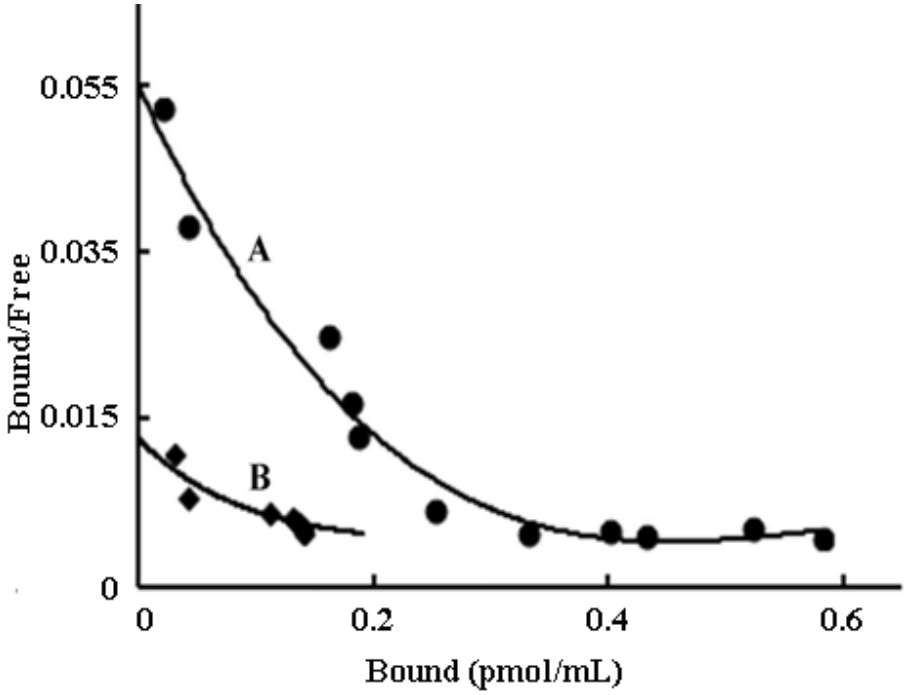

Figure-4.5: ***Equilibrium binding of estriol to gel filtered platelets in the presence and absence of the tamoxifen***

Gel filtered platelets were prepared from the normal platelet-rich plasma. The Scatchard plot of the equilibrium binding of estriol to the platelets in the presence and absence of tamoxifen (1.0nM) was used to constitute Scatchard plot. The amount of the bound and free was determined by Millipore gel filtration unit by enzyme linked immunosorbent assay (ELISA) as described in the Materials and Methods.

Curve A represents a Scastchard plot of equilibrium binding of estriol to the gel filtered platelets.Curve B shows the equilibrium binding of estriol in the presence of 1.0nM tamoxifen in identical binding mixtures.

4.3.6. "Cross-talk" between the receptors of DCN-2and estriol on the platelet membranes

As reported above, the synthesis of NO in the platelets from the AMI subjects treated with estriol was impaired in the AMI platelets when compared to that in the control counterpart. We have reported before the appearance of a stress induced protein (11kDa) in the circulation of the subjects identified to be DCN-2affected with ACS including AMI in all cases (Ghosh et al, 2011).

We have also reported before, first time ever, that the binding of an agonist to its own receptors may down regulate or up regulate the numbers of receptors of another agonist on the platelet membrane (Kahn et al, 1990). This down-regulation or the up-regulation of the receptor numbers on the platelet surface by one of agonists was affected by the binding of a different agonist to its own receptors of the two different agonists generally called "cross talk" between receptors (Kahn et al, 1990). To find out whether a similar kind of "cross talk"

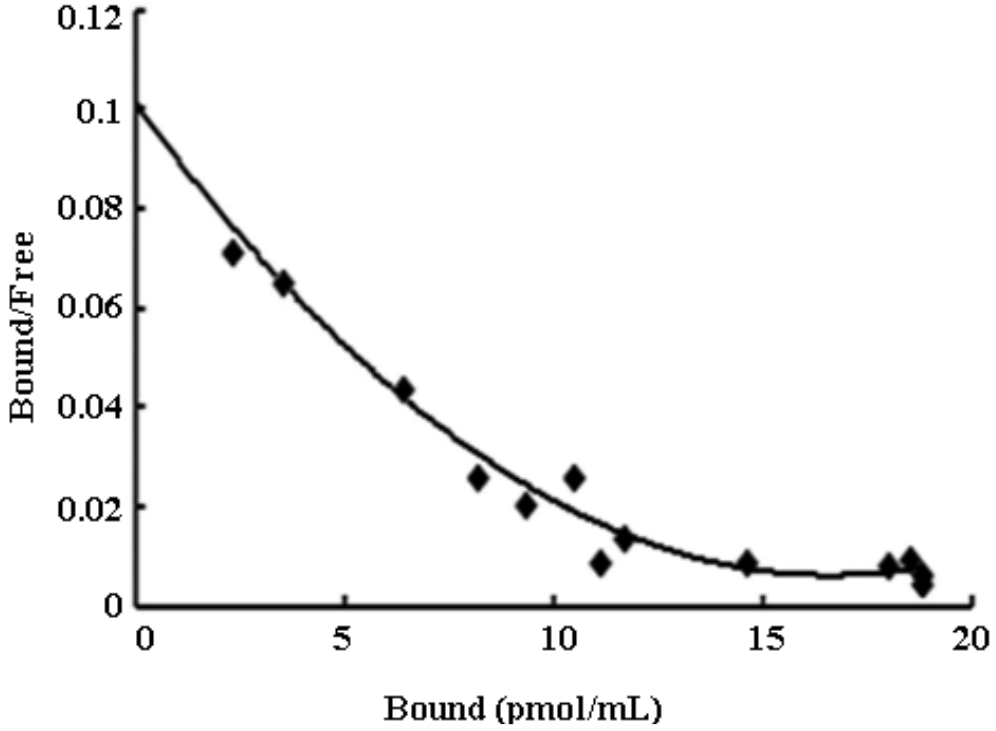

Figure-4.6: ***The Scatchard plot of the equilibrium binding of dermcidin isoform 2 (DCN-2)to the gel filtered normal platelets***
Typically gel filtered platelets (10^8 GFP/ml) were incubated with different concentrations of DCN-2. After the binding reached to the equilibrium (90min at 37˚C) the amount of DCN-2 (both bound and free) was determined by ELISA.

between the receptors of estriol and that of DCN-2 on the platelet surface resulted in the reduction of estriol receptors leading to the impaired NO synthesis by the steroid was

investigated. The binding characteristics of DCN-2 on the platelet surface were assessed by the Scatchard plot (Figure-4.6) suggesting that there were receptors of DCN-2 on the platelet surface (with K_d= 97.08, B_{max}= 24.89 X 10^3 molecules of protein/platelets).

When the binding of estriol on the control platelet surface was determined by the Scatchard plot (Figure-4.7) the analysis of plot produced K_d of estriol binding was 0.693 with B_{max}=1040 estriol binding sites/platelets. When DCN-2 was added to control PRP and incubated for 90 min and subsequently treated with estriol, the Scatchard plot of the DCN-2 treated platelets was carried out (Figure-4.7). It was found that the K_d of the estriol binding to platelets was 2.42 with B_{max} of only 640 estriol binding sites/ platelets. In other words, the binding of DCN-2 to its receptors down regulated the estriol receptor binding by 38.46 % due to a "cross-talk" between the different agonists.

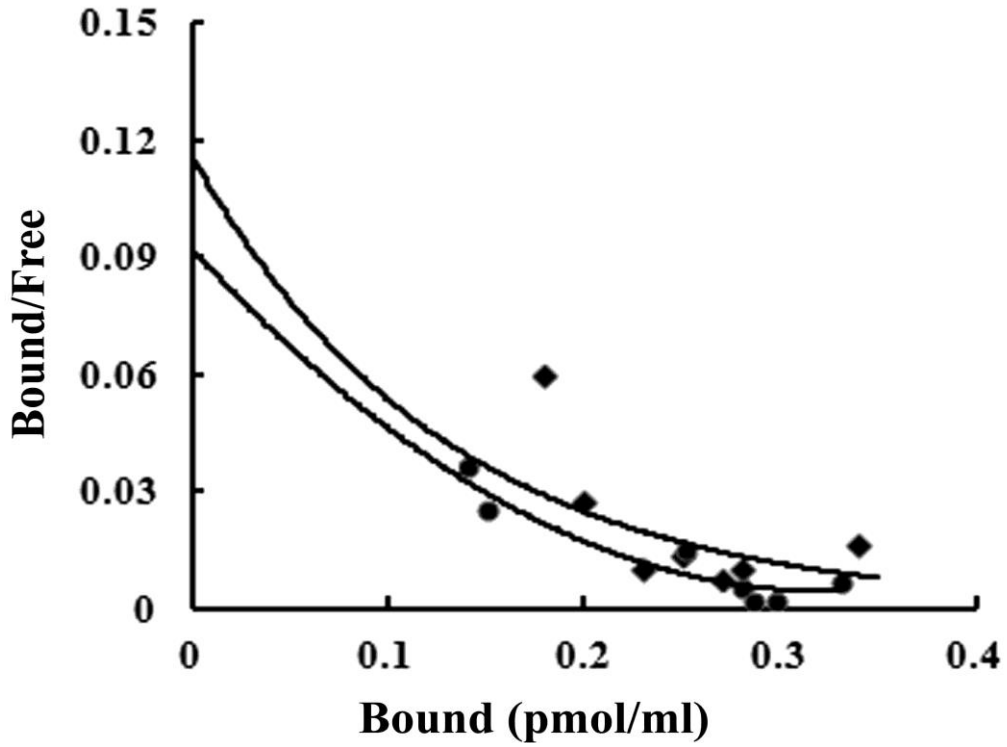

Figure-4.7: ***Changes in the equilibrium binding characteristics in Scatchard plot of estriol to the platelet in the presence and absence of DCN-2.***
Solid rhombus (-♦-) denotes binding of the estriol to the platelets in the absence of DCN-2. Solid circle (-●-) denotes binding of the estriol to the platelets in the presence of DCN-2.

4.4. Discussion

As described above estriol (one of estrogens) was reported to be the most potent inhibitor of platelet aggregation currently known. This steroid also occurs in the circulation of males albeit in much lower concentration (0.6nM). The concentrations of the estriol in plasma of the males are in the similar ranges for the inhibition of platelet aggregation. As middle aged males are the major victim of ACS and AMI an obvious issue of the failure of estriol to protect these victims with ensuing ACS or AMI remains unsolved in that whether this steroid, i.e. estriol has any useful role in the protection of the victims from the thrombosis under these conditions.

The activation of estriol mediated NO synthesis was due to a heterotrophic allosteric activation of the platelet membrane NOS that inhibits platelet aggregation. This allosteric activation of the platelet membrane NOS was determined, to be the consequence of estrogen receptor interaction of the enzyme which was never reported before and suggested a unique, and a new mode of action of an estrogenic hormone on platelet aggregation.

As described above the platelets from AMI patients synthesized much less amount of NO in the presence of estriol when compared to that in control platelets. It was found to be related to the presence of a stress induced protein in the circulation of AMI patient that causes a significant activation (Bank et al, 2014). Prior platelet activation may down regulate the estriol receptor number on the platelet surface and rendered estriol ineffective to inhibit platelet aggregation.

In this context, it might be mentioned here that DCN-2 has been reported to be a potent atherosclerotic agent in that the protein is simultaneously a pro-hypertensive agent as well as diabetogenic agent. Both hypertension and diabetes mellitus are two known major risk factors for atherosclerosis. This protein (MW 11kDa) is a potent inhibitor of all known forms of NOS and inhibited NO synthesis induced by estriol as described here, but the protein was also found to inhibit aspirin induced NO synthesis and thereby as in the case of estriol, DCN-2 made aspirin incapable of inhibiting platelet aggregation in AMI that is independent of the well-known effect of cyclooxygenase (Bank et al, 2014).

As this stress induced protein, i.e. DCN-2 is not an estrogen, the heterotropic down regulation of the estrogen receptor number and may be called as a case of negative “cross-

talk" between the receptors of DCN-2 and of estriol on the platelet surface. As human blood platelets do not synthesize protein the cross-talk between the receptors cannot be due to the stimulation or inhibition of the protein synthesis in platelets. This down regulated "cross-talk" could be hypothesized was due to the internalization of "spare receptor sites" from the platelet surface into the membrane bilayers. In this case DCN-2 was perhaps interacting with the estriol receptors on the platelet surface as in the case of tamoxifen, a well-known estrogen receptor blocker as reported above (Shiau et al, 1998).

It can be concluded that the appearance of DCN-2 in the circulation of the AMI subjects will severely reduce the estriol receptor numbers on the platelet surface and thereby rendering the steroid ineffective in the inhibition of platelet aggregation in this condition.

In the above context, it might be concluded here that the development of breast cancer significantly increased the risk of development of AMI (Hooning et al, 2007). As tamoxifen is sometime used in breast cancer, our results suggested that this compound should use with caution in the malignant condition in the case of tamoxifen that might instigate the development of AMI breast cancer.

CONCLUSION

CONCLUSION

Analysis of the above mentioned research works demonstrate the protective role of estrogenic metabolites. A direct effect of estrogens (estradiol and estriol) has been found on platelets as aggregation was directly inhibited in PRP and even in GFP. The molecular mechanism of this phenomenon describes the activation of platelet membrane protein. As platelets do not have nucleus the stimulation of the platelet membrane protein occurs through the non-genomic pathway. Estrogens are steroid molecule and they play important role as the regulator of various transcription factors and control various signaling pathway [Levin, 2005]. These results have demonstrated a new role of steroid molecule as the activator of protein or enzyme through the non-genomic pathway.

From here, it has also been found that estriol but not estradiol is more potent inhibitor of platelet aggregation though estradiol is denoted as the main estrogen with estrogenic activity. These results demonstrated that estriol, one of the most abundant ovarian hormones during pregnancy, was a potent stimulator of nitric oxide production by a platelet membrane associated protein of Mr. 69kDa, which resembles NOS-like activity. It can be concluded from the study that the cardio-protective effect of estriol mediated through the stimulation of NO synthesis is not due to the estrogenic effect of the steroid but mostly due to their ability to stimulate NO synthesis even in the absence of DNA in platelets.

The obtained results have concluded that estriol not inhibits platelet aggregation only but plays major role in the fibrinolysis through a novel NO mediated pathway also. Basically, formation of thrombus occurs as post-platelet aggregation mechanism due to formation of fibrin mass from platelet aggregation and activation of blood coagulation factors.

Thrombus formed after aggregation could be lysed by plasmin from plasminogen down [Cesarman-Maus et al, 2005]. Plasminogen got activated and synthesize plasmin by tPA or uPA [McFarlane, 1947]. Here, results clearly demonstrated that NO has the proteolytic activity and transform plasminogen to plasmin. The synthesized plasmin lyses the fibrin mass into FDPs.

To find out the possible therapeutic effect of estriol on AIHD the *in vitro* study was done collecting platelets from AIHD patients. Inability of estriol to inhibit platelet aggregation in AIHD platelets dragged the hypothesis to find out the causative agent which repress estriol to inhibit platelet aggregation. Occurrence of a high level of DCN-2 has been found in AIHD [Ghosh et al, 2010]. From the results obtained in the experiments, it can be concluded that the appearance of DCN-2 in the circulation of the AIHD subjects will severely reduce the estriol receptor numbers on the platelet surface and thereby rendering the steroid ineffective in the inhibition of platelet aggregation in this condition. The mode of action of DCN-2 resembles with the estrogen antagonist molecule tamoxifen.

In the above context, it might be concluded here that the development of breast cancer significantly increased the risk of developing AIHD [Hooning MJ et al, 2007]. As tamoxifen is sometime used in breast cancer, our results suggested that this compound should use with caution in the malignant condition in the case of tamoxifen that might instigate the development of AIHD breast cancer.

Regulation in the receptor populations of two different ligand corresponds the activity of one compound in the system. Here, the cross-talk between estriol and DCN-2 receptors regulate the effect of estriol on the inhibition of platelet aggregation

REFERENCES

REFERENCES

[1] Acharya K, Acharya R, Sinha AK. Inhibition of low Km phosphodiestrase of human blood platelets by nitric oxide. *Biosci Res Bull* 2001;17:47–53

[2] Adam SS, Key NS, Greenberg CS. "D-dimer antigen: current concepts and future prospects". *Blood* 2009;113 (13): 2878–2887

[3] Akarasereenont P, Tripatara P, Chotewuttakorn S, Palo T, Thaworn A. The effects of estrone, estradiol and estriol on platelet aggregation induced byadrenaline and adenosine diphosphate. *Platelets* 2006; 17:441–447.

[4] Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci U S A*. 1977 Aug;74(8):3203-7.

[5] Ashley EA, Niebauer J. Cardiology Explained. London: *Remedica*; 2004.

[6] Bank S, Jana P, Maiti S, Guha S, Sinha AK. Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction. *Sci Rep.* 2014;24;4:5804.

[7] Bennett JS, Vilaire G. Exposure of platelet fibrinogens by ADP and epinephrine. *J Clin Invest.* 1979 Nov; 64 (5): 1393-401.

[8] Bergstrom S, Danielsson H, and Samuelsson B. The enzymatic formation of prostaglandin E2 from arachidonic acid prostaglandins and related factors 32. *BiochimBiophysActa.* 1964;90: 207-210.

[9] Berliner J, Leitinger N, Watson A, et al. Oxidized lipids in atherogenesis: formation, destruction and action. *ThrombHaemost.* 1997;78:195–199. 18.

[10] Berk BC, Weintraub WS, Alexander RW. Elevation of C-reactive protein in "active" coronary artery disease. *Am J Cardiol*. 1990;65:168–172

[11] Bevers EM, Comfurius P, Zwaal RF. Platelet procoagulant activity: physiological significance and mechanisms of exposure. *Blood Rev*. 1991 Sep;5(3):146-54.

[12] Bhatia, Sujata K. (2010). Biomaterials for clinical applications (Online-Ausg. ed.). *New York: Springer*.

[13] Bhattacharjee KG, Bhattacharyya M, Halder UC, Jana P, Sinha AK. The roleof estrogen receptor statusin neutrophils and maspin synthesis throughnitric oxide production in human breast cancer. *J Breast Cancer* 2012; 15: 181–188.

[14] Bhattacharyya M, Karmohapatra SK, Bhattacharya G, Bhattacharya R, Sinha AK. The role of leucocytes in the acetyl salicylic acid (aspirin) induced nitric oxide synthesis in the production of interferon-α, a potent inhibitor of platelet aggregation and a thrombolytic agent. *J Thromb Thrombolysis* 2009;28(2):173-84

[15] Björkerud S, Björkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol.* 1996 Aug;149(2):367-80.

[16] Boesten LS, Zadelaar AS, van Nieuwkoop A, Gijbels MJ, de Winther MP, Havekes LM, van Vlijmen BJ. Tumor necrosis factor alpha promotes atherosclerotic lesion progression in APOE*3-

Leiden transgenic mice. *Cardiovasc Res.* 2005 Apr 1;66(1):179-85

[17] Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A*. 1990 Jan;87(2):682-5.

[18] Brouwer N, Chanulitrat W, Ferruzzi G, Sauls DL, Weinberg JB. Nitric oxide interactions with cobalamine: Biochemical and functional consequences. *Blood*. 1996; 88: 1857-1864

[19] Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *Br J Haemato*l. 2005;129(3):307-21.

[20] Carlsson LE, Santoso S, Spitzer C, Kessler C, Greinacher A. The alpha2 gene coding sequence T807/A873 of the platelet collagen receptor integrin alpha2beta1 might be genetic risk factor for the development of stroke in younger patients. *Blood*.1999 Jun 1;93(11):3583-6

[21] Carr ME Jr, Alving BM. Effect of fibrin structure on plasmin mediateddissolution of plasma clot. *Blood Coagul Fibrinolysis* 1995; 6:567–573.

[22] Chakrabarti S, Lekontseva O, Peters A, Davidge ST. 17beta-Estradiolinduces protein S-nitrosylation in the endothelium. *Cardiovasc Res* 2010;85:796–805.

[23] Chakraborty K, Khan GA, Banerjee P, Ray U, Sinha AK. Inhibition of human blood platelet aggregation and the stimulation of nitric oxide synthesis by aspirin. *Platelets* 2003;14:421–427.

[24] Chang Y, Bluteau D., DebiliN.,Vainchenker W. From hematopoietic stem cells to platelets. *Journal of Thrombosis and Haemostasis*, 5 (Suppl. 1): 318–327.

[25] Chesney CM, Piter DD, Byers LW, Mulrhead EE. Effect ofplatelet activating factor (PAF) on human platelets. *Blood*1982;59:582-585

[26] Choi AM, Alan J. Heme oxygenase-I: Function regulation and implication of a novel stress inducible protein in oxidant induced lung injury. *Am J Respir Cell Mol. Biol*. 1996; 15:9-19.

[27] Cleland WW. Dithiothreitol: a new protective reagent for SH groups. *Biochemistry* 1964; 3:480–482.

[28] Coles B, Bloodsworth A, Eiserich JP, Coffey MJ, McLoughlin RM, Giddings JC, Lewis MJ, Haslam RJ, Freeman BA, O'Donnell VB. Nitrolinoleate inhibits platelet activation by attenuating calcium mobilization and inducing phosphorylation of vasodilator-stimulated phosphoprotein through elevation of cAMP. *J Biol Chem.*2002 Feb 22;277(8):5832-40.

[29] Colman RW, Walsh PN Mechanisms of platelet aggregation. In: Colman RW, Hirsh J, Maeder VJ, SalzmanEW (eds). *Haemostasis and thrombosis* J.B. Lippincott, Philadelphia, PA, 1987; pp 594–605

[30] Cooley MH, Cohen P. Potassium transport in human blood platelets. J Lab Clin Med. 1967;70(1): 69-79

[31] Coronary Drug Project Research Group. The Coronary Drug Project: findings leading to modifications of its research protocol. *JAMA.*1970;214:1303-1313.

[32] Cox RD, Frank CW. Determination of nitrate and nitrite in blood and urine by chemiluminescence. *J Anal Toxicol* 1982;6:148–152

[33] Cremer SE, Kristensen AT, Reimann MJ, Eriksen NB, Petersen SF, Marschner CB, Tarnow I, Oyama MA, Olsen LH. Plasma and serum serotonin concentrations and surface-bound platelet serotonin expression

in Cavalier King Charles Spaniels with myxomatous mitral valve disease. *Am J Vet Res*. 2015 Jun;76(6):520-31.

[34] Croft SA, Samani NJ, Teare MD, Humpton KK, Steeds RP, Channer KS, Daly ME. Novel platelet membrane glycoprotein VI dimorphism is a risk factor for myocardial infarction. *Circulation*. 2001; 25;104(13):1459-63

[35] Dawson TM, Dawson VL, Snyder SH. A novel neuronal messenger molecule in brain: the free radical, nitric oxide. *Ann Neurol*. 1992 Sep;32(3):297-311.

[36] Degen, J.L. Genetic interactions between the coagulation and fibrinolytic systems. *Thrombosis and Haemostasis.* 2001;86, 130–137.

[37] Doyle MP, LePoire DM, Pickering RA. Oxidation of hemoglobin and myoglobin by alkyl nitrites inhibition by oxygen. *J Biol Chem.* 1981 Dec 10;256(23):12399-404.

[38] Durán MG, Gálvez GG, de Frutos T, Díaz Recaséns J, Casado S, López Farré A. 17 Beta-estradiol-stimulated nitric oxide production by neutrophils: effect on platelet activation. *Obstet Gynecol.* 2000 Feb;95(2):284-90.

[39] Dutta-Roy AK, Sinha AK. Purification and properties of prostaglandin E1/prostacyclin receptor of human blood platelets. *J BiolChemx* 1987;262(26):12685-91

[40] Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, Mathews AJ, Johnson KA, Smith RD, Phillips GN Jr, Olson JS. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry.* 1996 Jun 4;35(22):6976-83

[41] Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzymelabeled anti-immunoglobulin in antigen-coated tubes. *J Immunol* 1972;109(1):129–135.

[42] Esmon, C.T., Fukudome, K., Mather, T., Bode, W., Regan, L.M., Stearns-Kurosawa, J. & Kurosawa, S. Inflammation, sepsis, and coagulation. *Haematologica*. 1999;84, 254–259.

[43] Falk E. Dynamics in thrombus formation. *Ann N Y AcadSci* 667:204–223. doi:10.1111/j.1749-6632.1992;tb51618.

[44] Falk E. Plaque rupture with severe pre-existing stenosis precipitatingcoronary thrombosis. Characteristics of coronary atherosclerotic plaquesunderlying fatal occlusive thrombi. *Br Heart J*. 1983;50:127–134.

[45] Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation*. 1995;92:657–671

[46] Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M. Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev.* 2000 Dec;52(4):513-56.

[47] Farias-Eisner R, Chaudhuri G, Aeberhard E, Fukuto JM. The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J Biol Chem*. 1996 Mar 15;271(11):6144-51.

[48] Farndale RW, Hargreaves PG, Dietrich JL, Keogh RJ. Measurement of platelet arachidonic acid metabolism. *Methods Mol Biol*.;272:121-33

[49] Fazekas de St Groth S, Webster RG, Datyner A. Two new stainingprocedures for quantitative estimation

of proteins on electrophoretic strips. *BiochimBiophysActa* 1963; 71:377–391.

[50] Feldman PL, Griffith OW, Hong H, Stuehr DJ. Irreversible inactivation of macrophage and brain nitric oxide synthase by L-NG-methylarginine requires NADPH-dependent hydroxylation. *J Med Chem*. 1993 Feb 19;36(4):491-6.

[51] Feelisch M. The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *Journal of Cardiovascular Pharmacology*, 1991; 17, S25-S33

[52] Fisch C, Robin MA, Letteron P, Fromenty B, Berson A, Renault S, Chachaty C, Pessayre D. Cell-generated nitric oxide inactivates rat hepatocyte mitochondria in vitro but reacts with hemoglobin in vivo. *Gastroenterology*. 1996 Jan;110(1):210-20.

[53] Folcik, V. A., Nivar-Aristy, R. A., Krajewski, L. P., and Cathcart, M. K. *J. Clin. Invest*. 1995;96, 504–510

[54] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980; 288: 373-376

[55] Furchgott RF, Cherry PD, Zawadzki JV, Jothianandan D. Endothelial cells as mediators of vasodilation of arteries. *J Cardiovasc Pharmacol*. 1984;6 Suppl 2:S336-43.

[56] Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, and Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am CollCardiol*1998;1:352-358.

[57] Fuster V, Badimon J, Chesebro J H, Fallon J T. Plaque rupture, Thrombosis, and therapeutic implications. *Haemostasis*1996;26:269–284.

[58] Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque: part I: evolving concepts.*J Am CollCardiol.* 2005 Sep 20;46(6):937-54

[59] Gaffney PJ, Edgell T, Creighton-Kempsford LJ, Wheeler S, Tarelli E. "Fibrin degradation product (FnDP) assays: analysis of standardization issues and target antigens in plasma". *Br. J. Haematol*. 1995;90 (1): 187–94

[60] GangulyBhattacharjee K, Bhattacharyya M, Halder UC, Jana P, Sinha AK.Effect of progesterone receptor status on maspin synthesis via nitric oxideproduction in neutrophils in human breast cancer. *Breast Cancer* 2012

[61] Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature*. 1988 Nov 24;336(6197):385-8.

[62] George JN. Platelets. *Lancet*. 2000. Apr 29, 355(9214):1531-9. Review.

[63] Ghosh R, Karmohapatra SK, Bhattacharyya M, Bhattacharya R,Bhattacharya G, Sinha AK. The appearance of dermcidin isoform 2, a novelplatelet aggregating agent in the circulation in acute myocardial infarctionthat inhibits insulin synthesis and the restoration by acetyl salicylic acid ofits effects. *J Thromb Thrombolysis* 2011; 31:13–21.

[64] Goldblatt MW. A depressor substance in seminal fluid. *J SocChemInd* 1933;52:1056-1057.

[65] Gouva L, Tsatsoulis A. The role of estrogens in cardiovascular disease in the aftermath of clinical trials. *Hormones* (Athens) 2004;(3):171-83

[66] Grant JA, Scrutton MC. Novel alpha2-adrenoreceptors primarily responsible for inducing human platelet aggregation.*Nature*. 1979 Feb 22;277(5698):659-61.

[67] Gruether Ca, Barry BK, McNamara DB, Guether DY, Kadowitz PJ, Ignarrow IJ. Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprisside and a carcinogenic nitrosoamine. *J Cyclic Nucleotide Res*. 1979; 5(3):211-24

[68] Gupta R, Joshi P, Mohan V, Reddy KS, Yusuf S. Epidemiology and causation of coronary heart disease and stroke in India. *Heart*. 2008 Jan; 94(1):16-26.

[69] Hamberg M and Samuelsson B. Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc Natl AcadSci USA*. 1973;70: 899-903.

[70] Hamberg M, Svensson J, and Samuelsson B. Prostaglandin endoperoxides. A new concept concerning the mode of action of prostaglandins. *Proc Natl AcadSci USA*. 1974;71: 3824-3828.

[71] Harris TB, Ferrucci L, Tracy RP, et al. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med*. 1999;106:506–512.

[72] Haslam RJ, Taylor A.Effects of catecholamines on the formation of adenosine 3':5'-cyclic monophosphate in human blood platelets. *Biochem J*. 1971 Nov;125(1):377-9.

[73] Hauser IA, Johnson DR, Thévenod F, Goppelt-Strübe M. Effect of mycophenolic acid on TNF alpha-induced expression of cell adhesion molecules in human venous endothelial cells in vitro. *Br J Pharmacol*. 1997 Dec;122(7):1315-22.

[74] Haverkate F, Thompson S, Pyke S, et al. Production of C-reactive protein and risk of coronary events in stable and unstable angina. European concerted action on thrombosis and disabilities anginapectoris study group. *Lancet* 1997;349:462–6

[75] Hemminki E, Kennedy DI, Baum C, McKinlay SM. Prescribing of noncontraceptive estrogens and progestins in the US, 1974-86. *Am J Public Health*.1988;78:1478-1481.

[76] Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun*. 1988 Nov 30;157(1):87-94.

[77] Higuchi ML, Gutierrez PS, Bezerra HG, et al. Comparison between adventitial and intimal inflammation of ruptured and nonruptured atherosclerotic plaques in human coronary arteries. *Arq Bras Cardiol*. 2002;79:20–24.

[78] Hoffman M, Monroe DM 3rd. A cell-based model of hemostasis. *ThrombHaemost*. 2001;85(6):958-65.

[79] Hoffman MM, Monroe DM. Rethinking the coagulation cascade. *CurrHematol Rep*. 2005;4(5):391-6.

[80] Hogg N, Kalyanaraman B, Joseph J, Struck A, Parthasarathy S. Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett*. 1993 Nov 15;334(2):170-4.

[81] Holmsen H. Day J., Stormorken H (1969). The blood platelet release reaction. *Scand J Haematol* 8:3-26.

[82] Holvoet, P., Lijnen, H.R. & Collen, D. A monoclonal antibody specific for lys-plasminogen. *Journal of Biological Chemistry.*1985;260, 12106–12111.

[83] Hooning MJ, Botma A, Aleman BM, Baaijens MH, Bartelink H, Klijn JG, Taylor CW, van Leeuwen FE. Long-term risk of cardiovascular disease in 10-year survivors of breast cancer. *J Natl Cancer Inst.* 2007; 99:365–75.

[84] Houtkamp MA, de Boer OJ, van der Loos CM, van der Wal AC, Becker AE. Adventitial infiltrates associated with advanced atherosclerotic plaques: structural organization suggests generation of local humoral immune responses. *J Pathol*. 2001; 193:263–269.

[85] Howe AF, Groom T, Carter RG. Use of polyethylene glycol in the concentration of protein solutions. *Anal biochem*. 1964;9:443-53.

[86] Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J BiolChem* 1982; 257:2912–2919.

[87] Hwang SJ, Ballantyne CM, Sharrett AR, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases. The Atherosclerosis Risk in Communities (ARIC) study. *Circulation*. 1997; 96:4219–4225.

[88] Ignarro LJ, Wood KS, Byrns RE. Pharmacological and biochemical properties of endothelium derived relaxing factor (EDRF): Evidence of closely related to nitric oxide (NO) radical. *Circulation.* 1986; 74 (11): 287

[89] Ignarro LJ. Endothelium-derived nitric oxide: pharmacology and relationship to the actions of organic nitrate esters. *Pharm Res*. 1989 Aug;6(8):651-9.

[90] Ikeda Y, M Handa, K Kawano, T Kamata, M Murata, Y Araki, H Anbo, Y Kawai, K Watanabe, I Itagaki. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*. 1991; 87(4): 1234–1240.

[91] Ilveskero s, Siljander P, Lassila R. Procoagulant activity on platelets adhered to collagen or plasma clot. *ArteriosclerThrombVasc Biol*. 2001 21 (4):628-35.

[92] J. R. Vane. The mode of action of aspirin andsimilar compounds. *J Allergy Clin Immunol*,58:691-712, 1976.

[93] Jana P, Maiti S, Ghosh R, Ghosh TK, Sinha AK. Estriol, a stimulator of nitricoxide synthesis in platelets, and its role as the powerful inhibitor of plateletaggregation. *CardiovascEndocrino*l 2013; 2:50–54.

[94] Jay V. The Legacy of William Heberden. 1750 *Arch Pathol Lab Med*—Vol 124, December *2000*

[95] Jia L, Bonaventura C, Bonaventura J, Stamler SJ. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 1996;380:221–226

[96] Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. *Blood*. 2002;99(1):193-8.

[97] Jung SM, MoroiM.Platelets interact with soluble and insoluble collagens through characteristically

different reactions.*JBiol Chem*.1998 Jun 12;273(24):14827-37.

[98] Jung SM, MoroiM.Platelet collagen receptor integrin alpha2beta1 activation involves differential participation of ADP-receptor subtypes P2Y1 and P2Y12 but not intracellular calcium change.. *Eur J Biochem.* 2001 Jun;268(12):3513-22.

[99] Kahn NN, Sinha AK. Stimulation of prostaglandin E1 binding to humanblood platelet membrane by insulin and the activation of adenylate cyclase.*JBiolChem* 1990; 265:4976–4981.

[100] Kanowitz PJ. Evidence for the inhibitory role of guanosine 3, 5-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981;57:946–955

[101] Karmohapatra SK, Chakraborty K, Kahn NN, Sinha AK. The role of nitric oxide in aspirin induced thrombolysis in vitro and the purification of aspirin activated nitric oxide synthase from human blood platelets. *Am J Hematol* . 2007; 82(11):986-95.

[102] Karpatkin S (1969). Heterogeneity of human platelets. In. Metabolic and kinetic evidsence suggestive of young and old platelets. *J Clin Invest* Jun; 48(6): 1073-82

[103] Karpatkin S, Siskind GW (1967) Effect of antibody binding and agglutination on human platelet glycolysis: Comparision with thrombin and epinephrine. *Blood* 30(5).

[104] Kapatkin S (1972). Biochemistry and function of platelets. In: Hematology (Ed by Williams WJ, Beuter E, Erslev JA, Rundles RW) McGraw-Hill book company, *A Blakiston Publication USA* 1180.

[105] Kelm M, Feelisch M, Spahr R, Piper HM, Noack E, Schrader J. Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem Biophys Res Commun.* 1988 Jul 15;154(1):236-44.

[106] Kharitonov VG, Sundquist AR, Sharma VS. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J BiolChem* 1995; 270:28158–28164.

[107] Khetawat G, Faraday N, Nealen ML, Vijayan KV, Bolton E, Noga SJ, Bray PF. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood*2000; 95:2289–2296, 1.

[108] Kim KA, Ka SO, Moon WS, Yi HK, Lee YH, Kwon KB, Park JW, Park BH. Effect of dermcidin, an antimicrobial peptide, on body fat mobilization in normal mice. *J Endocrinol*. 2008 Jul;198(1):111-8

[109] Kim YM, Bergonia HA, Muller C, Pitt BR, Watkins WD, Lancaster JR Jr. Loss and degradation of enzyme bound heme induced by cellular nitroxide synthesis. 1995

[110] Kohchi K, Takebayashi S, Hiroki T, Nobuyoshi M. Significance of adventitial inflammation of the coronary artery in patients with unstable angina: results at autopsy. *Circulation.* 1985; 71:709–716.

[111] Kolev, K. &Machovich, R. Molecular and cellular modulation of fibrinolysis. *Thrombosis and Haemostasis*. 2003;89, 610–621.

[112] Kritzic M, Savage B, Nugent DJ, Santoso S, Ruggeri ZM, Kunicki TJ. Nucleotide polymorphisms in the alpha 2 gene define multiple allels that are associated with differences in platelet alpha 2 beta 1

density. *blood* 1998. Oct 1: 92(7):2382-8

[113] Kuhn, H., Belkner, J., Zaiss, S., Fahrenklemper, T., and Wohlfeil, S. (1994) *J. Exp. Med*. 179, 1903–1911

[114] Kunicki TJ, Orchekowski R, Annis D, Honda Y. Variability of integrin α2β1activity on human platelets. *Blood*. 1993 Nov 1; 82(9):2693-703

[115] LalauKeraly C, Vickers JD, Kinlough-Rathbone RL, Mustard JF. Involvement of phosphoinositide metabolism in potentiation by adrenaline of ADP-induced aggregation of rabbit platelets. *Biochem J*. 1987 Mar 15;242(3):841-7.

[116] Laine P, Naukkarinen A, Heikkila L, Penttila A, Kovanen PT. Adventitial mast cells connect with sensory nerve fibers in atherosclerotic coronary arteries. *Circulation.* 2000; 101:1665–1669.

[117] Lancaster JR Jr[1], Hibbs JB Jr. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc Natl Acad Sci U S A.* 1990 Feb;87(3):1223-7.

[118] Lancaster JR Jr. Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc Natl Acad Sci U S A.* 1994 Aug 16;91(17):8137-41.

[119] Lappano R, Rosano C, De Marco P, De Francesco EM, Pezzi V, Maggiolini M. Estriol acts as a GPR30 antagonist in estrogen receptor-negative breast cancer cells. *Mol Cell Endocrinol*. 2010 May 14;320(1-2):162-70. doi: 10.1016/j.mce.2010.02.006. Epub 2010 Feb 6.

[120] Levin ER. Integration of the extranuclear and nuclear actions of estrogen. *MolEndocrinol* 2005; 19:1951–1959.

[121] Leeuwenburgh, C., Rasmussen, J., Hsu, F., Mueller, D., Pennathur, S., and Heinecke, J. (1997) *J. Biol. Chem.* 272, 3520–3526

[122] Logvinenko NS. Aldosterone: the mechanisms of molecular action. *RossFiziolZhIm I M Sechenova* 2007; 93:746–761.

[123] Louet JF, LeMay C, Mauvais-Jarvis F. Antidiabetic actions of estrogen: insightfrom human and genetic mouse models. *CurrAtheroscler Rep* 2004;6: 180–185.

[124] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement bythefolin-phenol reagent. *J BiolChem* 1951; 1193:265–275.

[125] Maione F, Cicala C, Liverani E, et al. IL-17A increases ADP-induced platelet aggregation. *Biochem Biophys Res Commun* 2011; 408: 658-662.

[126] Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR, Lasser NL, et al.Estrogen plus progestin and the risk of coronary heart disease. *N EnglJMed* 2003; 349:523–534, 7.

[127] Marcus AJ,Zucker-Franklin D, Safier LB, Ullman HL (1966). Stusies on human platelet granules and membranes. *J ClinInvest* 45 (1): 14-28

[128] Marcus AJ, Sailer LB, Ullman HL, et al. Effects of acetylglyceryl ether phosphorylcholine on human platelet functionin vitro. *Blood* 1981 ;58:1027-1031

[129] Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite

and nitrate: nitric oxide is an intermediate. *Biochemistry.* 1988 Nov 29;27(24):8706-11.

[130] Maseri A, L'Abbate A, Baroldi G, et al. Coronary vasospasm as a possible cause of myocardial infarction: a conclusion derived from the study of ''preinfarction'' angina. *N Engl J Med*. 1978; 299:1271–1277.

[131] Matsudaira P (1987) Sequence from picomole quantities of proteinselectroblotted onto polyvinylidene difluoride membranes. *J BiolChem*262: 10035-10038. PubMed: 3611052.

[132] Mayer B, John M, Heinzel B, Werner ER, Wachter H, Schultz G, Böhme E. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett.* 1991 Aug 19;288(1-2):187-91.

[133] Michelson AD. *Platelets* (Elsevier Publishers) 3rd Edition, 2013. p 3.

[134] Michelson AD. How platelets work: platelet function and dysfunction. *J Thromb Thrombolysis*. 2003 16 (1-2): 7-12

[135] Mills DC. ADP receptors on platelets. *ThrombHaemost* 1996; 76(6):835-56

[136] Mittleman MA, Lewis RA, Maclure M, Sherwood JB, Muller JE. Triggering myocardial infarction by marijuana.*Circulation*. 2001 Jun 12;103(23):2805-9.

[137] Moake, J. L., N. A. Turner, N. A. Stathopoulos, L. H. Nolasco, and J. D. Hellums. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *J. Clin. Invest.*1986;78:1456-1461.

[138] Moake, J. L., N. A. Turner, N. A. Stathopoulos, L. Nolasco, and J. D. Hellums. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWFmultimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood*. 1988; 71:1366-1374.

[139] Moliterno DJ, Smyth SS. Tissue necrosis factor α and targeting its receptor in ischaemic heart disease. *Heart*. 2013 Sep;99(18):1305-6.

[140] Montoro-García S, Shantsila E, Lip GY. Potential value of targeting von Willebrand factor in atherosclerotic cardiovascular disease. *Expert Opin Ther Targets.* 2014 Jan;18(1):43-53.

[141] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991 Jun;43(2):109-42.

[142] Monroe DM, Hoffman M. What does it take to make the perfect clot?*ArterioscIerThrombVasc Biol.* 2006 Jan;26(1):41-8.

[143] Moroi M, Jung SM.Platelet glycoprotein VI: its structure and function. *Thromb Res.*2004;114(4):221-33

[144] Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity.* 1994 Nov;1(8):661-73.

[145] Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M et al. Heart disease and stroke statistics--2015 update: a report from the American Heart Association.

Circulation. 2015 Jan 27;131(4): e29-322.

[146] Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation*. 1989 Apr;79(4):733-43.

[147] Muller JE. Triggering of cardiac events by sexual activity: findings from a case-crossover analysis.*Am J Cardiol*. 2000Jul 20;86(2A):14F-18F.

[148] Murad F. The nitric oxide-cyclic GMP signal transduction systemfor intracellular and intercellular communications. *Rec Prog in Harm Res*. 1994; 49: 239-248

[149] Nachman RL, Marcus AJ. Platelet thrombosthenin : Subcellular localization and function. *J Clin Invest* 1967;46: 1380

[150] Nachman RL, Weksler BB. The platelet as an inflammatorycell. In: Weissman G, ed. The cell biology of Inflammation.Amsterdam: *Elsevier/North Holland*, 1980

[151] Nachmlas VT. Platelet and megakaryocyte shape change:triggered alterations in the cytoplasm. *SemHematol* 1983;20:261-281

[152] Nelken N, Coughlin S, Gordon D, Wilcox J. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 1991;88:1121–1127

[153] NeNelson HD, Humphrey LL, Nygren P, Teutsch SM, Allan JD. Postmenopausal hormone replacement therapy: scientific review. *JAMA* 2002;288(7):872-81.

[154] Nelson LR, Bulun SE. Estrogen production and action. *J Am Acad Dermatol*. 2001 Sep;45(3 Suppl):S116-24.

[155] O'Neil MJ, Smith lken N, Coughlin S, Gordon D, Wilcox J. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 1991;88:1121–1127

[156] Nelson DL, Cox M: *Principles of Biochemistry* (4th edition), Woth Publishers, pp 227-28

[157] A, Heckelman PE. The Merck index. 13th ed. WhitehouseStation: *Merck Research Laboratories*; 2006. pp. 660–661.

[158] Olefsky JM. Nuclear receptor minireview series. *J BiolChem*2001; 276:36863–36864.

[159] Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* 1987 Jun 11-17;327(6122):524-6.

[160] Pelzl L, Fakhri H, Umbach AT, Gawaz M, Paulmichl M, Lang F. Sgk1 sensitive pendrin expression in murine platelets. *Cell PhysiolBiochem*. 2013;32:210-20.

[161] Pang L, Xue HH, Szalai G, Wang X, Wang Y, Watson DK, Leonard WJ, Blobel GA, Ponez M. Maturation stage-specific regulation of megakaryopoiesis by pointed-domain Ets proteins. *Blood* 2006; 108: 2198–206.

[162] Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis in vitro. *J Clin Endocrinol Metab*. 2000 May;85(5):2057-67.

[163] Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction.*Circulation.* 2001 Jun 12;103(23):2810-5.

[164] Peterson, D. M., N. A. Stathopoulos, T. D. Giorgio, J. D. Hellums, and J. L. Moake. Shear-induced platelet aggregation requires von Willebrand factor and platelet membrane glycoproteins Ib and IIb-IIIa. *Blood.* 1987;69:625-628

[165] Raju U, Bradlow HL, Levitz M. Estriol-3-sulfate in human breast cyst fluid. Concentrations, possible origin, and physiologic implications. *Ann N Y Acad Sci*. 1990;586:83-7.

[166] Raum, D., Marcus, D., Alper, C.A., Levey, R., Taylor, P.D. &Starzl, T.E. Synthesis of human plasminogen by the liver. *Science*. 1980;208, 1036–1037.

[167] Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation*. 2001;103:491–495

[168] Ridker PM, Rifai N, Stampfer MJ, et al. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation*. 2000;101:1767–1772.

[169] Ridker PM, Rifai N, Pfeffer M, et al. Elevation of tumor necrosis factor- and increased risk of recurrent coronary events after myocardial infarction. *Circulation*. 2000;101:2149–2153.

[170] Rocha VZ and Libby P. Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol*.2009.55

[171] Royal College of General Practitioners' Oral Contraception Study: further analyses of mortality in oral contraceptive users. *Lance*t.1981;1:541-546.

[172] Rubbo H, Radi R, Trujillo M, Telleri R, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite dependent lipid peroxidation. *J Biol Chem* 1994; 269:26069-26075

[173] Rubbo H, Darley-Usmer V, Freeman BA. Nitric oxide regulation of tissue free radical injury. *Chem Res Toxicol.* 1996; 9:809-820.

[174] Ruggery ZM. Platelets in atherothrombosis. *Nat Med.* 2002 Nov; 8(11):1227-34

[175] Ryan KJ. Biochemistry of aromatase: significance to female reproductive physiology. *Cancer Res.* 1982 Aug;42(8 Suppl):3342s-3344s

[176] Sakuma I, Stuehr DJ, Gross SS, Nathan C, Levi R. Identification of arginineas a precursor of endothelium-derived relaxing factor. *Proc Natl AcadSciU S A* 1988; 85:8664–8667.

[177] Salzman EW, Levine L.Cyclic 3',5'-adenosine monophosphate in human blood platelets. II. Effect of N6-2'-o-dibutyryl cyclic 3',5'-adenosine monophosphate on platelet function.*JClin Invest.* 1971 Jan;50(1):131-41.

[178] Schafer AI, Vosper AJ. Solubility of nitric oxide in aqueous and non-aqueous solvents. *J BiolChem* 1979; 254(8): 2914-2917

[179] Scatchard G. The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* 1949;Volume 51, Molecular Interaction pages 660–672.

[180] Sharma S, Sharma SC. An update on eicosanoids and inhibitors of cyclooxygenase enzyme systems. *Indian J Exp Biol.* 1997 Oct;35(10):1025-31.

[181] Shaw AW, Vosper AJ. Solubility of nitric oxide in aqueous and non-aqueoues. *J Chem Soc Faraday*

Trans 1977; 1(8): 1239-1244

[182] Sheps DS, McMahon RP, Becker L, Carney RM, Freedland KE, Cohen JD, Sheffield D, Goldberg AD, Ketterer MW, Pepine CJ, Raczynski JM, Light K, Krantz DS, Stone PH, Knatterud GL, Kaufmann PG. Mental stress-induced ischemia and all-cause mortality in patients with coronary artery disease: Results from the Psychophysiological Investigations of Myocardial Ischemia study. *Circulation*. 2002;105(15):1780-4.

[183] Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*. 1998;23;95:927-37.

[184] Silzander P, Lassila R. Studies of adhesion dependent platelet activation distinct roles for different participating receptors can be dissociated by proteolysis of collagen. *ArteriosclerThrombVasc Biol*. 1999 Dec;19 (12):3033-43

[185] Sinha AK, Acharya K, Bhattachrya S, Mazumder S, Bauman WA, Kahn N. Prevention of thrombosis in vivo by nitric oxide. *Indian Physiol Allied Sci.* 1998; 52 (3):148-161

[186] Sixma JJ, van Zanten GH, Huizinga EG, van der Plas RM, Verkley M, Wu YP, Gros P, de Groot PG. Platelet adhesion to collagen: an update. *ThrombHaemost*.1997 Jul;78(1):434-8.

[187] Smith DC, Prentice R, Thompson DJ, Herrman WL. Association of exogenous estrogen and endometrial carcinoma. *N Engl J Med*.1975;293:1164-1167.

[188] Smith JB, Willis AL. Aspirin selectively inhibits prostaglandin production in human platelets. *Nat New Biol* 1971;231(25):235-7

[189] Soldan SS, varezRetuerto AI, Sicotte NL, Voskuhl RR. Immune modulationin multiple sclerosis patients treated with the pregnancy hormone estriol. *J Immunol* 2003; 171:6267–6274.

[190] Srinath Reddy K, Shah B, Varghese C, Ramadoss A. Responding to the threat of chronic diseases in India.*Lancet*. 2005 Nov 12; 366(9498):1744-9.

[191] Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, Doehmer J. Inhibition of cytochromes P4501A by nitric oxide. *Proc Natl Acad Sci U S A.* 1994 Apr 26;91(9):3559-63.

[192] Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FC, Hennekens CH. A prospective study of postmenopausal estrogen therapy and coronary heart disease. *N Engl J Med*. 1985; 313: 1044-1049.

[193] Stratford N, Britten K, Gallagher P. Inflammatory infiltrates in human coronary atherosclerosis. *Atherosclerosis.* 1986;59:271–276.

[194] Stary H, Chandler A, Dinsmore R, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of protein in macrophages in all phases of atherogenesis. Science atherosclerosis. A report from the Committee on Vascular Lesions of 1990;248:1009–1012. the Council on Atherosclerosis, *American Heart Association. ArteriosclerThrombVascBio*l1995; 15:1512–1531.

[195] Stary HC. An Atlas of Atherosclerosis: Progression and Regression. *NewYork: Parthenon Publishing Group*; 1999.

[196] Steering Committee of the Physicians Health Study Research Group. Finalreport on the aspirin component of the ongoingphysicians' health study. *N Engl J Med* 1989; 321:129–135.

[197] Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., and Steinberg, D. *Proc. Natl. Acad. Sci. U. S. A.* 1984;81, 3883–3887.

[198] Stewart GD, Skipworth RJ, Ross JA, FearonKCh, Baracos VE. The dermcidin gene in cancer: role in cachexia, carcinogenesis and tumour cell survival. *CurrOpinClinNutrMetab Care.* 2008 May;11(3):208-13.

[199] Stocker R. Induction of heme oxygenase as a defence against oxidative stress. *Free Radicals Res comm*. 1990; 9:101-112

[200] Struck AT, Hogg N, Thomas JP, Kalyanaraman B. Nitric oxide donor compounds inhibit the toxicity of oxidized low density lipoprotein to endothelial cells. *FEBS Lett*. 1995; 361: 291-294

[201] Strzemienski PJ, Dyer RM, Kenney RM. Effect of estradiol and progesterone on antistaphylococcal activity of neutrophils from ovariectomized mares. *Am J Vet Res*. 1987 Nov;48(11):1638-41.

[202] Tateson JE, Moncada S, Vane JR.Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets.*Prostaglandins.*1977 Mar;13(3):389-97

[203] Tiainen P, Pasanen A, Sormunen R, Myllyharju J. Characterization of recombinant human prolyl 3-hydroxylase isoenzyme 2, an enzyme modifying the basement membrane collagen IV. *J Biol Chem*.2008 Jul 11;283(28):19432-9.

[204] Trovati M, Anfossi G, Massucco P, Mattiello L, Costamagna C, Piretto V et al. Insulin stimulates nitric oxide synthesis in human platelets and through nitric oxide increases platelet concentrations of both guanosine 3, 5-monophosphate and adenosine 3, 5-cyclic monophosphate. *Diabetes* 1997; 46:742–749.

[205] van Dorp DA, Beerthuis RK, Nugteren DH, and Vonkeman H (1964) Enzymatic conversion of all-cis-polyunsaturated fatty acids into prostaglandins. *Nature (Lond*) 203: 839-841.

[206] Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 2000;20:1262–1275

[207] Vodyanik MA, Thomson JA, Slukvin, II. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. *Blood* 2006; 108: 2095–105.

[208] Wallentin L. $P2Y_{12}$ inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. *Eur Heart J.* 2009 Aug;30(16):1964-77.

[209] Warden SJ. Prophylactic use of NSAIDs by athletes: a risk/benefit assessment. *Phys Sportsmed*. 2010 Apr;38(1):132-8.

[210] Warner TD, Mitchell JA. Cyclooxygenase-3 (COX-3): filling in the gaps toward a COX continuum? *Proc Natl AcadSci U S A.* 2002 Oct 15;99(21):13371-3.

[211] Whittle BJ, Moncada S, Vane JR (1978) Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. *Prostaglandin*s 16:373–388.

[212] White JG. 1972 Platelet morphology and function. In: Hematology (Ed by Williams WJ, Beuter E, Erslev JA, Rundles RW) McGraw-Hill book company A *Blakiston Publication USA*. 1159

[213] White JG, Krivit W. The fine structural localization of adenosine triphosphatase in platelets and other blood cells. *Blood.* 1965;26: 554-568.

[214] White JG. The dense bodies of human platelets: Origin of serotonin storage particles from platelet granules. *Am J Pathol*. 1968;53 (5): 791-808.

[215] Wilcox JN, Scott NA. Potential role of the adventitia in arteritis and atherosclerosis. *Int J Cardiol*. 1996;54(suppl):S21–S35.

[216] Williams KJ, Tabas I. The response-to-retention hypothesis of atherogenesis reinforced. *CurrOpinLipidol.* 1998;9:471–474.

[217] Willich SN, Jimenez AH, Tofler GH, DeSilva RA, Muller JE. Pathophysiology and triggers of acute myocardial infarction: clinical implications. *ClinInvestig*. 1992;70 Suppl1:S73-8.

[218] Wink DA, Hanbauer I, Krishna MP, DeGraff W, Gamson J, Mitchell JB. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci USA*. 1993; 90:813-9817

[219] Wink DA, Hanbauer I, Laval F, Cook JA, Krishna MC, Mitchell JB. Nitric oxide protects against the cytotoxic effects of reactive oxygen species. *Ann N Y Acad Sci.* 1994 Nov 17;738:265-78.

[220] Wong, ND. "Epidemiological studies of CHD and the evolution of preventive cardiology." *Nature reviews. Cardiology*. 2014;11 (5): 276–89.

[221] Wright JH . "The Origin and Nature of the Blood Plates". *The Boston Medical and Surgical Journal* . 1906;154.

[222] Wright JH. "The histogenesis of blood platelets". *J Morphology*. 1910 (21): 263–278.

[223] Xu Y, Arenas IA, Armstrong SJ, Plahta WC, Xu H, Davidge ST. Estrogen improves cardiac recovery after ischemia/reperfusion by decreasing tumor necrosis factor-alpha. *Cardiovasc Res*. 2006 Mar 1;69(4):836-44.

[224] Xue B, Singh M, Guo F, Hay M, Johnson AK. Protective actions of estrogenon angiotensin II-induced hypertension: role of central nitric oxide. *Am JPhysiol Heart CircPhysiol* 2009; 297:H1638–H1646.

[225] Yamada E. The fine structure of the megakaryocyte in the mouse spleen. *Acta Anat.* (Basel). 1957;29: 267-290

[226] Yao SK, Akhtar S, Scott-Burden T, Ober JC, Golino P, Buja LH, Cassells W, Willerson JT. Endogenous and exogenous nitric oxide protect against intracoronary thrombosis and reocclusion after thrombosis. *Circulation.* 1995; 92(4):1005-1010.

[227] You HJ, Kim JY, Jeong HG. 17 beta-estradiol increases inducible nitricoxide synthase expression in macrophages. *BiochemBiophysResCommun* 2003; 303:1129–1134.

[228] Zucker MB. Platelets. In: Zweifach BW, Grant L, McCluskeyRT, eds. The inflammatory process. Vol 1. *New York: AcademicPress*, 1974:511-543.

LIST OF PUBLICATIONS

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Publications related my thesis:

1. **Pradipta Jana,** Sarbashri Bank, Smarajit Maiti, Shantanu Guha, Gausal Azam Khan, Asru Kumar Sinha. Estriol actuated restraint of platelet aggregation is repressed by binding of dermcidin isoform-2 to its receptor on platelets of the Acute Myocardial Infarction Patient. ***Cell Physiol Biochem*** [*in press*]

2. **Pradipta Jana**, Smarajit Maiti, Nighat N.Kahn, A. K. Sinha. "Estriol induced fibrinolysis due to the activation of plasminogen to plasmin by nitric oxide synthesis in platelets" ***Blood Coagulation & Fibrinolysis*** 2015 Apr;26(3):316-23.

3. **Pradipta Jana**, Rajeshwary Ghosh, Smarajit Maiti, Tamal Kanti Ghosh, A. K. Sinha. Estriol, a stimulator of nitric oxide synthesis in platelets and its role as the powerful inhibitor of platelet aggregation. ***Cardiovasc Endocrinol*** 2013, 2:50–54

Other publications:

4. Ganguly Bhattacharjee K, Bhattacharyya M, Halder UC, **Jana P**, Sinha AK. Effect of progesterone receptor status on maspin synthesis via nitric oxide production in neutrophils in human breast cancer. ***Breast Cancer***, 2014 Sep;21(5):605-13.

5. Sarbashri Bank, **Pradipta Jana**, Smarajit Maiti, Santanu Guha, and Asru Sinha. "Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction" ***Sci Rep*** 2014 Jul 24;4:5804.

6. Sarbashri bank, Rajeshwary Ghosh, P**radipta Jana**, Suman Bhattacharya, Asru K. Sinha. The diagnosis of high altitude illness by the determination of plasma dermcidin isoform 2 levels by enzyme linked immunosorbent assay. ***Clin. Lab.*** 2014;60:1187-1191

7. Rajeswary Ghosh, Udayan Ray, **Pradipta Jana**, Rabindra Bhattacharya, Debipriya Banerjee, Asru K Sinha. "Reduction of death rate due to acute myocardial infarction in subjects with cancers through systemic restoration of impaired nitric oxide" ***Plos One*** 9(2): e88639

8. Rajeshwary Ghosh, Mau Bhattacharyya, Gausal Khan, Somashree Chakraborty, Rabindra Bhattacharya, Uttam K. Maji, **Pradipta Jana**, Asru K. Sinha. Diagnosis of Essential

Hypertension in Humans by the Determination of Plasma Renal Cortexin using Enzyme-Linked Immunosorbent Assay. ***Clin Lab*.** 2013;59(5-6):475-81

9. Karabi Ganguly (Bhattacharjee), Mau Bhattaharyya, Umesh Chandra Halder, **Pradipta Jana**, Asru K. Sinha. The "Cross Talk" between the Receptors of Insulin, Estrogen and Progesterone in Neutrophils in the Synthesis of Maspin through Nitric Oxide in Breast Cancer ***Int J Biomed Sci***, 2012(8):129-139

10. Karabi Ganguly (Bhattacharjee), Mau Bhattaaharyya, Umesh Chandra Halder, **Pradipta Jana**, Asru K. Sinha. "The role of estrogen receptor status in neutrophils on maspin synthesis through nitric oxide production in human breast cancer" ***J Breast Cancer*.** 2012;15(2):181-188.

11. Rajeswary Ghosh, **Pradipta Jana**, Asru K Sinha. "The Control of Hyperglycemia in Alloxan Treated Diabetic Mice through the stimulation of Hepatic Insulin Synthesis due to the Production of Nitric oxide",***Exp Clin Endocrinal Diabetes***, 2012, 120(3):145-51.

Conference Proceedings:

1. **Jana P**, Sinha A. "Cross talk" between the receptors of dermcidin isoform-2 and estriol in resistance of the effect of estriol in platelet aggregation in myocardial infarction**.** Abstracts of the **HAA 2014 Annual Scientific Meeting. Perth, Australia**. Page-447. P236. October18-22, 2014
2. **Pradipta Jana,** Asru K. Sinha**.** "Cross talk" between the receptors of dermcidin isoform-2 and estriol in resistance of the effect of estriol in platelet aggregation in myocardial infarction. **Indian Ocean Rim Laboratory Haematology 2014. Mandurah, Australia.** Pg-17. **http://www.labhaem2015.org/assets/Uploads/Jana-e-posterAPP-Cross-talk.pdf**. October 15-17, 2014.
3. **Pradipta Jana,** Asru K. Sinha "Purification and characterization of a platelet membrane bound nitric oxide synthase activated by estriol and progesterone due to heterotropic allosteric activation" was given in **ISARCON2013, Trissur, India**
4. **Pradipta Jana,** Suman Bhattacharya, Asru K. Sinha. The preventive role of estrogens as antiatherosclerotic hormone through its antidiabetic, anti-hypertensive and antiplatelet effects on the development of the coronary heart disease (CHD). Indian Society for Atherosclerosis Research Conference2012, **Chidambaram, India**

Estriol, a stimulator of nitric oxide synthesis in platelets, and its role as the powerful inhibitor of platelet aggregation

Pradipta Jana[a], Smarajit Maiti[c], Rajeshwary Ghosh[a], Tamal K. Ghosh[b] and Asru K. Sinha[a]

Women, before menopause, are known to be resistant to the development of acute ischemic heart disease (AIHD). As the inhibition of platelet aggregation is reported to prevent incidences of AIHD, the effects of estradiol and estriol on ADP-induced platelet aggregation in platelet-rich plasma were determined. It was found that it was not estradiol, the most potent estrogenic hormone, but estriol, less potent than estradiol, that had a minimum inhibitory concentration (MIC) of 0.6 nmol/l for 100% inhibition of ADP-induced platelet aggregation. In contrast, the MIC of estradiol was 2.0 nmol/l ($P<0.005$, $n=40$). The stimulation of nitric oxide (NO) by 0.6 nmol/l estriol in platelet-rich plasma was 0.55 nmol/10^8 cells/h and the stimulation by the 2.0 nmol/l estradiol was 0.179 nmol/10^8 cells/h. Treatment of intact platelets with 0.05% Triton X-100 released a membrane NO synthase in the supernatant that had basal K_m of 5.28 mmol/l with V_{max} of 0.029 nmol NO/mg/h. The treatment of the supernatant with 0.6 nmol/l estriol decreased the K_m to 3.42 mmol/l with increased V_{max} to 0.337 nmol NO/mg/h. These results showed that estriol was one of the most potent inhibitors of platelet aggregation with MIC that was in subnanomolar ranges, which is lower than any other inhibitors currently known and suggested that estriol might prevent AIHD in women before menopause. *Cardiovasc Endocrinol* 2:50–54

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Keywords: acute ischemic heart disease, estriol, nitric oxide, platelet aggregation

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Introduction

The aggregation of platelets by aggregating agents such as ADP, L-epinephrine, collagen, or thrombin is an essential physiologic event in the life-saving blood coagulation process (see Colman and Walsh [1] for comprehensive references). In contrast, excessive platelet aggregation on the site of atherosclerotic plaque rupture on the coronary artery, particularly by ADP in humans, has been reported to result in the development of acute ischemic heart disease (AIHD), commonly known as 'heart attack'. The formation of thrombus (a microaggregate of platelets embedded in fibrin mass) on the wall of the coronary artery blocks the normal blood circulation in the heart muscle and may lead to the development of a life-threatening condition [2–4].

As the above-mentioned platelet-aggregating agents may lead to the formation of thrombus on the site of atherosclerotic plaque rupture, the inhibition of the platelet aggregation by several factors present in the circulation is known to counteract the excessive platelet aggregation that might be crucial for the prevention of AIHD. Among these humoral factors capable of inhibiting platelet aggregation are prostacyclin [5], insulin [6], and interferon-α [7]. These factors, including prostacyclin, insulin, and interferon-α, have been reported to be some of the most potent inhibitors of platelet aggregation in that these compounds are capable of inhibiting platelet aggregation at nmol/l concentrations. Although prostacyclin has been reported to inhibit platelet aggregation by increasing the platelet cyclic AMP level, interferon-α and insulin are reported to inhibit platelet aggregation by the stimulation of nitric oxide (NO) synthesis induced by these compounds in platelets [7]. NO, thus formed, is not only reported to increase both cyclic AMP [8] and cyclic GMP [9] in platelets for the inhibition of platelet aggregation, but the oxide has also been reported to activate plasminogen to plasmin, which results in the dissolution of intraplatelet fibrinogen bridges that is essential for the aggregation of platelets [7]. The plasmin-induced lysis of the fibrinogen bonds resulted in the inhibition of platelet aggregation in the absence of an increase in the cyclic nucleotide level in platelets.

It is well known that women, before menopause, are resistant to the development of AIHD [10,11].

In the above context, where the inhibition of the platelet aggregation might lead to the prevention of AIHD, it could be expected that the humoral estrogens in women before the onset of menopause were involved in the inhibition of platelet aggregation for the prevention of the condition [11]. However, no reports on the effect of

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estrogens on the inhibition of platelet aggregation *in vitro* are available.

The basic issue related to the inhibition of platelet aggregation by estrogens was further complicated by the fact that for the expression of the effect of estrogen, the presence of DNA in the target cell is needed [12]. As human blood platelets do not contain DNA, no alternative mechanism for the estrogen-induced inhibition of platelet aggregation independent of DNA in these cells is currently available.

Here, we report that the inhibition of platelet aggregation in platelet-rich plasma (PRP) by estradiol, the most potent estrogenic steroid, as well as by estriol, which is reported to be one of the most abundant albeit considerably less potent estrogenic hormones than estradiol itself in women [13], stimulated platelets to synthesize NO. We further report that estriol is one of the most powerful inhibitors of platelet aggregation currently known, and the inhibition of platelet aggregation was mediated by the stimulation of NO synthesis because of the activation of a membrane-bound nitric oxide synthase (NOS) in platelets independent of DNA.

Materials and methods

Ethical clearance

The study, which used blood samples from normal volunteers, was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology (Calcutta).

All participating volunteers were asked to sign an informed consent form before they were requested to donate blood.

Chemicals

Estriol, estradiol, ADP, and Triton X-100 were obtained from Sigma Aldrich (St Louis, Missouri, USA). The estrogens were dissolved in 0.9% NaCl. ADP used in the study was dissolved in distilled water and the pH was adjusted to 7.4 at 0°C just before use and discarded after use.

The solvents used for preparing stocks were freshly prepared.

Selection of volunteers

The volunteers selected (women = 20, men = 20; $n = 40$) were between 25 and 40 years of age. None of the volunteers had a history of diabetes mellitus nor systemic hypertension and had never had any cardiovascular nor cerebrovascular disease. The participants had no life-threatening infections at the time of investigation and had not been hospitalized for any condition at least 6 months before their blood samples were collected. All volunteers were asked to stop using any medications including acetyl salicylic acid (aspirin) for at least 4 weeks before they consented to donate blood.

Female volunteers donated their blood when they were in the mid-follicular phase of their menstrual cycle. The estradiol and estriol concentration ranged between 37–53 and 24–30 pmol/l, respectively. None of the female volunteers had ever received any contraceptive medication.

Collection of blood from the volunteers and the preparation of platelet-rich plasma and gel-filtered platelet

Peripheral blood sample (30 ml) was obtained by venipuncture using 19-G siliconized needles, collected in plastic tubes, and anticoagulated by mixing one volume of sodium citrate with 10 volumes of the blood by gentle inversion. The final concentration of the anticoagulant was 13 mmol/l. The blood samples were centrifuged at 200*g* for 15 min at room temperature (23°C). The supernatant fraction, PRP, was collected and used for further studies. The cell-free blood plasma was prepared by centrifuging the anticoagulated blood sample at 10 000*g* for 30 min at 0°C.

The gel-filtered platelet (GFP) were prepared from the blood of a single donor (200 ml) using the method described previously [14]. Both estradiol and estriol present in the plasma were removed from platelets by sepharose gel filtration.

Platelet aggregation

The aggregation of platelets was studied using 2.0 μmol/l ADP as the aggregating agent with an aggregometer as described before [15]. To determine the effect of estriol or estradiol as the inhibitor of platelet aggregation, different concentrations of the estrogens were added to PRP and were incubated for 45 min at 37°C before the aggregation of platelets was initiated by using ADP. The minimum inhibitory concentration (MIC) of either estriol or estradiol was defined as the amount of the compound that completely inhibited platelet aggregation induced by 2.0 μmol/l ADP as described above.

Determination of nitric oxide synthesis

The synthesis of NO was determined by the conversion of oxyhemoglobin into methemoglobin as described previously [7]. The formation of NO in the reaction mixture was verified independently using the chemiluminescence method [16].

Assay of nitric oxide synthase

Typically, a reaction mixture containing 0.5–0.8 mg of the protein preparation as the source of NOS was incubated with 10 μmol/l L-arginine in the presence of 2 mmol/l $CaCl_2$ in a total mixture of 1.0 ml in Tyrode's buffer, pH 7.4, as described previously [15]. The formation of NO in

the supernatant was quantified using the method described above.

Treatment of gel-filtered platelet with Triton X-100

In a certain phase of the study, it was necessary to release platelet membrane proteins as the source of NOS. For this purpose, GFP was prepared from 200 ml of blood from a single donor. The GFP was treated with 0.05% Triton X-100 (v/v) for 30 min at 0°C with occasional gentle shaking. We have reported previously that the treatment of GFP with a low concentration of the detergent resulted in the release of platelet membrane proteins without dissolving the whole platelets [14]. The supernatant thus obtained was centrifuged at 30 000*g* for 60 min at 0°C. The clarified supernatant was concentrated to 1.0 ml [14] and dialyzed against 0.9% NaCl overnight. The dialyzed supernatant was used as the source of NOS.

Results

The inhibition of ADP-induced platelet aggregation by estriol and estradiol in platelet-rich plasma

The treatment of PRP from normal volunteers with 2.0 μmol/l ADP resulted in the complete aggregation of platelets (Fig. 1a). When the same PRP was pretreated with different amounts of estriol as indicated, and incubated for 45 min at 37°C, and subsequently treated with 2.0 μmol/l ADP, it was found that the MIC of estriol that completely inhibited platelet aggregation induced by ADP was 0.6 nmol/l (Fig. 1a). In contrast, when the same PRP preparation was incubated with different amounts of estradiol under identical conditions, it was found that the MIC of estradiol for the ADP-induced platelet aggregation was 2.0 nmol/l ($P < 0.005$, $n = 40$) (Fig. 1b), indicating that estriol was more than two-fold powerful inhibitor of platelet aggregation when compared with estradiol under identical conditions.

Synthesis of nitric oxide in platelet-rich plasma induced by estriol or by estradiol

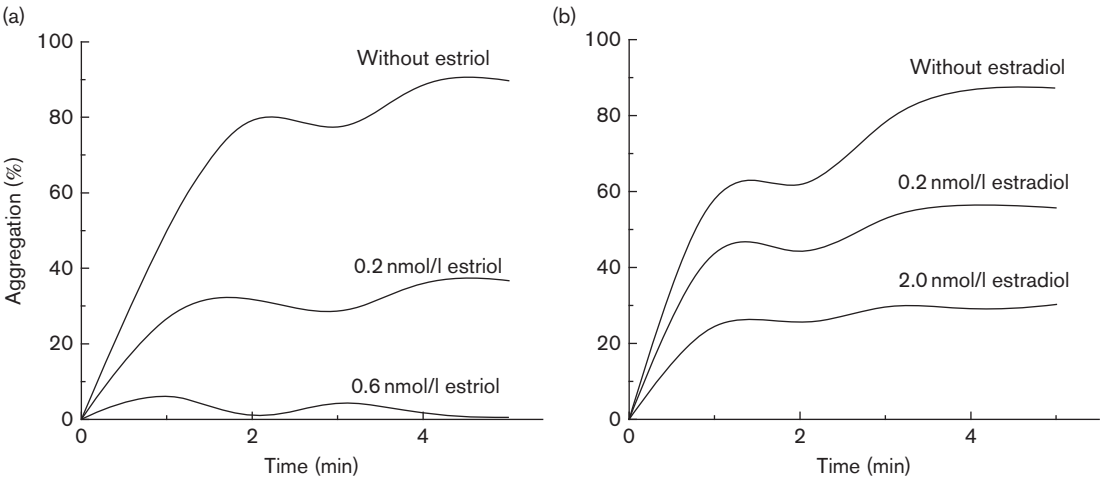
To determine the role of NO in the inhibition of platelet aggregation by the estrogens, the production of NO induced by both of these steroids in PRP was determined (Fig. 3). Although both estriol and estradiol could stimulate NO produced in PRP, the former steroid was a more potent inducer of NO synthesis in platelets than the latter.

Although the maximal synthesis of NO in the presence of estriol in PRP was achieved at 0.6 nmol/l, the maximal production of NO in PRP occurred at 2.0 nmol/l estradiol (Fig. 3). The amount of NO produced at 2.0 nmol/l estradiol in PRP was found to be 0.179 nmol/10^8 cells/h and was 0.55 nmol/10^8 cells/h when treated with 0.6 nmol/l estriol. These results indicated that estriol was a greater than three-fold powerful stimulator of NO synthesis than that produced by estradiol in the same platelet preparation for the maximal inhibition of platelet aggregation that was induced by ADP.

Mechanism of estrogen-induced nitric oxide synthesis in platelets

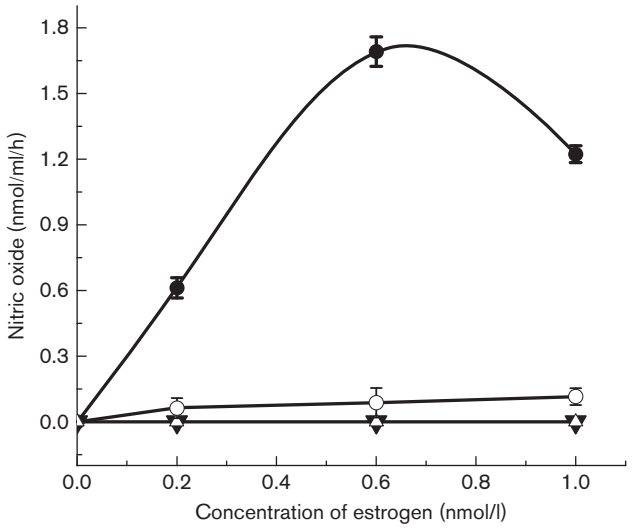
As human platelets do not have DNA it cannot synthesise NOS, an enzyme protein. As such, it was believed that the blood plasma might contain NOS that catalyzed the formation of NO from L-arginine (the substrate of NOS). However, no NOS activity in the presence or absence of estradiol or estriol could be found in the human blood plasma (Fig. 2).

Fig. 1



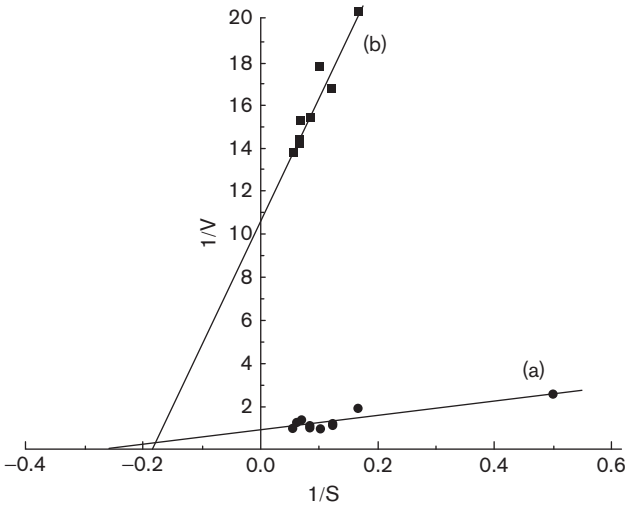
ADP-induced platelet aggregation inhibition by estriol (a) or by estradiol (b) in platelet-rich plasma. Before the initiation of platelet aggregation induced by 2.0 μmol/l ADP, the platelet-rich plasma was incubated with or without different concentrations of estriol or estradiol as indicated for 45 min at 37°C. The profiles of platelet aggregation are typical from more than 40 different experiments using 40 different individuals.

Fig. 2



Stimulation of nitric oxide (NO) synthesis by estriol or estradiol in platelet-rich plasma (PRP). PRP from five different volunteers was incubated with different concentrations of estrogens as indicated for 45 min at 37°C. After incubation, NO synthesis in the PRP was determined as described in the Materials and methods section. Whereas the solid circles (–●–) represent estriol, the open circle (–○–) show estradiol. The formation of NO is indicated by the hollow triangle (–△–) in PRP treated without estriol or estradiol and in PRP in the presence of estriol or estradiol with 10 mmol/l N^G-nitro-L-arginine methyl ester. The solid inverted triangles (–▼–) show the formation of NO in PFP without estriol or estradiol.

Fig. 3



Lineweaver–Burk plots of the supernatant of Triton X-100-treated gel-filtered platelet in the presence and absence of estriol. The gel-filtered platelets were treated with 0.05% Triton X-100 and the supernatants from the incubation mixture were dialyzed as described in the Materials and methods section. The dialyzed supernatant was used as the source of nitric oxide synthase. Line (a) represents the Lineweaver–Burk plot of the dialyzed supernatant in the presence of 0.6 nmol/l estriol; line (b) represents the Lineweaver–Burk plot of the same dialyzed supernatant in the absence of the added estriol.

Efforts were next made to determine whether platelet membrane might contain basal NOS activity that was activated by the estrogens as shown in Fig. 2.

We have reported previously that the treatment of intact platelets with a low concentration of Triton X-100 (0.05% v/v) released membrane proteins in the supernatant without dissolving the whole platelet as described in the Materials and methods section.

When the dialyzed supernatant was tested for the presence of NOS, the basal activity of the protein in the dialyzed preparation was found to be present. A Lineweaver–Burk plot of the NOS in the dialyzed supernatant was constructed in the presence and absence of 0.6 nmol/l estriol.

Lineweaver–Burk plots of the NOS activity showed that in the absence of the added estriol, the K_m was 5.28 mmol/l with a V_{max} 0.029 nmol NO/mg/h (Fig. 3, line (b)). When the same platelet supernatant was treated with 0.6 nmol/l estriol, K_m was found to be reduced to 3.42 mmol/l with a concomitant increase of V_{max} to 0.337 nmol NO/mg/h (Fig. 3, line (a)).

In other words, the basal NOS activity of the platelet membrane protein was stimulated ≈10-fold in the presence of 0.6 nmol/l estriol.

Discussion

It is generally believed that all steroids, including estrogens, mediated their effects through the synthesis or inhibition of protein in the target cells, that is, those cells that are capable of expressing the effects of the steroid [12].

As such, for the expression of the steroids' effect, the presence of both DNA and the nuclear steroid receptors is needed [17]. However, we have reported that the effects of estriol [18] and progesterone [19], as well as NO itself, led to the synthesis of maspin, an antibreast cancer protein in neutrophils, which suggests that NO was 'acting' like the second messenger in these cases.

In some cases, it has been described that estriol at 100 nmol/l can synergize the increased platelet aggregation [20], but the amount of estriol (100 nmol/l) was much higher than the concentrations that showed the highest NO synthesis by estriol (0.6 nmol/l) in our study. It was also found that the synthesis of NO was decreased when the concentration of estradiol or estriol was increased beyond the optimum level of estrogens for the maximal synthesis of NO in platelets.

Our results, as described above, showed that NOS activity induced by estradiol or estriol did not involve the participation of DNA but probable nongenomic activity of the estrogen β receptor [21] (Fig. 3). Our results also showed that not estradiol, but estriol was the more powerful inhibitor of platelet aggregation in PRP.

Indeed, it was found that estriol was not only a more powerful inhibitor of platelet aggregation than estradiol (MIC $\approx$ 2.0 nmol/l) but is one of the most powerful inhibitors of platelet aggregation currently known (MIC $\approx$ 0.6 nmol/l), in that no other inhibitors of platelet aggregation were reported to completely inhibit (100%) platelet aggregation at subnanomolar concentrations as a result of stimulation of NO synthesis in platelets. It was found that 0.55 nmol NO/10^8 cells/h was produced by 0.6 nmol/l estriol and 2.0 nmol/l estradiol synthesized 0.179 nmol NO/10^8 cells/h (Fig. 1a and b).

In this context, it should be mentioned that extensive trials using estradiol to prevent AIHD in menopausal women failed to reduce the occurrence of the deadly condition in the participants [10]. The basis of this trial, however, was an extension of estrogen replacement therapy for the prevention of AIHD, which itself is only phenomenological in nature. In some other cases, investigators claimed that endogenous estradiol may play a role in increased platelet aggregation by ADP or adrenaline [20]. However, inhibition of platelet aggregation in GFP that is free of endogenous estradiol occurred using a minimal concentration of estradiol (2 nmol/l) as in the case of PRP. The investigation erroneously presumed that the effect of estrogens on the inhibition of AIHD was mediated by estradiol. We found that not estradiol but estriol was the major inhibitor of platelet aggregation that might prevent AIHD in women. Recent studies further showed that estradiol may actually be a thrombogenic agent *in vivo* by increasing platelet aggregation [22].

However, the beneficial effect of estradiol could be related to its effect as an antihypertensive [23] and an antidiabetogenic [24] agent that could result in the control of atherosclerosis but was not necessarily related to the inhibition of platelet aggregation *in vivo*. Our study also showed that the estrogenic effects of estradiol or estriol were not necessarily related to their ability to inhibit platelet aggregation, which has a major effect in the prevention of AIHD as supported by numerous studies using acetyl salicylic acid [25]. Perhaps, the use of estriol might have produced a better outcome in the prevention of AIHD compared with that obtained using estradiol [10].

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Conflicts of interest

There are no conflicts of interest.

References

1 Colman RW, Walsh PN. Mechanisms of platelet aggregation. In: Colman RW, Hirsh J, Maeder VJ, Salzman EW, editors. *Haemostasis and thrombosis*. Philadelphia: J.B. Lippincott; 1987. pp. 594–605.
2 Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, *et al*. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; **1**:352–358.
3 Falk E. Dynamics in thrombus formation. *Ann N Y Acad Sci* 1992; **667**: 204–223.
4 Fuster V, Badimon J, Chesebro JH, Fallon JT. Plaque rupture, thrombosis, and therapeutic implications. *Haemostasis* 1996; **26**:269–284.
5 Whittle BJ, Moncada S, Vane JR. Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. *Prostaglandins* 1978; **16**:373–388.
6 Trovati M, Anfossi G, Massucco P, Mattiello L, Costamagna C, Piretto V, *et al*. Insulin stimulates nitric oxide synthesis in human platelets and through nitric oxide increases platelet concentrations of both guanosine 3, 5-monophosphate and adenosine 3, 5-cyclic monophosphate. *Diabetes* 1997; **46**:742–749.
7 Bhattacharyya M, Karmohapatra SK, Bhattacharya G, Bhattacharya R, Sinha AK. The role of leucocytes in the acetyl salicylic acid (aspirin) induced nitric oxide synthesis in the production of interferon-α, a potent inhibitor of platelet aggregation and a thrombolytic agent. *J Thromb Thrombolysis* 2009; **28**:173–184.
8 Acharya K, Acharya R, Sinha AK. Inhibition of low Km phosphodiesterase of human blood platelets by nitric oxide. *Biosci Res Bull* 2001; **17**:47–53.
9 Kanowitz PJ. Evidence for the inhibitory role of guanosine 3, 5-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 1981; **57**: 946–955.
10 Nelson HD, Humphrey LL, Nygren P, Teutsch SM, Allan JD. Postmenopausal hormone replacement therapy: scientific review. *JAMA* 2002; **288**: 872–881.
11 Gouva L, Tsatsoulis A. The role of estrogens in cardiovascular disease in the aftermath of clinical trials. *Hormones (Athens)* 2004; **3**:171–183.
12 Levin ER. Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 2005; **19**:1951–1959.
13 O'Neil MJ, Smith A, Heckelman PE. *The Merck index*. 13th ed. Whitehouse Station: Merck Research Laboratories; 2006. pp. 660–661.
14 Dutta-Roy AK, Sinha AK. Purification and properties of prostaglandin E1/prostacyclin receptor of human blood platelets. *J Biol Chem* 1987; **262**:12685–12691.
15 Chakraborty K, Khan GA, Banerjee P, Ray U, Sinha AK. Inhibition of human blood platelet aggregation and the stimulation of nitric oxide synthesis by aspirin. *Platelets* 2003; **14**:421–427.
16 Cox RD, Frank CW. Determination of nitrate and nitrite in blood and urine by chemiluminescence. *J Anal Toxicol* 1982; **6**:148–152.
17 Olefsky JM. Nuclear receptor minireview series. *J Biol Chem* 2001; **276**:36863–36864.
18 Ganguly Bhattacharjee K, Bhattacharyya M, Halder UC, Jana P, Sinha AK. The role of neutrophil estrogen receptor status on maspin synthesis via nitric oxide production in human breast cancer. *J Breast Cancer* 2012; **15**: 181–188.
19 Ganguly Bhattacharjee K, Bhattacharyya M, Halder UC, Jana P, Sinha AK. Effect of progesterone receptor status on maspin synthesis via nitric oxide production in neutrophils in human breast cancer. *Breast Cancer* 2012; [Epub ahead of print].
20 Akarasereenont P, Tripatara P, Chotewuttakorn S, Palo T, Thaworn A. The effects of estrone, estradiol and estriol on platelet aggregation induced by adrenaline and adenosine diphosphate. *Platelets* 2006; **17**:441–447.
21 Khetawat G, Faraday N, Nealen ML, Vijayan KV, Bolton E, Noga SJ, Bray PF. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood* 2000; **95**:2289–2296, 1.
22 Manson JE, Hsia J, Johnson KC, Rossouw JE, Assaf AR, Lasser NL, *et al*. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003; **349**:523–534, 7.
23 Xue B, Singh M, Guo F, Hay M, Johnson AK. Protective actions of estrogen on angiotensin II-induced hypertension: role of central nitric oxide. *Am J Physiol Heart Circ Physiol* 2009; **297**:H1638–H1646.
24 Louet JF, LeMay C, Mauvais-Jarvis F. Antidiabetic actions of estrogen: insight from human and genetic mouse models. *Curr Atheroscler Rep* 2004; **6**:180–185.
25 Steering Committee of the Physicians Health Study Research Group. Final report on the aspirin component of the on going physicians health study. *N Engl J Med* 1989; **321**:129–135.

Estriol-induced fibrinolysis due to the activation of plasminogen to plasmin by nitric oxide synthesis in platelets

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Estriol, an oestrogen, at 0.6 nmol/l was reported to inhibit ADP-induced platelet aggregation through nitric oxide synthesis. As nitric oxide has been reported to cause fibrinolysis due to the activation of plasminogen to plasmin, the role of estriol as a fibrinolytic agent was investigated. Also, the mechanism of estriol-induced nitric oxide synthesis in anucleated platelets was investigated. The estriol-induced lysis of platelet-rich plasma (PRP) clot was determined by photography of the clot lysis and by the assay of fibrin degradation products in the lysate and was obtained by SDS-PAGE. Nitric oxide was determined by methemoglobin method. The platelet membrane protein was isolated from the platelets by using Triton X-100 (0.05% v/v). The binding of estriol to the protein was determined by Scatchard plot by using an ELISA for estriol. Estriol at 0.6 nmol/l was found to lyse the clotted PRP due to fibrinolysis that produced fibrin degradation products in the lysate. The amino acid analysis of the platelet membrane protein, which resembles with nitric oxide synthase (NOS) activity, was activated nearly 10-fold over the control in the presence of estriol and was identified to be a human serum albumin precursor (Mr. 69 kDa) that binds to estriol with Kd_1 of 6.0×10^{-9} mol/l and 39 ± 2 molecules of estriol bound the NOS molecule. The estriol-induced nitric oxide is capable of inducing fibrinolysis of the clotted PRP. The binding of estriol to platelet membrane NOS activated the enzyme in the absence of DNA in the platelet. *Blood Coagul Fibrinolysis* 26:316–323

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Introduction

The aggregation of platelets is a life-saving physiologic event that plays an essential role in blood coagulation process (reference [1], for comprehensive literatures). In contrast, excessive platelet aggregation particularly at the site of atherosclerotic plaque rupture on the coronary artery is reported to result in the coronary artery disease (CAD) due to the formation of thrombus (a microaggregate of platelets embedded in fibrin mass) on the plaque rupture site. Thrombus thus formed obstructs the normal blood circulation that is essential for the physiologic activity of the musculature of the heart, and the blockade might precipitate CAD due to the thrombosis [2,3].

The dissolution of the thrombus on the coronary artery was reported to help the restoration of the normal blood circulation in the heart and may consequently resolve the ensuing condition [4]. For this purpose, various thrombolytic agents are therapeutically used [5,6]. It should be mentioned here that the thrombolytic agents are all fibrinolytic agents that are capable of converting plasminogen to plasmin (a serine protease, which is responsible for the fibrinolytic effect of these agents) [7]. Although nitric oxide is not an enzyme, and known to be a potent inhibitor of platelet aggregation [8], the inorganic compound has been reported to convert plasminogen to plasmin in the absence of any cofactors or cells and was found to cause fibrinolysis [8–11].

Various compounds and agents including acetylsalicylic acid (aspirin) [11], interferon-α [8], insulin [12] which are reported to stimulate nitric oxide synthesis in platelets, and nitric oxide itself, are all reported to be efficacious fibrinolytic agents through the activation of plasminogen (Mr. 89 kDa) to plasmin (Mr. 75 kDa) due to excision of a peptide chain 'excision peptide', (Mr. 14 kDa) from plasminogen molecule probably due to the nitric oxide induced breakage of cross-strand disulfide (S–S) bridges [11].

It has also been reported that the efficacy of aspirin in the prevention of CAD was not only related to the inhibition of platelet aggregation due to the inhibition of cyclooxygenase alone but was also due to the aspirin-induced stimulation of nitric oxide synthesis in platelets that resulted in the activation of plasminogen to plasmin in the plasma leading to the dissolution of the formed thrombus on the arterial wall [11].

As estriol was found to be one of the most potent inhibitor of platelet aggregation at 0.6 nmol/l through the synthesis of nitric oxide in platelets, studies were carried out to determine the role of estriol, if any, as a fibrinolytic agent on ex-vivo clotted platelet-rich plasma (PRP).

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Materials and methods

Ethical clearance

The protocol used in the study involved normal humans and was carried out in accordance with the Helsinki agreement, which was approved by the Internal Review Board, Sinha Institute of Medical Science & Technology, Calcutta, West Bengal. All volunteers signed informed consent form before they were included in the study.

Chemicals and antibody

Estriol (purity 98%), goat anti-rabbit immunoglobulin G-alkaline phosphatase and fibrinogen were obtained from Sigma-Aldrich. Polyclonal antibody against estriol was obtained from Thermo Scientific. Maxisorp plates for ELISA were obtained from Nunc, Roskilde, Denmark. All other chemicals used were of analytical grade.

Selection of volunteers

The study was conducted by involving both male and female volunteers ($n = 40$, men 30, women 10) between the ages of 20 and 40 years. They had no history of systemic hypertension or diabetes mellitus. They were also clarified for any life-threatening infectious diseases if they had. The volunteers did not suffer from cardiovascular or cerebrovascular diseases and had not been hospitalized for any reason at least for 6 months before they participated in the study. Women volunteers who had never received any contraceptive medications were asked to donate their blood in the mid-follicular phase of their menstrual cycle. No medications including acetyl salicylic acid (aspirin) were not taken by the participants at least for 4 weeks before they donated blood.

Preparation of platelet-rich plasma and platelet-free plasma from blood samples

Blood samples (20 ml) were obtained by venipuncture by using 19-gauge siliconized needles, collected in plastic vials, anticoagulated by using sodium citrate (13 mmol/l final concentration) as described before [13]. The PRP and platelet-free plasma (PFP) were prepared from the anticoagulated blood samples as described [13].

Synthesis of nitric oxide in platelets in the presence or absence of estriol

Incubation mixtures containing PRP were incubated with 10 μmol/l of L-arginine in the presence or absence of estriol in different times. In control experiments, nitric oxide synthase (NOS) activity in the PFP was similarly treated with the steroids under identical conditions. In our preliminary experiments, it was found that estriol was able to synthesize maximal nitric oxide when PRP was incubated at 37°C. After incubation of either PRP or PFP with 0.6 nmol/l estriol at 37°C, the synthesis of nitric oxide was determined by the conversion of oxyhaemoglobin to methaemoglobin by the spectral changes of the absorption maxima at 525 and 630 nm as described earlier [8]. Appropriate control experiments were carried out by incubating PRP with 10 μmol/l of L-arginine in the absence of the added oestrogen. The amount of nitric oxide formed in the presence of estriol was calculated by subtracting nitric oxide formation in the control experiment. The quantitation of nitric oxide was independently verified by chemiluminescence method [14].

Preparation of gel-filtered platelets

Gel-filtered platelets (GFPs) were prepared by gel filtration of PRP on Sepharose CL-2B and suspended in Tyrode's buffer without Ca^{2+}, pH 7.4 as described before [15].

The lysis of the clotted platelet-rich plasma in the presence of added estriol

The PRP or PFP was clotted after incubation with estriol in the presence of $CaCl_2$ (10 mmol/l) in silicon-coated glass tubes as described earlier [11]. The clot lytic activity of the added estriol (0.6 nmol/l) was recorded by non-weighted photography and by the determination of the accumulated fibrin degradation products in the lysate as described below at different times. Experiments were also carried out by adding 0.1 mmol/l L-NAME (N^G-nitro-L-arginine methyl ester) to PRP to inhibit estriol-induced nitric oxide synthesis in platelets [16] before the PRP or PFP was treated with estriol and subsequently clotted by $CaCl_2$.

Determination of fibrin degradation products, FDP_1 and FDP_2, by the estriol-induced fibrinolysis of the clotted platelet-rich plasma

The fibrinolysis of the clotted PRP or PFP was determined by determining the amounts of fibrin degradation product-1 (FDP_1) and fibrin degradation product-2 (FDP_2) accumulated in the clot lysate by SDS-PAGE of the lysate [17]. Both FDP_1 and FDP_2 bands were excised out of the gel after the electrophoresis, and amounts of the proteins were determined as described by Lowry *et al.* [18]. Appropriate control experiments were run by using similar Vol of only 0.9% NaCl without estriol to determine the accumulation of FDP_1 and FDP_2 in the clot that might be formed due to clot retraction and not by fibrinolysis.

Isolation of protein from the intact platelet membranes by using Triton X-100

Triton X-100 (0.05% v/v) was added to the GFP suspension in Tyrodes' buffer, pH 7.4, prepared from 200 ml of blood obtained from a single donor and incubated at 0°C with occasional shaking for 30 min as described before [15]. The supernatant was subsequently collected and was dialyzed against 0.9% NaCl overnight to remove the detergent at 4°C. Release of nitric oxide was catalysed by the dialyzed protein and was determined as described below.

Typically, nearly 0.6 mg of protein in the supernatant was incubated with 10 μmol/l of L-arginine containing 2.0 mmol/l $CaCl_2$ in 2.5 ml reaction mixture with 0.6 nmol/l estriol for 45 min at 37°C and the release of nitric oxide was determined as described above.

Lineweaver–Burk plot of the electrophoretically purified protein with NOS-like activity (described below) was constructed in the presence and absence of estriol.

SDS-PAGE of the released proteins in the supernatant from the Triton X-100 treated gel-filtered platelet

The dialyzed supernatant from the Triton X-100 treated platelets was electrophorosed on SDS-polyacrylamide gel and the protein bands were stained by Coomassie brilliant blue [19]. The protein bands in an identical gel, but not stained, were excised out from the gel. Each protein band was separately excised, triturated in 0.9% NaCl and clarified by centrifugation and dialyzed as described earlier. The NOS activity of each of the eluted and dialysed protein bands was subsequently determined.

The 69-kDa protein band in the gel that released nitric oxide was reduced by using dithiothreitol (DTT) and electrophorosed on SDS polyacrylamide to determine the subunit composition of the protein [20].

The amino acid sequence analysis of the protein with nitric oxide releasing ability described earlier

The amino acid sequence analysis of the electrophoretically purified protein, first in the presence and then in the absence of SDS (as described above), which showed stimulated release of nitric oxide in the presence of 0.6 nmol/l estriol, was performed at Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer. The protein sample was processed as instructed by the service provider.

Binding of estriol to the electrophoretically purified protein (Mr. 69 kDa) with nitric oxide producing ability

The details of the binding of estriol to a platelet membrane protein that had the ability to release nitric oxide as described earlier were carried out by the methods that we have described recently [21]. Briefly, the binding of estriol to the electrophoretically purified protein was carried out by separating the bound oestrogen from the unbound oestrogen by using micro glass fiber cation (GF/C) membrane filters by using Millipore filtration [22]. The amount of the estriol was quantitated by ELISA using estriol antibody [23]. The specific binding was determined by subtracting nonspecific binding from the total binding.

Scatchard plot analysis

Scatchard plot [24] of the equilibrium binding of estriol to the electrophoretically purified protein with nitric oxide producing activity was determined by the ELISA of the steroid as described before [23].

Statistical analyses

The results shown are mean ± standard deviation (SD) of at least five different experiments using the blood samples from five different donors each in triplicate.

The significance '*P*' of the results was determined by Student's *t*-test, and *P* value less than 0.001 was considered to be significant ($n = 15$). The coefficient of correlation '*r*' was determined by Pearson test by using GraphPad Prism software.

Results

Effect of estriol on the synthesis of nitric oxide in platelet-rich plasma

To determine the synthesis of nitric oxide in PRP, mol/l nitric oxide assay was done in the presence of 0.6 nmol/l estriol at different times at 37°C. As described in Fig. 1, the maximum synthesis of nitric oxide induced by 0.6 nmol/l estriol was achieved after 45 min of incubation.

The estriol-induced fibrinolysis of the clotted platelet-rich plasma due to the stimulation of nitric oxide synthesis in platelets

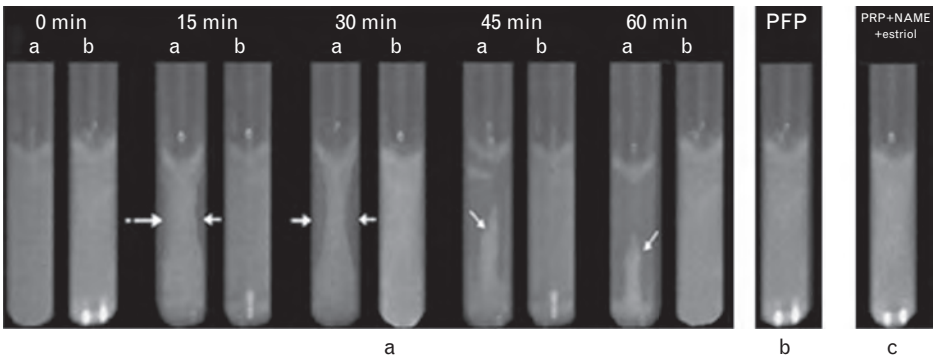
Experiments were carried out to determine the role of estriol, if any, on fibrinolysis of the clotted PRP induced by nitric oxide released in platelets through plasmin formation [11].

Incubation of clotted PRP with 0.6 nmol/l estriol was found to result in the gradual increase of the clot lysis from 0 to 60 min at 37°C as shown in the tubes marked 'a' at different times (Fig. 1a), and it was found that at 60 min, the lysis of the PRP clot was maximally achieved. In control experiments in which the PRP was clotted only in the presence of 0.9% NaCl (vehicle for estriol), no clot lysis could be seen in the tubes marked 'b' at different times (Fig. 1a).

When PFP was clotted in the presence of 0.6 nmol/l estriol, no clot lysis could be found (Fig. 1b). The addition of 0.1 mmol/l L-NAME that inhibited nitric oxide synthesis in the platelets incubated with 0.6 nmol/l estriol resulted in the complete failure of the oestrogen to lyse the clotted PRP (Fig. 1c).

When the lysates were obtained from the above experiment after different times of incubation, and the accumulation of FDP_1 and FDP_2 in the lysate were determined (Fig. 2), the amounts of both FDP_1 and FDP_2 in the lysate were found to be increased with the increase of the incubation time, and at 60 min, the maximal amounts of both FDP_1 and FDP_2 were found to be accumulated in the lysate (Fig. 2). In control experiments in which an equal Vol of 0.9% NaCl was used instead of estriol and the PRP was clotted by $CaCl_2$, no such increase in the amounts of FDP_1 and FDP_2 due to

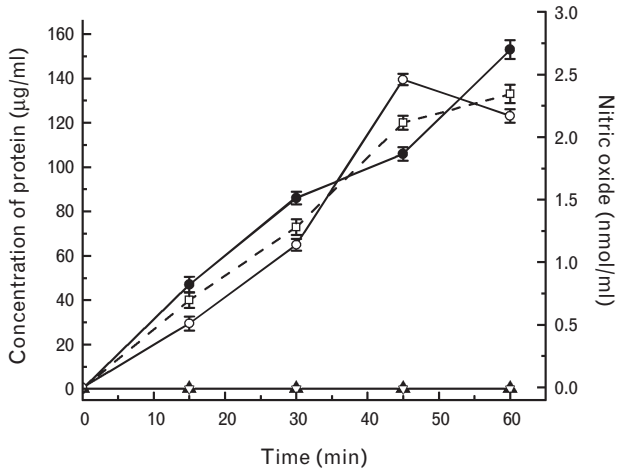
Fig. 1



The lysis of the clotted platelet-rich plasma in the presence of 0.6 nmol/l estriol at different times. (a) The lysis of clot at different times is shown by the white arrow (→) in the tube marked 'a'. The tubes marked 'b' show no clot lysis at different times in the absence of the oestrogen. In these tubes, only vehicle (0.9% NaCl) was used. The tubes were incubated for different time as indicated and the lysis was recorded by photography as described in Materials and Methods section. (b) The absence of clot lysis in the presence of 0.6 nmol/l estriol in the case of the clotted platelet-free plasma (PFP) under identical condition. (c) The effect of addition of L-NAME to the PRP on the clot lysis in the presence of 0.6 nmol/l estriol. The figure is a typical representative of at least five more similar experiments using blood from 10 different volunteers.

fibrinolysis could be found, thus excluding the possibility that the observed PRP clot lysis was due to the clot retraction in which no accumulation of either FDP_1 or FDP_2 in the lytsate takes place. The correlation of coefficient '*r*' between the nitric oxide synthesis and the appearance of FDP_1 and FDP_2 was determined to be 0.9774 and 0.9087.

Fig. 2



Accumulation of fibrin degradation products (FDP_1 and FDP_2) in the lysate and synthesis of nitric oxide in the presence 0.6 nmol/l estriol at different times. The appearance of hollow circles (—○—) describes the production of nitric oxide at different times as indicated. The solid circles (—●—) or the continuous line represents FDP_1 and hollow squares (—□—) or the discontinuous line shows the FDP_2, respectively, at different times as indicated. Solid triangles (—▲—) show the absence of FDP_1 and FDP_2 in the lysate in the presence of both estriol and 0.1 mmol/l L-NAME at different times. The hollow inverted triangles (—▽—) demonstrate that the accumulation of FDP_1 and FDP_2 in the lysate in the absence of estriol wherein only equal Vol of only 0.9% NaCl was used and incubated under identical conditions. Mean ± SD of at least five experiments each in triplicate using blood samples from five different volunteers. After 60 min incubation of estriol, the level of FDP_1 and FDP_2 was significantly different ($P < 0.001$, $n = 15$) from the level FDP_1 and FDP_2 in the absence of estriol after 60 min.

The mechanism of estriol-induced stimulation of nitric oxide release in platelets

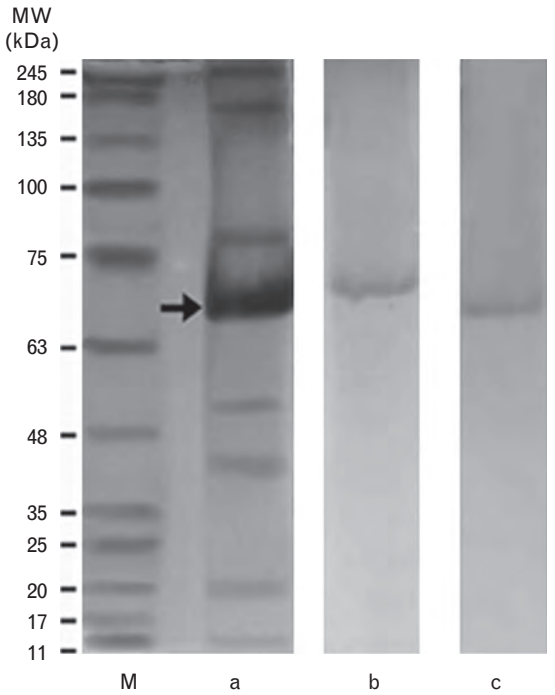
As human blood platelets do not contain DNA, the role of estriol in the nitric oxide production in PRP or in GFP through the stimulation of NOS requires a nongenomic mechanism (i.e. DNA independent) for the oestrogens' effects in the inhibition of platelet aggregation due to nitric oxide production as reported before [21] or in the fibrinolysis of the PRP clot through estriol-induced nitric oxide production as described above.

And, as such, efforts were made to determine whether platelet membrane might contain a protein that could be activated in the presence of the oestrogens to release nitric oxide in PRP or in GFP as reported before [21]. To test the possibility of increase in nitric oxide in the platelet membrane, GFP that could be stimulated by estriol was treated with 0.05% Triton X-100 as described in Materials and Methods section. Experiments were carried out to find whether the treatment of intact platelets with the low amount of Triton X-100 that has been reported before to result in the release of membrane proteins in the supernatant without dissolving the whole platelets [15] might show the presence of basal nitric oxide releasing ability in the released proteins.

Characterization of the platelet membrane proteins with nitric oxide releasing activity that was stimulated by estriol in the supernatant released by the treatment of platelets by Triton X-100

The supernatant obtained from the Triton X-100 treated platelets was electrophorosed on polyacrylamide gel with

Fig. 3



PAGE of the supernatant from the Triton X-100 treated gel filtered platelet suspension. Lane a: SDS-PAGE of the supernatant from GFP treated with Triton X-100 stained with Coomassie blue. The arrow shows the protein band of 69 kDa. Lane b: PAGE of the protein band in the Lane a (black arrow) that was excised out and reelectrophoresed in the absence of SDS. Lane c: The protein from Lane b was isolated, treated with dithiothreitol and electrophoresed in SDS-polyacrylamide gel. Lane M: Marker proteins in SDS polyacrylamide gel. Protein bands in all cases were stained by coommassie brilliant blue.

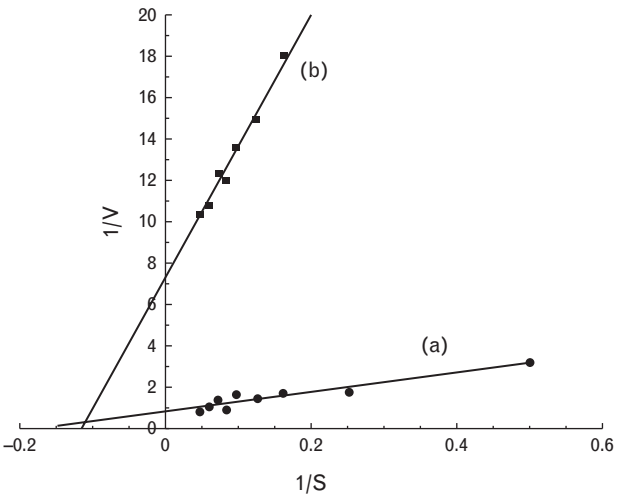
SDS, which showed the presence of several major protein bands when stained with Coomassie blue (Fig. 3a). These protein bands were separately excised out from another identical gel that was not stained. The eluted protein from each band in the gel was triturated in 0.9% NaCl, clarified and dialyzed as described in Materials and Methods section. Only the 69-kDa protein band showed enhanced NOS activity when treated with 0.6 nmol/l estriol. The eluted protein of 69 kDa from the gel was next concentrated [25] and reelectrophorosed on polyacrylamide gel in the absence of SDS that again demonstrated the presence of a single protein (Fig. 3b). The protein band was subsequently eluted from the gel and reduced by using DTT [20] and reelectrophorosed on SDS-polyacrylamide gel. The reduction of protein did not show the presence of any subunits suggesting that the 69-kDa platelet protein with NOS activity was probably a single polypeptide chain (Fig. 3c).

When the electrophoretically purified protein was treated with either estriol or estradiol, the synthesis of nitric oxide from L-arginine in the presence of Ca^{++} in the reaction mixture could be demonstrated. However, it was found that the amounts of nitric oxide produced in the presence of 0.6 nmol/l estriol were more than three-fold (1.77 nmol nitric oxide formed/mg protein/h) than that produced in the presence of similar amounts of estradiol (0.49 nmol of nitric oxide formed/mg of protein/h) under identical conditions, indicating that estriol was a more potent stimulator of the platelet membrane NOS than estradiol.

Lineweaver–Burk plot of the NOS activity of the protein purified by the repeated electrophoresis (Fig. 3b) as described above in the presence and absence of estriol demonstrated that K_m of L-arginine in the absence of added oestrogen was 8.637 mmol/l with V_{max} equal to 16.33 nmol nitric oxide formed/mg protein/h (Fig. 4, line-b). In contrast, when the same protein was treated with 0.6 nmol/l estriol, the K_m was found to be reduced to 6.093 mmol/l with corresponding increase of V_{max} to 154.2 nmol nitric oxide formed/mg protein/h, which indicates that the rate of nitric oxide release was stimulated by nearly 10-fold in the presence of 0.6 nmol/l estriol over the basal nitric oxide releasing ability (Fig. 4, line-a).

The amino acid sequence of the 69-kDa protein with the nitric oxide releasing ability is shown in Fig. 5. The Uniprot data bank matching of the amino acid sequence of the purified platelet protein (Mr. 69 kDa) identified to

Fig. 4



Lineweaver–Burk plots of the nitric oxide synthase activity of the electrophoretically purified 69-kDa protein in the presence and absence of estriol. The 69-kDa (Mr.) protein was purified by PAGE first in the presence and then in the absence of SDS. The purified protein was studied for its nitric oxide synthase activity by Lineweaver–Burk plots in the presence and absence of 0.6 nmol/l estriol added to the reaction. Line A: Lineweaver–Burk plot of the 69-kDa protein in the presence of 0.6 nmol/l estriol in the reaction. Line a and Line b represents Lineweaver-Burk plot of the 69-kDa protein in the presence and absence of 0.6 nmol/l estriol respectively.

Fig. 5

MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAF
AQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTV
ATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTA
FHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAAC
LLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAE
FAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKEC
CEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLG
MFLYEYARRHPDYSVVLLLRLAKTYETTKEKCCAAADPHECYAKVFDEFK
PLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRN
LGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCT
ESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALV
ELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQ
AALGL

The amino acid sequence of the 69-kDa protein with nitric oxide synthase activity. The electrophoretically purified protein with 69 kDa Mr. was used for the determination of the amino acid sequence. The details of the procedures and the Uniprot data bank of the protein by matching the amino acid sequence have been described in Materials and Methods section.

be Human serum albumin precursor (Uniprot ID: P02768) is shown in Fig. 5.

The equilibrium binding of estriol to the electrophoretically purified protein and Scatchard plot analysis of the binding characteristics

The binding profile of estriol to the electrophoretically purified protein (Fig. 3b) from platelets showed a saturable binding characteristic and the presence of specific estriol-binding sites in the protein, as determined by the subtraction of the nonspecific binding from the total binding (Fig. 6a).

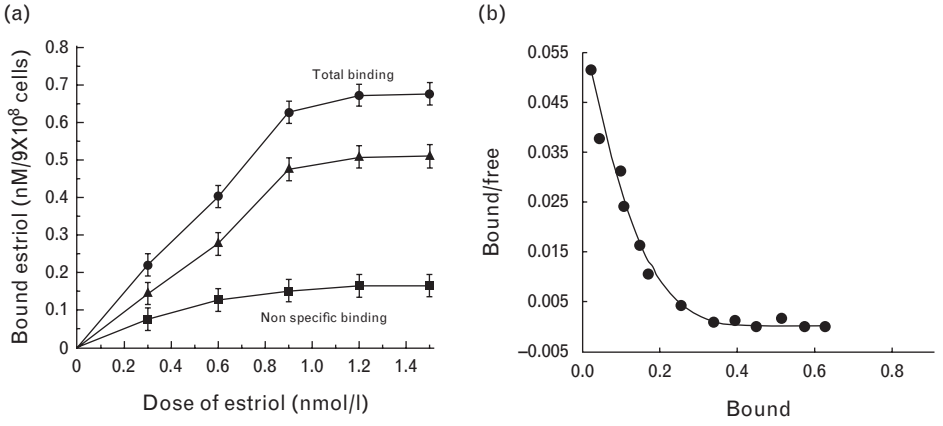
Scatchard plot of equilibrium estriol binding characteristics to the purified platelet protein produced a curvilinear profile of estriol binding to the purified 69-kDa protein suggesting the presence of heterogenous binding sites of the oestrogen in the protein molecule with one high affinity ($Kd_1 = 6.002$ nmol/l) low capacity ($n_1 = 39 \pm 2$ molecules estriol/molecule of protein) and one low affinity ($Kd_2 = 243$ nmol/l) high capacity ($n_2 = 1.4 \times 10^3$ molecules estriol/molecule of protein) binding sites of estriol in the Mr. 69 kDa protein (Fig. 6b).

Discussion

These results demonstrated that estriol, one of the most abundant ovarian hormones, was a potent stimulator of nitric oxide production by a platelet membrane associated protein of Mr. 69 kDa, which resembles NOS-like activity. As described in the Results, the binding of estriol to this 69-kDa protein resulted in the nearly 10-fold stimulation of the basal nitric oxide production ability of the protein. And, as such, it can be inferred that the 69-kDa protein, identified to be human serum albumin precursor (Fig. 5), might not only bind to estriol with high affinity ($Kd_1 = 6002$ nmol/l), but also the binding of the oestrogen to this protein resulted in the stimulation of the basal nitric oxide production by nearly 10-fold. In this context, it was also noted that the Kd_1 of estriol binding to 69-kDa protein was in ranges similar to the optimal concentration of the oestrogen for the maximal inhibition of platelet aggregation as reported earlier [21]. The low affinity ($Kd_2 = 243$ nmol/l) with high capacity binding sites (n_2) probably indicated nonspecific binding of fatty acids like molecules by the human serum albumin precursor.

ERα or ERβ binding to oestrogen resulting in upregulation of NOS gene expression and nitric oxide production is reported [26], which may be ruled out in our present investigation because platelet is an anucleated cell. The 69-kDa protein was apparently acting like a 'receptor protein' for the production of the nitric oxide induced by

Fig. 6



The equilibrium binding of estriol to the 69-kDa protein with nitric oxide synthase activity that was stimulated by estriol. (a) The nonspecific and specific binding of estriol to the 69-kDa protein was determined. Values obtained from total binding and nonspecific binding were significantly different when $P < 0.001$, $n = 15$. (b) Scatchard plot of the equilibrium binding to the 69-kDa protein was determined by separating the bound estriol from the unbound by using GF/C filters and the quantity of the estriol was determined by ELISA.

estriol in the absence of DNA in human blood platelets. These results suggested a non genomic expression of the oestrogen effect in anucleated platelets apparently in the absence of nuclear receptors or DNA, which are currently believed to be involved in the expression of the steroid effect at least in the nucleated cells [26]. There is a possibility of nitrosothiol formation with nitric oxide and the 69-kDa protein, as predicted to be a human serum albumin precursor that is highly *cys* rich [27]. Oestrogen binding to albumin followed by structural modifications may also promote the sustained release of nitric oxide, which is able to perform further physical functions [28].

The steroids mediate their effects through the expression or through the inhibition of specific protein synthesis via hormone receptor element (HRE) in the DNA [29]. We have reported before that both estriol and rogesterone were capable of inducing 'maspin' synthesis, an anti-breast cancer protein in the nucleated cells, through the synthesis of nitric oxide in the nucleated cells [23]. Interestingly, as we have reported here, estriol also mediates its effect through nitric oxide synthesis in the inhibition of platelet (anucleated cells) aggregation either in PRP or in GFP [8], which might suggest that nitric oxide was acting like the 'messenger' of these steroids in these cases apparently without the synthesis of proteins. In the above context, it should also be mentioned that the effect of aldosterone that has an essential role in the electrolyte balance in the kidney function apparently was capable of mediating its effect in a DNA-independent way [30].

Estriol at 0.6 nmol/l was found to be a potent inhibitor of platelet aggregation as described before [21], and it was also a thrombolytic agent in the postplatelet aggregation event of thrombus formation as shown in Figs 1 and 2.

As described earlier, the NOS-like activity of the platelet membrane protein by estriol was three times greater than estradiol, which is known to be a more potent estrogenic metabolite than estriol, and it proves that the estrogenic potency of oestrogen was not necessarily related to any physiologic activities [31], and our result suggested that the antithrombotic property of estriol could have a more important role in the prevention of CAD than that of the estradiol in women before the onset of menopause.

In the above context, the role of estriol-induced nitric oxide release that only reported to result in the inhibition of platelet aggregation, but as described here nitric oxide, was also found to be a potent thrombolytic agent through the activation of plasminogen to plasmin (a serine protease) through the excision of 14-kDa Mr. peptide from plasminogen. Then, the formation of plasmin was independent of Hageman factor dependent slow pathway for the systemic production of plasmin [8]. And, as such, the estriol effects as both the inhibitor of platelet aggregation and a thrombolytic agent that is similar to those of acetyl salicylic acid, currently known to be one of the most beneficial compounds against the CAD. Perhaps, estriol could have similar beneficial effects against CAD due to thrombus formation on the coronary artery wall.

Finally, it should also be mentioned here that nitric oxide is the only compound currently known that inhibits platelet aggregation when it increases. The decrease of the basal nitric oxide level actually led to the aggregation of platelets even in the absence of ADP [32].

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Colman RW, Walsh PN. Mechanisms of platelet aggregation. In: Colman RW, Hirsh J, Maeder VJ, Salzman EW, editors. *Haemostasis, thrombosis*. Philadelphia, PA: JB Lippincott; 1987. pp. 594–605.

2 Falk E. Dynamics in thrombus formation. *Ann N Y Acad Sci* 1992; **667**:204–223.

3 Fuster V, Badimon J, Chesebro JH, Fallon JT. Plaque rupture, thrombosis, and therapeutic implications. *Haemostasis* 1996; **26**:269–284.

4 Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *J Biol Chem* 1982; **257**:2912–2919.

5 Sherry S. Mechanism of fibrinolysis. In: William WJ, Beutler E, Ersler A, Rundels RW, editors. *Hematology*. Mcgraw-Hill; 1977. p. 1924.

6 Fletcher AP, Alkjaersig N, Lewis M, Tulevski V, Davies A, Brooks JE, *et al.* A pilot study of urokinase therapy in cerebral infarction. *Stroke* 1976; **7**:135–142.

7 Summaria L, Hsieh B, Robbins KC. The specific mechanism of activation of human plasminogen to plasmin. *J Biol Chem* 1967; **242**:4279–4283.

8 Bhattacharyya M, Karmohapatra SK, Bhattacharya G, Bhattacharya R, Sinha AK. The role of leucocytes in the acetyl salicylic acid (aspirin) induced nitric oxide synthesis in the production of interferon-α, a potent inhibitor of platelet aggregation and a thrombolytic agent. *J Thromb Thrombolysis* 2009; **28**:173–184.

9 Yao SK, Akhtar S, Scott-Burden T, Ober JC, Golino P, Buja LM, *et al.* Endogenous and exogenous nitric oxide protect against intracoronary thrombosis and reocclusion after thrombolysis. *Circulation* 1995; **92**:1005–1010.

10 Sinha AK, Acharya K, Bhattacharya S, Mazumder S, Bauman WA, Kahn NN. Prevention of thrombosis in vivo by nitric oxide. *Ind J Physiol Allied Sci* 1998; **52**:148–161.

11 Karmohapatra SK, Chakraborty K, Kahn NN, Sinha AK. The role of nitric oxide in aspirin induced thrombolysis in vitro and the purification of aspirin activated nitric oxide synthase from human blood platelets. *Am J Hematol* 2007; **82**:986–995.

12 Chakraborty K, Sinha AK. The role of insulin as an antithrombotic humoral factor. *Bioessays* 2004; **26**:91–98.

13 Chakraborty K, Khan GA, Banerjee P, Ray U, Sinha AK. Inhibition of human blood platelet aggregation and the stimulation of nitric oxide synthesis by aspirin. *Platelets* 2003; **14**:421–427.

14 Cox RD, Frank CW. Determination of nitrate and nitrite in blood and urine by chemiluminescence. *J Anal Toxicol* 1982; **6**:148–152.

15 Dutta-Roy AK, Sinha AK. Purification and properties of prostaglandin E1/prostacyclin receptor of human blood platelets. *J Biol Chem* 1987; **262**:12685–12691.

16 Sakuma I, Stuehr DJ, Gross SS, Nathan C, Levi R. Identification of arginine as a precursor of endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A* 1988; **85**:8664–8667.

17 Carr ME Jr, Alving BM. Effect of fibrin structure on plasmin mediated dissolution of plasma clot. *Blood Coagul Fibrinolysis* 1995; **6**:567–573.

18 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement by the folin-phenol reagent. *J Biol Chem* 1951; **1193**:265–275.

19 Fazekas de St Groth S, Webster RG, Datyner A. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochim Biophys Acta* 1963; **71**:377–391.

20 Cleland WW. Dithiothreitol: a new protective reagent for SH groups. *Biochemistry* 1964; **3**:480–482.

21 Jana P, Maiti S, Ghosh R, Ghosh TK, Sinha AK. Estriol, a stimulator of nitric oxide synthesis in platelets, and its role as the powerful inhibitor of platelet aggregation. *Cardiovasc Endocrinol* 2013; **2**:50–54.

22 Kahn NN, Sinha AK. Stimulation of prostaglandin E1 binding to human blood platelet membrane by insulin and the activation of adenylate cyclase. *J Biol Chem* 1990; **265**:4976–4981.
23 Bhattacharjee KG, Bhattacharyya M, Halder UC, Jana P, Sinha AK. The role of estrogen receptor statusin neutrophils and maspin synthesis through nitric oxide production in human breast cancer. *J Breast Cancer* 2012; **15**:181–188.
24 Scatchard G. The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* 1949; **51**:660–672.
25 Ghosh R, Karmohapatra SK, Bhattacharyya M, Bhattacharya R, Bhattacharya G, Sinha AK. The appearance of dermcidin isoform 2, a novel platelet aggregating agent in the circulation in acute myocardial infarction that inhibits insulin synthesis and the restoration by acetyl salicylic acid of its effects. *J Thromb Thrombolysis* 2011; **31**:13–21.
26 You HJ, Kim JY, Jeong HG. 17 beta-estradiol increases inducible nitric oxide synthase expression in macrophages. *Biochem Biophys Res Commun* 2003; **303**:1129–1134.
27 Kharitonov VG, Sundquist AR, Sharma VS. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J Biol Chem* 1995; **270**:28158–28164.
28 Chakrabarti S, Lekontseva O, Peters A, Davidge ST. 17beta-Estradiol induces protein S-nitrosylation in the endothelium. *Cardiovasc Res* 2010; **85**:796–805.
29 Levin ER. Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 2005; **19**:1951–1959.
30 Logvinenko NS. Aldosterone: the mechanisms of molecular action. *Ross Fiziol Zh Im I M Sechenova* 2007; **93**:746–761.
31 Soldan SS, varez Retuerto AI, Sicotte NL, Voskuhl RR. Immune modulation in multiple sclerosis patients treated with the pregnancy hormone estriol. *J Immunol* 2003; **171**:6267–6274.
32 Banerjee D, Mazumder S, Sinha AK. The role of inhibition of nitric oxide synthesis in the aggregation of platelets due to the stimulated production of thromboxaneA2. Blood Coagul Fibrinolysis (in press).

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Original Paper

Estriol Actuated Restraint of Platelet Aggregation is Repressed by Binding of Dermcidin Isoform-2 to its Receptor on Platelets of the Acute Myocardial Infarction Patient

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Key Words
Acute Myocardial Infarction • Dermcidin Isoform-2 • Platelet aggregation • Nitric Oxide • Estrogen

Abstract
Background: Estriol inhibits platelet aggregation by activating nitric oxide synthase (NOS). Furthermore, estriol could not inhibit platelet aggregation and synthesize NO in PRP from the acute myocardial infarction (AMI) patients. An investigation was carried out to determine the mechanism of NO production in platelets and to find the reason of failure to inhibit platelet aggregation in AMI platelets with estrogen. ***Methods:*** Dermcidin isoform-2 (DCN-2) and the platelet membrane NOS was purified by gel electrophoresis. The binding characteristics of estriol to platelets were determined by ELISA. ***Results:*** NOS was treated with 0.6nM estriol resulted in the increased synthesis of NO by 8 folds through allosteric activation, compared to control. Estriol activation of NOS could be inhibited by tamoxifen, an estrogen receptor antagonist. The treatment of AMI platelets with 0.6nM estriol failed to increase NO synthesis followed by platelet aggregation with 2.0μM ADP due to impaired estriol binding to the platelets. Control platelets incubated with DCN-2 which appears in the circulation of AMI resulted in the reduced estriol binding sites/platelets by 1.6fold with dissociation control in the similar ranges. ***Conclusion:*** The failure of estriol to inhibit platelet aggregation via NO synthesis was due to "cross-talk" between DCN-2 and estriol on the platelet surface in AMI.

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Introduction

Platelet aggregation by different platelet aggregating agents, including ADP, *l*-epinephrine, collagen or thrombin has a critically important role in physiologic events in the life saving blood coagulation process [reference 1 for comprehensive literatures]. In contrast, an excessive platelet aggregation on the atherosclerotic plaque rupture site in the coronary artery is reported to develop into thrombus formation that has been demonstrated to obstruct the normal circulation of the blood in the musculature of the heart, leading to acute coronary syndrome (ACS) as well as acute myocardial infarction (AMI), known to be the major killers of the human race [2]. The inhibition of platelet aggregation by several humoral factors, including insulin [3], prostacyclin [4], estriol [5] and pharmacological compound like acetyl salicylic acid (aspirin) through their ability to inhibit platelet aggregation are considered to produce a counter veiling effect on the excess platelet aggregation leading to homeostasis and consequently might help to prevent the occurrence of ACS [6].

It is generally accepted that the expression of the steroid effects, including estrogens (of which estriol is one) in the hormone responsive cells is mediated through the binding of the steroid to one of the nuclear receptors [7]. Binding of the agonist changed the conformation of the nuclear receptor that in consequence binds to a specific sequence in the DNA known as hormone responsive element (HRE) that results either in the expression or in the inhibition of the gene that results in the synthesis or in the inhibition of protein(s) that mediates the steroid effect in the cells [7]. The validity of this assertion in the effect of estriol (one of the estrogens) in the stimulation of nitric oxide (NO) synthesis as a potent inhibitor of platelet aggregation in platelets may actually pose a special problem in that we have recently reported that, estriol, at 0.6nM probably acted, to the best of our knowledge, as the most potent inhibitor of human platelet aggregation currently known through the production of NO in platelets [5]. As human blood platelets do not contain DNA, the effect of estriol in the inhibition of platelet aggregation through the NO synthesis catalyzed by nitric oxide synthase (NOS) cannot be mediated through the DNA dependent expression of the NOS gene in the platelets.

In the context of estriol as a potent inhibitor of platelet aggregation, the obvious question was, if estriol was such a potent inhibitor of platelet aggregation why did the steroid fail to inhibit platelet aggregation in the development of ACS or AMI in man?

We report herein the mechanism of the resistance of the platelet aggregation from the AMI subjects was due to the systemic appearance of dermcidin isoform 2 (DCN-2) [8] in the circulation that conferred the resistance of platelets to estriol from the AMI subjects due to the "cross talk" between the receptors of DCN-2 and estrogen on the platelet surface.

Materials and Methods

Ethical clearance for human subjects

Platelet rich plasma (PRP) both from a control and from the subjects with AMI was obtained. Appropriate protocol according to Helsinki agreement was approved by the Internal Review Board, Sinha Institute of Medical Science & Technology, Calcutta, West Bengal. All volunteers signed informed consent forms before they were included in the study. Informed consent forms were also signed by the control subjects.

As the subjects affected with AMI were not always physically fit to sign an informed consent form, the collection of blood was carried out by attending physician of the Department of Cardiology, Calcutta Medical College, Kolkata with the consent from next of kin of the patient and in the presence of legal councilor to the patients. The blood (5.0ml) was withdrawn only after the attending physician obtained consent from next of kin of the patient and decided that the withdrawing of the blood sample was safe and permissible, both for the confirmation of the occurrence of AMI by plasma troponin-I level and of platelet aggregation studies.

Chemicals and antibody

Estriol (98% purity) and goat anti-rabbit immunoglobulin G-alkaline phosphatase were obtained from Sigma-Aldrich. Polyclonal antibody against estriol was obtained from Thermo Scientific. Maxisorp plates for enzyme linked immunosorbent assay (ELISA) were obtained from Nunc, Denmark. All other chemicals used were of analytical grade.

Selection of volunteers

Subjects who participated in the study did not have any history of systemic hypertension or diabetes mellitus or were suffering from any life threatening infection. The participants did not suffer from cardiovascular or cerebrovascular diseases and no record had been found to be hospitalized for any reason at least for 6 months before they participated in the study. The volunteers were asked not to take any medications including acetyl salicylic acid (aspirin) for at least 4 wks before they donated blood. None of the female volunteers had ever received any contraceptive medications.

The selection of AMI patients

The blood sample was withdrawn from both ST elevated AMI (STEMI) and non ST elevated AMI (nSTEMI)-UA patients. However, when such differentiation was not possible, the subject was not included in the study. The presence of Q wave in the EKG was routinely followed for the patient selection. At presentation all AMI patients had characteristics of chest pain for AMI for more than 120 min. The occurrence of AMI was confirmed by the determination of troponin-I by collecting blood samples within 6h of hospitalization before the initiation of any cardiac therapy. The blood sample was drawn from 30 AMI Patients (M=15, F=15) with an equal number of age and sex matched control volunteers.

Preparation of platelet-rich plasma (PRP) and platelet free plasma (PFP) from blood samples

Blood samples (5.0 ml) were obtained by venipuncture by using 19 gauge siliconized needles, collected in plastic vials, anti-coagulated by using sodium citrate (13mM final concentration). The PRP and PFP were prepared from the anticoagulated blood samples as described before [9].

Production of NO in platelets in the presence or absence of estriol

Incubation mixtures containing PRP were incubated with 10μM of *l*-arginine in the presence or absence of different concentrations of estriol with triplicate experiments for each sample. To determine NO production in the PFP in the control experiment, the platelet free plasma was similarly treated with the steroids under identical conditions. In our preliminary experiment, it was found that the estrogens were able to maximally produce NO after 45 min incubation of the platelet preparations at 37°C. After incubation of either PRP or PFP for 45 min at 37°C in the presence of the estrogens, the production of NO was determined by the conversion of oxyhemoglobin to methemoglobin by the spectral changes of the absorption maxima at 525 and 630nm as described before [6]. Production of NO was confirmed by chemiluminescence method [10].

Aggregation of platelets

Platelet aggregation both in control and AMI platelets were determined by using 2.0μM ADP as the aggregating agent with an aggregometer as described before [9]. Aggregation of AMI platelets and in control platelets was determined in equal number of age and sex matched volunteers (n=30, M=15, F=15) with triplicate experiments in each . To find out if the estriol has any role to inhibit both **control** and AMI platelets, with 0.6nM estriol was added to PRP as described before [5].

Isolation of estriol binding protein from the intact platelet membranes by using Triton X-100

Gel filtered platelets (GFP) from PRP were prepared by using a Sepharose 6B column as described before [5]. The GFP suspension in Tyrodes' buffer, pH 7.4, was prepared from 200ml of blood obtained from a single donor. Triton X-100 (0.05% v/v) was added to the GFP and incubated at 0°C with occasional shaking for 30 min as described before [11]. Synthesis of NO catalyzed by the dialyzed protein from the GFP suspension supernatant was determined as described below. The platelet supernatant thus obtained was electrophoresed first in the presence of sodium dodecyl sulfate (SDS) in polyacralamide gel and the protein bands were stained by coommassie brilliant blue [12]. In an identical experiment where the same

supernatant was similarly subjected to SDS gel electrophoresis, but was not stained by coommassie blue, the protein bands were excised from the gel and separately triturated in 0.9% NaCl, dialyzed overnight against 0.9% NaCl and was concentrated by using polyethylene glycol [13] the NOS activity of the preparation was determined as described before [14].

Typically, ≈27μg/ml protein in the supernatant was incubated with 10μM of *l*-arginine containing 2.0mM $CaCl_2$ in 2.5ml reaction mixture with 0.6nM estriol for 45 min at 37°C and the production of NO was determined as described above.

Characterization of the estriol binding protein

The platelet membrane protein, thus obtained, was re-electrophoresed in polyacralamide gel in the absence of SDS was dialyzed, concentrated as described above. NOS activity with 0.6 nM estriol was determined in crude and in each step of purification of the protein.

The isolated and dialyzed protein from the gel was also subjected for its purity by micro-capillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer at Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology.

Determination of allosteric modulation of the platelet membrane protein for NO production

The platelet membrane protein purified by repeated electrophoresis as described above was treated with 0.6nM estriol, and the activity of NOS was determined by using different concentrations of *l*-arginine (the substrate of NOS) to determine V_{max} for the assessment of the effect of estriol, as a possible heterotropic allosteric activator [15].

Determination of the effect of estriol on tamoxifen treated NO production in protein

The production of NO by the protein was studied by using tamoxifen, a well known antagonist of estrogen receptor [16] to determine whether the compound can nullify the effect of estriol on NO production. Typically the purified NOS from the platelet membrane preparation, as described above was treated with different concentrations of the estrogen receptor antagonist and after incubation for 45 min at 37°C, the production of NO in the reaction mixture was determined as described.

The Scatchard plot analysis

The binding characteristics of estriol to platelets in the presence or absence of Tamoxifen or DCN-2 were carried out by the Scatchard plot analysis [17] of the equilibrium binding of the agonist.

Statistical Analysis

The results shown are mean±standard deviation (SD); the significance (p) of the results was determined by the student t-test. The coefficient of correlation (''r'') was determined by Pearson test. The dissociation constant (Kd) and the number of the dermcidin binding sites (B_{max}) in the Scatchard plot analyses were determined by Microsoft Office Excel.

Results

Purification of estriol binding protein in platelet membrane

To find out the purity of the protein that produce NO due to binding with the estriol, the assay for NO production was done at each step of the protein purification as described in the Methods and Materials. It was found that in PRP (crude) the amount of NO formed 0.025nmol NO/mg protein and final product from the re-electrophoresis of the protein was found to be purified about 22,814 fold when compared with the PRP (Table 1).

Determination of allosteric modulation of the NOS activity of the protein in the presence of estriol

To find out whether the steroid could activate the enzyme as a heterotrophic allosteric activator when the basal level synthesis of NO was determined in the absence of the added estriol, it was found that the synthesis of NO production showed V_{max}=0.42 nmol NO/ml with

Table 1. Summary of the protein purification steps

Steps of the purification	Total Protein (mg)	Specific activity (nmol NO produced/mg protein)	Fold Purifiction	Yield (%)
1. Platelet rich plasma (PRP)	61.3	0.025		
2. Gel filtered platelets (GFP)				
3. Dialyzed protein from GFP suspension (Triton X-100 treated)	12.23	0.125	5	19.95
4. Electrophoresed protein	0.038	40.52	1621	0.061
	0.0027	570.37	22814.8	0.004

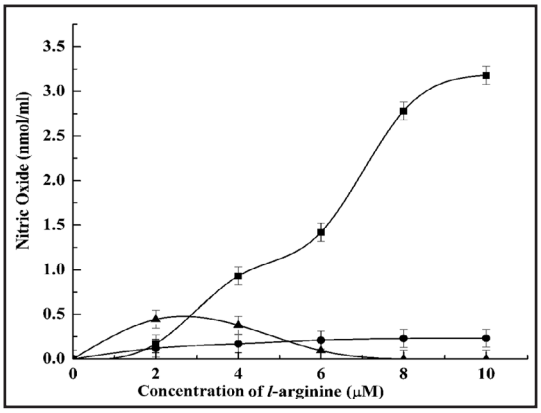

Fig. 1: Allosteric modification of the platelet membrane (NOS) prepared in the presence and absence of estriol and tamoxifen. Platelet NOS was specified to homogeneity as described in the Materials and Methods. The purified membrane protein was treated with 0.6nM estriol and incubated for 45 min at 37°C. The enzyme preparation was next incubated with different concentrations of substrate (*l*-arginine) and synthesis of NO was determined. The solid squares (-■-) = NOS treated with estriol (0.6nM) in the absence of tamoxifen. Solid circles (-●-) = Control experiment in the absence of both estriol and tamoxifen. Solid triangles (-▲-) represent the effect of tamoxifen (1.0nM) in the presence of estriol (0.6nM). The result shown are mean±S.D. of at least 10 different experiments by using blood samples from different donors.

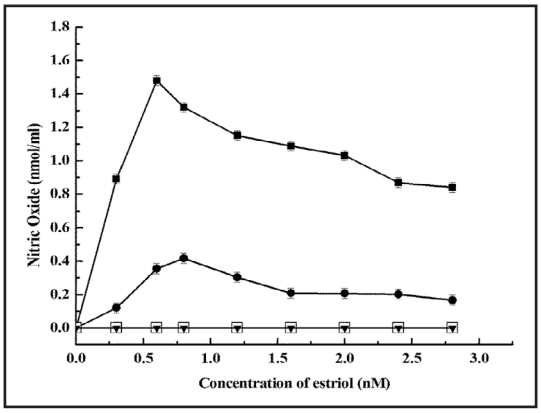

Fig. 2: Effect of tamoxifen on estriol induced NO production in platelet membrane. The purified NOS from the platelet membrane was incubated with different concentrations of estriol as indicated. After incubation for 45 min at 37°C the synthesis of NO was determined. The synthesis of NO by the NOS preparation was also carried out in the presence of *l*-NAME. The solid squares (-■-) = The production of nitric oxide in the platelet membrane NOS preparation at different concentrations of estriol. Solid circles (-●-) = The production of NO in the presence of tamoxifen (1nM) treated platelet membrane NOS prepared in the presence of different concentrations of estriol. Hollow squares (-□-) = Control experiment in the absence of estriol and tamoxifen. Inverted solid triangles (-▼-) = the estriol (0.6nM) induced production of NO in the presence of 0.1mM *l*-NAME (N^G-Nitro-L-arginine methyl ester). 2 The result shown are mean±S.D. of 30 different experiments (triplicate each time) by using blood samples from 30 different donors.

K_m=1.14μM (Fig. 1). When the same preparation was incubated with 0.6nM estriol the NO production in the estriol treated NOS demonstrated V_{max}=3.16nmol NO/ml with K_m=6.32 μM (Fig. 1). In other word the rate of synthesis of the NO was increased by 8 folds in the presence of the steroid in the absence of new protein synthesis.

To determine whether the role of estrogen on the platelet surface NOS through the interaction of the steroid with the platelet surface receptors, pre-incubation of platelet with 1nM tamoxifen, an estriol receptor antagonist [16] for 15min before the addition with the estrogen for 45 min resulted the inhibition of NO synthesis with V_{max}=0.44nmol/ml with K_m=0.76 μM (Fig. 1).

Inhibition of estriol induced NO synthesis in the presence of estrogen receptor inhibitors

Different concentrations of estriol (0.3nM, 0.6nM, 0.8nM, 1.2nM, 1.6nM, 2.0nM, 2.4nM, 2.8nM) were incubated to find out the ability to stimulate the NOS and tamoxifen (0.1nM, 1nM, 10nM and 100nM) in another set were pre-incubated to find out its ability to inhibit estriol binding to the receptor and subsequent synthesis of NO. It was found that tamoxifen at 1nM concentration is able to inhibit NO synthesis maximally. The profile of the NO synthesis by estriol in the absence and presence of tamoxifen shows a difference in the production of NO from 1.54±0.043nmol/3 X 10^8 platelets to 0.42±0.011nmol/3 X 10^8 platelets respectively (Fig. 2).

As DCN-2, an inhibitor of NOS [8] has been reported to be present in the circulation in ACS and AMI when the estriol induced NO synthesis was determined in platelets, in AMI subjects, it was found that the synthesis of NO was decreased to 0.067±0.006nmol NO/3 X 10^8 platelets that contrasted the synthesis of 1.49±0.032nmol NO/3 X 10^8 platelets in the presence of 0.6nM estriol (Fig. 3).

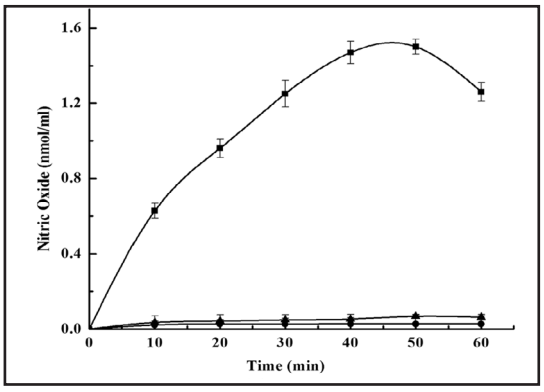

Fig. 3: Stimulation of NO in platelets from the subjects with acute myocardial infarction (AMI) compared to normal platelet rich plasma. Platelet-rich plasma from the subjects with AMI was prepared (n=30, M=15, F=15). Platelet rich plasma was also prepared from age and sex matched male and female normal subjects. The platelet-rich plasma preparations were treated with 0.6nM estriol for 45 min at 37°C. Solid squares (-■-) = The production of nitric oxide in different times in normal platelet rich plasma induced with 0.6nM estriol. Solid triangles (-▲-) = The production of NO in AMI platelet rich plasma in the presence of 0.6nM estriol. Solid circles (-●-) = The production of NO at different times in the absence of estriol with added equal of vehicle (0.9% NaCl).

The impairment of estriol induced NO synthesis in platelets from the subjects affected with AMI and the resistance of the platelets from AMI to the inhibitory effect of the estrogen in ADP induced platelet aggregation

When the PRP from AMI subjects was treated with 0.6nM estriol and the aggregation of platelets was determined in the presence of 2.0μM ADP, it was found that the AMI platelet was not inhibited that contrasted the inhibition of platelet aggregation by 100% induced by the same amount of estriol in the presence of equimolar ADP under identical conditions in control PRP (Fig. 4).

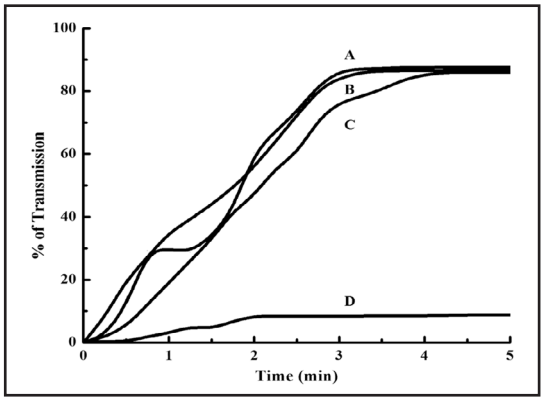

Fig. 4: The effect of estriol on the ADP induced platelet aggregation in PRP from normal volunteers and from the subjects affected with AMI. Platelet-rich plasma (PRP) was prepared from both AMI subjects (n=30, M=15, F=15) and from age and sex matched normal volunteers. The aggregation of platelets was initiated by treating the PRP with 2.0μM ADP and the aggregation was followed up to 5 min as shown. A= the aggregation of AMI platelets without estriol, B= aggregation of normal PRP without estriol , C= aggregation of AMI platelets incubated with estriol, D= aggregation of normal PRP incubated with estriol.

Changes in binding characteristics in the Scatchard plot analysis of estriol to the protein in the presence and absence of tamoxifen

The Scatchard plot analysis of estriol to the protein showed the shifting of the curvilinear plot towards the left side of the X axis (Fig. 5). This shifting profile signified the change in estriol binding to the protein was

due to the treatment of the protein (NOS) with tamoxifen.

The heterogeneous binding sites on curvilinear binding profile of the 69 kDa protein to the estriol in the absence of tamoxifen showed one high affinity (Kd_1=8.77nM) and one low capacity (n_1=51±2 molecules estriol/molecule of protein) and one low affinity (Kd_2=243nM) high capacity (n_2=1.4X10^3 molecules estriol/molecule of protein) receptor population.

In the presence of tamoxifen, the binding of estriol to the protein with a curvilinear binding profile showed heterogeneous binding sites with a high affinity (Kd_1=15.19nM) and a low capacity (n_1=27±2 molecules estriol/molecule of protein) and one low affinity (Kd_2=8.77nM) high capacity (n_2=104 molecules estriol/molecule of protein) binding sites.

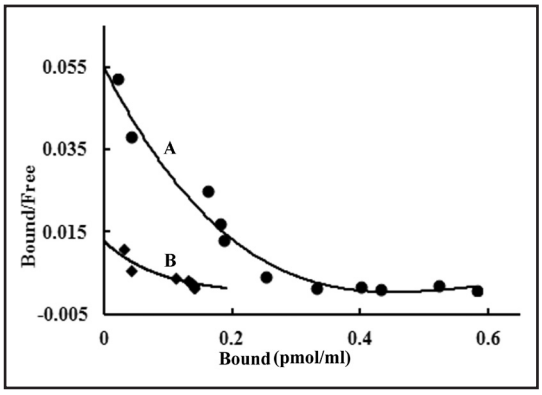

Fig. 5: Equilibrium binding of estriol to gel filtered platelets in the presence and absence of the tamoxifen. Gel filtered platelets were prepared from the normal platelet-rich plasma. The Scatchard plot of the equilibrium binding of estriol to the platelets in the presence and absence of tamoxifen (1.0nM) was used to constitute Scatchard plot. The amount of the bound and free was determined by Millipore gel filtration unit by enzyme linked immunosorbent assay (ELISA) as described in the Materials and Methods. Curve A represents a Scastchard plot of equilibrium binding of estriol to the gel filtered platelets. Curve B shows the equilibrium binding of estriol in the presence of 1.0nM tamoxifen in identical binding mixtures as described.

"Cross-talk" between the receptors of DCN-2 and estriol on the platelet membranes

As reported above, the synthesis of NO in the platelets from the AMI subjects treated with estriol was impaired in the AMI platelets when compared to that in the control counterpart. We have reported before the appearance of a stress induced protein (11kDa) in the circulation of the subjects identified to be DCN-2 affected with ACS including AMI in all cases [8].

We have also reported before, first time ever, that the binding of an agonist to its own receptors may down regulate or up regulate the numbers of receptors of another agonist on the platelet membrane [18]. This down-regulation or the up-regulation of the receptor numbers on the platelet surface by one of agonists was affected by the binding of a different agonist to its own receptors of the two different agonists generally called "cross talk" between receptors [18]. To find out whether a similar kind of "cross talk" between the receptors of estriol and that of DCN-2 on the platelet surface resulted in the reduction of estriol receptors leading to impaired NO synthesis by the steroid was investigated. The binding characteristics of DCN-2 on the platelet surface were assessed by the Scatchard plot (Fig. 6) suggesting that there was receptors of DCN-2 on the platelet surface (with K_d= 97.08, B_{max}= 24.89 X 10^3 molecules of protein/platelets).

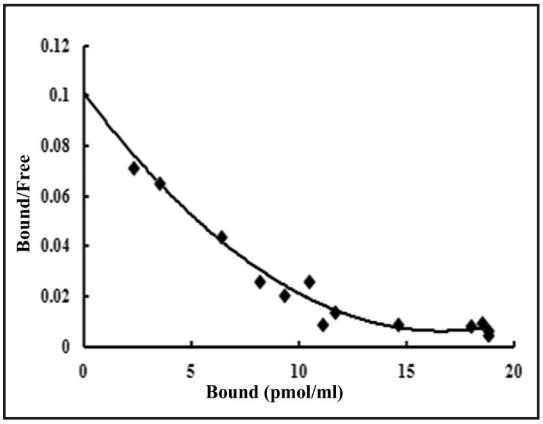

Fig. 6: The Scatchard plot of the equilibrium binding of dermcidin isoform 2 to the gel filtered normal platelets. Typically gel filtered platelets (108 GFP/ml) were incubated with different concentrations of dermcidin isoform 2. After the binding reached to the equilibrium (90min at 37°C) the amount of DCN-2 (both bound and free) was determined by ELISA.

When the binding of estriol on the control platelet surface was determined by the Scatchard plot (Fig. 7) the analysis of plot produced K_d of estriol binding was 0.693 with

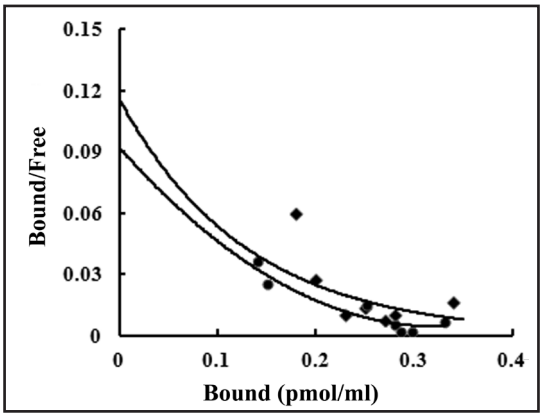

Fig. 7: Changes in the equilibrium binding characteristics in Scatchard plot of estriol to the platelet in the presence and absence of DCN-2. Solid rhombus (-♦-) denotes binding of the estriol to the platelets in the absence of DCN-2. Solid circle (-●-) denotes binding of the estriol to the platelets in the presence of DCN-2.

B_{max}=1040 estriol binding sites/platelets. When DCN-2 was added to control PRP and incubated for 90 min and subsequently treated with estriol, the Scatchard plot of the DCN-2 treated platelets was carried out (Fig. 7). It was found that the K_d of the estriol binding to platelets was 2.42 with B_{max} of only 640 estriol binding sites/ platelets. In other words the binding of DCN-2 to its receptors down regulated the estriol receptor binding by 38.46 % due to a "cross-talk" between the different agonists.

Discussion

As described above estriol (one of estrogens) was reported to be the most potent inhibitor of platelet aggregation currently known. This steroid also occurs in the circulation of males albeit in much lower concentration (0.6nM). The concentrations of the estriol in plasma of the males are in the similar ranges for the inhibition of platelet aggregation. As middle aged males are the major victim of ACS and AMI an obvious issue of the failure of estriol to protect these victims with ensuing ACS or AMI remains unsolved in that whether this steroid, i.e. estriol has any useful role in the protection of the victims from the thrombosis under these conditions.

Some researchers have described before that in case of murine platelets nucleic acids express protein in the presence of stimulator [19]. The activation of estriol mediated NO synthesis was due to a heterotrophic allosteric activation of the platelet membrane NOS that inhibits platelet aggregation. This allosteric activation of the platelet membrane NOS was determined, to be the consequence of estrogen receptor interaction of the enzyme which was never reported before and suggested a unique, and a new mode of action of an estrogenic hormone on platelet aggregation.

As described above the platelets from AMI patients synthesized much less amount of NO in the presence of estriol when compared to that in control platelets. It was found to be related to the presence of a stress induced protein in the circulation of AMI patient that causes a significant activation [20]. Prior platelet activation may down regulate the estriol receptor number on the platelet surface and rendered estriol ineffective to inhibit platelet aggregation.

In this context, it might be mentioned here that DCN-2 has been reported to be a potent atherosclerotic agent in that the protein is simultaneously a pro-hypertensive agent as well as diabetogenic agent. Both hypertension and diabetes mellitus are two known major risk factors for atherosclerosis. This protein (MW 11kDa) is a potent inhibitor of all known forms of NOS and inhibited NO synthesis induced by estriol as described here, but the protein was also found to inhibit aspirin induced NO synthesis and thereby as in the case of estriol, DCN-2 made aspirin incapable of inhibiting platelet aggregation in AMI that is independent of the well known effect of cyclooxygenase [20].

As this stress induced protein, i.e. DCN-2 is not an estrogen, the heterotropic down regulation of the estrogen receptor number and may be called as a case of negative "cross-talk" between the receptors of DCN-2 and of estriol on the platelet surface. As human blood platelets do not synthesize protein the cross-talk between the receptors cannot be due to the stimulation or inhibition of the protein synthesis in platelets. This down regulated "cross-talk" could be hypothesized was due to the internalization of "spare receptor sites" from the platelet surface into the membrane bilayers. In this case DCN-2 was perhaps interacting with the estriol receptors on the platelet surface as in the case of tamoxifen, a well known estrogen receptor blocker as reported above [16].

Conclusion

It can be concluded that the appearance of DCN-2 in the circulation of the AMI subjects will severely reduce the estriol receptor numbers on the platelet surface and thereby rendering the steroid ineffective in the inhibition of platelet aggregation in this condition.

In the above context, it might be concluded here that the development of breast cancer significantly increased the risk of development of AMI [21]. As tamoxifen is sometime used in breast cancer, our results suggested that this compound should use with caution in the malignant condition in the case of tamoxifen that might instigate the development of AMI breast cancer.

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Disclosure Statement

The authors report no conflicts of interest.

References

1 Colman RW, Walsh PN: Mechanisms of platelet aggregation; in Colman RW, Hirsh J, Maeder VJ, Salzman EW (eds): Haemostasis and thrombosis J.B. Lippincott, Philadelphia, PA. 1987, pp 594–605.
2 Fuster V, Badimon J, Chesebro JH, Fallon JT: Plaque rupture, Thrombosis, and therapeutic implications. Haemostasis 1996;26:269–284.
3 Chakraborty K, Sinha AK: The role of insulin as an antithrombotic humoral factor. Bioessays 2004;26:91-98.
4 Whittle BJ, Moncada S, Vane JR: Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species. Prostaglandins 1978;16:373–388.
5 Jana P, Maiti S, Ghosh R, Ghosh TK, Sinha AK: Estriol, a stimulator of nitric oxide synthesis in platelets, and its role as the powerful inhibitor of platelet aggregation. Cardiovascular Endocrinology 2013;2:50–54.
6 Karmohapatra SK, Chakraborty K, Kahn NN, Sinha AK: The role of nitric oxide in aspirin induced thrombolysis in vitro and the purification of aspirin activated nitric oxide synthase from human blood platelets. Am J Hematol 2007;82:986-895.
7 Levin ER: Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005;19:1951-1959.
8 Ghosh R, Karmohapatra SK, Bhattacharyya M, Bhattacharya R, Bhattacharya G, Sinha AK: The appearance of dermcidin isoform 2, a novel platelet aggregating agent in the circulation in acute myocardial infarction that inhibits insulin synthesis and the restoration by acetyl salicylic acid of its effects. J Thromb Thrombolysis 2011;31:13-21.

9 Chakraborty K, Khan GA, Banerjee P, Ray U, Sinha AK: Inhibition of human blood platelet aggregation and the stimulation of nitric oxide synthesis by aspirin. Platelets 2003;14:421–427.
10 Cox RD, Frank CW: Determination of nitrate and nitrite in blood and urine by chemiluminescence J Anal Toxicol 1982;6:148–152.
11 Dutta-Roy AK, Sinha AK: Purification and properties of prostaglandin E1/prostacyclin receptor of human blood platelets. J Biol Chem 1987;262:12685-12691.
12 Fazekas de St Groth S, Webster RG, Datyner A: Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. Biochim Biophys Acta 1963;14;71:377-391.
13 Howe AF, Groom T, Carter RG: Use of polyethylene glycol in the concentration of protein solutions. Anal Biochem 1964;9:443-453.
14 Jana P, Maiti S, Kahn NN, Sinha AK: Estriol-induced fibrinolysis due to the activation of plasminogen to plasmin by nitric oxide synthesis in platelets. Blood Coagul Fibrinolysis.2015;26:316-323.
15 Nelson DL, Cox M: Principles of Biochemistry (4th edition), Woth Publishers, 2004, pp 227-228
16 Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL:
The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;23;95:927-937.
17 Scatchard G: The attractions of proteins for small molecules andions. Annals NY Acad Sci 1949;51:660–672.
18 Kahn NN, Sinha AK: Stimulation of prostaglandin E1 binding to human blood platelet membrane by insulin and the activation of adenylate cyclase. J Biol Chem 1990;25;265:4976-4981.
19 Pelzl L, Fakhri H, Umbach AT, Gawaz M, Paulmichl M, Lang F: Sgk1 sensitive pendrin expression in murine platelets. Cell Physiol Biochem 2013;32:210-220.
20 Bank S, Jana P, Maiti S, Guha S, Sinha AK: Dermcidin isoform-2 induced nullification of the effect of acetyl salicylic acid in platelet aggregation in acute myocardial infarction. Sci Rep 2014;24;4:5804.
21 Hooning MJ, Botma A, Aleman BM, Baaijens MH, Bartelink H, Klijn JG, Taylor CW, van Leeuwen FE: Long-term risk of cardiovascular disease in 10-year survivors of breast cancer. J Natl Cancer Inst 2007;99:365–375.