Prevention of Acute Ischemic Heart Disease by Using Estrogenic Metabolites

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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. 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 of 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]. Both atherosclerosis and inflammation bring about the narrowing of the coronary artery, and along these lines diminishing the nutrients, O2 and minerals in the bloodstream [Fuster V et al, 2005]. However, platelet aggregation has critically 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]. The thrombus thus formed could physically block the normal circulation of the blood in the heart muscles which may lead to the development of 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 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).

Demographic reports from all over the world have suggested that the occurrence of the 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 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. 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]. Another disturbing news emerged: estrogens increased the risk of CAD in men, and oral contraceptives increased the risk of CAD and stroke in middle-aged women [Coronary Drug Project Research Group, 1970]. 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]. 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. 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 up regulate 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 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 NN et al, 1990].

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. 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. The treatment of PRP from normal volunteers with 2.0μM ADP resulted in the complete aggregation of platelets. 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 that completely inhibited platelet aggregation induced by ADP was 0.6nM. 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) indicating that estriol was >2-fold powerful inhibitor of platelet aggregation when compared to estradiol under identical conditions. 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. 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. Lineweaver-Burk plots of the NOS activity demonstrated the characterisation of the protein that platelet supernatant was treated with 0.6nM estriol Km was found to be reduced to 3.42mM with concomitant increased Vmax to 0.337nmol NO/mg/h in respect to Km 5.28mM and Vmax 0.029nmol NO/mg/h in the absence of estriol. Inhibitory property of estriol on platelet aggregation has been published in internationally reputed peer reviewed journal *Cardiovasc Endocrinol* 2013; 2:50–54 (Lippincott Williams & Wilkin.)

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). 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. Incubation of clotted PRP with 0.6nM estriol was found to result in the gradual increase of the clot lysis from 0min to 60min at 37°C. 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. When platelet free plasma was clotted in the presence of 0.6nM estriol no clot lysis could be found. 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. NOS that resides on the platelet membrane was isolated and purified. The binding property of estriol to the protein has demonstrated that K_d value with 6.002nM. The fibrinolytic property of estriol has been published in the journal *Blood Coagul & Fibrinol*;2015, 26:315-323 (Lippincott Williams & Wilkin), an internationally reputed peer reviewed journal.

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 AIHD in man?

We report herein 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) (Ghosh et al, 2011) in the circulation that conferred the resistance of platelets to estriol from the AIHD

subjects due to the "cross talk" between the receptors of DCN-2 and estrogen on the platelet surface. DCN-2, an inhibitor of NOS has been reported to be present in the circulation in AIHD. When the estriol induced NO synthesis was determined in platelets, in AIHD subjects, it was found that the synthesis of NO was decreased to 0.067 ± 0.006nmol NO/3 X 108 platelets that contrasted the synthesis of 1.49 ± 0.032nmol NO/3 X 108 platelets in the presence of 0.6nM estriol. When the PRP from AIHD 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 AIHD 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. It was found that the mean value of aggregation in AIHD platelets ($87.1\pm4.4\%$ of transmission) did not change much ($83.29\pm3.81\%$ of transmission) due to incubation with 0.6nM estriol. Whereas, in control platelets (age and sex matched with AIHD samples), the mean aggregation of platelets ($82.64\pm8.29\%$ of transmission) has been changed to $12.86\pm4.21\%$ of transmission. The binding characteristics of DCN-2 on the platelet surface were assessed by the Scatchard plot suggesting that there were receptors of DCN-2 on the platelet surface (with K_d= 97.08, B_{max}= 24.89 X 103 molecules of protein/platelets). When the binding of estriol on the control platelet surface was determined by the Scatchard plot 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. It was found that the Kd of the estriol binding to platelets was 2.42 with Bmax 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. The study on the "cross-talk" between DCN-2 and estriol accepted in the internationally reputed peer reviewed journal *Cell Physiol & Bichem* (Karger Publishers)

Analysis of the above mentioned research works demonstrate the protective role of estrogenic metabolites. A direct effect of estrogens has been found on platelets. As platelets do not have nucleus the stimulation of the platelet membrane protein occurs through the non-genomic pathway. These results have demonstrated a new role of steroid molecule as the activator of protein or enzyme through the non-genomic pathway. 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. 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. 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.

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