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NUTRITION, LIPID PEROXIDES, PROSTANOIDs AND PLATELET FUNCTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Kirkwood Arthur Pritchard, Jr. B.A., M.S.

****

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1983

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DEDICATION

This dissertation is dedicated to my loving parents, Dr. Kirkwood A. Pritchard and Dr. Edythe C. Pritchard who supported and encouraged me during my graduate career.
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PUBLICATIONS

Alterations of prostacyclin-thromboxane ratio in
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Pritchard, A.J. Merola and R.V. Panganamala. Prostaglandins

Influence of dietary vitamin E on platelet thromboxane A2 and
vascular prostacyclin I2 in rabbit. K.A. Pritchard Jr.,

Restoration of prostacyclin/thromboxane A2 balance in
diabetic rat: Influence of dietary vitamin E. C.W. Karpen,
K.A. Pritchard Jr., J.H. Arnold, D.G. Cornwell, and R.V.

ABSTRACTS


Hypercholesterolemic (HC) platelet dependence on calcium for thromboxane (TxB2) and 12-hydroxyeicosatetraenoic acid (HETE) release. KA Pritchard Jr., NJ Greco, and RV Panganamala. Clinical Chemistry 29(6):1227, 1983.
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The prostaglandin story began through the work of Kurzrok and Lieb who demonstrated that fresh human semen caused rhythmic contraction and relaxation of myometrium (1). Further studies on the biological effects of human seminal fluid demonstrated a lowering of blood pressure (2,3). Coldblatt and Von Euler independently demonstrated that the depressor action of seminal plasma was not due to the compounds histamine, acetycholine or substance P (3,4). Von Euler, thinking that the agent responsible for these physiological effects originated in the prostate named the lipid-soluble acidic substances prostaglandin and vesiglandin (5). However, Eliasson demonstrated that the prostaglandin in semen was not from the prostrate gland but was made by the seminal vesicules and secreted into the seminal fluid (6,7,8). After the origin of prostaglandin was determined and prostaglandin isolation techniques were established, the purification and characterization of prostaglandin species E and F were performed by Bergstrom and Sjovall (9,10,11). Rapidly the isolation and purification of prostaglandin series E and F from various tissues were carried out with Bergstrom et al. being the greatest contributor (12,13).
The isolation and purification of prostaglandins led to various quantitation techniques such as isotopic dilution, mass spectrometry (14, 15), the combined gas chromatography-mass spectrometry (GC-MS) (16), radioimmunoassay (RIA) (17, 18) and high performance liquid chromatography (19). Because of the earlier work in isolation and purification the above techniques brought about a tremendous wealth of information in prostaglandin metabolism. Prostaglandins were isolated from a variety of tissues in man, animal and nature. The striking fact about prostaglandins is not only their ubiquity in nature but also their great variety of biological activity. The biological activities have become a long list of which hemostasis and atherosclerosis are but two.

The in vitro studies of Van Dorp et al. (20) and Bergstrom (21) demonstrated that PGE₂ could be synthesized from arachidonic acid by sheep vesicular glands. Bergstrom demonstrated the conversion of dihomo-gamma-linolenic acid (20:3, W-9) to PGE₁ and eicosapentaenoic acid (20:5, W-3) to PGE₂ which eventually lead to the concept of families or series in prostaglandin synthesis. The next major accomplishment in prostaglandin metabolism was the discovery by Vane that aspirin inhibited prostaglandin formation by blocking cyclooxygenase (22). Smith et al. demonstrated that aspirin could inhibit the synthesis of prostaglandins in human platelets (23). Soon after this, Hamberg and Samuelsson isolated the endoperoxide, PGG₂ and PGH₂ (24).
The endoperoxides have a wide variety of actions which are dependent on the tissues being examined. The endoperoxides have short half-lives ($T_1/2 = 4$ to $5$ minutes) and would aggregate platelets and contract smooth muscle.

The next major finding in prostaglandin metabolism was by Hamberg, Svensson and Samuelsson (25, 26, 27). Over a period of 2 years the elucidation of formation and structure of the thromboxanes, the most potent aggregatory and vasoconstrictor known at that time, were performed. Using $^{14}$C-arachidonic acid with human platelets they demonstrated the conversion of arachidonic acid into three products, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and a new prostaglandin metabolite which they named PHD and later renamed thromboxane B$_2$ (TxB$_2$). However, none of these compounds could account for the strong aggregating activity of the supernatant from aggregated platelets when combined with indomethacin treated platelets. Using elaborate trapping experiments an unstable intermediate ($T_1/2 = 32$ seconds) between PGG$_2$ and TxB$_2$ was isolated and identified with a structure of thromboxane A$_2$ (TxA$_2$). These works as well as others, firmly established the proaggregatory role of arachidonic acid metabolism in hemostasis and thrombosis.

However, it wasn't until 1976 that Vane and co-workers discovered an unstable antiaggregatory metabolite of arachidonic acid from an enzyme isolated from arterial tissue.
Moncada and Vane named the compound PGX and found it to be 30-40 times more potent than PGE$_1$ in inhibiting platelet aggregation (29). PGX was found to relax coronary (30) and splanchnic vascular strips in vitro (31). In vivo studies demonstrated that PGX could dilate vascular beds (32,33) and exhibit a strong and thrombotic activity (34,35). It was Pace-Asciak who first described the formation of 6-keto-PGF$_1\alpha$ from endoperoxide metabolism in rat stomach fundus (37). From this knowledge Johnson et al. (36) were able to determine the structure of PGX which was renamed prostacyclin (PGI$_2$) (a collaboration between Upjohn and Wellcome Research Labs). The approved name of epoprostanol is synonymous with PGI$_2$; however, PGI$_2$ will be used throughout this dissertation.

As mentioned earlier TXA$_2$ is a proaggregatory and vasoconstricting arachidonic acid metabolite. In addition it has been shown to directly or indirectly decrease cyclic AMP in PGE$_1$ stimulated platelets (38). Prostacyclin on the other hand is antiaggregatory, vasodilatory and a stimulator of adenylcyclase activity in platelets (39). The exact role of cyclic AMP in preventing platelet aggregation is not yet clear but it is generally accepted that increasing cyclic AMP levels decreases and even reverses platelet aggregation (39). While most of the work on cyclic AMP and hemostasis is concerned with the platelets the likelihood of a cyclic AMP role in endothelium may also exist (40) with modulation of
the phosphodiesterase as a control point. One can see the potential for hemostasis lies in the proper balance of TxA$_2$/PGI$_2$ and cyclic AMP metabolism. In fact much of the research in the late 1970's and early 1980's has been concerned with manipulation of the TxA$_2$/PGI$_2$ ratio to prevent thrombotic events and atherosclerosis.

ARACHIDONIC ACID METABOLISM IN PLATELETS

The arachidonic acid cascade for platelets as well as arterial metabolism is shown in Figure 1. The metabolic fate of arachidonic and in platelets is determined by two oxygenase systems. The cyclooxygenase-thromboxane synthetase microsomal enzyme system (41) and the lipoxygenase enzyme (42,43). Cyclo-oxygenase catalyzes the oxygenation of arachidonic acid with two molecules of O$_2$. One O$_2$ molecule forms the cyclic endoperoxide at carbons 9 and 11 while the other becomes the hydroperoxy group at carbon 15 of the PGG$_2$. PGG$_2$ can be reduced to PGH$_2$ by reducing the hydroperoxide at carbon 15 to a hydroxy. Either of the endoperoxides are available to the thromboxane synthetase which is also believed to be in close proximity of the cyclo-oxygenase (41). TxA$_2$ is formed by converting the cyclic endoperoxide at 9 and 11 carbons to an oxane oxetane ring system. TxA$_2$ is a short lived (T1/2 = 32 seconds) active metabolite which converts to the 9,11-dihydroxy-oxetane ring system, the
Figure 1. Arachidonic acid cascade in platelets and artery.
biologically inactive metabolite TxB\textsubscript{2}. It has been shown that TxB\textsubscript{2}, formerly PHD, is produced at the same rate as HHT. TxA\textsubscript{2} probably is not an intermediate for HHT formation (44) although conflicting data does exist (45). The main end metabolites of arachidonic acid in platelets are TxB\textsubscript{2}, HHT and MDA from the cyclo-oxygenase-thromboxane synthetase system. The other major prostaglandins formed from the endoperoxides are PGE\textsubscript{2}, PGF\textsubscript{2a} and PGD\textsubscript{2}. PGD\textsubscript{2} has been shown to be an inhibitor of aggregation (46,47) by binding at a specific receptor (49) on adenyl cyclase (48). Smith et al. (49) demonstrated that the endoperoxides released from platelets into the surrounding plasma are readily converted to PGE\textsubscript{2} (34%), PGD\textsubscript{2} (24%) and PGA\textsubscript{2} (16%). Given that PGD\textsubscript{2} inhibits aggregation Smith et al. (49) proposed that aggregation might be controlled by either increasing the production of PGD\textsubscript{2} or administering a stable analogue of PGD\textsubscript{2}. However, PGE\textsubscript{2} has been shown to greatly reduce the effectiveness of PGI\textsubscript{2}, PGE\textsubscript{1}, and PGD\textsubscript{2} in inhibiting aggregation. PGE\textsubscript{2} exhibits a dual role in aggregation. At higher concentrations it will inhibit aggregation by saturating the PGE\textsubscript{1} receptor on adenyl cyclase. But at low concentrations it will reduce the effectiveness of PGI\textsubscript{2}, PGE\textsubscript{1} and PGD\textsubscript{2} to inhibit aggregation (50). PGF\textsubscript{2a} has been shown to have no effect on platelet aggregation when administered intravenously (51).
Hamberg and Samuelsson isolated 12-HETE from washed platelets in 1974 (25). The lipoxygenase enzyme responsible for the conversion of arachidonic acid to 12-HETE was found in the supernatant of disrupted platelets (51). When semipurified preparations of platelet lipoxygenase were used arachidonic acid was converted to 12-L-hydroperoxy-5,8,11,14-eicostetraenoic acid (12-HPETE) indicating that 12-HPETE was reduced to 12-HETE by some component or enzyme in the crude platelet preparation (52,53). Nugteren described the fatty acid specificity of platelet lipoxygenase as requiring double bonds at carbons 8 and 11 and that the activity appeared to be related to the distance from the methyl end rather than the carboxyl end of the fatty acid (52). In contrast to Nugteren's work, Ho et al. found lipoxygenase activity in the particulate portion of disrupted platelets (54).

Lipoxygenase products have been discovered in skin psoriasis and may be involved (55). Okuma has described some patients with myeloproliferative diseases as having platelets deficient in lipoxygenase (56). 12-HETE has been shown to be chemotactic for neutrophilic leukocytes and eosinophils (57,58,59,60). Others have implicated lipoxygenase products as having an inhibitory effect on cyclo-oxygenase (61), diglyceride lipase (62) and prostacyclin synthetase (63). Moncada (63) has shown that prostacyclin synthetase is inhibited by 15-HPETE. 15-HPETE has also been shown to
inhibit platelet aggregation by arachidonate, PGH$_2$, and the 9-methano-analogue of PGH$_2$ or collagen but not thrombin (64). This lead Turk et al. (65) to investigate the role of 12-HPETE inhibition of prostacyclin synthetase. Although he discovered that synthetic 12-HPETE inhibited prostacyclin synthetase as did 15-HPETE, he was unable to demonstrate that intact platelets generated and released sufficient 12-HPETE to inhibit prostacyclin synthetase. This lead him to conclude that in the in vivo situation intact platelets destroy the hydroperoxides before they could be released. Bryant (66,67) described 12-HETE production that was dependent on the activity of selenium glutathione (GSH) peroxidase which was regenerated from the hexose monophosphate (HMP) shunt's NADPH. If the GSH peroxidase was depleted by destruction or lack of reducing NADPH from the HMP shunt then 12-HPETE production would increase. This could have two effects. Arachidonic acid could be made more available to lipoxygenase by 12-HPETE's inhibition of cyclo-oxygenase (61). Another way could be to enhance the activity of the lipoxygenase enzyme (61). However this process(es) occurs, increasing 12-HPETE production leads to other lipoxygenase products such as 8,9,12-trihydroxy-eicosatrienoic acid and 8,11,12-trihydroxy-eicosatrienoic acid (THETE) and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (HPEA) (66,67,68).
One might be able to study 12-HPETE production and its inhibition on prostacyclin synthetase by inhibiting the GSH peroxidase and cyclo-oxygenase in intact platelets. The effects of 12-HPETE on platelet aggregation and prostaglandin production could be examined by either promoting its production or by the absence of its production. The latter has been a problem for some time due to lack of a specific inhibitor for lipoxygenase. Previously described inhibitors which block lipoxygenase such as ETYA (69), NDGA, phenidone (70) and BW755C (71) also block cyclo-oxygenase with similar potency. Inhibitors which are partially selective for lipoxygenase over cyclooxygenase are 5,8,11-ETA (72) and acetone phenylhydrazone (73) and offer some advantage over the others. However, Wilhelm, Sprecher et al. (74) in 1981 described the synthesis and in vitro effects of some specific acetylenic fatty acids lipoxygenase inhibitors. In light of this paper and the one following it by Sun et al. (75) more definitive studies on the role of lipoxygenase in platelets as well as in other tissues may be achieved.

The inhibitory action of 12-HPETE may be due to the free OH· generated by the breakdown of the hydroperoxy group (76). Protection against the OH· damage could be afforded by natural antioxidants (77) or free radical scavengers (78). The inhibitory role of 12-HPETE on prostacyclin-synthetase could be examined with respect to aorta in light of a HETE product
formed in arterial tissue (79). Dutilh (80) implies that 12-HETE formation plays a role in irreversible aggregation by altering the environment around the platelets after release from the platelets. As mentioned earlier 12-HETE has chemotactic activity. But what happens to the 12-HETE after the neutrophils are exposed should be of great interest. Stenson and Walker (81) demonstrated that radio-labeled 12-HETE was taken up by neutrophils as well as further hydroxylated. The incorporation of hydroxylated fatty acids into neutrophil's phospholipids may perturb the membrane sufficiently for activation by membrane modulation. The fact that 12-HETE is further hydroxylated by the neutrophils may indicate a different role of cell to cell communication with 12-HETE.

THE ROLE OF VASCULAR ENDOTHELIUM AND PROSTACYCLIN

The vascular endothelium serves three important functions for the vascular system. One, the endothelium supplies small nutrients to the subendothelial structures. Two, it prevents inappropriate substances from entering the vascular tissues. And three, the vascular endothelium presents a non-thrombogenic surface to the blood. Factors which make the endothelium non-thrombogenic are: electrostatic repulsion of blood platelets; surface ADPase enzymes (82) to prevent accumulation of platelet derived ADP;
heparin-proteoglycans which provide not only a negative static charge for repulsion but also sequester free Ca\(^{+2}\); provides thrombin binding sites for thrombin deactivation (83); and lastly the endothelium produces prostacyclin in order to inhibit and reverse platelet aggregation.

In health, sufficient prostacyclin is produced to balance the TxA\(_2\) produced by platelets. However, when vascular tissue becomes diseased as in plaque formation, prostacyclin is decreased and the production of platelet TxA\(_2\) goes unchecked. The ability of prostacyclin to maintain vascular integrity rests on its profound effects on platelets and vascular tissue. Prostacyclin inhibits platelet aggregation (28,34) and causes dilation of vascular vessels (31,32). It has three major actions on aggregating platelets. Prostacyclin will limit the adhesion of platelets which may damaged the endothelium. Prostacyclin causes aggregated platelets to disaggregate. Shape change in activated platelets is reversed by prostacyclin. All of these actions by prostacyclin are accompanied by an increase in platelet cyclic AMP content. These physical changes in platelets can be mimicked by adding dibutryl-cyclic AMP (38). Prostacyclin does not cause as great an increase in cyclic AMP in endothelium as in platelets which may be due to an extra-active phosphodiesterase which breaks down cyclic AMP as soon as it is formed (39). However, prostacyclin in the presence of isobutylmethylxanthine, a phosphodiesterase
inhibitor, does increase cyclic AMP in vascular tissue (39). Therefore, the role of the vascular phosphodiesterase may be to protect against a negative feedback mechanism with prostacyclin and adenyl cyclase (39). Increasing cyclic AMP levels in vascular tissue should decrease deacylation reactions which would decrease available arachidonic acid for prostacyclin synthesis.

Evidence of the separate roles of prostacyclin and endothelium in hemostasis was presented by Hoak in a recent review (84). When prostacyclin synthesizing capability was removed from the vascular tissue by aspirin treatment, the basal levels of platelet adherence to endothelium did not increase. Only in the pro-thrombotic state with thrombin-induced platelets did platelet adherence increase from 4% to 60%. This indicates that even in a situation where the endothelium has lost its capacity to produce prostacyclin there are still some characteristics of the endothelium which are present for protection. But when it is overwhelmed by a pro-thrombotic state as with thrombin-induced platelets this base level of protection is quickly overwhelmed. Upon adding exogenous prostacyclin to the thrombin-induced platelet-endothelium system platelet adherence is reduced, indicating the role and importance of prostacyclin.
Besides making prostacyclin the endothelium also binds thrombin with high affinity (83). It also appears to speed-up the binding of thrombin to antithrombin III. Thus the endothelium aids in removing thrombin which could activate platelets. Recently evidence was presented for the production of a necessary co-factor by the endothelium which promotes the activation of Protein C (85,86). Protein C has anticoagulant activities by inactivating clotting factors Va and VIIIa.

As mentioned before prostacyclin synthetase is inhibited by lipid peroxides (62,64). Disease states such as diabetes (87,88,89) and vitamin E deficiency (90) may increase circulating lipid peroxides. The inhibition of prostacyclin synthesis has been demonstrated by Karpen et al. in diabetes (87) and vitamin E deficiency (90). There was a 50% drop in aortic prostacyclin from vitamin E deficient rats when compared to vitamin E supplemented rats. Diabetes in streptozotocin diabetic rats resulted in a 50% drop in aortic prostacyclin produced when compared to control rats. However, when vitamin E was added to the diet the streptozotocin diabetic rat aortas produced nearly equal amounts of prostacyclin as the control aortas. Clearly, prostacyclin synthesis is altered by disease and dietary state of the animal.
In addition to vitamin E deficiency, adding cholesterol to the diets of rabbits has inhibited the recovery of prostacyclin production in deendothelialized and reendothelialized areas of previously injured rabbit aorta (91). The addition of cholesterol to rabbit diets tend to be the causal factor for the decrease in prostacyclin production (92).

ROLE OF CYCLIC AMP IN PLATELET FUNCTION

The first evidence of prostaglandins mediating platelet function by increasing cyclic AMP was presented by Kloeze in 1966 (93). Earlier, it was known that cyclic AMP was a weak inhibitor of platelet aggregation (94). Later, dibutryl-cyclic AMP was found to be a stronger inhibitor of platelet aggregation probably due to its ability to cross membrane and resistance to enzymatic hydrolysis by phosphodiesterase (95,96). Not only does cyclic AMP inhibit aggregation it also increases calcium sequestering in the platelet (97). The reverse is also true in that anything which promotes calcium release decreases cyclic AMP levels in the platelets.

Physiologically anything that promotes cyclic AMP formation in the platelet should inhibit aggregation. Low concentrations of cyclic AMP inhibit secondary functions of
platelet aggregation such as phospholipase activity, platelet secretion and second-phase irreversible aggregation (cyclo-oxygenase dependent). Higher levels of cyclic AMP inhibit primary functions of platelet aggregation, shape change and adhesion to surfaces (noncyclo-oxygenase dependent)(98,99). The potency of antiaggregatory prostaglandins can be directly correlated to the levels of cyclic AMP (100). PGI$_2$ is the strongest with PGD$_2$ greater than PGE$_1$ in inhibition of platelet aggregation (99). The ability of these prostaglandins to stimulate cyclic AMP production in platelets is in the same order: PGI$_2$>PGD$_2$>PGE$_1$.

TxA$_2$ inhibits platelet adenyl cyclase (101). By using a TxA$_2$ synthetase inhibitor it was shown that the inhibition of PGI$_2$-stimulated cyclic AMP levels by PGH$_2$ was in fact dependent on the TxA$_2$ synthesis (99, 102). TxA$_2$ generation is able to block nearly all of PGI$_2$-stimulated cyclic AMP production. Platelet basal levels of cyclic AMP are not decreased by TxA$_2$. Only when cyclic AMP levels are increased do you see the lowering effect of TxB$_2$ on cyclic AMP levels (101). As of yet there has been no evidence of TxA$_2$ lowering arterial cyclic AMP levels.

One advantage of inhibiting thromboxane synthetase directly is that the cyclo-oxygenase in still functional (103). The endoperoxides produced by the cyclo-oxygenase leave the platelet and can be used by the arterial tissue to produce PGI$_2$. Thus the endogeneous arterial endoperoxides
are supplemented by the platelet endoperoxides in making PGI₂. In turn the augmented increase in PGI₂ would inhibit platelet aggregation.

THE SOURCE OF ARACHIDONIC ACID

Platelets are easily activated by many stimuli. The result of the stimuli is platelet aggregation, granule release and prostaglandin production. The first two responses are able to take place even when prostaglandin production is blocked (104,105,106). However, prostaglandin production is probably the most significant in terms of biochemical response to stimuli. Prostaglandin production is directly dependent on the availability of arachidonic acid (107,108,109). Therefore, the understanding of how arachidonic acid might be released from platelet phospholipids and what are the controlling factors in such a release are of great importance. This is an area of current intense research. There seem to be more than one system present in a species and definitely between species (110,111).

Purified phospholipase C (PLC) from Clostridium perfringens (Welchii) was found to induce platelet aggregation (112,113). Speculation on the mode of PLC action were from increased calcium permeability (112) to modification of the receptors by altering the phospholipid environment (113).
Later it was demonstrated that phospholipase (PLA\(_2\)) initiated platelet aggregation which was inhibited by aspirin indicating the participation of the cyclo-oxygenase system in PLA\(_2\) aggregation (114). From this information it is apparent that phospholipases are involved in platelet aggregation.

In 1977 Rittenhouse-Simmons, Deykin et al. presented the influence of ATP deprivation (115) and Ca\(^{+2}\) dependence on arachidonic acid mobilization in human platelets (116). They concluded that PLA\(_2\) was not a latent enzyme in this process but actually required a rise in cytoplasmic Ca\(^{+2}\) (4 mM) to be activated. They further concluded that the release of arachidonic acid from PC and PI was dependent on ATP levels. Earlier work by Tyson (117) proposed that phosphatidic acid could act as an ionophore to promote Ca\(^{+2}\) mobilization. PA is formed from diglyceride (DG) and ATP by the enzymatic action of diglyceride kinase (DGK). Thus the loss of arachidonic acid release with low ATP levels fits nicely with the sequential roles of DG and DGK to produce PA.

Lapetina and associates have demonstrated that stimulation of horse platelets with thrombin (118,119,120) and platelet activating factor (PAF) (121) results in an increase in PA content. The nearly universally accepted theory of arachidonic acid release starts with the action of phospholipase C (PLC) on PI. There is much experimental evidence to support the first step. Rittenhouse-Simmons described the production of diglyceride from PI in stimulated
platelets (122) as well as Bell and Majerus (123). Lapetina demonstrated that the fall in PI is reflected in the increase in PA, the combined effect of PLC and DGK (119, 112). Broekman et al. demonstrated a fall in PI content and changes in PA content after thrombin stimulation but contrary to Lapetina the loss of PI was greater than the gain in PA (124). Legarde et al. split on the role of PLA$_2$ and PLC in platelet activation. Instead they felt that it might be important to look at the action of these enzymes with respect to their location and phospholipid environment (125). On this one point of PI metabolism by PLC and possibly PLA$_2$, most everyone is in agreement. The subsequent reactions that lead to the final release of arachidonic acid is where the greatest arguments develops.

Mauco in 1978 proposed that PI was metabolized to DG; DG to MG; and MG to AA by the combined hydrolysis reactions of PLC and diglyceride lipase (DGL) (111). Rittenhouse-Simmons described an indomethacin and hydroperoxy fatty acid sensitive (DGL) which could be protected by adding glutathione (122). She postulates that the inhibition of indomethacin on DGL is a result of HPETE build. Bell and Majerus (123) have used the disappearance of PI and appearance of monophosphateinositol as the evidence for PLC activity as mentioned before. At the same time they claim that the appearance of arachidonic acid suggests the presence of DGL. They claimed that the time course of PI decrease
parallels arachidonic acid release. In addition Bell and Majerus claimed that they saw no increase in PA as the Lapetina group suggests. Further work by Prescott and Majerus with human platelets and fatty acid composition of PI demonstrated that the loss of PI and the resynthesis could not possibly follow the "PI cycle" theory because the fatty acid composition in the resynthesis of PI is completely different from that implied by the PI cycle (126). The fact that platelet phospholipids are capable of undergoing deacylation-reacylation reactions further supports Majerus' DGL theory.

However, Billah, Lapetina and Cuatrecasas in 1981 discovered a PLA$_2$ which was specific for PA in horse platelets (127). It has optimal activity at pH 7.0 and 10 uM Ca$^{+2}$ and is completely inhibited by EGTA. They claim that this enzyme is completely different from the other PLA$_2$ which act preferentially on PE and PC under alkaline pH, mM Ca$^{+2}$ concentrations, and deoxycholic acid for activity. In addition they are unable to demonstrate a DGL in their membrane preparations. Earlier, Billah and Lapetina described conditions for the alkaline PLA$_2$ and PLC which were mutually exclusive (128). In 1981 Lapetina et al. further discussed the role of this PA specific PLA$_2$ (129). Additions to the "PI cycle" theory are that the PLA$_2$ acts on PA to release arachidonic acid and lysophosphatidic acid an equally potent Ca$^{+2}$ ionophore. The increase in intracellular Ca$^{+2}$
blocks resynthesis of PI from PA which counters one of Majerus' criticism (130). Majerus and co-workers main criticisms of Lapetina and co-workers are: 1) time course too short for definition of precursor product relationship; 2) PA returning to PI is not true because the fatty acid composition of PI after thrombin stimulation has no specific relationship to PI fatty acid composition prior to thrombin stimulation; 3) lyso-PA of the second layer of PI cycle is 1-lysophosphatic acid not 2-lysophosphatic acid; and 4) the PLA2 described by Lapetina is unique to horse platelets and not to found in human (130). Another problem with studying the fatty acid release is that it is an extremely short process which is nearly complete within 1 minute and 50% complete in 15 seconds.

As you can see there are species differences which increase the confusion about precursor product relationships. Majerus tries to explain the release of arachidonic acid from PI by citing rates which are in agreement with the appearance of arachidonic acid and well in excess of PLA2 rates (139). From the present state of the research one can be certain that many more papers will be written covering the precursor product relationships and phospholipid metabolism in stimulated platelets.
ROLE OF PLATELET-ACTIVATING FACTOR

As early as 1971 it was demonstrated that a soluable product from stimulated leukocytes could destroy platelets (131). Benveniste et al. developed a routine method for isolating this substance and began characterization (132). Benveniste et al. described this platelet-activating factor (PAF) as a 2-acyl-glycerol-3-phosphoryl choline in 1977 (133). Later Benveniste and co-workers described PAF as a 1-lysophosphatidyl choline based on lipase work (134,135) but it wasn't until 1979 when Demopolus et al. proposed the 1-O-alkyl-2-acetyl-3-phosphoryl choline that PAF's structure was elucidated (136). Benveniste and co-workers soon after reported on the characterization of PAF (137) confirming the work of Demopolus. There was a good deal of research on PAF from various laboratories between the years 1971 and 1979 before the structure of PAF was known. In order to avoid confusion in the literature with previous work, the new name of PAF was proposed to be PAF-acether rather than AGEPC which stands for the individual letters from the standard nomenclature (138). However, in this dissertation PAF will be used rather than PAF-acether. In order to distinguish PAF from other stimulators (arachidonic acid, thrombin, ADP or prostaglandins) three criteria have to be met in order to confirm the presence of PAF (137):
1) aggregates platelets in presence of aspirin or indomethacin and ADP scavengers;

2) have similar elution pattern similar to the PAF extract of stimulated hog leukocyte or synthetic PAF on silicic acid TLC or HPLC;

3) is inactivated by PLA₂ and resistant to lipase A₁ (from *Rhizopus aiztizus*).

PAF is a derivative released from the membrane phospholipids by basophils (139,140,141), macrophages (142,143,144), neutrophils (146,148,150), and platelets (105,134) after stimulation by various agents. PAF was first demonstrated to be released by rabbit basophils from IgE-serum antigen-antibody reactions (140). Murine macrophages produce PAF upon stimulation by degranulating agents 48/80 and complement C₃a and C₅a (147). Neutrophils produce PAF during phagocytosis (146,148) and is associated with lysosomal enzymes (149) and the presence of calcium (149,150). In a mixed peritoneal cell population it was discovered that PAF was produced by the macrophages and not the mastocytes (142). The mastocytes upon stimulation release their granule packets which in turn are phagocytized by the macrophages initiating PAF production (142). Charo et al. presented evidence that high concentrations of thrombin and collagen could aggregate platelets in the presence of aspirin and ADP scavengers (151). From this information Chignard, Benveniste and co-workers concluded that PAF might
be involved. They examined the supernatants of aggregated rabbit and human platelets and discovered that PAF was produced (134). From experiments designed to examine platelet response to PAF, inhibition of PAF stimulation and PAF production they concluded that PAF does directly cause aggregation of platelets and is not a by-product of aggregation (105), thus indicating a third pathway. The third pathway theory is not without resistance by several investigators (152,153,154) but it does explain the aggregation of platelets in the presence of cyclo-oxygenase inhibitors and ADP scavengers, the two accepted pathways of aggregation. The criticism of PAF being the third pathway is based on inhibitors which block glycolysis, respiration, increase cyclic AMP or block cyclo-oxygenase (152). Arguments against using CP/CPK to inhibit ADP induced aggregation are based on claims that the enzymes alone prevent second wave aggregation but not secretion (151,156). Rao and co-workers maintain that PAF aggregation is ADP and TxB₂ dependent; in addition, its action is through membrane modulation (153,154). Membrane modulation is a term used to describe a new pathway of aggregation. PAF fits the requirement of an activator of membrane modulation because it binds to the platelet membrane and activates the membrane. Further enhancement of membrane modulation is achieved by the binding of epinephrine. Kloprogge et al. based their arguments on PAF-induced release of serotonin (5HT), beta
thromboglobulin (beta-TG), and gamma-neuramidase (gamma-N) in the presence of ASA and/or CP/CPK (155). Because second wave aggregation and secretion by PAF were almost totally inhibited by ASA and CP/CPK as well as ETYA and indomethacin, it was felt that PAF induced aggregation depends on arachidonic acid metabolism and ADP secretion (155). These findings are in sharp contrast to much of the current literature on PAF. Whether or not PAF follows a third pathway in platelet aggregation, it is still one of the most potent platelet aggregating agents known to date. The specificity of PAF is severely restricted. Any change in the basic structure markedly decreases its potency and/or completely destroys its activity (157,158,159). By changing the 2-acetyl group to a 2-butryl or 2-formyl, complete loss of activity is achieved (160). Removing the P-O-C group of PAF at the phosphoryl choline and substituting a P-C, the activities of the PAF analogue are reduced 30 times compared to synthetic PAF (157). The natural fatty acids at the number 1 position are octadecyl or hexadecyl. Some investigators have explored the role of saturation at the number one position (160,161). Increasing chain length of C16 to C18 at the number one position decrease the activity by about 6 times (159,160). Removing the methyl groups from the choline residue decreased platelet reactivity by about 300 times. Lyso-PAF has about 5000 times less activity than synthetic PAF (158,159). Besides PAF's platelet aggregation
properties, PAF also produces in guinea pigs and rats, an inflammation response which is about 1000 to 10,000 times more than histamine on a mole to mole basis (159). PAF has been implicated in the anaphalactoid reaction (162). The bronchiconstriction and pulmonary hypertention in anaphylaxis is now thought to be attributable to PAF's action on platelet-induced Txa₂ formation (163,164,165,166). Because rat platelets are non-responsive to PAF only hypotension results when PAF is administered (159). Again when PAF is administered in rabbits thrombocytopenia results indicating platelet activation (164).

Recently there has been evidence presented that various tissue microsomal preparations from rats are capable of producing PAF from precursor material (166,167). Thus it appears that tissue damage alone may initiate PAF formation besides that which the blood components are able to produce.

A natural mechanism for modulating the activity of PAF must exist for such an active compound. Indeed as of late there are many (168,172). PAF is degraded to lyso-PAF by neutrophiles and then reacylated preferentially with arachidonic acid, thereby removing two active agents (179). Platelets deacylate and reesterify at the number 2 position with long chain fatty acids (lineoleate preferred) destroying PAF's activity (171,172). In rabbits there is a plasma enzyme capable of destroying PAF (172 and personal observations).
Shaw et al. has shown that when platelets are stimulated with PAF many changes take place (173,174). Arachidonic acid is lost from PI and PC with an increase in diacylglycerol and phosphatidic acid (173). The authors claimed that these results would indicate that a PLC-DGL mechanism is initiated by PAF. As mentioned earlier, Lapetina demonstrated PAF-induced PA accumulation which is evidence for the involvement of the PLC-DGK mechanism (121). Benveniste et al. provide evidence for a PLA₂ activation by PAF (175). The lipoxygenase system in platelets (176) and leukocytes (177) is stimulated by PAF. Cyclic AMP accumulation in platelets is inhibited by PAF probably by inhibiting the adenylate cyclase activity (178).

PAF has the profound capability to initiate both aggregation and inflammation response (increased vascular permeability). The implications are that PAF may play a very important role in atherosclerosis. The increased vascular permeability would compromise the integrity of the vascular bed allowing greater lipid invasion. The activation of platelets would increase TxA₂ production and granule release with vasoactive amines adding to the atherosclerosis process.
LIPID PEROXIDATION

By now free radical reactions and lipid peroxides in the living system are well accepted (179,180). There has been much work on lipid peroxidation in areas from food industry to the biological cellular systems. The concept connecting lipid peroxidation and atherosclerosis first developed when Glavind (181) reported on lipid peroxides in human atheroma plaque. This work was criticized as being an artifact induced by technique. But the work of Iwakami (182), designed to counter these criticisms, clearly established conjugated diene hydroperoxides in the lipids of human atheroma and lipid peroxides in atherosclerotic rabbit aortas. Peroxidative damage has been linked to endothelial damage and loss of arterial integrity (183). The fact that 15-HPETE (62,63) and 12-HPETE (64) inhibit prostacyclin synthetase indicates that other lipid peroxides may do the same. The work of Nordoy (184) demonstrated that high lipid peroxides in human LDL fractions are able to inhibit endothelial cell cultures production of prostacyclin. When this work was repeated by Szekzlik et al. it was found that LDL fractions prepared by Nordoy's method had artifactually high lipid peroxides (185,186). By removing calcium ions with EDTA and having BHT present, LDL fractions could be prepared which contained little lipid peroxides. Of course the LDL-induced decrease in prostacyclin production was less
than LDL prepared by Nordoy's method. Yet there were still some patients whose LDL fractions, free from isolation-induced lipid peroxides, could inhibit prostacyclin production (186). Okuma demonstrated increased lipid peroxides in the aorta of vitamin E deficient rat which correlated inversely with the ability of the aortas to produce prostacyclin (187). The consequences of loss of prostacyclin production and endothelial damage leads to increased platelet deposition and atherosclerosis (183). Damage to the endothelium allows increased plasma lipid invasion supplying the lipid material in plaques. There are many reports now of physiological states and/or disease states which can be linked to lipid peroxides in plasma (186,188,189,190,191). Some of the states which have been associated with increased lipid peroxides are: aging, cerebrovascular disorders (188); rheumatoid arthritis (189); diabetes (190,191), retinopathy, burn injury (190); and cardiovascular disease (186). In addition, evidence of a prooxidant stress in diabetes can be seen from increased levels of dehydroascorbic acid which can not be corrected by increasing ascorbic acid intake (192,193,194).

Measurement of lipid peroxidation has been achieved using three very different techniques. The oldest and probably the most frequently used is the thiobarbituric acid (TBA) test (195). Malonyldialdehyde combines with thiobarbituric acid in 1 to 2 ratio respectively producing a
chromophor which can be measured by following the absorbance at 532 nm (188). Enhancement of TBA testing can be achieved by using fluorescence technique. The chromophor is excited at 532 nm and the fluorescence is measured at 548 nm (196, 197). Although this test is very sensitive it lacks specificity in measuring lipid peroxidation. This has led to the investigation of other products of lipid peroxidation which Tappel addresses in his review (180). When breakdown products of lipid peroxidation react with amino acids a conjugated Schiff's base is formed which possesses characteristic absorbances in the ultraviolet (UV) and visible regions. In a review by Tappel (180) fluorescent products of amino acids in proteins and enzymes, phospholipids (amine portions) and nucleic acids (DNA) all with characteristic spectrum are discussed.

It is very difficult to isolate the lipid peroxide fluorescent products from samples and care must be taken not to induce their formation while handling the samples. In testing for the presence of lipid peroxides by either of these methods the sample is destroyed. Following the time course of disease and lipid peroxide accumulation become difficult requiring larger sample sizes.

Another consequence of lipid peroxidation of polyunsaturated fatty acids (PUFA) is chain breakages. Thus when malonyldialdehyde is released from (PUFA) a hydrocarbon corresponding to the rest of the chain is also released.
Linoleate will release ethane while arachidonate will release pentane. In 1974 Riely et al. based on these observations described a gas chromatograph (GC) method for measuring ethane evolution from CCl₄-induced lipid peroxidation of mouse tissue (198). Tappel and associates have been monitoring pentane evolution in whole animal models as well as in individual tissue samples (180). The use of this method should increase as people become better acquainted with the system. Following pentane and ethane evolution in animal populations is a noninvasive technique of assessing the lipid peroxide state which promises to be quite useful in the future.

**CHOLESTEROL**

The association between hyperlipemia and atherosclerosis has been clearly established. Shattil and Cooper have extensively examined the effect of hypercholesterolemia on reticulocytes (199,200). From this work they developed an *in vitro* model system for studying the effects of cholesterol on platelet function (201). By incubating platelets for up to 5 hours with phosphotidylcholine liposomes of varying cholesterol content they were able to demonstrate an exchange of cholesterol between platelets. Not only did these platelets exchange cholesterol in a concentration dependent manner but after the exchange their response to aggregating
agents were clearly and drastically altered. The cholesterol to phospholipid ratio (C/PL) of normal platelets were altered either by increasing platelet C/PL with cholesterol-rich liposomes or decreasing platelet C/PL with cholesterol-poor liposomes. In each case these altered platelets responded quite differently to aggregating agents. The cholesterol enriched platelets were 35-fold more sensitive to epinephrin and 15-fold more sensitive to ADP than the cholesterol normal platelets. The increased platelet response was followed by aggregation and 5-HT release. In later work Shattil, Cooper et al. (202) demonstrated a concomittant decrease in membrane fluidity as measured by the hydrophobic fluorescent probe following fluorescence polarization. Incorporation of cholesterol was allowed to continue for 20 hours and demonstrated an increase in microviscosity. There was a direct correlation between C/PL and microviscosity. By decreasing membrane fluidity with cholesterol a higher degree of order is achieved in the membrane. They speculated that this more highly ordered membrane must somehow increase the transfer of information of stimulation secondary to the binding of epinephrine because the number of binding sites were not altered by the incorporation. From these works they concluded that the lipid composition of platelets was influenced by the plasma lipid environment. In an attempt to more closely tie this concept to hyperbetalipoproteinemia the C/PL of plasma LDL and platelets were examined from
hyperbetalipoproteinemia patients and normal individuals (203). They found a good correlation between the C/PL of LDL and C/PL of platelets. However a direct cause and effect relationship could not be established by incubating normal platelets with the plasma of hyperbetalipoproteinemia patients. The aggregation and 5HT-release responses of hypercholesterol platelets from hyperbetalipoproteinemia patients were similar to the in vitro liposomal model described earlier.

In 1979, Tremoli et al. reported a direct correlation between arachidonic acid-induced (20 uMol/L) TxB₂ production from washed platelets and serum cholesterols (204). In 1980, Stewart, Gerrard and White (205) thoroughly examined the effect of cholesterol incorporation in platelets on arachidonic acid metabolism. They concluded that there was an enhanced release of arachidonic as well as an enhanced metabolism of arachidonic acid at the cyclo-oxygenase level. This is in contrast to the work of Kawaguchi, Ishibashi and Imai from Japan who demonstrated that hypercholesterolemic rabbits platelets have an enhanced arachidonic acid metabolism at the level of thromboxane synthetase and that the release by either PLA₂ or PLC-DGL is unaltered (206,207). In addition, Kawaguchi et al. demonstrated that there was an overall decrease in arachidonic acid in PI, PS, and PE with an increase in palmitoleic acid (16:1) in these same phospholipid fractions (206). The loss of 20:4 was not
discovered in the cholesterol ester fraction of the platelets. Cholesterol ester content in platelets from hypercholesterolemic rabbits was 70 ug/mg protein as compared to 8 ug/mg protein of normal rabbit platelets (206). In 1982 Kramer et al. examined the arachidonic acid release in human platelets and concluded that elevated cholesterol content promotes enhanced arachidonic acid release by the phospholipase resulting in increased prostaglandin production (208).

Some investigators have presented evidence that an increased C/PL does not always indicate increased platelet activity. Such is the case reported by Owen et al. on platelet composition and aggregation in liver disease (209). His aggregation results correlated inversely with the C/PL ratio and that was probably due to decreases in arachidonic acid availability. O'Brien reported on "exhausted platelets" being increased in hypercholesterolemic patients blood (210). Exhausted platelets have at some point been activated and then disaggregated yet continue to circulate with only a portion of their ability to aggregate available. Platelet aggregates have been measured by the method of Wu and Hoak (211) in hypercholesterolemic rabbits (212). Administration of aspirin decreased the number of platelet aggregates implying increased arachidonic acid metabolism in platelets from hypercholesterolemic rabbits (212).
Circulating platelet aggregates imply a role of arterial prostacyclin in cholesterol feeding. The generation of prostacyclin in aortas from hypercholesterolemic rabbits has been reported as increased (213), decreased (214) and no change or recovery (215). As mentioned earlier LDL fractions of cardiac patients plasma were able to inhibit prostacyclin production by endothelial cell cultures. The hyperlipemic states induced by feeding cholesterol to rabbits should inhibit arterial prostacyclin production. However, this is probably complicated by the aorta's ability to adapt to periods of stress such as gross hypercholesterolemia.

DIABETES MELLITUS AND LIPOPROTEIN LIPASE

Diabetes mellitus is an energy storage disease caused by a decreased or total loss of insulin production resulting in hyperglycemia, glucosuria, polyuria, ketonuria, thirst, hunger, emaciation, acidosis, ketosis and hyperlipemia. Vasculopathy is one of the major sequelae of diabetes mellitus resulting from atherosclerosis (216,217,218,219,220), microangiopathy (216,221,222) and thrombosis (216,221,222,223). These vascular complications are due to many factors. Platelets are hyperactive in diabetes mellitus with greater aggregation and TxA2 generation upon stimulation (87,90). Vascular tissues produce less PGI2 in diabetes mellitus than normal vascular tissue (87,88,89,90). Thus
vascular lesions are more frequent due to the imbalance of 
$\text{TxA}_2/\text{PGI}_2$ (87). In addition to the imbalanced platelet and 
vascular prostaglandin metabolism diabetes causes an increase 
in plasma lipids (224,225). There are many factors involved 
in increasing circulating lipids such as increased ingestion 
of foods, increased hepatic production of lipids and 
decreased deposition and utilization of lipids. Each of 
these areas are influenced by other factors. The exact 
reason for hyperlipemia in diabetes mellitus is still not 
clear. If food ingestion is held constant hyperlipemia due 
to overeating could be ruled out. Diabetes mellitus causes 
hyperlipemia even when food ingestion is not a factor, as in 
fasting state (226). This indicates that the defect in lipid 
metabolism could be at another local. The clearance of 
plasma lipids is almost exclusively due to lipoprotein lipase 
(LPL). This enzyme is found universally throughout the body 
and indications are that different forms of the LPL exist 
(227). The liver contains another lipase, hepatic 
triglyceride lipase (HTGL) as well as LPL. HTGL activity is 
not inhibited by 1.0 M NaCl and does not require an 
apoprotein activator while LPL activity does (227). On this 
basis they can clearly be distinguished. LPL and HTGL 
activities are inducible by insulin and feeding. Heart LPL 
has a high affinity for chylomicron triglycerides while 
adipose LPL has a low affinity thus insuring postprandial 
saturation of adipose LPL and constant saturation of heart
LPL (227).

As mentioned earlier LPL activity can be induced by insulin. Conversely without insulin LPL activity is lost (225). Insulin causes an increase in LPL activity by increasing the synthesis and secretion of LPL from adipose tissue (228,229). Other investigators have isolated and cultured cells from the stromal-vascular fraction of rat epididymal adipose tissue (229). They have demonstrated that exposing these cells to heparin causes an increased release of preformed enzyme. So it is clear that in insulin deficiency LPL release could be lost. After LPL is secreted it is used locally for hydrolysis of lipids in that tissue. LPL is releasable from the tissues by high doses of heparin. LPL from different sources have similarities. The study of LPL from a plentiful source could be used to make inferences of LPL in general. Such is the case with milk LPL and bovine aortic LPL (230). Stability of milk LPL was shown to be increased by the lipid components of endothelial cells (231). LPL activity is dependent on apoprotein C-II of VLDL proteins (227,232). Apo C-II enables LDL to bind to VLDL and to exert its hydrolysis action. Plasma from uremic patients inhibits LPL activity either directly (233) or blocks the binding of Apo C-II. In streptozotocin-induced diabetic rats there is an abundance of cofactor for LPL activity yet hyperlipemia exists (234). This points to a loss of LPL activity either by enzyme defect or decrease in LPL production. There are
conflicting reports on LPL activity in diabetes mellitus. LPL activity is influenced by the nutritional status of the animal as well as the type of diet. Conflicting results between these studies could well be attributed to differences in the external factors of diet, fasting, non-fasting, time of animal sacrifice etcetera (235). In one study, muscle LPL activity was found to be decreased in diabetes mellitus (226). While in another study no change in muscle LPL was found allowing the investigators to conclude that hypertriglyceridemia as a result of insulin deficiency is not a simple function of decreased tissue LPL activity (236).

Diabetic rat plasma has higher MDA than control rat plasma as measured by the thiobarbituric acid test (87). In addition they also have higher triglycerides. Diabetic rats placed on vitamin E supplemented chow had lower triglycerides than diabetic rats on vitamin E normal chow (87). Kotze et al. reported on the influence of ascorbic acid on LPL activity which was decreased in heart but increased in adipose (237,238,239). Kotze et al. (238) demonstrated that although LPL activity in ascorbic acid deficient hearts was low in vitro addition of ascorbic acid increased LPL activity. The possibility exists that LPL activity is lost due to peroxidative damage and that this damage is decreased through the use of lipid soluble and hydrophilic antioxidants.
VITAMIN E DEFICIENCY

Vitamin E deficiency can be induced in rats (240) and rabbits (241) by maintaining them on vitamin E-deficient diets. Platelets from vitamin E-deficient rats are highly sensitive to aggregation and produce large amounts of PGE₂ (242,243). Collagen-stimulated platelets from vitamin E-deficient rats produce more TxA₂ even though TxA₂ conversion from exogenous arachidonic acid is unchanged (90). Enhanced deacylation of platelet phospholipids and enhanced lipoxygenase activity has been observed in vitamin E-deficient rabbits (244,255). Arterial PGI₂ production is suppressed in vitamin E-deficient rats (187). The low PGI₂ production in the aorta of vitamin E-deficient rats has been correlated to increased tissue peroxidation (187).

Vitamin E deficiency in rabbits produces an increase in bound lysosomal enzyme activity as well as free lysosomal enzymes in the muscles (180). The contribution of macrophage or leukocyte lysosomal enzymes in the muscle tissue is highly likely due to the observed increase of these cells in the muscle tissues of vitamin E-deficient rabbits (246). Vitamin E deficiency in rabbit produces a nutritional muscular dystrophy due to this increase in lysosomal enzymes. This process can be delayed by N,N'-diphenyl-p-phenylenediamine(DPPD) a synthetic antioxidant unrelated to vitamin E (247) but, not halted (180). PGI₂ synthesis in
aorta of vitamin E deficient rabbits was found to be lower than vitamin E supplemented and DPPD supplemented rabbits by Chan et al. (247). The loss in arterial PGI₂ synthesis is inversely related to lipid peroxides in the tissues (248). DPPD an antioxidant yielded the highest PGI₂ production in aorta but, did not suppress the NADPH-oxidase system in the liver of the rabbits (247).

Steiner et al. has examined the role of vitamin E on platelet aggregation and release (249). Since the time that prostaglandin production was found to proceed by a peroxidative process it became popular to study platelets and antioxidants. Many investigators have felt that the inhibition of vitamin E was due to its antioxidant properties. Steiner and co-workers have demonstrated that vitamin E and its fully oxidized quinone are able to inhibit platelet aggregation with almost equal potency. This means that vitamin E probably plays a different role other than antioxidation. The phytol side chain of vitamin E is able to interact with arachidonic acid making it less free for metabolism (250). In 14C-arachidonic acid labeled platelets the loss in 14C-labeled phospholipids was significantly greater in platelets from E-deficient rabbits than E normal rabbits implying a phospholipase action (244). These results cast doubt on the antioxidant role of vitamin E in platelets. One of the faults of these studies is that they do not take into account the in vivo actions of vitamin E. There may be
a dual role of vitamin E in vivo; membrane stabilization with close association of the phytol side chain with PUFA as well as the antioxidant properties which would preserve the tissues against lipid peroxidation. The peroxides build up in the body and promote platelet hyperactivity. Vitamin E deficiency in rabbits in our lab has demonstrated that vitamin E-deficient rabbit platelets produce more prostaglandins when stimulated with thrombin and collagen but not arachidonic acid when compared to vitamin E-supplemented rabbit platelets.
STATEMENT OF PROBLEM

Diabetes, vitamin E deficiency, and hypercholesterolemia are three disease states which lead to atherosclerosis. Diabetes is accompanied by a hyperlipemia which accelerates the atherosclerosis by altering platelet function. Vitamin E deficiency induces alterations in the platelet and arterial prostaglandin metabolism which lead to atherosclerosis. Hypercholesterolemia also alters platelet and arterial prostaglandin metabolism and function which lead to greater atherosclerosis. The purpose of this dissertation is to 1) examine the cause of hyperlipemia in the diabetic rat and to determine if vitamin E plays a role in decreasing the hyperlipemia, 2) detect changes in platelet prostaglandin metabolism and function, and changes in vascular prostaglandin metabolism in vitamin E deficiency in the rabbit, and 3) determine changes in platelet and vascular prostaglandin metabolism in hypercholesterolemia in rabbit.
METHODOLOGY

The following five sections will use the same materials and methodology. The methods in this section will apply to all subsequent experimental sections.

MATERIALS

The following chemicals and materials were purchased from Sigma Chemical Company, St. Louis, Missouri.

- Dextran (ave. mol. weight = 80,000)
- Bovine achilles-tendon collagen
- Bovine thrombin
- Streptozotocin
- Bovine albumin
- Bovine gamma-globulins
- Gelatin from calf skin Type IV 60 Bloom
- Trizma base

The following materials and chemicals were purchased from Nu Chek Prep (Elysian, MN).

- Triolein (T-325)
- Arachidonic acid (U-71-A)
The following radioactive compounds were purchased from either New England Nuclear Corporation (NEN) (Boxton, Mass.) or Amersham Corporation (AmC) (Arlington, Ill.) as indicated.

1-\(^{14}\)C-arachidonic acid, [52.7 mCi/mmol] NEN

Thromboxane B\(_2\), [5,6,8,9,11,12,14,15-\(^3\)H(N)],

[150 Ci/mmol] NEN, AmC

Prostaglandin E\(_2\), [5,6,8,11,12,14,15-\(^3\)H(N)],

[100 Ci/mmol] NEN, AmC

6-keto-prostaglandin \(F\)\(_{1a}\)

6-[5,8,9,11,12,14,15-\(^3\)H(N)], [100 Ci/mmol] NEN, AmC

12-L-hydroxy-5,8,10,14-eicosatetraenoic acid,

[5,6,8,9,11,12,14,15-\(^3\)H(N)], [40 Ci/mmol] NEN

triolein [carboxyl-\(^{14}\)C], [100 Ci/mmol], NEN

5-hydroxytryptamine [side chain -2-\(^{14}\)C(N)] creatine sulfate, [50Ci/mmol], AmC

adenosine 3',5'-cyclic phosphoric acid,

[\(^{125}\)I]-2'-O-succinyl-(iodotyrosine methyl ester)-

[3010/uCi/ug], NEN
The following prostaglandins were supplied by Dr. John Pike of UpJohn.

6-keto-PGF\textsubscript{1a}
Thromboxane B\textsubscript{2}
PGF\textsubscript{1a}
PGF\textsubscript{2}(trimethane salt)
PGD\textsubscript{2}
PGE\textsubscript{2}
PGE\textsubscript{1}

The following hydroxy fatty acids were supplied by Dr. R. Bryant of George Washington University, D.C.

12-L-hydroxyeicosatetraenoic acid
15-L-hydroxyeicosatetraenoic acid.

The following antibodies were supplied by Dr. L. Levine, Brandis University, Waltham, Mass.

AB-6-keto-PGF\textsubscript{1a}
AB-Tx\textsubscript{B2}
AB-PGE\textsubscript{2}
AB-HETE
Imdomethacin was purchased from Merck and Co., Inc. (Rahway, NJ).
Soluble calfskin collagen was purchased from Millipore Corp. (Freehold, NJ) and Chemical Dynamic Inc. (Freehold, NJ).
Imipramine HCl was obtained from Geigy Pharmaceuticals Division of Geigy Chemical Corp. (Ardsley, NY).
Sodium Bentabarital (1 grain/mL) was purchased from Butler Co. (Columbus, Ohio).
Keto-Diastix urine reagent strips were purchased from Ames Division, Miles Laboratories (Elkhart, IN).
Charchol (Norit-A) was purchased from Fisher Scientific Co. (Fairlawn, NJ).
Unopette platelet/leukocyte count (1:100 dilutors were purchased from Becton Dickinson (Rutherford, NJ).
Alpha-D-tocopherol was purchased from Eastman Kodak Co. (Rochester, NY).
PAF-acether (alpha-Lecithin, beta-acetyl, gamma-O-alkyl) was purchased from Cabiochem Behring Corp. (San Diego, California).
Cyclic AmP antibody was purchased from Kew Scientific (Columbus, Ohio).
METHODS

Collagen Preparation

The preparation of collagen was developed by Charles W. Karpen, Ph.D. The suspension of bovine tendon collagen was prepared by a modified method of Nakanishi et al. (251). One gram of bovine tendon collagen was homogenized in 50 mL of normal saline at 4 C. The mixture was then centrifuged at 810 x g for 15 minutes. The collagen suspension is removed from the middle of three layers: the bottom sediment layer; the middle collagen suspension; and the top surface film. Protein concentration was determined by the method of Lowry (252) with standards made from pre-assayed soluble calf skin collagen. An albumin standard curve and collagen standard curve are shown in Figure 2.

Cholesterol Assay

Total plasma cholesterol was determined on an Abbott ABA 100 analyzer with Abbott Cholesterol reagent a modified method of Allain et al. (253). The cholesterol esters are hydrolysed by a cholesterol esterase and free cholesterol is oxidized with a cholesterol oxidase to cholest-4-en-3-one and H$_2$O$_2$. The hydrogen peroxide reacts with 4-amino-antipyrine in the presence of phenol to form a quinonemine chromophor which can be measured at 500 nm. The reaction comes to completion in 10 minutes.
Figure 2. Standard curves for albumin and collagen (ug/mL) Lowry protein assay.
Triglyceride Assay

Total plasma triglycerides were performed on an Abbott ABA 100 with Abbott triglyceride reagent. The reagent is based on coupled enzymatic procedure where the triglyceride is cleaved by a lipase in the presence of a bile salt (254). The free glycerol is acted on in a series of coupled reactions with glycerol kinase, pyruvate kinase, and lactate dehydrogenase. Quantitation is achieved by following the decrease in absorbance at 340 nm when NADH is converted to NAD⁺.

Vitamin E Assay

Vitamin E was quantitated in plasma, platelets and various tissues by modified method of Kayden et al. (255) and Hatam and Kayden (256). Plasma was obtained from platelet-free-plasma. Washed platelets were prepared as before except that an aliquot of approximately 1-2x10⁹ platelets was pelleted and the supernatant aspirated off. Plasma and platelets were stored under N₂ capped, sealed, with parafilm and kept at -20 C until analysis could be performed. Tissues were homogenized with either a Polytron homogenizer or a no clearence morter-pestle, all at 4 C. Aliquots were removed for protein determination by method of Lowry (252). The procedure is outlined below.

1. To sample (plasma, platelets, or tissue) add 2 mL of ethanolic 1% ascorbic acid solution.
2. Place tubes in a 70 C water bath for 2 minutes, then add 0.3 mL of ethanolic saturated KOH.
3. Incubate tubes at 70 C for 30 minutes.
4. Remove and cool tubes in ice.
5. Add 1 mL of distilled water followed by 4 mL hexane (HPLC grade or purified).
6. Vortex for 1 minute and remove hexane layer and place in precleaned 1 dram screw cap vials.
7. Evaporate hexane under N2 and add 200 uL of HPLC grade methanol, cap under N2, vortex, and store at -20 C until analysis.

Analysis is performed on a Beckman Altex 420 Dual pump HPLC using a Beckman Altex Ultrasphere-ODS-15cmx4.6mm reverse-phase column and isocratic solvent system of 100% HPLC grade methanol. Vitamin E content of unknowns is compared to authentic vitamin E standards made from weighing a known quantity of vitamin E and dissolved in HPLC grade ethanol.

Absorbance of vitamin E is at 194 nm on a Hitachi Model 100-10 variable wavelength detector, and integrated on an Altex Model C-R1A peak integrator. Vitamin E has a retention time of 3.7-4.0 minutes with this system.

Statistical Analysis of Data

The students t test was used to indicate significance between means (257, 258). An F test was used to determine if the variances between groups were significantly
different. If the variances were proved to be significantly different the computational formula for degrees of freedom of Dixon and Massey (259) was used. Data is presented as mean +/- SEM (standard error of the mean). The difference between means are expressed as two-tailed t test to compute p values. A p value of 0.05 is taken as being significant. The initials NS indicate that the means are not significantly different.

**Preparation of Platelet-Rich-Plasma**

Two methods for preparing platelet-rich-plasma (PRP) were used depending on the purpose of the study and the animal. PRP was prepared in rats, rabbits and humans using EDTA as the anticoagulant (9.2 parts blood:0.8 parts EDTA). PRP was prepared in rats using 3.8 gr/dL trisodium citrate (9 parts blood:1 part citrate). Aggregation studies on any animal or subject required PRP to be prepared with Na$_3$ citrate.

During the hypercholesterolemic rabbit study preparation of PRP (Na$_2$EDTA) from the hypercholesterolemic rabbits was plagued by red cell hemolysis. This problem was solved by examining the milliosmolalities (Osmo) of the anticoagulants. Trisodium citrate (3.8 gr/dL) yielded an Osmo of 348 while Na$_2$EDTA (2.6 gr/dL) yielded an Osmo of 167. The Osmo of rabbit serum was found to be 310. The fragile hypercholesterolemic red cells were bursting due to hypotonic shock. In light of this information a new EDTA
anticoagulant solution was prepared using Na$_4$EDTA (2.93 gr/dL) and adjusting the pH to 7.2. This new EDTA anticoagulant yielded an Osmo of 360, prevented red cell hemolysis and provided satisfactory PRP preparations when used with hypercholesterolemic rabbits. The standard methods for preparation of PRP for the different animals are presented below.

Rats under pentobarbital anesthesia were exsanguinated by opening the thoracic cavity and performing cardiac puncture. Blood was drawn into the following proportions (9 parts blood:1 part 3.8 gr/dL Na$_3$citrate) or (9.2 parts blood:0.8 parts Na$_4$EDTA pH 7.2).

Rabbits were drawn either by using Na$_4$EDTA or Na$_3$citrate in the same proportions as described for rats. Blood was removed by entering the main ear artery with a 21 gauge scalp vein needle and dripping the blood into a conical centrifuge tube containing the appropriate anticoagulant. Prior to sacrifice by exsanguination the rabbits were anesthetized with ether. At sacrifice the thoracic cavity was opened and the blood withdrawn by cardiac puncture into a 30 cc syringe containing the appropriate and adequate amount of anticoagulant.

Human venous blood was drawn into a 50 cc syringe and dispensed into a centrifuge tube containing the appropriate and adequate anticoagulant.
Anticoagulated rat, rabbit and human blood was centrifuged in a Sorvall GLC-2B centrifuge at 250xg (1500 rpm) for 15 minutes; 200xg (1180 rpm) for 20 minutes; and 250xg (1500 rpm) for 15 minutes respectively. The red cells and white cells sediment leaving the cloudy PRP above.

**Preparation of Washed Platelets**

The PRP was removed with a plastic tipped pipet and dispensed in a plastic ultracentrifuge tube. The PRP was centrifuged in a Sorvall RC2-B centrifuge at 1980xg (4000 rpm) for 20 minutes at 4 C. Platelet-free-plasma (PFP) was poured off and saved for further analysis. The platelet button was resuspended with a gentle push-pull technique through a plastic tipped pipet in 5 mL of either Tris-NaCl-EDTA or Krebs-Henseleit buffer. After resuspension an additional 5 mL of the appropriate buffer was added and the resuspended platelets mixed by gentle inversion (about 15 times). The resuspended platelets were again centrifuged in a Sorvall RC2-B centrifuge, 1980xg (4000 rpm) for 15 minutes at 4 C. This procedure is referred to as washing platelets. In earlier work rabbit and human platelets were washed 1 and 2 times respectively in Tris-NaCl-EDTA buffer before the final resuspension in Krebs-Henseleit buffer. Rat platelets were washed one time in Krebs-Henseleit buffer before the final resuspension in Krebs-Henseleit buffer. Later when calcium was added to the Krebs-Henseleit buffer all animals and human platelets were
washed twice with Tris-NaCl-EDTA buffer.

Krebs-Henseleit buffer was prepared as follows:

Krebs stock solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>70.0 g/L</td>
</tr>
<tr>
<td>KCl</td>
<td>3.5 g/L</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>3.0 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.6 g/L</td>
</tr>
</tbody>
</table>

QS to 1 liter with distilled water and store at 4°C.

Working Krebs-Henseleit buffer was prepared fresh daily.

Krebs stock solution 10.00 mL

Dextrose 0.18 g

NaHCO₃ 0.21 g

QS to 100 mL with distilled water and aiseate with O₂/CO₂ 95%/5% before use.

When calcium was used in the Krebs-Henseleit buffer 13.2 mg of CaCl₂·2H₂O was added to 90 mL of distilled water followed by the dextrose, NaHCO₃ and then the Krebs stock solution. Final concentration of CaCl₂ was 0.94 mM.

Platelet counts are determined on the washed platelet resuspensions using Unopette platelet/WBC dilutors, a hemocytometer, and a phase contrast microscope. The final platelet count was adjusted to 200,000 platelets/μL using Krebs-Henseleit buffer. Volumes of 0.5 mL were used for platelet incubation studies at 37°C with arachidonic acid, collagen, thrombin and platelet-activating-factor (PAF).
Prostaglandin Assay

RIA

Antibodies were raised for TxB₂, 6-keto-PGF₁α, PGE₂ and HETE by Dr. Levine, Brandis University, Waltham, Mass. Cold prostaglandins were furnished by Dr. J. Pike, Upjohn. Cold HETE was furnished by Dr. R. Bryant, George Washington University, Washington, D.C. Tritiated prostaglandins and HETE were purchased from New England Nuclear or Amersham.

The following standard method for all prostaglandin measurements is presented in Table 1. The only difference between the assays is that TxB₂, PGE₂ and 6-keto-PGF₁α are carried out in Tris-Albumin buffer pH 7.6 while HETE must be carried out in Tris-Gelatin buffer, pH 7.8. Albumin has a higher binding affinity for HETE than charcoal-dextran and therefore the charcoal cannot separate free label from the bound label.

| Table 1 |

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Bzero</th>
<th>Blank</th>
<th>Standard</th>
<th>Unknown</th>
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</thead>
<tbody>
<tr>
<td>Standards</td>
<td>----</td>
<td>----</td>
<td>200uL</td>
<td>-------</td>
</tr>
<tr>
<td>Unknowns</td>
<td>----</td>
<td>----</td>
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<tr>
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<td>200uL</td>
<td>200uL</td>
<td>200uL</td>
<td>200uL</td>
</tr>
<tr>
<td>Antiserum</td>
<td>200uL</td>
<td>----</td>
<td>200uL</td>
<td>200uL</td>
</tr>
</tbody>
</table>
**TxB\(_2\) and 6-keto-PGF\(_{1a}\)**

The TxB\(_2\) and 6-keto-PGF\(_{1a}\) RIA methods were established by C.W. Karpen, Ph. D. for his doctoral dissertation (260). The same methods are used to study platelet and aorta prostaglandin metabolism in this dissertation.

**PGE\(_2\)**

Antibody dilution was fixed at 1:3500 in a 200 uL aliquot (final concentration was 1:11,500). Determination of PGE\(_2\) from unknown samples was performed using external standards of known PGE\(_2\) quantity. Cross reactivities against PGE\(_2\) for the antibody were determined; arachidonic acid=0.00045%; PGF\(_{1a}\)=0.76%; PGF\(_{2a}\)=0.31%; PGD\(_2\)=0.051%; TxB\(_2\)=0.0078% and 6-keto-PGF\(_{1a}\)=0.040%.

Separation of bound from free label was accomplished by using a charcoal-dextran-Tris-Albumin mixture. A quantity of 0.2500 g of activated charcoal and 0.025 g of dextran were mixed with 50 mL of Tris-Albumin buffer. A volume of 0.5 mL of the charcoal slurry was pipetted into each tube. The tubes were lightly vortexed and centrifuged at 3000 rpm for 20 minutes in a Sorvall GLC-2 centrifuge, 4 C. The supernatant was poured off into a minivial and mixed with 5.5 mL of ACS II xylene scintillation cocktail and counted in a Beckman LS 8100 spectrophotometer. PGE\(_2\) controls were found to be CL=258 pg/200 uL, 9.6% C.V. and CH 656 pg/200 uL, 10.6% C.V. PGE\(_2\) determinations from aortic samples were performed by removing an aliquot from the Tris-NaCl
supernatant of the aorta sample and diluting it with an appropriate volume of Tris-Albumin buffer. Two hundred microliters of this dilution was used for analysis.

Platelet PGE$_2$ determinations were performed by removing an appropriate volume (200 uL) of the resuspended prostaglandin residue and adding an appropriate volume of Tris-Albumin buffer to the RIA tube to make a total volume of 200 uL.

**PGE$_2$ Assay Linear Recovery: Dilution Study**

An unknown quantity of platelet synthesized PGE$_2$ was analyzed by RIA. A series of interrelated dilutions were constructed with the assayed PGE$_2$ solution and the common matrix (Tris-Albumin) with no PGE$_2$ (262). Verification of the specificity of the PGE$_2$ RIA assay is achieved when the data from the interrelated samples are examined. If the assay is measuring PGE$_2$ then the plot of the actual data versus the theoretical data should yield a straight line with a slope of 1. The results can be seen in Figure 3.

The slope of the plot is 1.006, very nearly 1.0. A correlation r=0.9990 indicates a high degree of linearity. The y intercept of -10.63 indicates that the PGE$_2$ assay has a negligible amount of bias in its assay of the interrelated samples. This information indicates that the PGE$_2$ assay is a highly sensitive assay with an excellent one to one correlation with a biologically obtained unknown.
Figure 3. Interrelated sample of PGE₂ recovery.
HETE

Standards were made from the cold HETE supplied by Dr. R. Bryant. Concentration of the stock HETE solution was determined by measuring the absorbance at 235 nm; \( E_{235} = 28,000 \) in ethanol. An antibody dilution in Tris-Gelatin, pH 7.8 of 1:3500 was used to deliver the antibody in an aliquot of 200 \( \mu \)L. The final antibody dilution was 1:11,500 in a total volume of 600 \( \mu \)L. Separation of bound from free label was accomplished by using a dextran-charcoal-Tris-Gelatin slurry. Dextran and charcoal were added in the same proportions as with the slurry in Tris-Albumin. The charcoal slurry should be kept on ice while stirring. Cross reactivities for HETE antibody are: 12-hydroxyheptadecatrienoic acid (HHT), 8.6%; arachidonic acid, 0.085%; PG\(_D_2\), <0.0017%; PGE\(_2\), <0.0046%; PGF\(_2\)\(_a\), <0.0019%; thromboxane B\(_2\), <0.014%; vitamin E, <0.0004%. The detection limit for HETE in this assay is 0.2 picomoles. Interassay variability, evaluated with the use of two pooled platelet controls, was 8.2% and 9.1% (CV) for the high and low controls respectively.

Prostaglandin Extraction Efficiency

Prostaglandin extraction with diethyl ether were also examined by using tritiated prostaglandins. An aliquot of TxB\(_2\), PGE\(_2\) or HETE (10,000-20,000 cpm) was pipetted into separate and individual WPS incubations (1x10\(^8\) platelets/500 \( \mu \)L). The platelets were activated with collagen for ten
minutes, terminated with 100 uL 1 N HCl and prostaglandins extracted with multiple diethyl ether extractions. The results are shown below:

\[
PGE_2 \% \text{ Recovery First extraction } 90.95 +/- 1.43(6) \\
\text{Second extraction } 6.73 +/- 1.40(6) \\

TxB_2 \% \text{ Recovery First extraction } 85.50 +/- 1.16(6) \\
\text{Second extraction } 6.98 +/- 0.63(4) \\

HETE \% \text{ Recovery First extraction } 91.70 +/- 0.38(4) \\
\text{Second extraction } 6.43 +/- 0.34(4)
\]

**Platelet Prostaglandin Production In Rat, Rabbit and Human**

RIA of TxB_2, PGE_2 and HETE can be performed on a single sample of WPS making it possible to examine in greater detail and depth the response of platelet to various stimuli. The activities of cyclo-oxygenase (CO) and thromboxane synthetase (TxS) can be examined by quantitating PGE_2 (CO) and TxB_2 (CO and TxS). The activity of the lipoxygenase enzyme (LO) of platelets after stimulation can be examined by quantitating HETE. Analysis of rabbit platelets was performed with TxB_2, PGE_2 and HETE after stimulation with thrombin, collagen arachidonic acid and PAF. Analysis of rat platelets were performed with TxB_2, PGE_2 and HETE after stimulation with arachidonic acid. A thorough examination of rabbit platelets prostaglandin production in response to thrombin, collagen, arachidonic acid, PAF and calcium was carried out. An examination of TxB_2 and HETE production in human platelets was carried out.
with thrombin collagen and arachidonic acid.

Previously it was demonstrated that rat platelet (WPS) demonstrated a maximum for TxB\textsubscript{2} production at 5-10 uM arachidonic acid in 10 minute and 1 minute incubations (260). PGE\textsubscript{2} increased throughout the range of 1-36 uM arachidonic acid in 10 minute incubations. In one minute incubations PGE\textsubscript{2} leveled off after 2 uM arachidonic acid. Rat platelets were examined further with 1.1 to 8.8 uM arachidonic acid at 2 and 5 minute incubations to define the production of TxB\textsubscript{2}, PGE\textsubscript{2} and HETE. In Figure 4, TxB\textsubscript{2} and PGE\textsubscript{2} level off after 2 uM arachidonic acid in 2 minute incubations. However, the production of HETE continues increasing throughout the range of arachidonic acid much more than TxB\textsubscript{2} and PGE\textsubscript{2}. In 5 minute incubations HETE increases linearly with increasing arachidonic acid concentration (Figure 5), TxB\textsubscript{2} plateaus at about 5 uM while PGE\textsubscript{2} increases linearly with increasing arachidonic acid concentrations (Figure 6). In conclusion, HETE production in rat platelets is dominant over TxB\textsubscript{2} and PGE\textsubscript{2} at 2 and 5 minute incubations (Figure 4,5,6). TxB\textsubscript{2} production at 5 minutes with increasing concentrations of arachidonic acid confirm previous work by C.W. Karpen, Ph.D. (260).

The production of rabbit platelet CO and LO products can be seen in Figure 7. Figure 7 demonstrates the time dependent formation of HETE, TxB\textsubscript{2} and PGE\textsubscript{2} from thrombin (3.0 U/mL) stimulation. HETE formation is delayed with TxB\textsubscript{2}
Figure 4. Arachidonic acid concentration dependent production of HETE, TxB₂ and PGE₂ from rat platelets (2 minutes).
Figure 5. Arachidonic acid concentration dependent production of HETE from rat platelets (5 minutes).
Figure 6. Arachidonic acid concentration dependent production of TxB₂ and PGE₂ from rat platelets (5 minutes).
Figure 7. Time dependent production of HETE, TxB$_2$, and PGE$_2$ from rabbit platelets stimulated with 3 U/mL thrombin.
rising rapidly and then leveling off. PGE$_2$ formation is a slow steady climb. Figure 8 demonstrates the thrombin concentration dependence of HETE, TxB$_2$ and PGE$_2$ in 10 minute incubations. As can be seen all CO and LO products increase with increasing concentration of thrombin. Figure 9 demonstrates the formation of HETE, TxB$_2$ and PGE$_2$ as a function of platelet concentration and thrombin (3 U/mL). The normal platelet concentration use in the studies in this dissertation was 200,000 platelet/uL ($1\times10^8$ platelets/500 uL). All three products rise appropriately with increasing concentration of platelets. Figure 10 demonstrates the time dependent formation of HETE, TxB$_2$, and PGE$_2$ from collagen stimulated rabbit platelet. TxB$_2$ is completely formed in 5 minutes. PGE$_2$ again increases slowly with time. HETE increases with time although much faster than TxB$_2$ or PGE$_2$. Figure 11 demonstrates the effects of platelet concentration on HETE, TxB$_2$ and PGE$_2$ formation with collagen stimulation for 10 minutes. It is interesting to see TxB$_2$ and PGE$_2$ remaining parallel until the platelet concentration is twice the normal incubation concentration. HETE is formed in an increasing linear fashion as platelet concentration increases.

Figure 12 demonstrates the effect of increasing arachidonic acid concentrations in 2 minute incubations on TxB$_2$, HETE and PGE$_2$. There is a rapid increase in TxB$_2$ which plateaus at about 10 uM after which it decreases
Figure 8. Thrombin concentration dependent formation of HETE, TxB2 and PGE2 from rabbit platelets (10 minutes).
Figure 9. Concentration of rabbit platelets versus production of HETE, TxB₂ and PGE₂ after stimulation with thrombin 3 U/mL.
Figure 10. Time dependent production of HETE, TxB₂ and PGE₂ from collagen (300 ug/mL) stimulated rabbit platelets.
Figure 11. Concentration of rabbit platelets versus production of HETE, TxB₂, and PGE₂ after stimulation with collagen 300 ug/mL (10 minutes).
Figure 12. Arachidonic acid concentration dependent production of TxB₂, HETE and PGE₂ from rabbit platelets (2 minutes).
gradually. HETE increases linearly with increasing concentrations of arachidonic acid as does PGE$_2$ only to a lesser extent. Figure 13 demonstrates the effects of low concentrations of arachidonic acid (2 uM) on rabbit platelets as a function of time. HETE is slow to be produced initially then as TxB$_2$ production falls off HETE production increases. PGE$_2$ production increases with time but at a slower rate than HETE. Figure 14 shows the effect of high concentrations of arachidonic acid (30 uM) as a function of time. Again HETE formation is slow to respond while the rate of TxB$_2$ begins to slow but, not as great as in 2 uM arachidonic acid. PGE$_2$ increases slowly to higher levels than with 2 uM arachidonic acid as would be expected.

In conclusion rabbit platelets make HETE in greater amounts than PGE$_2$ and TxB$_2$ at high concentrations of thrombin (Figure 8) collagen (Figure 10) and arachidonic acid (Figure 14). In shorter incubation times TxB$_2$ production is greater than HETE production with thrombin (Figure 7) and arachidonic acid (Figure 13) while at longer incubation times HETE is produced in greater amounts with thrombin (Figure 8) and arachidonic acid (Figure 14). All platelet products increase with increasing platelet concentrations in response to thrombin (Figure 9) and collagen (Figure 11).
Figure 13. Time dependent formation of HETE, TxB₂, and PGE₂ from rabbit platelets stimulated with 2 μM arachidonic acid.
Figure 14. Time dependent formation of HETE, TxB_2 and PGE_2 from rabbit platelets stimulated with 30 μM arachidonic acid.
The effects of PAF on TxB2, PGE2 and HETE production require the presence of Ca\(^{2+}\). A calcium concentration of 0.94 mM was chosen because it approximates what would be found as ionized calcium in the blood. In Figure 15 increasing concentrations of PAF initiate a small increase in TxB2 and PGE2 but a large increase in HETE production in 10 minute incubations. One minute incubations with increasing PAF concentrations and 0.94 mM Ca\(^{2+}\) yield similar curves in 10 minutes but to a lesser extent and can be seen in Figure 16. Increasing TxB2 production with respect to time in response to PAF at 10\(^{-6}\), 10\(^{-8}\) and 0 M concentration can be seen in Figure 17. Increasing PGE2 production with respect to time and PAF can be seen in Figure 18. Increasing HETE production with respect to time and PAF can be seen in Figure 19. In order to better understand calcium's role on rabbit platelets with other activators of platelet aggregation calcium of increasing concentration was incubated with thrombin, collagen and arachidonic acid. All activators were kept at very low levels to examine the effect of Ca\(^{2+}\) rather than the activator effect. In Figure 20, thrombin at 0.2 U/mL, 2 minute incubations, an increase in HETE and TxB2 begins as low as 8.0x10\(^{-5}\)M calcium. With no added thrombin nonspecific increases in TxB2 and HETE due to stirring and calcium alone is not seen until one reaches the level of 1 mM Ca\(^{2+}\). With a low concentration of collagen in 10 minute incubations (Figure 21) it can be seen
Figure 15. PAF concentration dependent formation of HETE, TxB\(_2\) and PGE\(_2\) from rabbit platelets (10 minutes).
Figure 16. PAF concentration dependent formation of HETE, TxB₂ and PGE₂ from rabbit platelets (1 minute).
Figure 17. Time dependent production of TxB₂ from rabbit platelets stimulated with PAF (10⁻⁶, 10⁻⁸ and 0 M).
Figure 18. Time dependent production of \( \text{PGE}_2 \) from rabbit platelets stimulated with PAF (10\(^{-6}\), 10\(^{-8}\) and 0 M).
Figure 19. Time dependent production of HETE from rabbit platelets stimulated with PAF ($10^{-6}$, $10^{-8}$ and 0 M).
Figure 20. Calcium concentration influence on low thrombin (0.2 U/mL) stimulation of rabbit platelets (10 minutes).
Figure 21. Calcium concentration influence on low collagen (120 µg/mL) stimulation of rabbit platelets (10 minutes).
that HETE is activated at lower calcium concentration 
\(5.0 \times 10^{-5} \text{M}\) than \(\text{TxB}_2\) \(1 \times 10^{-4} \text{M}\). With no added collagen 
though it can be seen that there is a gradual increase in 
HETE production from \(1 \times 10^{-4}\) to \(1 \times 10^{-3} \text{M}\) while \(\text{TxB}_2\) production 
does not begin until \(1 \times 10^{-3} \text{M}\) is reached. Using free 
arachidonic acid at \(30 \ \mu\text{M}\) and 2 minute incubations (Figure 22) we see a dramatic increase in HETE production through the 
full range and with a jump at \(1 \times 10^{-3} \text{M}\) calcium. In contrast 
\(\text{TxB}_2\) does not change that much throughout the calcium range. 
It must be remembered that \(30 \ \mu\text{M}\) arachidonic acid is a 
saturating level of substrate for thromboxane synthetase. 
This points to a calcium activation of the lipoxygenase 
pathway in rabbit platelets.

In conclusion the influence of calcium on platelet \(\text{TxB}_2\) 
and HETE production is quite dramatic. In the case of 
thrombin (Figure 20) we see a sudden increase in HETE up to 
10 fold at levels of calcium greater than \(1 \ \text{mM}\) and beyond 
what one would expect to be ionizable levels of calcium. 
\(\text{TxB}_2\) increases nearly 8 fold through this range. Again with 
collagen (Figure 21) HETE increases 10 fold while \(\text{TxB}_2\) 
gradually increases 6 fold. Arachidonic acid demonstrates 
clearly that the effect of calcium on platelet arachidonic 
acid metabolism rests in the LO pathway (Figure 22). When 
\(\text{PAF}\) is used it can be seen that clearly the arachidonic acid 
metabolite produced in greatest quantities is HETE with 
respect to time (Figure 17, 18 and 19) and concentration
Figure 22. Calcium concentration influence on 30 uM arachidonic acid stimulation of rabbit platelets (2 minutes).
Examination of rabbits on different diets was undertaken with this information as background. The models used were hypercholesterolemic rabbit and vitamin E supplemented and deficient rabbits. The analysis of platelet prostaglandin production was followed in light of these experiments in order to ascertain whether platelets could be influenced by dietary factors.

Characterization of human platelet TxB$_2$ and HETE production was carried out with thrombin, collagen and arachidonic acid with respect to concentration and time. These studies were carried out with the assistance of C.W. Karpen, Ph.D. in preparation for the study on platelets from Type IIa hypercholesterolemic patients from Dr. Falco's lipid clinic. In figure 23 HETE and TxB$_2$ are produced in equal amounts up to 0.5 U/mL beyond which TxB$_2$ appears to level off with HETE production is in excess of TxB$_2$. The time dependent formation of TxB$_2$ and HETE are shown in Figure 24. TxB$_2$ is made in greater amounts than HETE between 1 and 6 minutes at which point HETE equals TxB$_2$ formation. In Figure 25 more TxB$_2$ is made than HETE through 10 to 18 ug/mL of collagen. While at 300 ug/mL they are made in equal amounts. TxB$_2$ and HETE production in response to 168 ug/mL collagen can be seen in Figure 26. TxB$_2$ is made in greater quantities than HETE throughout the time span. Arachidonic acid concentration studies on TxB$_2$ and
Figure 23. Thrombin concentration dependent formation of HETE and TxB₂ from human platelets (10 minutes)
Figure 24. Time dependent formation of TxB₂ and HETE from human platelets stimulated with thrombin (0.5 U/mL).
Figure 25. Collagen concentration dependent formation $\text{TxB}_2$ and HETE from human platelets (10 minutes).
Figure 26. Time dependent formation of TxB₂ and HETE from human platelets stimulated with collagen (168 μg/mL).
HETE (Figure 27) demonstrate a slowing of TxB₂ in response to increasing arachidonic acid while HETE production lags until about 5 uM and thereafter increases extremely fast. Figure 28 shows HETE and TxB₂ production in response to 9 uM arachidonic acid with respect to time. TxB₂ production levels off at about 5 minutes while HETE production increases quickly up to 5 minutes and then increases at a slower rate.

In conclusion HETE production is greater than TxB₂ only when the CO-TxS enzyme systems are overwhelmed as in the cases of high thrombin concentrations (Figure 23) and arachidonic acid (Figure 27 and 28). Collagen preferentially initiates TxB₂ production over HETE except at extremely high concentrations (300 ug/mL) and long incubation times (>12 minutes).

Prostaglandin Production in Rabbit Aorta

Previously PGI₂ production as measured by 6-keto-PGF₁α RIA in rat aorta was shown to increase with time of incubation and was on the order of 50-60 pmoles/mg tissue weight (260). The rabbit aorta makes about 10 times less PGI₂/mg tissue weight than the rat aorta using the same method of analysis. In addition PGI₂ production in the rabbit aorta varies throughout the length of the rabbit aorta. The lower abdominal region (A) makes the most PGI₂ with the middle abdominal region (M) next and the thoracic region (T) making the least. This can be seen in Figure 29.
Figure 27. Arachidonic acid concentration dependent formation of HETE and TxB\(_2\) human platelets (10 minutes).
Figure 28. Time dependent formation of HETE and TxB₂ from human platelets stimulated with arachidonic acid (9 μM).
Figure 29. 6-keto-PGF$_{1a}$ and PGE$_2$ generation from rabbit aorta.
PGE₂ production is measurable but very low at 90 minutes. When 27 uM arachidonic acid is added to the aorta pieces PGI₂ production is increased in the same manner. However, the abdominal arachidonic acid metabolism to 6-keto-PGF₆α far exceeds that of the middle abdominal or thoracic regions. In contrast when rat aorta is incubated with arachidonic acid 6-keto-PGF₆α levels typically reached about 200 pmoles/mg tissue (87).

In conclusion rabbit aorta have a regional response of PGI₂ production and limited capacity to produce PGI₂ when compared to rat PGI₂ production.

**Cyclic AMP Assay**

Cyclic AMP is assayed by RIA a modified method of Steiner (263) developed with antibody purchased from Kew Scientific (Columbus, Ohio). The samples analyzed contain 1x10⁸ platelet in Krebs-Henseleit buffer 500 uL. One mL of 5% trichloroacetic acid (TCA) is used to kill the reaction. PRP can be used but the TCA may not yield complete precipitation of plasma proteins. In addition plasma lipids may not completely precipitate and for these reasons a PPP blank should be performed.

**Preparation of Sample**

1. To 1x10⁸ platelets (500 uL) in 13x100mm test tube add 1.0 mL 5% TCA after appropriate incubation time.

2. Vortex gently.
3. Centrifuge sample at 3000 RPM for 25 minutes 4 C to pellet platelet protein.

4. Quantitatively transfer 1 mL of TCA-platelet supernatant to a clean 13x100mm test tube.

5. Add 3 mL of water saturated diethyl ether vortex for 30 seconds.

6. Allow ether and aqueous layers to separate.

7. Aspirate the ether layer taking care not to remove any aqueous solution.

8. Repeat steps 5 to 7, two times.

9. Place tubes in a 50-55 C water bath for 10 minutes to boil off residual ether.

10. Add 0.1 mL of 1 M Na-Acetate buffer, pH 6.5 to stabilize the cyclic AMP in the sample. The total volume is now 1.1 mL.

11. Samples were stored at -20 C until assayed.

RIA Procedure

The contents of one vial are quantitatively transferred to 5.0 mL of 0.05M Na Acetate buffer, pH 6.2, containing 0.1% bovine serum albumin. Label tracer adenosine 3',5'-cyclic phosphoric acid, [125I]-2'-O-succinyl (iodotyrosine methyl ester)- (125I-CAMP-TME) was purchased from New England Nuclear (Boston, Mass.) in 1.0 uCi quantities. Label, 125I-CAMP-TME, was diluted in 0.05 M Na-Acetate buffer, pH 6.2, containing 0.25% gamma-globulins such that a 50 uL aliquot would yield 8000-10,000 CPM.
Samples and standards are pipetted into 12x75 mm test tubes kept on ice. The antibody binds non-acetylated cyclic AMP with a 10-fold lower affinity than the acetylated species. Therefore the cyclic AMP of the samples and standards are acetylated with a mixture of acetic anhydride and tri-ethylamine (2:5), 10 uL. Acetylation is done in the hood after the samples and standards are removed from ice and brought to room temperature (10 minutes). The 10 uL of acetylating reagent is injected to the bottom of the tube while vortexing. After acetylation the samples are kept at room temperature for 10 minutes and then returned to ice. Then 0.30 mL of 0.05M Na-Acetate buffer, pH 6.2 are added to all tubes. An additional 50 uL of the same buffer is added to the blank tubes. Label was added to all tubes in a 50 uL volume. After the label was added, 50 uL of the antibody was injected into all tubes except the blanks. All tubes were gently vortexed, covered with aluminum foil to prevent photolysis of the constituents in the assay tubes and stored at 4 C for an optimum time of 18 hours. After the 18 hour incubation 2.5 mL of 60% saturated ammonium sulfate solution (195 g in 500 mL distilled water) was added to all tubes using a Cornwall syringe. Each tube was vortexed vigorously and then incubated for 20 minutes on ice. This technique is called ammonium sulfate precipitation which separates free label from bound label. After the 20 minute incubation on ice the tubes were centrifuged at 3000 rpm in a Sorvall
GLC-2 centrifuge at 4°C for 20 minutes. The supernatant was poured off. The tubes were wiped clean and free from excess solution and allowed to dry. The tubes were counted in a Searle automatic gamma counter, Searle Analytic, Inc., 3 channel counter model number 12-85. Quantitation of cyclic AMP levels is accomplished by calculating \% Bound of the standards, constructing a plot of \%B versus log concentration and reading of unknowns.

In order to better understand the animal models used in the research, time, and concentration curves were constructed for rat (Figure 30 and 31), rabbits (Figure 32 and 34) and humans (Figure 34 and 35) platelet production of cyclic AMP. WPS were incubated at different times and different concentrations of PGE₁. Based on these results a 30 second incubation was chosen to be used for concentration studies with and without isobutylmethylxanthine (IBMX) (25 uM). In the rat a 30 second incubation was found to yield to maximal cyclic AMP levels with 0.31 uM PGE₁. By using a standard time of 30 seconds and 0.31 uM PGE₁ the production of cyclic AMP is on the increasing side of production and near maximal production. Using higher concentrations of PGE₁ might saturate the platelets and abolish any differences in production rates of cyclic AMP. On the other hand using less than 0.31 uM PGE₁ could yield a highly variable result due to under stimulation. Adding IBMX, a phosphodiesterase inhibitor, induces a dramatic increase
Figure 30. Time and PGE<sub>1</sub> concentration dependent cyclic AMP formation from rat platelets.
Figure 31. PGE\(_1\) concentration dependent cyclic AMP formation from rat platelets with and without 25 uM IBMX (30 seconds).
Figure 32. Time and PGE$_1$ concentration dependent cyclic AMP formation; IBMX with 0.31 uM PGE$_1$ from rabbit platelets.
Figure 33. PGE₁ concentration dependent cyclic AMP formation from rabbit platelets with and without IBMX (30 seconds).
Figure 34. Time and PGE$_1$ concentration dependent cyclic AMP formation from human platelets; IBMX with 0.31 uM PGE$_1$.

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Figure 35. PGE$_1$ concentration dependent cyclic AMP formation from human platelets with and without IBMX (30 seconds).
in cyclic AMP levels in the rat and human. The increase in
cyclic AMP in rabbit WPS incubated with IBMX and PGE$_1$ is not
as striking.

**MDA: Estimation of Lipid Peroxides**

Plasma lipid peroxides were estimated by a modified
method of Satoh (180,196). A volume of 0.5 mL non-EDTA
plasma (197) is precipitated with 2.5 mL of 20% TCA. The
precipitate is vortex gently and allowed to stand at room
temperature for 5 minutes. The precipitate is pelleted by
centrifugation at 2000 rpm for twenty minutes in a Sorvall
GLC-2. The supernatent is poured off and 2.5 mL of 0.05 M
H$_2$SO$_4$ is added to wash the precipitate by vortexing. The
resuspended precipitate is pelleted once more by
centrifugation as before. This process is repeated one more
time. The last supernatant wash is poured off and 2.5 mL of
0.05 M H$_2$SO$_4$ and 3.0 mL of the TBA reagent (0.2 g TBA/dL,
40% saturated Na$_2$SO$_4$ solution). The precipitate and mixture
is vortexed, placed in a 100 C water bath for 30 minutes in
order to develop the color.

The tubes are cooled in an ice bath for 5 minutes. The
developed color is extracted with 4 mL of n-butanol by
vortexing for 30 seconds. The tubes are centrifuged to make
a clean interface between the aqueous and organic phases.
The n-butanol is removed. The fluorescence was measured by
excitation at 532 nm and emission at 548 nm on an
Aminco-Bowman Spectrophotofluorometer. The excitation and
Figure 26. Fluorometric excitation and emission of MDA-TBA reaction product.
emission spectra can be seen in figure 36.

Analysis of plasma from the CDE rats were examined by spectrophotometry and spectrophotofluorometry (Figure 37). The correlation between the two methods was found to be quite good, with fluorescence yielding slightly higher values. Icteric (green tint) plasma yielded slightly higher results when measured by spectrophotometry. Hemolysis was found not to have any biased effect between the two methods of measurement. MDA measurement by fluorometry (Figure 38) yields a 10 fold increase in sensitivity over spectrophotometry (Figure 39). It is less susceptible to interferences by icteric plasma.

MDA-Tissues

In addition to plasma MDA measurements, tissue MDA was performed. The tissue was homogenized using either a motor driven mortar and pestle or by use of Polytron homogenizer. The tissues analyzed were heart and liver. Not all tissues were analyzed in each animal model system but the same basic procedure exists for all the tissues. Tissues were homogenized with either a KCl solution or a NH₄Cl/NH₂OH pH 8.0. No differences in the MDA production were detected between the two solutions.

Lipoprotein Lipase Assay

Lipoprotein lipase is measured by the principle of enzymatic hydrolysis of 1-¹⁴C-oleate from ¹⁴C-triolein glycerol-lecithin emulsion diluted in heat inactivated
Figure 37. Linear regression of MDA determination: Fluorometry versus spectrometry.
Figure 38. Standard curve 0.1-1.0 MDA nMol/mL: Fluorometry
Figure 39. Standard curve 1-10 MDA nMol/mL: Spectrometry
serum-albumin buffers (264-268). The reaction is terminated by adding methanol-chloroform-heptane (1.41:1.25:1, V/V/V) followed by potassium carbonate borate buffer. The free \(^{14}\text{C}\)-oleate is preferentially extracted into the methanol-water upper phase. An aliquot of the methanol-water upper phase is removed for counting in a liquid scintillation counter. Quench correction is made for all samples and results are calculated based on specific activity of the \(^{14}\text{C}\)-triolein substrate and time of incubation.

Tissue samples were taken from rats after exsanguination and rinsed in Tris-NaCl buffer (pH 8.0) prior to weighing. After weighing they were quickly frozen in liquid nitrogen and stored at -70 C until analysis. All tissues were chopped and minced while frozen and then combined with buffers prior to homogenation.

All tissue samples were homogenized 3-7 times with 10 second bursts with a Polytron homogenizer in a \(\text{NH}_4\text{Cl-} \text{NH}_3\text{OH}\) buffer pH 8.0 while on ice. An aliquot of the tissue homogenates was removed and extracted two times with acetone (5 mL) and one time with ether (5 mL), except adipose tissue homogenate which was extracted 3 times with acetone (5 mL) and 2 times with ether (5 mL). The acetone powders were dried under vacuum dessication overnight. Once in the dry acetone powder state lipoprotein lipase is stable for one week at room temperature and months frozen at -20 C. The
acetone powders were resuspended in 0.073 M Tris-HCl buffer pH 7.4 such that aliquots would contain 5-8 mg of the original wet tissue weight for liver, heart and muscle. Adipose acetone powders were resuspended with the serum-albumin buffer in order to maintain the protein concentration of reaction mixture. Adipose acetone powders were resuspended such that about 15 mg of the original wet tissue weight would be contained in each aliquot. The substrate was prepared according to method of Nilsson-Ehle and Schotz (264) and is as follows.

1. 500 mg triolein was placed in a 25 mL counting vial.
2. 35 mg of egg lecithin was added to the same vial.
3. A quantity of $^{14}$C-triolein (NEC-674) (New England Nuclear, Boston, Mass) (0.05 mCi/99.7 mCi/mMol) was added to the vial such that the specific activity equalled 136,000 cpm/uMol of triolein (0.052 uCi/uMol triolein)
4. 10 grams of fresh glycerol was added
5. The mixture was homogenized with a Polytron homogenizer 10 times (30 seconds on, 30 seconds off). The mixture was kept on ice during this process.
6. The vial was capped under N$_2$ and allowed to stand overnight to let the bubbles clear. The homogenized mixture must clear prior to use.
7. The next day after it was verified that the glycerol triolein concentrated substrate was clear, 1 volume of
concentrated substrate was combined with 4 volumes of Tris-HCl buffer 0.2 M (pH 8.0) containing 3% (W/V) bovine albumin and 1 volume of serum. The working substrate was finally prepared for use by vortexing the working substrate vigorously for 5 seconds.

8. The reaction mixture was composed of 0.1 mL enzyme or buffer and 0.1 mL of working substrate. Start of incubation was taken at addition of working substrate. Incubations were carried out at 31°C with gentle shaking.

9. Final reaction concentration was:

- 5.66 uMol/mL Triolein
- 0.35 uMol/mL Lecithin
- 1.0% (W/V) Albumin
- 8.5% (V/V) Serum
- 8.5% (W/V) Glycerol

10. Reactions were carried out for 30 minutes at 37°C with gentle shaking. Reactions were stopped by addition of 3.25 mL of methanol-chloroform-heptane (1.41:1.25:1, v/v/v) followed by 1.05 mL of 0.1 M potassium carbonate-borate buffer (pH 10.5).

11. All tubes were stoppered, vortexed vigorously for 15 seconds and then centrifuged for 15 minutes at 3000 x g. A 1 mL aliquot of the methanol-water upper phase was added to 10 mL ACSII Amersham scintillation cocktail and counted in a Beckman LS8100 with quench correction.
EFFECT OF STREPTOZOTOCIN INDUCED DIABETES ON LIPOPROTEIN LIPASE IN RATS AND ENHANCED LIPOPROTEIN LIPASE ACTIVITY IN DIABETIC RATS BY DIETARY VITAMIN E.

INTRODUCTION

Lipoprotein Lipase

Previously it was observed that rats made diabetic had high plasma lipids (87). Further, these high plasma lipids could be lowered by increasing the levels of the lipid-soluble antioxidant vitamin E in the diet. Kotze et al. demonstrated that heart muscle lipoprotein lipase (LPL) of baboons was altered by dietary levels of the water-soluble antioxidant vitamin C (238). Baboons on high vitamin C diets (34 mg/Kg body mass/day) had lower triglycerides than baboons on scorbutogenic diets. Heart muscle LPL activity was reported to be lower in vitamin C fed baboons than vitamin C-deficient baboons. However, the LPL activity of adipose tissue was higher in vitamin C fed baboons than deficient baboons (239). In addition in vitro studies on LPL with vitamin C demonstrated enhanced activity of LPL when samples of heart muscle from vitamin C-deficient baboons was incubated with vitamin C (238). This evidence strongly suggests that an allosteric modulation of LPL is taking place in vitro (238). The in vivo studies indicate that vitamin C plays other roles in LPL modulation, tissue
specific synthesis, and enhanced activation or allosteric modulation (238).

In diabetic rats on vitamin E-control diet not only were plasma lipids increased but also were found to contain high levels of lipid peroxides as measured by MDA. Tappel in his recent review on lipid peroxidation described the inactivation of enzymes by lipid peroxides which form a Schiffs base with free amino groups of the protein (180). LPL may also be inactivated by lipid peroxides. As mentioned earlier, supplementation of diets with vitamin E (200 mg/Kg diet) afforded a dramatic decrease in plasma lipids in the diabetic rats. Not only was there a decrease in plasma lipids but also a decrease in lipid peroxides in vitamin E-supplemented diabetic rats over vitamin E-normal diabetic rats. LPL activity may be preserved in diabetes if lipid peroxides are lowered by vitamin E.

It is with these concepts in mind that the study of LPL with respect to dietary vitamin E supplementation and lipid peroxides was undertaken.

EXPERIMENTAL DESIGN

Male Sprague-Dawley rats (160-200 gr) under light ether anesthesia, were injected through a tail vein with streptozotocin (80 mg/Kg, 0.1 mL) in 0.05 M citrate buffer, (pH 4.5), or with citrate alone. Diabetic rats demonstrated
glucosuria within 3 days of injection. Animals were housed three to a cage and fed standard Purina Lab Chow for 4 weeks. At that time controls and diabetic rats were placed on their respective diets. Diabetic rats were separated equally by weight into two groups. One group received the standard vitamin E-defined diet (30 mg tocopherol-acetate/Kg diet) prepared by ICN as the control rats while the other group received a vitamin E-supplemented diet (200 mg tocopherol-acetate/Kg diet). The animals were sacrificed after 10 weeks on defined diet. Tissues were removed, rinsed in Tris-NaCl pH 8.0, weighed, frozen in liquid nitrogen and kept at -70 C until analysis. Blood was removed and saved for cholesterol and triglyceride determinations.

Diabetic rats (D) had greater triglycerides than control (C) and diabetic vitamin E rats (E) as can be seen in Figure 40. However, E rats did not obtain a lowering of triglycerides to the level of C rats. In Figure 41 the plasma vitamin E status of D and E rats are equivalent and significantly higher than C rats. However this equivalent plasma vitamin E status did not protect the D rats from plasma lipid peroxides as measured by MDA can be seen in Figure 42. As a result lipid peroxides which are probably generated in the tissues were released into the plasma. In Figure 43 the liver vitamin E status of E rats is significantly increased over C and D rats. As can be seen in Figure 44 liver lipid peroxides were significantly
Figure 40. Plasma triglycerides (mg/dL) from C, D, and E rats.
Figure 41. Plasma vitamin E levels (ug/mL) from C, D, and E rats.
Figure 42. Plasma MDA values (nmoles/mL) from C, D, and E rats.
Figure 43. Liver vitamin E content (mg/gram tissue) from C, D, and E rats.
Figure 44: Liver MDA values (nmoles/gram tissue) from C, D, and E rats.
greater in D rats than E rats. Due to variability in the assay, the p value between C versus D was calculated to be 0.0775 just over the lowest level of acceptable significance in a two-tailed test (p<0.05). However, it is felt that although the lipid peroxide measurement was statistically not significantly different, there is sufficient evidence to say that D rat livers contained more lipid peroxides than E rats and in a qualified sense more than the C rats.

The effects of diabetes and diabetes with vitamin E supplementation on rat liver lipase activity can be seen in Figure 45. Liver lipase activity (total hepatic triglyceride lipase: THTGL) is composed of hepatic triglyceride lipase (HOTGL) and lipoprotein lipase (LPL). LPL activity can be inhibited by 1M NaCl thus providing a means by which to distinguish LPL from HTGL. THTGL is significantly lower in D rats than in C and E rats, while there is basically no difference between C and E. HTGL activity in D and E rats is significantly lower than C rats. There is a slight increase in HTGL activity of E rats over D rats but not enough to indicate any significance. Thus, when HTGL is subtracted from THTGL the result is an increase in LPL in E rats over C and D rats. Diabetic rats fed vitamin E exhibit more LPL activity than D rats. This increase in LPL activity may be due to increased synthesis of the enzyme, greater protection of the enzyme due to lower tissue or plasma MDA levels or both.
Figure 45. Liver lipase activity (Total, Hepatic triglyceride lipase and Lipoprotein lipase: nmoles FFA released/mg tissue/hour) from C, D, and E rats.
Other tissues were analyzed for LPL activity. Heart LPL activity (Figure 46) showed no differences in LPL between C, D, and E rats. There was a general increase in muscle LPL activity (Figure 47) C < D < E rats. However statistically there was no difference between C and D rat muscle LPL activity. An equivalent adipose LPL activity (Figure 48) in D and E rats which was significantly greater than C rats.

In conclusion the major point of LPL increase seems to be liver and muscle activities. Liver LPL activity shows a striking increase in E rats over C and D rats. Muscle LPL activity is increased in E rats over C and D rats. The combination of the increase in LPL activity of these metabolic organs may be enough to lower the blood lipids. The plasma vitamin E status of D rats is equivalent to E rats probably at the expense of much needed tissue vitamin E to control lipid peroxidation in tissues. E rats were fed high levels of vitamin E which resulted in a lowering of plasma lipid peroxides. Tappel in his recent review (180) stated that plasma vitamin E content is directly proportional to the intake of vitamin E and that the tissue levels are proportional to the log of vitamin E intake (180). Bieri et al. says that approximately a 10-fold intake is required to double the plasma vitamin E and that the tissue vitamin E increases in a linear fashion (269). Most tissue concentrations tend to level off after a few
Figure 46. Heart lipoprotein lipase activity (nmoles FFA released/mg tissue/hour) from C, D, and E rats.
Figure 47. Muscle lipoprotein lipase activity (nmoles FFA released/mg tissue/hour) from C, D, and E rats.
Figure 48. Adipose lipoprotein lipase activity (nmoles FFA released/mg tissue/hour) from C, D, and E rats.

C vs D $p<0.01$
C vs E $p<0.05$
D vs E NS
weeks after which the only tissue that continues to increase is adipose (269). Liver is very active metabolically and in a disease state would need more protection from endogenous lipid peroxidation than controls. Supplementing diets with vitamin E results in an increase in plasma vitamin E levels which may saturate the tissue providing this protection. The result of vitamin E in the tissue is greater LPL activity which lowers triglyceride levels in the E rats when compared to the D rats.
INFLUENCE OF DIETARY VITAMIN E ON PLATELET THROMBOXANE $\Delta_2$
AND VASCULAR PROSTACYCLIN ($PGI_2$) IN RABBIT

INTRODUCTION

Vitamin E deficiency symptoms in rabbit are a nutritional muscular dystrophy accompanied by an invasion of macrophages into the muscles and an increase in lysosomal enzyme activity (180, 246). Scott in his review on vitamin E has stated that vitamin E deficiency symptoms are secondary to the widespread nonspecific damage produced by peroxidative chain reactions (270). Platelet function and platelet prostaglandin production are known to be altered by free radical scavengers and reducing agents (76, 77). Vitamin E and vitamin E quinone in high doses has been shown to be almost equally effective in blocking aggregation (249). This may be due to the surfactant properties of vitamin E rather than antioxidant properties (245, 249, 250). However, vitamin E supplementation in diets has demonstrated that vitamin E-deficient rabbit platelets release more $^{14}$C-arachidonic acid from phospholipids than vitamin E-normal rabbit platelets (244). This increased release indicates a more active phospholipase. Phospholipase activity requires disulfide bridges (271) and vitamin E
deficiency may activate -SH groups to form disulfide bridges which increase the phospholipase activity (90). Influence of vitamin E on cyclic AMP may alter phospholipase activity. Steiner et al. have shown that vitamin E inhibits platelet phosphodiesterase (249) allowing cyclic AMP to accumulate.

Vitamin E inhibits platelet aggregation and release when added in diet or in vitro (77,249,272,273). However vitamin E in vivo seems to be a better inhibitor of aggregation and release than in vitro (77,270) indicating in vitro vitamin E may not be as available as in vivo vitamin E. This may be due to a specific carrier of vitamin E which carries vitamin E to deeper membranes in cells and distributes it equally (274). In vitro inhibition of aggregation requires millimolar concentrations of vitamin E while in vivo dietary supplementation of vitamin E inhibits at much lower levels when compared to vitamin E deficient states. Vitamin E inhibits aggregation due to ADP, epinephrine, collagen, thrombin and arachidonic acid (77,272,275,276). Hope et al. has demonstrated decreased prostaglandin synthesis in serum of clotted rat blood due to vitamin E supplementation when compared to vitamin E deficient rats (243). Vitamin E inhibits in vivo prostaglandin synthesis during blood clotting and in in vitro stimulation of washed platelets from vitamin E supplemented rats when compared to vitamin E deficient rats (90). These results were also confirmed in vitamin E supplemented and deficient rabbits (277).
Diets and Animals

Vitamin E deficient diet was formulated by ICN Pharmaceutical Co. according to the method of Tappel and Zalkin (241). The vitamin E-supplemented (E) diet was the vitamin E-deficient (-E) diet supplemented with 1g of vitamin E-acetate per Kg diet. Male New Zealand white rabbits 4 to 6 weeks old of various weights were placed on +E and -E diets for 3 to 4 weeks until deficient animals reached a muscular dystrophic state. Animals were housed in individual cages and diets fed ad libitum.

Methods

Under no anesthesia blood was drawn from main ear artery with a 21 gauge scalp vein needle set into either EDTA anticoagulant (WPS) or citrate (PRP) when rabbits were kept for further analysis. When rabbits were sacrificed, the rabbits were anesthetized with ether. Chest was opened and blood drawn into EDTA anticoagulant from heart for WPS studies. The aorta was quickly removed, rinsed free from blood and cleaned on a Tris-NaCl ice block prepared the day before. The advential tissue was gently teased and cut off the aorta with fine tweezers and fine scissors. Care was taken so as not to disrupt the aortic tissue in the cleaning process.

Washed platelets (WPS) were prepared as described earlier. Platelet incubations in 13x100mm test tubes contained $1 \times 10^8$ platelets in 500 uL Krebs-Henseleit buffer.
Platelet incubations were performed at 37 C with thrombin, collagen, arachidonic acid and PAF. Thrombin was added in a Krebs-Henseleit buffer. Collagen was added in normal saline. Arachidonic acid was added in 5 uL volume, ethanol base. PAF was added in 2.8 uL volume, ethanol base. Incubations were terminated with 100 uL 1N HCL and PG's extracted with ether as described earlier. The RIA of the different PG's were performed as described earlier.

Aorta pieces were cut, gently blotted dry with cotton gauze and then weighed on a Mettler AC 88 top loading balance. Pieces were placed in 16x125mm test tube washed 5 minutes with Tris-NaCl buffer, pH 8.0. An appropriate volume of the Tris-NaCl buffer was added prior to incubation in 37 C with stirring. Appropriate aliquots were removed at timed intervals and pipetted into Tris-Albumin buffer pH 7.6 for RIA of 6-keto-PGF$_1$. Determination of vitamin E status in -E and +E rabbits plasma and platelets was performed as described earlier.

RESULTS

Effect of Diet: Rabbits and Plasma Vitamin E Levels

The -E and +E rabbits demonstrated a change in weight throughout the time course of the study as can be seen in Figure 49. The -E rabbits were on the average heavier than
Figure 49. Weight gain of vitamin E supplemented and deficient rabbits.
the +E in the beginning and as time progressed the -E rabbits weight gain leveled off while the +E rabbits continued to gain weight. The -E rabbits developed a dietary muscular dystrophy and eventually if allowed would continue to lose weight. Rabbits on vitamin E-deficient diets had significantly lower plasma vitamin E levels (-E=15.1+/−2.82 μg/mL (6): +E =56.0+/−6.38 μg/mL (4): p<0.01).

Platelet vitamin E levels were also significantly lower (-E<0.05 μg/10⁹ platelets and +E=0.56 μg/10⁹ platelets). These levels were achieved after rabbits were on diets for 3-4 weeks.

Platelet Prostaglandin Production

Previous studies with vitamin E deficient rabbits in Dr. Panganamala's laboratory by E.T. Gwebu demonstrated increased HETE production in platelets from -E rabbits over +E rabbits (278). In addition they found the HETE production to be dependent on platelet protein concentration in the incubation. The results of this study are based on platelet counts of 1x10⁸ platelets per incubation as described earlier. Work based on platelet count methods and RIA quantitation of TxB₂ from stimulated platelets demonstrated that -E platelet produced more TxB₂ when stimulated by thrombin and collagen (277). Figure 50 shows that -E platelet are more sensitive to low concentrations of thrombin (0.8 U/mL) than +E platelets. However when higher concentrations of thrombin (3.0 U/mL) were used stimulation
Figure 50. TxB₂ production from thrombin (0.8 U/mL and 3.0 U/mL, 10 minutes) challenged +E and -E rabbit platelets.
Figure 51. TxB2 production from bovine tendon collagen (720 µg/mL) stimulated E and -E rabbit platelets.
differences were abolished. Collagen stimulation 300 ug/ml of -E platelets caused a significant stimulation of TxB_2 production can be seen in figure 51. The fact that there were no significant differences in TxB_2 production from exogenous arachidonic acid at 2 μM or 30 μM indicates that there is no real difference between the cyclooxygenase-thromboxane synthetase enzyme systems (Figure 52).

Recently the earlier work was reexamined because of the development of PGE_2 and HETE antibodies. Due to the efforts of Nicholas J. Greco, a serotonin (5-HT) release assay was developed and which will be reported in greater detail by him at a later date. The results of -E and +E 5-HT release are reported here in part. Figure 53 shows that TxB_2 and HETE are significantly increased in -E platelets over +E platelets in thrombin stimulation (1 U/mL, 10 minute incubation). No differences in PGE_2 are detected between -E and +E. At a much lower concentration of collagen (120 μg/mL, Figure 54) than before (300 μg/mL, Figure 51) -E platelet still produce more TxB_2 as well as PGE_2 and HETE than -E platelet in a 10 minute incubation. Figure 55 demonstrates that the cyclooxygenase-thromboxane synthetase systems in the -E and +E platelets are essentially the same as was suspected earlier from Figure 50. However, at saturating levels of arachidonic acid (30 μM, 2.0 minutes incubation) HETE is significantly increased in -E platelets.
Figure 52. TxB₂ production from arachidonic acid (2 uM, 0.5 minutes; and 30 uM, 2 minutes) stimulated E and -E rabbit platelets.
Figure 53. TxB₂, PGE₂ and HETE production from thrombin (1 U/mL, 10 minutes) challenged +E and -E rabbit platelets.
Figure 54. TxB₂, PGE₂ and HETE production from calfskin soluble collagen (120 μg/mL, 10 minutes) stimulated +E and -E rabbit platelets.
Figure 55. TxB2, PGE2 and HETE production from arachidonic acid (2 uM, 0.5 minutes; and 30 uM, 2 minutes) stimulated +E and -E rabbit platelets.
over +E while the cyclo-oxygenase and thromboxane products are essentially equivalent. These results support the original observations by Gwebu (278). These results demonstrate vitamin E's role in modulating synthesis of prostaglandins and lipoxygenase products in platelets with thrombin, collagen, and arachidonic acid. Vitamin E deficiency produces a hyperactive platelet response which is reflected in increased TxB₂, HETE and PGE₂ in the case of collagen. These results imply that there is an increased supply of arachidonic acid from the phospholipids of the platelet membrane in -E platelets made available to the cyclooxygenase thromboxane synthetase and lipoxygenase enzyme systems.

Platelets release granule constituents upon stimulation with various agents. Rabbit platelets are very sensitive to PAF and release their granule packets at concentration as low as 1x10⁻⁹ M in washed platelets. Shaw et al. has reported that TxB₂ and 5-HT are released in parallel upon stimulation with PAF (173). Figure 56 shows the effect PAF has on TxB₂ and HETE production in -E and +E rabbit platelets. TxB₂ and HETE production are significantly increased in -E platelets at 1x10⁻⁶ M PAF, 0.94 mM Ca⁺² and 10 minute incubation. Vitamin E modulates aggregation responses to PAF in vitro and in vivo as can be seen in Figure 57. A control rabbit fed standard lab chow has platelets that are changed from irreversible aggregation to
Figure 56. TxB₂ and HETE production from PAF (1x10⁻⁶ M, 0.94 mM Ca²⁺, 10 minutes) stimulated +E and -E rabbit platelets.
Figure 57. Aggregation of control rabbit PRP with in vitro vitamin E; in vivo +E and -E rabbit PRP to PAF at (1x10^-8 M and 1x10^-7 M).
reversible by preincubation of platelets with 1.3 mM vitamin E. The vitamin E supplemented and deficient rabbits PRP's aggregated differently to PAF with -E PRP being more active at $1 \times 10^{-8}$ M and $1 \times 10^{-7}$ M. This hyperactivity in -E PRP is reflected not only in aggregation but also in % 5-HT release as can be seen in Figure 66. PRP is much less sensitive to PAF than WPS. WPS respond differently to PAF in the presence of 0.94 mM Ca$^{+2}$. In a single pair of -E and +E rabbit WPS preparation 5-HT release was examined with and without Ca$^{+2}$ (0.94 mM). Figure 59 shows that -E WPS release more 5-HT than +E WPS without Ca$^{+2}$ and also that 0.94 mM Ca$^{+2}$ greatly reduces the differences in 5-HT release in 2.0 minute incubations. Vitamin E then clearly modulates platelet function in release mechanisms as well as prostaglandin and HETE production.

It has been demonstrated that platelet function is modulated by vitamin E. Arterial prostaglandin production should also be affected by vitamin E status. Table 2 shows that -E rabbit thoracic aorta releases more PGI$_2$ as measured by 6-keto-PGF$_{1a}$ than +E rabbit thoracic aorta. Contrary to this observation Chan and Leith (248) showed decreases in arterial PGI$_2$ production from -E rabbits. This difference in results may be explained by the way PGI$_2$ production per tissue weight is expressed. In this study wet tissue weight was used to calculate 6-keto-PGF$_{1a}$ production per mg tissue weight. However, Chan and Leith used dry delipidized and
Figure 58. Percent 5-HT release from +E and -E rabbit platelet-rich-plasma in response to increasing PAF concentration challenges.
Figure 59. Percent 5-HT release from +E and -E rabbit washed-platelet-suspensions in response to PAF with and without 0.94 mM Ca\(^{+2}\).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>60 min</th>
<th>90 min</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-E Rabbit Thoracic Aorta(4)a</td>
<td>2.38 +/- 0.36</td>
<td>2.88 +/- 0.40</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+E Rabbit Thoracic Aorta(4)</td>
<td>1.35 +/- 0.4</td>
<td>1.65 +/- 0.15</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*a number of animals*
deendothelialized arterial tissue to express their 6-keto-PGF$_{1a}$ production per mg tissue weight. Here -E rabbit aorta tissue showed an increase in 6-keto-PGF$_{1a}$ production while -E rat aorta tissue has demonstrated a decrease in 6-keto-PGF$_{1a}$ production (90). These differences may be explained by the time dependency of the two animal models. Rabbits are made deficient in 3-4 weeks and cannot survive much longer than this time.

In order to observe lipid peroxide effects on PGI$_2$ synthesis the rats had to be maintained for 10-12 weeks on -E diet. Lipid peroxides formed may require to be present in circulating lipoproteins for a prolonged time in order to decrease endothelial PGI$_2$ synthesis (185). So in a shorter time period of vitamin E depletion, the vascular wall may even produce more PGI$_2$ in order to compensate for the effects of elevated TxA$_2$ in -E animals. In example, genetically hypertensive rats produce more PGI$_2$ compared to normotensive animals (279).
INFLUENCE OF DIETARY CHOLESTEROL ON PLATELET AND VASCULAR PROSTAGLANDIN METABOLISM IN RABBIT

INTRODUCTION

Increased platelet prostaglandin production has been associated with hypercholesterolemia since the late 1970's. The in vitro studies of Shattil and Cooper (201, 202, 203) have established the effect of exogenous cholesterol on developing cholesterol-rich hyperactive platelets while cholesterol-poor platelets demonstrate increased stability. However, they were unable to increase human platelet cholesterol content with human plasma LDL fractions (203). Thus in hypercholesterolemia the increase in plasma cholesterol does not directly affect the platelets. The platelet may pick up cholesterol at a different point in its life span. The megakaryocyte may be the source of active cholesterol incorporation in the platelet (280, 281, 282). The means by which the platelet obtains cholesterol may make a difference in platelet function. In 1979 Tremoli demonstrated a direct correlation between serum cholesterol levels and platelet production of TxB₂ in response to 20 uMol/L arachidonic acid in Type IIa hypercholesterolemic patients (204). Stuart et al. claimed that in vitro incorporation of cholesterol causes an increase in the release of arachidonic acid from phospholipids of
cholesterol-rich platelets (205). While most investigators have reported on the enhanced activity of platelets from hypercholesterolemic patients and animals, other investigators have pointed out that this may not always be the case.

Gryglewski et al. (92) claim that arachidonic acid metabolism to TxB₂ in platelets from atherosclerotic rabbits was not increased until 3 months on diet. Owen et al. have reported on inversely related platelet C/PL with aggregation results in patients with liver disease (209). It is possible in this case the decrease in platelet response was due to a loss of platelet arachidonic acid. O'Brien also reported on exhausted platelets which continue to circulate with only a portion of their original activity (210). Clearly there are exceptions to the rule that platelets from hypercholesterolemic patients and animals are hyperactive.

The reports of a cholesterol effect on aortic prostacyclin have also been inconsistent. Voss et al. recently reported on increase PGI₂ production in rabbits on cholesterol diets (283, 284). Dembinski-Keic et al. has reported on PGI₂ production in rabbit aorta which was decreased (214) and either no change or was fully recovered after cholesterol feeding (215). Gryglewski et al. reported on an initial loss then recovery of aortic PGI₂ synthesis after cholesterol feeding in rabbits (92).
It was with these works in mind that the study of 2%, 1% and 0.1% cholesterol fed rabbits was undertaken. Historically the 2% cholesterol fed rabbit model was used to study the influx of cholesterol lipid in the rabbit aorta. This is a very high dose of cholesterol which develops plague in a short time. Tremoli et al. have claimed to have fed 2% cholesterol for 1 month to rabbits in order to study platelet function in hypercholesterolemic rabbits (285,286). Other investigators have studied the process of plague formation with respect to atherogenic diets with and without cholesterol (287). Gryglewski and Dembinski-Keic use 1 gram cholesterol with 3 gram olive oil per day in order to make rabbits hypercholesterolemic (92, 214, 25). For these reasons a 1% cholesterol diet was used to make rabbits hypercholesterolemic. Later in order to bring plasma cholesterol levels in line with those usually associated with human hypercholesterolemia a 0.1% cholesterol diet was used to make rabbits hypercholesterolemic.

**Diet and Animals**

Cholesterol diet 2%, 1% and 0.1% was formulated by ICN Pharmaceutica Co. Powdered cholesterol was pulverized with standard laboratory chow matched by batch and lot to make the atherosclerotic diets and control diets without cholesterol. Male New Zealand white rabbits 1.5-2.5 kg were housed individually and fed diet ad libitum.
Methods

Under no anesthesia blood was drawn from main ear artery with a 21 gauge scalp vein needle set into either EDTA anticoagulant for WPS preparation or citrate for PRP preparations. When rabbits were sacrificed, the rabbits were anesthetized with ether. Chest was opened and blood drawn from heart into EDTA anticoagulant for WPS studies. The aorta was quickly removed, rinsed free from blood and cleaned on a Tris-NaCl ice block prepared the day before. The adventitial tissue was teased and cut off with fine tweezers and fine scissors. Care was taken so as not to disrupt the aortic tissue during the cleaning process.

Washed platelets were prepared as described earlier. Platelet incubations in 13x100 mm test tubes contained $1 \times 10^8$ platelets in 500 uL Krebs-Henseleit buffer. Platelet incubations were performed at 37 C with thrombin, collagen, arachidonic acid and PAF. Collagen was added in normal saline. Arachidonic acid was added in 5 uL of ethanol. PAF was added in 2.8 uL of ethanol for WPS studies and 0.25% Albumin-Tris-NaCl pH7.4 for PRP aggregation and 5HT release studies. WPS incubations were terminated with 100 uL 1N HCl and PG's were extracted with diethyl ether (BHT added) as described earlier. RIA of the various PG's and HETE were performed as described earlier.
Aorta pieces were cut, gently blotted dry with cotton gauze and then weighed on a Mettler AC 88 top loading balance for wet tissue weight. Pieces were placed in 16x125 mm test tubes washed for 5 minutes with Tris-NaCl buffer, pH 8.0. An appropriate volume of the Tris-NaCl buffer was added prior to incubation at 37 C with stirring. Appropriate aliquots were removed at time intervals and pipetted into Tris-Albumin buffer pH 7.6 for RIA of 6-keto-PGF$_{1a}$ and PGE$_2$.

Determinations of cholesterol, triglyceride, MDA, vitamin E in plasma and tissues were performed as described earlier.

RESULTS

Effects of Diet on Rabbits

Control rabbits (C) and experimental rabbits (E) exhibited no change in weight gain or loss throughout the time course of the studies with any of the three diets. Physical changes such as corneal arcus, xanthoma and xanthelasma were apparent on 2% E and 1% E but not with 0.1% E in the time frame studies. The skin of rabbits on 2% and 1% cholesterol became very thick with diffuse interdermal cholesterol deposition. Plasma cholesterol were increased in 2% E, 1% E and 0.1% E over C plasma cholesterol (Table 3). Rabbits eat about 170 grams of lab chow per day.
<table>
<thead>
<tr>
<th></th>
<th>CHOLESTEROL mg/dL</th>
<th>TRIGLYCERIDE mg/dL</th>
<th>TIME ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>71 +/- 22.6 (8)</td>
<td>123 +/- 15.3 (8)</td>
<td>3 MONTHS</td>
</tr>
<tr>
<td>2% E</td>
<td>1916 +/- 184 (8)</td>
<td>114 +/- 27.6 (8)</td>
<td>&quot;</td>
</tr>
<tr>
<td>C</td>
<td>55 +/- 10.6 (5)</td>
<td>125 +/- 14.7 (5)</td>
<td>4 MONTHS</td>
</tr>
<tr>
<td>1% E</td>
<td>1202 +/- 163 (5)</td>
<td>55 +/- 11.2 (5)</td>
<td>&quot;</td>
</tr>
<tr>
<td>C</td>
<td>100 +/- 10 (4)</td>
<td>244 +/- 68 (4)</td>
<td>2 WEEKS</td>
</tr>
<tr>
<td>0.1% E</td>
<td>183 +/- 39 (4)</td>
<td>256 +/- 35 (4)</td>
<td>&quot;</td>
</tr>
<tr>
<td>1% E</td>
<td>1773 +/- 168 (4)</td>
<td>314 +/- 86 (4)</td>
<td>&quot;</td>
</tr>
<tr>
<td>C</td>
<td>78 +/- 14 (4)</td>
<td>104 +/- 25 (4)</td>
<td>4 WEEKS</td>
</tr>
<tr>
<td>0.1% E</td>
<td>326 +/- 44 (4)</td>
<td>142 +/- 35 (4)</td>
<td>&quot;</td>
</tr>
<tr>
<td>1% E</td>
<td>2237 +/- 126 (4)</td>
<td>226 +/- 98 (4)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Although rabbits were fed chow *ad libitum* the maximum dispensed per day was 250 grams. This meant that 2%E could ingest 5 grams of cholesterol per day: 1%E-2.5 grams/day, and 0.1%E-0.25 gr/day. As can be seen from Table 3 2%E rabbits have a high plasma cholesterol at 3 months feeding. The 1%E rabbit obtained equally high plasma cholesterols at 2 and 4 weeks but by 3 months they adjust to lower levels of plasma cholesterol. The 0.1%E rabbits cholesterol values are twice control rabbit at 2 weeks which increases to 4 times control values at 4 weeks. Triglycerides are unremarkable except for 3 month 1%E rabbits which are significantly lower than C rabbits. The result of feeding these levels of cholesterol is a profound altering of platelet prostaglandin metabolism, while vascular prostaglandin metabolism is not as dramatically altered. The lipoprotein profiles of C, 1%E and 0.1%E are shown in Figure 60. As can be seen 1%E profile is more intense indicating increased levels of lipid in all fractions but basically a normal electrophoretic pattern.

**Other Measured Parameters of Control and Cholesterol Rabbits**

Because the 2.0% cholesterol rabbit model was new to the research in Dr. Panganamala's laboratory many other tests were performed simply to better characterize the rabbit model. This information is collected in Table 4. Plasma vitamin E is higher in 2.0%E rabbits. But vitamin E content of the platelets is significantly lower. These results are
Figure 60. Lipoprotein electrophoresis profiles of control (C), 0.1%E and 1%E rabbit plasma; origin to the left, migration to the right.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>2.0% E</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E plasma (ug/mL)</td>
<td>4.48 +/- 0.35(12)</td>
<td>21.8 +/- 1.86(12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>platelet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ng/10⁹ platelets)</td>
<td>186 +/- 27  (5)</td>
<td>77 +/- 17     (5)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Cholesterol plasma (mg/mL)</td>
<td>66.2 +/- 6.5  (12)</td>
<td>2010 +/- 134 (12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ug/10⁹ platelets)</td>
<td>47.4 +/- 2.56 (7)</td>
<td>78 +/- 6.09   (6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>reticulocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ug/10⁹ cells)</td>
<td>95.3 +/- 4.51 (5)</td>
<td>165.2 +/- 13.6 (5)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Platelet size (microns)</td>
<td>4.4 +/- 0.257 (6)</td>
<td>3.0 +/- 0.063 (6)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
in agreement with the diabetic rat model which has lower platelet vitamin E (87) as a result of hyperlipemia while having higher plasma vitamin E levels than the control rat. The plasma cholesterol content is increased as mentioned earlier (Table 3). The platelet cholesterol content is increased which may be the causative factor in displacing vitamin E from the platelet. Reticulocyte cholesterol content is increased as would be expected. Platelet size has been used as measure of platelet age (288). Older platelets are smaller than younger platelets. Cholesterol platelets are significantly smaller as measured by a Coulter Counter platelet profiler.

The 1% cholesterol rabbit platelets were analyzed for vitamin E content with respect to time on diet. Control rabbit platelets were analyzed at the same time. The results can be seen in Figure 61. However, there is a discrepancy between the results obtained in the 2% rabbit studies and the 1% rabbit studies. The values obtained in the 2% rabbit study was performed by HPLC as described earlier except that a one point calibration point was used as an external standard. This one point was set at such a level as to fall between our lowest samples and our highest samples. Plasma contains about 20 times more vitamin E per injection than platelets therefore the results at the low end of absorbance were artificially inflated. The 1% rabbit study was performed with actual vitamin E standards throughout the
Figure 61. Vitamin E content of control (C) and 0.1% E rabbit platelets with respect to time (ng/10⁹ platelets).
entire range and standard curves were prepared for the low end and the high end of the vitamin E ranges. Analysis of vitamin E in this manner yielded greater control at low levels of vitamin E and at high levels of vitamin E.

Platelet Studies

In the 2%E rabbit model thrombin, collagen and arachidonic acid were used for platelet challenges. Anti-TxB$_2$, Anti-PGE$_2$ and Anti-6-keto-PGF$_{1a}$ were the only RIA antibodies developed and in possession at the time this part of the research was being done. Medium thrombin (1.6 U/mL) stimulation of washed platelets yielded low TxB$_2$ and PGE$_2$ in 2%E when compared to C (Figure 62). However, in low thrombin 0.8 U/mL no differences in TxB$_2$ and PGE$_2$ production were detected (Figure 63). Bovine tendon collagen at two levels of concentration 764 ug/mL and 382 ug/mL were used to challenge C and 2%E platelets. TxB$_2$ production was significantly decreased in 2%E platelets when challenged with 764 ug/mL and 384 ug/mL (Figure 64). WPS were challenged with saturating levels (30 uM) and stimulating levels (2 uM) arachidonic acid. Analysis of 30 uM (2.0 minutes) arachidonic acid challenge revealed a decrease in TxB$_2$ production with no change in PGE$_2$ production when compared to C (Figure 65). Stimulating levels at 2 uM arachidonic acid (0.5 minutes) demonstrated a significant decrease in TxB$_2$
Figure 62. TxB$_2$ and PGE$_2$ production from thrombin (1.6 U/mL, 10 minutes) stimulated rabbit platelets.

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Figure 63. TxB₂ and PGE₂ production from thrombin (0.8 U/mL, 10 minutes) stimulated rabbit platelets.
Figure 64. TxB₂ production from bovine tendon collagen (382 and 764 μg/mL, 70 minutes) stimulated rabbit platelets.
Figure 65. PGE$_2$ production from bovine tendon collagen (382 and 764 µg/mL, 70 minutes) stimulated rabbit platelets.
production in 2%E (Figure 66). No PGE\textsubscript{2} analysis was performed of 2 uM arachidonic acid stimulated platelets due to low levels of PGE\textsubscript{2} production in 0.5 minutes. Analysis of aortic PGI\textsubscript{2} production as measured by 6-keto-PGF\textsubscript{1\alpha} revealed that PGI\textsubscript{2} production in the 2%E rabbits were significantly elevated over C at 30, 60 and 90 minute incubations (Figure 67).

The 2%E rabbits could have ingested up to 5 grams of cholesterol per day. This is an extremely high dose of cholesterol which may be toxic. The physical changes as well as the biochemical changes in these animals bare this out. The fact that there were lower TxB\textsubscript{2} and PGE\textsubscript{2} in some of the tests may possibly be explained by the fact that CO and TxS are membrane bound enzymes. The enzyme activities of CO and TxS could very well be altered by an increase in the membrane cholesterol. Alternatively there could be an overall loss of arachidonic acid as Kawaguchi et al. demonstrated in rabbits (206). Pitas et al. (289) fed high saturated fats and cholesterol to dogs and discovered an overall drop in arachidonic acid from PC, PI, PS, PE and plasmologen PE. Oleic acid synthesis is increased in response to cholesterol feeding (290). The increase in oleic acid synthesis is a direct result of an increase in the number of oleate acyl-CoA acceptors due to cholesterol feeding. Cholesterol becomes a sink for fatty acids in forming cholesterol esters. LCAT activity is increased which removes fatty acids from the
Figure 66. TxB₂ and PGE₂ from arachidonic acid challenged rabbit platelets (30 μM, 2 minutes and 2 μM, 0.5 minutes).
Figure 67. 6-keto-PGF$_{1a}$ production from control (C) and 2%E rabbits.
phospholipids. They in turn circulate back to the liver and are reesterified. When cholesterol is in such excess as in the 2%E rabbits an overwhelming supply of acyl acceptors may be available for fatty acids resulting in a drain on membrane fatty acids as in the canine (289) and rabbit platelets examples (206).

Increases in aortic PGI$_2$ synthesis in cholesterol fed rabbits have been demonstrated by Voss et al. (283, 284). Voss claims that plaques can make more PGI$_2$ in response to cholesterol in order to compensate for the induced stress. This is similar to results obtained by Gryglewski et al. (92) who reported an initial loss and then recovery of PGI$_2$ synthesis in response to cholesterol feeding.

The 2%E rabbits yielded PGI$_2$ results that were in conflict with investigators mentioned earlier. The increase in PGI$_2$ as a result of feeding 2% cholesterol for 3 months may have positioned the PGI$_2$ synthesizing capacity in the recovery period of Gryglewski (92). To investigate this hypothesis and to slow the influx of cholesterol into the rabbits a 1% cholesterol supplemented chow was chosen. Prostacyclin production in rabbits is very low. The ability of aorta to make PGI$_2$ in response to exogenous arachidonic
acid was also explored by adding 27 uM arachidonic acid to the incubation. Rabbits were sacrificed at 2, 4, and 8 weeks. The results on PGI$_2$ production of 1% cholesterol feeding over this time span are shown in Figure 68. As can be seen no differences were detected at 2, 4 or 8 weeks either with endogenous or exogenous arachidonic acid. These results complicated the research in that neither a decrease nor an increase could be demonstrated in the hypercholesterolemic rabbits. Large visible plaque formation was observed at 4 and 8 weeks in 1% rabbit aorta. Small plaque could be seen only rarely in 2 week 1% rabbit aortas. Another set of rabbits were placed on control diets and cholesterol diets to be sacrificed at 1 month and 5 months. The results of this study can be seen in Figure 69. Clearly there is an effect with long term cholesterol feeding.

There is a significant drop in aortic PGI$_2$ synthesis as measured by 6-keto-PGF$_{1 \alpha}$-RIA. The 1%E rabbits were severely affected by cholesterol feeding. Physical changes were apparent in 5 month 1%E rabbits. They had large diffuse areas of plaque throughout their aorta. Their hearts had diffuse areas of white lipid, deposited in the muscle which did not have the consistency of normal fat. The aorta was extremely thick and stiff when compared to the control aorta. The major organs had visible evidence of cholesterol deposition in the liver, lung, kidney and skin. Corneal arcus was quite predominant. The effect of long term 1%
Figure 68. 6-keto-PGF₁α production from control (C) and 1%E rabbit aorta in 60 minutes with and without arachidonic acid (27 μM).
Figure 69. 6-keto-PGF$_{1\alpha}$ production from control and 1% E rabbit aorta at 4 weeks and 5 months (60 minute incubations).
cholesterol feeding did decrease PGI$_2$ synthesis in aortic tissue. These results indicate that a recovery as proposed by Gryglewski (92) is not occurring in this animal model. There was no initial loss of PGI$_2$ production at 2, 4 and 8 weeks. These results are in agreement with Dembinski-Keic (215). The 2%E rabbits demonstrated an increase in PGI$_2$ synthesis and indicates that the results from that model is in agreement with Voss et al. (283, 284).

The 1%E rabbits were examined at 4 months for the effects of diet on platelet function. By this time HETE-RIA was firmly established in the laboratory and afforded a fast, simple means of assessing platelet LO pathway. In the 2%E rabbit studies 1.6 U/mL thrombin was the highest concentration used. In the 1%E rabbit studies 3.0 U/mL thrombin was added to examine the effects of high thrombin challenge on platelet function. Bovine tendon collagen preparation was replaced by calfskin soluble collagen preparation for 600 ug/mL collagen challenge. Calfskin soluble collagen provided high concentrations of collagen in small volumes and stimulated the platelets to produce more TxB$_2$ and PGE$_2$ than bovine tendon collagen preparations. Thrombin at 3.0, 1.6 and 0.4 U/mL in 10 minutes incubation with 1%E platelets produced significantly less TxB$_2$ than C platelets (Figure 70). PGE$_2$ production was equivalent between C and 1%E platelets at all three concentrations of thrombin (Figure 71). In Figure 70 1%E platelets challenged
Figure 70. TxB₂ production from thrombin stimulated control and 1%E rabbit platelets.
Figure 71. PGE₂ production from thrombin stimulated control and 1% E rabbit platelets.
with 1.6 U/mL thrombin for 10 minutes had significantly less TxB₂ and PGE₂. It appears that decreasing dietary cholesterol from 2% to 1% decreased the inhibition of cholesterol on PGE₂ synthesis (CO) but not TxB₂ synthesis (TxS).

HETE produced by 1%E platelets in response to 3.0, 1.6 and 0.4 U/mL thrombin are significantly less than C platelets (Figure 72). When thrombin concentrations are increased from 0.4 to 3.0 U/mL in 10 minute incubations more TxB₂, PGE₂ and HETE are produced at each level of thrombin in response. The C platelets produce more TxB₂ and HETE than 1%E platelets at all 3 concentrations of thrombin. PGE₂ is produced in equivalent amounts in C and 1%E platelet at each thrombin concentration. In contrast less PGE₂ was produced by 2%E platelets than C platelets challenged with 1.6 U/mL but not 0.4 U/mL. It appears that decreasing dietary cholesterol from 2% to 1% has removed some effect on CO but not TxS enzymes when platelets are challenged with thrombin.

The calfskin soluble collagen (CSSC) challenge at 600 ug/mL (Figure 73) elicited much greater TxB₂ production than bovine tendon collagen at 382 and 764 ug/mL (Figure 64). PGE₂ production in response to CSSC was unchanged from that produced by bovine tendon collagen (BTC). TxB₂ and HETE were significantly reduced in 1%E platelets when challenged with 600 ug/mL CSSC. PGE₂ production was not significantly different in response to 600 ug/mL CSSC.
Figure 72. HETE production from thrombin stimulated control and 1%E rabbit platelets 10 minute incubations.
Figure 73. TxB₂, PGE₂ and HETE from calfskin soluble collagen stimulated control and 1% rabbit platelets (10 minutes).

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Arachidonic acid challenge at 30 uM for 2.0 minutes produced basically the same results in 1%E as in 2%E. TxB₂ from 1%E is significantly reduced when compared to C (Figure 74). PGE₂ from 1%E and C are produced in equivalent amounts (Figure 74). However, HETE from 1%E TxB₂ is significantly reduced when compared to C (Figure 74). When platelets are challenged with 2 uM arachidonic acid TxB₂ is significantly reduced in 1%E compared to C (Figure 75). This is similar to results obtained from C and 2%E platelets with 2 uM arachidonic acid (Figure 66). PGE₂ and HETE are produced by 1%E and C platelets with no significant differences demonstrated (Figure 75).

Aggregation Studies

Control and 1%E platelets were isolated for aggregation studies. 1%E plasma with cholesterol values reaching 2000 mg/dL make it an unsuitable medium to follow aggregation. Therefore washed platelets were prepared as described earlier but the final resuspension of platelets in Krebs-Henseleit buffer was altered to include 10% citrated plasma from a control rabbit. This procedure provides all the necessary clotting cofactors in a sufficient level to allow aggregation to take place. The interference by hypercholesterolemic plasma is removed allowing aggregation to be followed by transmittance. Washed platelets behave differently than PRP preparations in aggregation. They may lose the ability to respond as the time from isolation increases.
Figure 74. TxB₂, PGE₂ and HETE from 30 μM arachidonic acid challenged control and 1%E rabbit platelets (2 minutes).
Figure 75. TxB2, PGE2 and HETE from 2 μM arachidonic acid stimulated control and 1%E rabbit platelets (0.5 minute).
From Figure 76a it can be seen that although C and 1%E platelet aggregate to the same degree it takes 1%E platelets about 0.8 minutes longer to go to complete aggregation in response to 0.4 U/mL thrombin. Washed platelets do not respond to ADP as well as PRP preparations. In Figure 76b 180 uM ADP produces the same degree of aggregation in the control as 15 uM ADP (Figure 76c). The difference between C WPS and 1%E WPS can also be seen in that 1%E WPS preparations are almost completely non-responsive to ADP at either concentration. ADP at 180 uM yields 33% in C 15% in 1%E. ADP at 15 uM yields 35% in C and 5% in 1%E. This indicates that 1%E WPS are less responsive than C WPS to aggregating agents thrombin and ADP. The high levels of plasma cholesterol may cause the membrane properties to change in the platelet and may alter the receptors to thrombin and ADP.

Time Study Platelet Prostaglandins and PAF

During the time that aortic PGI₂ synthesis was being explored with respect to time on diet. Platelets were also analysed. The time study with platelet prostaglandins met with limited success. The reasons for such results are many. One source is that small groups of rabbits were ordered at different times which resulted in a mixed group of different age and weights. Each group had different basal levels of platelet prostaglandin production. However, enough information was extracted to allow a plot of results in the
Figure 76. Aggregation profiles of control and 1% E rabbit WPS in response to thrombin and ADP.
form of 1%E results as a percentage of control results (Figure 77). The handling of the data in this way removes the influence of group variation and to some extent day to day variation. The rabbits used for aortic PGI\textsubscript{2} synthesis were sacrificed making repeat analysis of platelet prostaglandins impossible. By removing blood from the main ear artery platelets may be tested many times from the same animal. The information received for such a study could characterize the platelet prostaglandin response with less variation. In addition purchasing rabbits in one large order insures a more homogeneous group. The rabbits could then be divided up with respect to litter mates, weight, date of birth and date of weaning which provides considerably more control over the animal population. As can be seen from Figure 77 1%E WPS were inhibited 45\% at 2 weeks, 45\% at 4 weeks and 68\% at 8 weeks. The inhibition is attributed to dietary cholesterol as has been seen before with 2%E and 1%E. Thus far all platelet stimulating agents tested resulted in 2\% and 1%E platelets being less reactive than control platelets as measured by prostaglandin production.

**Platelet Cyclic AMP**

Cyclic AMP was analyzed in control and 1%E platelets by the methods described earlier. Platelets were challenged for 30 seconds prior to halting synthesis of cyclic AMP with 1.0 mL of 5\% trichloracetic acid. The platelet challenges were:

(1) Tris-NaCl buffer pH 7.2 - measures basal levels of
Figure 77. % Inhibition of control TxB₂ in response to thrombin 3.0 U/mL, 10 minutes.
cyclic AMP;

(2) 0.31 uM PGE$_1$ - measures stimulated levels of cyclic AMP reflects both synthesis and degradation of cyclic AMP by phosphodiesterase;

(3) IBMX at 25 uM for 5 minutes preincubation on ice to inhibit phosphodiesterase prior to adding Tris-NaCl buffer pH 7.2 - reflects basal synthesis without degradation; and

(4) IBMX (25 uM, 5 minutes on ice) prior to adding 0.31 uM PGE$_1$ - measures cyclic AMP synthesis without degradation.

The results of the analysis can be seen in Figure 78. The LSE platelets have a decreased ability to synthesize cyclic AMP with and without IBMX which indicates that the problem is not an extra active phosphodiesterase. It is significant that LSE platelets are unable to synthesize cyclic AMP in that if the platelets were to be activated then the natural antiaggregatory prostaglandin PGI$_2$ might not be able to reverse the activation process. Platelets which are unable to respond to antiaggregatory stimuli because of a loss in the ability to synthesize cyclic AMP become prothrombotic platelets relative to platelets which do respond to PGI$_2$. 

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Figure 78. Cyclic AMP production in control and 1% E rabbit platelets at 4 weeks feeding in response to the indicated stimuli.
PAF and Hypercholesterolemia in Rabbits

During the time that the 4 month 1%E study was being conducted PAF was made commercially available by Calbiochem-Behring Corp. (San Diego, California). As mentioned earlier, PAF requires exogenous Ca\(^{+2}\) to be present in WPS in order to initiate prostaglandin production. Calcium Chloride-\(2\text{H}_2\text{O}\) was added to Krebs-Henseleit buffer to make calcium 0.94 mM which approximates ionizable levels of Ca\(^{+2}\) in plasma. In addition this level of Ca\(^{+2}\) barely initiated TxB\(_2\) and HETE production when platelets were simply stirred without the aggregating agents. Addition of aggregating agents would induce platelet prostaglandin and HETE production which in turn would be more attributable to the agent than the nonspecific effects of calcium.

Preliminary studies with PAF demonstrated than 1%E platelets were more sensitive to PAF than control platelets. This was found to be true in one minute incubations (Figure 79 and 80) as well as 10 minute incubations (Figure 81 and 82). Short incubation times demonstrate CO-TxS activity more than LO activity as was seen earlier with arachidonic acid in Figures 13 and 14. PAF incubations with calcium alter the dominance of CO-TxS over LO in short incubations times as was seen earlier in Figure 16. However, if one compares the rise in HETE to the rise in TxB\(_2\) from 1 to 10 minute incubations it can be seen that HETE quadruples its one minute incubation value while TxB\(_2\) only doubles. So generally speaking the
Figure 79. TxB₂ production from control and 1%E rabbit platelets from PAF, calcium 0.94 mM, 1 minute incubation.
Figure 80. HETE production from control and 1% E rabbit platelets from PAF, calcium 0.94 mM, 1 minute incubation.
Figure 81. TxB₂ production from control and 1%E rabbit platelets for PAF, calcium 0.94 mM, 10 minute incubations.
Figure 82. HETE production from control and 1% E rabbit platelets from PAF, calcium 0.94 mM, 10 minute incubations.
longer the incubation time the more the prostaglandin and HETE values will reflect the LO pathway. Additional studies which are yet to be fully explored are the synergistic effects of PAF with other aggregating agents. PAF may have a synergistic role with collagen in activating platelets.

Thrombin is a very potent platelet activator and in the experiments so constructed overwhelms the platelet such that any action which may be attributable to PAF is masked.

Extremely low levels of thrombin in the presence of Ca$^{+2}$ and PAF may hold promise. Table 5 shows that 1%E platelets produce nearly equal amounts of TxB$_2$ and almost 2 times more HETE when calcium is present in 10 minute incubations.

Addition of PAF with thrombin does not change this pattern much except that 1%E is decreased when paired to C which may or may not be significant. Collagen at 120 µg/mL, 5 times lower than in prior 10 minute incubations yields surprising results. TxB$_2$ production is increased 45-60% when PAF is present with collagen. HETE is not only increased when PAF is present but 1%E produces more HETE with collagen alone as well as when PAF is present. This appears to be true with collagen in 2 minute incubations as well. In 2 minutes C is greater than 1%E with respect to TxB$_2$ and HETE is greater in 1%E than C which is consistent with the 10 minute data. PAF does not seem to play much of a synergistic role with 0.4 U/mL thrombin at 2 minutes either. Thus hypercholesterolemic platelets in the presence of calcium produce more TxB$_2$ and
Table 5

Calcium 0.94 mM, Krebs-Henseleit, O₂/CO₂(95/5), (mean of 2)

<table>
<thead>
<tr>
<th></th>
<th>TxB₂ (pmoles/10⁸ platelets)</th>
<th>HETE (pmoles/10⁸ platelets)</th>
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</thead>
<tbody>
<tr>
<td><strong>10 minute incubations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>246</td>
<td>1158</td>
</tr>
<tr>
<td>0.4 U/mL Thrombin</td>
<td>242</td>
<td>1905</td>
</tr>
<tr>
<td>0.4 U/mL Thrombin + 1x10⁻⁸M PAF</td>
<td>264</td>
<td>179</td>
</tr>
<tr>
<td>120 ug/mL collagen</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>120 ug/mL collagen + 1x10⁻⁸M PAF</td>
<td>139</td>
<td>151</td>
</tr>
<tr>
<td><strong>2 minute incubations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 U/mL Thrombin</td>
<td>233</td>
<td>183</td>
</tr>
<tr>
<td>0.4 U/mL Thrombin + 1x10⁻⁸M PAF</td>
<td>236</td>
<td>188</td>
</tr>
<tr>
<td>120 ug/mL Collagen</td>
<td>53</td>
<td>51</td>
</tr>
<tr>
<td>120 ug/mL Collagen + 1x10⁻⁸M PAF</td>
<td>82.5</td>
<td>110</td>
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</tbody>
</table>
HETE with collagen and collagen-PAF stimulation. In addition HETE is produced more in thrombin stimulated E platelets than C when calcium is present.
Low Cholesterol Diet Studies and Platelet $\text{TxB}_2$ and HETE

Rabbits were purchased as before. A low level cholesterol diet of 0.1% (w/w) cholesterol supplemented chow was formulated by ICN as well as 0.0% and 1.0% (w/w) cholesterol supplemented chows. Rabbits were fed diets ad libitum as before but for four weeks. At the end of this time, blood was withdrawn from main ear artery as described earlier into EDTA and citrate anticoagulants for WPS and PRP studies respectively. Platelet $\text{TxB}_2$ and HETE were measured as described earlier from WPS in the absence and presence of 0.94 mM $\text{Ca}^{2+}$ in Krebs-Henseleit buffer. WPS were challenged with thrombin, collagen, arachidonic acid and PAF. PAF-induced serotonin release studies were made possible by Nicholas J. Greco. Aortic prostacyclins were not studied due to lack of differences in prior studies.

RESULTS

Decreased $\text{TxB}_2$ from 1% E platelets in response to thrombin at 1.6 U/mL were confirmed in these experiments as can be seen in Figure 83. $\text{TxB}_2$ from 0.1%E platelets was not significantly different from C platelets while being significantly increased over 1.0%E platelets. A decrease in HETE production from 1.0%E platelets detected at 4 months feeding was not detected in this 4 week feeding period. This indicates that a time dependent alteration of platelet
Figure 83. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit platelets stimulated with thrombin 1.6 U/mL, 10 minutes.
function with cholesterol feeding may exist. The addition of 0.94 mM Ca\(^{+2}\) in WPS alters TxB\(_2\) and HETE profiles. In Figure 84 no significant differences in TxB\(_2\) and HETE production can be seen between C, 1%E and 0.1% E platelets when challenged with thrombin 1.6 U/mL in the presence of 0.94 mM Ca\(^{+2}\) from C platelets was 2.05 times TxB\(_2\) without Ca\(^{+2}\), while 1%E increased 2.65 times and 0.1%E increased 1.96 times. This may indicate that in 1%E platelets a reserve of arachidonic acid exists which can be stimulated preferentially which C and 0.1%E do not. Alternatively, these results may indicate that 1%E platelets are much more sensitive to Ca\(^{+2}\) than are C and 0.1%E which results in more arachidonic acid being released. In Figure 85 low levels of thrombin at 0.4 U/mL are used to challenge platelets. HETE is significantly greater from 1%E platelets than C. The HETE results from 0.1%E are highly variable making any conclusion impossible. However when this same level of thrombin is used in the presence of calcium the entire profile changes. Now 0.1%E platelets produce more TxB\(_2\) than 1%E while 1%E platelets produce more HETE than C and 0.1% E platelets (Figure 86). TxB\(_2\) is increased 6.16 times in C, 5.56 times in 1%E and 7.09 times in 0.1%E when Ca\(^{+2}\) is present. HETE is increased 11.55 times in C, 12.55 times in 1%E, and 11.30 times in 0.1%E. Thus TxB\(_2\) is stimulated more in 0.1%E than C and 1%E when Ca\(^{+2}\) is present. On the other hand HETE is stimulated more in 1%E than C or 0.1%E. There is a shift in increasing TxB\(_2\)
Figure 84. TxB$_2$ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with thrombin 1.6 U/mL, 10 minutes, calcium 0.94 mM.
Figure 85. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with thrombin 0.4 U/mL, 10 minutes.
Figure 86. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with thrombin 0.4 U/mL, 10 minutes, calcium 0.94 mM.
production from 0.1%E platelets to an increased HETE production from 1%E platelets when cholesterol in the diet is increased. High dietary cholesterol may inhibit the CO-TxS pathway as was seen before in 4 month studies. There is a relative drop in HETE from 0.1%E platelets which although not significantly lower than C platelets is significantly lower than 1%E platelets. One would expect that an increase in TxB2 would be accompanied by an increase in HETE. It could be that low dietary cholesterol perturbs the membrane just enough to enhance the CO-TxS pathway over the LO pathway.

**Effects of Length of Time on Diet and Thrombin in Platelet challenges**

A time study was undertaken to detect changes in platelet TxB2 production from thrombin challenges as a function of time on diet. As can be seen in Figure 87 there are no significant differences between C, 1.0%E and 0.1%E throughout the concentration range. However, in 4 weeks there is a distinct and significant drop in TxB2 production from 1.0%E platelets at 1.6 U/mL and 3.0 U/mL thrombin. When calcium was used with thrombin all TxB2 differences were lost. The 1%E rabbit had cholesterol values in excess of 1500 mg/dL at 2 weeks and 2000 mg/dL at weeks. The small increase in 0.1%E plasma from 183 mg/dL at 2 weeks to 326 mg/dL did not alter the 0.1%E platelets response to thrombin significantly in the time frame. Thus the high plasma levels of cholesterol in 1.0%E appear to have decreased 1%E
Figure 87. TxB$_2$ production from increasing concentrations of thrombin (10 minutes), at time feeding periods 2 weeks and 4 weeks.
Platelets ability to product TxB₂ in response to thrombin stimulation.

Collagen

Collagen challenges at 120 ug/mL result in TxB₂ and HETE being produced in greater amounts by 0.1%E platelets than C and 1%E (Figure 88). Collagen at 120 ug/mL with Ca⁺² barely activates C and 1%E platelets with 0.1%E platelets being more active. Previously 600 ug/mL collagen was used to stimulate platelets. This low collagen concentration was chosen to demonstrate the effects of Ca⁺². In Figure 89 collagen at 120 ug/mL with 0.94 mM Ca⁺² tremendously stimulates TxB₂ and HETE production in C, 1%E and 0.1%E. The profiles are different than before. TxB₂ is produced most by 0.1%E than 1%E over C platelets. But HETE is produced more by 1%E than C platelets. Although 0.1%E platelets produced less HETE than 1%E platelets the level of significance was p<0.10 which is not considered significant. This decrease although not significant may indicate that 0.1%E platelets preferentially metabolize arachidonic acid to TxB₂ at the expense of HETE, while 1%E platelets with a restricted CO-Txs pathway shunt released arachidonic acid through the LO pathway.

Arachidonic Acid

Exogenous arachidonic acid challenges circumvent the phospholipase release steps and examines the CO-Txs and LO pathways directly. As can be seen in Figure 90 the CO-Txs is
Figure 88. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with 120 µg/mL collagen, 10 minutes.
Figure 89. TxB$_2$ and HETE production from C, 1% E and 0.1% E rabbit WPS stimulated with 120 ug/mL collagen, 10 minutes and 0.94 mM calcium.
Figure 90. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with 2 uM arachidonic acid (0.5 minutes).
restricted in 1%E as reflected by the TxB2 values. HETE at 0.5 minutes is not made in significant amounts to adequately reflect the LO pathway. When Ca+2 is added (Figure 91). The differences in TxB2 production are abolished and HETE production increases slightly. Calcium stimulates platelets enough to make C, 1%E and 0.1%E behave in a similar manner in a short incubation time with low levels of exogenous arachidonic acid. High saturating levels of arachidonic acid, 30 uM, as seen in Figure 92 demonstrate that the CO-TxS system is inhibited by high dietary cholesterol (1%E) and that the inhibition is partially reflected in the HETE production even though not significantly different from C. However, when Ca+2 is present the Co-TxS and LO product production is altered (Figure 93). There is a decrease in TxB2 production from C platelets when Ca+2 is present. TxB2 production is 1%E platelets is slightly increased while 0.1%E remains unchanged. The HETE production is increased in all 3 but not to the same extent. C platelet HETE increases 2.25 times, 1%E, 4.12 times; and 0.1%E, 3.0 times. There is a tremendous increase in HETE production from 1%E platelets when challenged with 30 uM arachidonic acid and 0.94 mM Ca+2. When Ca+2 is present excess arachidonic acid may be shunted into the LO pathway even at the expense of TxB2 as in the case of the control platelets. When the CO-TxS enzyme system has some restriction or inhibition the TxB2 reaches maximal levels with excess arachidonic acid acted on by the LO
Figure 91. TxB₂ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with 2 μM arachidonic acid, 0.5 minute, and 0.94 mM calcium.
Figure 92. TxB$_2$ and HETE production from C, 1%E and 0.1%E rabbit WPS stimulated with 30 µM arachidonic acid (2 minutes).
Figure 93. TxB₂ and HETE production from C, 1% E and 0.1% E rabbit WPS stimulated with 30 µM arachidonic acid (2 minutes) and 0.94 mM calcium.
pathway. Platelets from hypercholesterolemic rabbits in the presence of Ca$^{+2}$ demonstrate increased arachidonic acid metabolism through the LO pathway as reflected in increased HETE production.

In conclusion platelets have been stimulated with thrombin, collagen and arachidonic acid in the absence and presence of 0.94 mM Ca$^{+2}$. Ca$^{+2}$ stimulates the platelets to produce more TxB$_2$ and HETE. Platelets from hypercholesterolemic rabbit exhibit increased arachidonic and metabolism when Ca$^{+2}$ is present. It appears that high dietary cholesterol causes a restriction in the CO-TxS which results in low TxB$_2$ production such as the low TxB$_2$ results from 1.6 U/mL thrombin without Ca$^{+2}$, and 2 μM arachidonic acid without Ca$^{+2}$. When these two tests are performed in the presence of Ca$^{+2}$ the differences in TxB$_2$ are abolished. HETE appears to be the main product in platelets which are activated in the presence of Ca$^{+2}$. HETE is increased in platelets where the CO-TxS pathway is restricted as in with 1%E platelets. Low dietary cholesterol results in rabbits with platelets which are primarily active in the CO-TxS pathway as reflected by the increase in TxB$_2$.

PAF and Hypercholesterolemia in Rabbits

Platelets from C, 0.1%E and 1%E were challenged with PAF at 1.0x10$^{-8}$M and 1.0x10$^{-6}$M in the presence of Ca$^{+2}$ 0.94 mM. PAF does not cause platelets to produce prostaglandins or
HETE in significant amounts in the absence of Ca\(^{+2}\). Calcium causes platelets to produce HETE in significant levels which were unattainable in early incubation times. However, CO-TxS activity through TxB\(_2\) production is reflected mainly in short incubation time even though HETE is increased above levels normally found in one minute. Longer incubation times reflect the LO pathway in the presence of Ca\(^{+2}\) as is expected even without calcium present.

One minute WPS incubations show TxB\(_2\) (Figure 94) and HETE (Figure 95) levels at background, 1x10\(^{-8}\) M and 1x10\(^{-6}\) M PAF. TxB\(_2\) is elevated in 1\%E platelets at both concentrations of PAF. HETE is elevated in 1\%E platelets at both concentrations of PAF as well. At short incubation times 0.1\%E platelets do not respond significantly different than C platelets. In the first examination of one minute incubations then 0.1\%E platelet do not appear to be different in their response to PAF than the control platelets. However, with longer incubation times (10 minute) and 1.0x10\(^{-6}\) M PAF 0.1\%E platelets produce more TxB\(_2\) than C platelets (Figure 96). In contrast 0.1\%E platelets do not produce significantly more HETE than control platelets (Figure 97). These results are consistent with other platelet challenges which indicate that 0.1\%E platelet prostaglandin response is through the CO-TxS pathway. The 1\%E platelets produce more TxB\(_2\) than C at 1.0x10\(^{-8}\) M and 1.0x10\(^{-6}\) M PAF. In short incubation times 0.1\%E platelets do
Figure 94. TxB$_2$ production from C, 0.1%E and 1%E rabbit WPS, 0.94 mM calcium with PAF, 1 minute.
Figure 95. HETE production from C, 0.1%E and 1%E rabbit WPS, 0.94 mM calcium with PAF, 1 minute.
Figure 96. TxB₂ production from C, 0.1% E and 1% E rabbit WPS, 0.94 mM calcium with PAF, 10 minutes.
Figure 97. HETE production from C, 0.1% E and 1% E rabbit WPS, 0.94 mM calcium with PAF, 10 minutes.
not respond differently than C platelets. In first
examination 0.1%E platelet do not appear to be any different
from C platelets with respect to PAF stimulation. However,
with longer incubation times of 10 minutes 0.1%E platelets
produce more TxB₂, but not HETE than C platelets with 1x10⁻⁶M
PAF. The 1%E platelets are very sensitive to PAF with respect
to TxB₂ and HETE at 1 minute and 10 minute incubations.

In conclusion PAF stimulates significantly more TxB₂ and
HETE production in WPS from 1%E rabbits than control or 0.1%E
rabbits in short incubation times. The response of 0.1%E
rabbit platelets to PAF is similar to control rabbit
platelets in short incubation times. However in longer
incubation times and high PAF concentrations, 0.1%E platelets
will produce more TxB₂ than control platelets. HETE is
unaffected in 0.1%E platelets by PAF indicating once again
that low cholesterol diets cause enhanced CO-TxS activity in
platelet from mildly hypercholesterolemic rabbits.

PAF also stimulates granular release of serotonin. 5-HT
release is easily quantitated by loading PRP platelets with
¹⁴C-5-HT. Platelets will take up the ¹⁴C-5-HT and upon
challenge will release granule packets in proportion to the
strength of the stimulus. Results of 5-HT release can be
seen in Figure 98. Surprisingly, 5-HT release is decreased
in 1%E PRP at both concentrations. The 0.1%E PRP release
significantly less 5-HT than C at only 2.5x10⁻⁷M PAF.
Differences between C and 0.1%E 5-HT release are lost at
Figure 98. % 5-HT release from C, 1% E and 0.1% E rabbit PRP stimulated with PAF, 2 minute incubations.
higher concentrations of PAF. The 1% E PRP does respond to different concentrations of PAF. The release of 5-HT is dependent on the effective concentration of PAF. The PRP of the 1% E is very lipid laden and PAF could be absorbed non-specifically by the high lipids reducing its effective concentration. In order to examine this problem washed platelets were made and 5-HT examined in the absence of plasma lipid. These results will be discussed in more detail by Nicholas J. Greco at a later date. The preliminary results indicate that the differences between 0.1% E are reduced but that the decreased release of 5-HT by 1.0% E WPS is still present at 1x10^-8 M PAF.

On the surface there appears to be a breakdown between TxB2 and HETE generation and 5-HT release. Previous studies by Shattil and Cooper (201, 202) and Stuart et al. (205) have demonstrated that platelets made cholesterol-rich with liposomes produced more TxB2 and released more 5-HT than cholesterol-normal platelets. However, these studies were examining the effect of in vitro cholesterol incorporation in the platelet. In vivo cholesterol incorporation may result in placement of cholesterol in membranes too deep for in vitro incorporation to achieve (291). From the evidence presented in vivo cholesterol loaded platelets as in the 2% E rabbit results in a significant decrease in TxB2 production with all challenges of platelets. The 1% E platelets also have decreased TxB2 generation in response to various
stimuli. Only the 0.1%E demonstrates increased TxB2 production with collagen. Clearly there are degrees of cholesterol incorporation and effects on platelets with varying levels of dietary cholesterol intake. Cholesterol may stabilize the deeper platelet membranes near CO-TxS system and at the same time allow for greater arachidonic acid release from plasma membrane phospholipids which would result in increased HETE production. If cholesterol is stabilizing platelet membranes then this could explain the results of decreased 5-HT release. The vitamin E-supplemented rabbits also had decreased 5-HT release in response to PAF. Cholesterol and vitamin E affect membranes in a similar fashion by decreasing the fluidity and increasing the microviscosity (202, 249). However, vitamin E is considered as having antioxidant properties than cholesterol (272). If cholesterol is stabilizing the inner platelet membranes and enhancing the plasma membrane sensitivity to calcium then these results from 2%E, 1%E and 0.1%E could be explained.

Human Hyperlipemia and Platelet Studies

Patient donors were obtained from Dr. Falco's lipid clinic through the Clinical Research Center, Rhoades Hall, University Hospital, OSU. Patients and age matched controls signed informed consent forms prior to having blood withdrawn. Blood was collected into a 50cc syringe and dispensed into 15cc conical centrifuge tube with EDTA
Anticoagulant. Blood was processed as described earlier to isolate PRP from which WPS was prepared. Platelets were resuspended in Krebs-Henseleit buffer. Platelets were counted by phase contrast microscopy and adjusted to 200,000 platelet/μL. Incubations contained 1x10⁸ platelets in 500 μL volume. Thrombin was added in Krebs-Henseleit buffer. Calfskin soluble collagen was added in a normal saline solution. Isolation and quantitation of TxB₂ and HETE was as described earlier. The results of four experiments can be seen in Tables 6 and 7.

Table 6, experiment number 1 compares TxB₂ and HETE results of an age matched control to a type IIa familial hypercholesterolemic patient. The patient was 58 years old and had heart by-pass surgery five years earlier for coronary atherosclerosis. At the time of analysis the patient and control were off all medications for 4 weeks prior to blood drawing. Thrombin results show that the patient's (Pt) platelets made more TxB₂ and HETE at all levels of thrombin and times of incubation. Collagen results indicate that the patient platelets also produce more TxB₂ and HETE than control platelets throughout the collagen concentrations and time range. However, the increase in HETE induced by collagen is probably not as significant as in thrombin stimulation.
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<tbody>
<tr>
<td></td>
<td>TxB₂</td>
<td>HETE</td>
<td>TxB₂</td>
<td>HETE</td>
</tr>
<tr>
<td>Thrombin</td>
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<tr>
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<td>169</td>
<td>84.3</td>
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<td>322</td>
<td>155</td>
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<tr>
<td>0.5 U/mL(8.0 min)</td>
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<td>373</td>
<td>264</td>
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<tr>
<td>2.0 U/mL(8.0 min)</td>
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<td>491</td>
<td>736</td>
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<td>12.5 ug/mL(8.0 min)</td>
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<td>15.1</td>
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TxB₂ and HETE reported as pmoles/10⁸ platelets
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<tr>
<td></td>
<td>$TxB_2$</td>
<td>$HETE$</td>
</tr>
<tr>
<td></td>
<td>$C$  $PT$</td>
<td>$C$  $PT$</td>
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<tr>
<td>Thrombin</td>
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<tr>
<td>0.125 U/mL (8.0 min)</td>
<td>67  164</td>
<td>70  142</td>
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<td>187  267</td>
<td>200  189</td>
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<td>365  541</td>
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<td>12.5 ug/mL (8.0 min)</td>
<td>8.9  10.2</td>
<td>9.9  12.1</td>
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<tr>
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<td>35.3  31.1</td>
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<td>189  240</td>
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<tr>
<td>210</td>
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<tr>
<td>Triglyceride</td>
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$TxB_2$ and $HETE$ reported as pmoles/10^8 platelets
In experiment number 2 the type IIa patient was 25 years old had apparent tendon xanthoma on the hands. The patient's platelets produced more TxB2 and HETE from thrombin challenges in longer incubation times and lower thrombin concentrations. When the results from thrombin at 2.0 U/mL and 8.0 minutes incubations are examined more closely the TxB2 results appear to be at a maximum level; therefore, any excess arachidonic acid would be metabolized by the LO pathway which is seen as increased HETE values when compared to control. Collagen challenges result in increased TxB2 and HETE only in lower incubation times. There are no differences in HETE at low concentrations or in short incubations.

In table 7 experiment three the patient's a type IIa familial hypercholesterolemia patient. He was 25 years old and over signs of hypercholesterolemia such as tendon xanthomas. Platelet TxB2 and HETE are increased in the patient in low thrombin concentrations and long incubation times. Higher levels of thrombin do not show differences in TxB2 or HETE. Collagen stimulation shows the patient with increased TxB2 only at high levels of collagen. HETE production with collagen shows no differences between control and patients.

In experiment four, the patient is a type IV hyperlipoproteinemia patient. The patient is 45 years old, obese, drinks alcohol, smokes and had complained of chest pain prior to admission. Family doctor discovered
hyperlipoproteinemia problem with routine analysis of cholesterol and triglycerides. The patient was not on any medication prior to blood drawing. Thrombin challenges show that the patient's platelets produce more TxB2 at all concentrations and times. HETE production in the patient platelets is unremarkable from control platelet HETE production. Collagen studies show that the patient platelets produce more TxB2 in all challenges and HETE in only the high concentration at lower incubation times.

In conclusion the results from these hyperlipoproteinemia patients do not parallel the results from 2%E and 1%E rabbits and collagen. The results indicate that the 2%E and 1%E rabbit models are useful in studying long term severely hypercholesterolemia on platelet function and aortic prostacyclin. But, 0.1%E rabbit model is more useful in studying mild hypercholesterolemia which approximates the levels seen in humans.
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