HYPERCHOLESTEROLEMIA AND PLATELET SENSITIVITY TO
THROMBIN-INDUCED AGGREGATION IN WHOLE BLOOD

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ABSTRACT

HYPERCHOLESTEROLEMIA AND PLATELET SENSITIVITY TO THROMBIN-INDUCED AGGREGATION IN WHOLE BLOOD

Charles E. Hammond, II, M. S.
Morehead State University, 1989

Hypercholesterolemia is a risk factor which is associated with an increased incidence of cardiovascular disease. Hypercholesterolemia is correlated with the formation of atherosclerotic lesions which result in ischemia of vital tissues.

In the normal hemostatic response, platelets respond to a variety of activating molecules which facilitate their aggregation at sites of injury to the vessel wall. In hypercholesterolemic individuals, there is an altered physiology of the endothelial lining of blood vessels and of blood platelets. This physiological change is believed to result in a response-to-injury reaction at the vessel wall. The biochemical injury is thought to be induced by high levels of LDL cholesterol in the plasma. High levels of LDL cholesterol are positively correlated with changes in the lipid composition of the membranes of platelets and endothelial cells.

These changes are also correlated with an increased sensitivity of isolated, washed platelets to thrombin binding. However, an increased platelet sensitivity to

thrombin has never been documented in whole blood studies. It has also been found that other types of blood cells participate physically and biochemically with platelets in the aggregation process. Therefore, this study focused on changes in platelet sensitivity to thrombin-induced aggregation in whole blood, as measured in a whole blood impedance aggregometer.
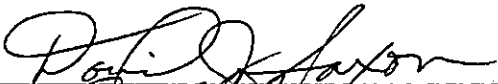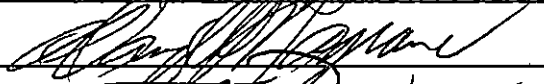
Hypercholesterolemia was induced in rats following 8 weeks of a high cholesterol diet containing 5% cholesterol and 1% sodium taurocholate. The attainment of high levels of plasma cholesterol were confirmed at this time by performing enzymatic assays on plasma derived from the animals' blood. Following 8 weeks of this diet, aggregometry measurements were taken from their whole blood and compared with results from a normal diet group of rats. A 0.15 unit threshold of thrombin induced aggregation in the whole blood of 70% of the high cholesterol diet group, while this same level of thrombin only induced aggregation in the whole blood of 20% of the normal diet group. The reduced threshold of the high cholesterol diet group was associated with an increase in plasma levels of total cholesterol and LDL cholesterol, cholesterol, and a decreased level of HDL cholesterol.
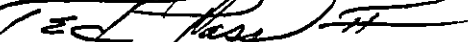
The high cholesterol diet group's blood showed a significantly greater maximal increase in impedance (p <

0.05) in response to the addition of 0.15 units of thrombin than occurred in blood from the normal diet group. The high cholesterol diet group's platelets also aggregated at a significantly faster rate ($p < 0.025$) than did the platelets from the normal diet group.

Therefore the hypercholesterolemia did have a role in altering the sensitivity of platelets to thrombin-induced aggregation. It is hoped that this study provides additional insight into the role of platelets in atherogenesis.

Accepted by: _____, Chairman

ACKNOWLEDGEMENTS

I wish to express my appreciation for the help, the patience, and the inspiration shown me by the members of the faculty of the Department of Biological and Environmental Sciences, especially the members of my committee, Dr. David Saxon, Dr. Ted Pass, and Dr. David Magrane. They have each, by their example and their kind encouragement, provided me with the inspiration to set for myself higher goals in the pursuit of excellence and achievement in a career in higher education.

Thanks are also due to Dr. Gerald DeMoss, who, as chairman of the department, assisted in the funding for this research.

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LITERATURE REVIEW

INTRODUCTION:

Cardiovascular disease is the number one cause of death in the United States. The primary cause of cardiovascular disease is atherosclerosis, a thickening of the vessel wall which can obstruct the flow of arterial blood, resulting in ischemia of heart, brain or other organs. This ischemia often leads to the death of the individual. A number of factors which can contribute to the formation of atherosclerotic lesions have been identified, and these are usually associated with lifestyles or diseases, including smoking, diabetes mellitus, hypercholesterolemia, and hypertension (Ross, 1986).

Changes in the proteins of the vessel wall have been shown to occur as a direct result of prolonged hyperglycemia. It has been hypothesized that, as a result of these changes, a chain of events leading to atherosclerosis often occurs (Cerami et al, 1987).

The incidence of high levels of blood cholesterol, mainly low-density-lipoprotein (LDL) cholesterol, has been shown to be a major contributor to atherosclerotic plaque formation (Lipid Research Clinics Program, 1984). A high blood cholesterol level, or hypercholesterolemia,

often results from the habitual ingestion of foods which
are rich in cholesterol, although there are some
individuals who develop hypercholesterolemia due to a
genetic deficiency of low-density-lipoprotein receptors
(Goldstein & Brown, 1983). Most cases of
hypercholesterolemia are related to diet, and one recent
estimate is that 36% of all American adults are
candidates for medical intervention to prevent
cardiovascular disease (Sempos et al, 1989). In order to
understand how hypercholesterolemia can lead to the
formation of atherosclerotic lesions, we must discover
how cholesterol and other factors affect the types of
cells which are involved in arterial lesions.

Normally, the arterial wall consists of a single,
internal layer (tunica intima) of flat endothelial cells
overlying a thin basal lamina consisting of connective
tissue. Within the basal lamina are small numbers of
monocytes or macrophages. These are believed to serve in
a scavenger capacity for the removal of blood
cholesterol, which is absorbed by endothelial cells and
transcytosed to the underlying monocytes (Steinberg,
1987). Smooth muscle cells are the predominant cell type
of the next layer, or tunica media. The third arterial
layer is the tunica adventitia, which consists mostly of
connective tissue.

In a microscopic cross-section of an atherosclerotic artery, the most obvious characteristic differentiating it from a normal artery is a massive proliferation of smooth muscle cells in the tunica intima. The intima will also have an increased amount of intracellular and extracellular lipid. This type of lesion can develop over a number of years, beginning in childhood in humans. In the arteries of children, macroscopic structures known as fatty streaks are common. They consist of increased amounts of lipid, macrophages and smooth muscle cells in the intima, directly underneath the endothelial lining. These appear in the same areas which, in later life, have a high incidence of atherosclerotic lesions. Animal studies have supported the theory that the fatty streaks are the early stages of atherosclerosis (Ross, 1986).

THE RESPONSE-TO-INJURY HYPOTHESIS:

Atherosclerotic lesion formation is associated with hypercholesterolemia and a number of other risk factors. Balloon angioplasty of arteries, which can remove the endothelial lining of the artery in the area where the procedure is performed, has provided information relating a role for physical injury to endothelium in atherogenesis.

When the endothelial layer is removed from the

Intima, the subendothelial layer is exposed to the blood, and blood platelets quickly attach to exposed collagen fibers and monocytes and release a number of products, including platelet-derived growth factor (PDGF). PDGF induces blood monocytes to migrate into the intima and differentiate into macrophages, white blood cells which usually migrate into injury sites, acting as phagocytes to destroy invading bacteria. As these macrophages become situated under and between endothelial cells, they become swollen with cholesterol.

The PDGF also acts upon the smooth muscle cells of the tunica media, inducing their proliferation in the subendothelial layer. The increased volume of cells below the endothelial layer can proliferate, leading to a marked stenosis of the arterial lumen. Such a stenosis in a coronary artery can lead to myocardial infarction.

PDGF is probably not only produced by platelets. It has been found that agents which strongly resemble PDGF are produced by endothelial cells and macrophages. Endothelium production of the PDGF-like mitogen is enhanced when its cellular overturn is increased in such situations as the loss of a small number of adjacent cells in the intima. In small, localized injury sites, the endothelium is able to overcome such small losses quickly through mitotic replacement. This mitotic

overturn _in vivo_ is normally very slow. However, when monocultures of endothelial cells are grown, their overturn rate is slightly increased, with a concomitant increase in the production of the mitogen. It is therefore thought that endothelial cells growing in an artificial culture exist in a state resembling cells which have been continually exposed to an injury, thus they produce the PDGF-like mitogen in response (Ross, 1986).

It has been proposed that hypercholesterolemia can induce in endothelial cells a continual state of biochemical injury in which the integrity of the cells' physiology is somehow compromised, leading to the release of chemoattractants which cause an increased number of macrophages to migrate into the intima. High LDL levels can also produce endothelial injury due to the toxic effects of oxidation products of lipoproteins upon endothelium (Hennig & Chow, 1988). The increased amount of cholesterol in the blood which is transcytosed to macrophages may induce them to produce the PDGF-like mitogen, thus causing smooth muscle cells in the intima to proliferate. However, cholesterol-laden macrophages may cause smooth muscle cell proliferation indirectly by interposing themselves between endothelial cells, thereby exposing the subendothelium to the blood, and

promoting the attachment and activation of platelets to subendothelial collagen; these activated platelet aggregates will then release PDGF (Ross, 1986).

THE ROLE OF PLATELETS IN ATHEROSCLEROSIS:

Many studies have shown that when the intimal wall is denuded of endothelial cells, platelets will attach to the collagen fibers of the basal lamina and become activated, and will degranulate. Degranulation indicates the release of vesicle-bound substances such as fibronectin, fibrinogen, von Willebrand's factor, thrombospondin, and PDGF. Adenosine diphosphate (ADP) is also released. ADP causes an increase in intracellular Ca^{++} levels. An increase in extracellular Ca^{++} precedes a plethora of reactions necessary for platelet shape change, degranulation, and aggregation. One reaction which occurs is the calcium-calmodulin complex-dependent formation of contractile cytoskeletal proteins. The filamentous proteins are constructed from their globular monomers already present in the cytoplasm within approximately fifteen seconds of the intracellular Ca^{++} increase. These contractile proteins function in the shape change reaction of activated platelets (Phillips et al, 1985).

A minimal level of calcium is also necessary for the

activation of the complex of glycoproteins IIb and IIIa.
This complex is the membrane-bound adhesive receptor for
fibrinogen. When fibrinogen binds this receptor, it
facilitates platelet aggregation by binding one platelet
at each end of the molecule. Platelet aggregates can
thereby be formed through the action of fibrinogen links
(Siess, 1989).

The normal physiological concentration of calcium is
approximately 1 millimolar (mM). It has been found that
the extracellular concentration of free Ca^{++} must be at
least 0.01 mM for maintenance of the glycoprotein IIb-
IIIa complex. While the ADP-induced increase in
intracellular Ca^{++} is necessary for the initial
activation, extracellular levels below this minimal level
are inhibitory to the activity of the fibrinogen receptor
(Shattil & Brass, 1985).

The glycoprotein IIb-IIIa complex is also able to
bind to von Willebrand factor (vWF), an endothelial cell
protein released upon endothelial cell injury. VWF binds
the glycoprotein complex at one end, and binds to
subendothelial collagen at the other end. The result is
a platelet monolayer covering the denuded arterial wall.

The adherance of platelets to the arterial wall,
followed by their aggregation and subsequent release of
PDGF, is believed to be a major contributor to

atherogenesis. It has been shown that when
thrombocytopenic rabbits are exposed to endothelial
injury by angioplasty, there is no occurrence of
atherosclerosis (Hawiger et al, 1985). It has not yet
been determined which factor produced by platelets is
responsible for plaque production, but PDGF is believed
to play a pivotal role (Steinberg, 1987).

Therefore the mechanisms of platelet activation
certainly must be explained. In the hemostatic capacity
of platelets, many factors are known to interact. When a
vessel wall is injured, platelets first adhere to the
site of injury through vWF, then undergo a change of
shape. As they aggregate through the action of
fibrinogen, they then degranulate (Siess, 1989). Other
platelets which then pass by the injury site may also
adhere to the vessel wall and to each other. The
formation of the hemostatic plug is often completed by
the production of a fibrin mesh which envelops the
platelets around the injury site, thereby forming a blood
clot (Hawiger et al, 1985).

The development of the fibrin network is dependent
upon the action of the prothrombinase enzyme complex
located on the membrane surface of platelets. This
enzyme, in its active state, catalyzes the cleavage of
prothrombin to produce thrombin. Thrombin is itself an

enzyme which catalyzes the conversion of fibrinogen to the protein fibrin (Siess, 1989). The prothrombinase complex consists of two proteinaceous subunits. The larger subunit is Factor Va, a 16.8 kilodalton glycoprotein located on the surface of the platelet membrane.

Factor Va (activated Factor V) is derived from Factor V (inactive), a blood-borne profactor. It is cleaved by thrombin released by activated platelets to produce Factor Va. The activated Factor Va has a high affinity for the platelet membrane surface, so it attaches to platelets. Once attached to the phospholipid surface, Factor Va acts as a receptor for the other prothrombinase subunit, a blood-borne enzyme called Factor X. Factor X, when activated by attachment to the Va receptor, has an enhanced ability to cleave prothrombin at two identical amino acid sequences, producing thrombin (Mann et al, 1985). It has been found that in hypercholesterolemic rabbits, there is a significant amount of Factor Va located within the the tissues of the aorta. It has been hypothesized that some physiological change which occurs as a result of hypercholesterolemia causes smooth muscle cells to produce Factor Va. This isolated aorta tissue from hypercholesterolemic rabbits has been shown to have

significant Factor Va activity, in that it can, upon activation by thrombin or other vessel constituents, catalyze the conversion of Factor X into Factor Xa (Rodgers et al, 1988).

The increase of thrombin thus produced can have several direct physiological effects. As was just described, thrombin has enzymatic effects which activate the prothrombinase complex, resulting in the production of fibrin, which is part of the hemostatic response called coagulation.

Thrombin also has a hormone-like effect on platelets. Glycoprotein Ib, a membrane-bound receptor on platelet membranes, binds thrombin at one of thrombin's non-enzymatic sites. When thrombin is bound to this receptor, it activates the adhesion receptors (receptors for vWF and fibrinogen) of platelets. The exact mechanism of action is unclear, but an intracellular rise in Ca^{++} occurs within 0.25 seconds of thrombin-initated platelet activation (Siess, 1989). As was previously stated, the increase in intracellular calcium is correlated with many of the reactions which occur in platelet activation. This intracellular rise in calcium is then responsible for increased activation of the glycoprotein IIb-IIIa complex (adhesion receptor) which facilitates platelet aggregation through linkages with

fibrinogen. Platelet aggregates often form at the site
of a vessel wall injury without initiating the
coagulation process; instead, they will become activated
at the injury site, then begin to release PDGF (Siess,
1989).

All of these physiological interactions which have
been mentioned refer to the activities of platelets as we
understand them, both in hemostasis and in atherogenesis.
Much of what is known about the pathogenesis of
atherosclerosis has resulted from the study of changes
which occur in the wall of biochemically injured
vessels. There have also been many recent discoveries
concerning the effects of hypercholesteolemia upon
platelet activity. Much of this work has shown that
platelets have an increased tendency to undergo
aggregation and activation in hypercholesterolemic
animals. There are several mechanisms by which this
change in platelet activity is thought to occur.

Cholesterol is a natural constituent of plasma
membranes. Its presence within the membranes' lipid
bilayer acts as a control of the fluidity of the membrane
constituents. Another major factor affecting fluidity is
the fatty acid composition of membrane phospholipids.

According to Ross (1986), increased levels of blood
cholesterol may induce the state of biochemical injury

In endothelial cells by cholesterol's increased infusion into their membranes, thus changing their fluidity and in some way altering their biochemistry. This type of alteration has been found to occur in platelet membranes.

Berlin et al (1987) found that a high level of blood cholesterol in rabbits was associated with an increased level of cholesterol within the membranes of their platelets, and a decrease in platelet membrane fluidity. Also associated with hypercholesterolemia is a change in the fatty acid composition of the platelet membrane phospholipids. One of the most common changes which occurs in hypercholesterolemic animals' platelets is a reversal of the arachidonic-to-oleic acid ratio (Winocour et al, 1987).

Another way hypercholesterolemia may change platelet activity is by causing an alteration in the life cycle of platelets in circulation. Megakaryocytes, the progenitor cells of platelets which are located in bone marrow, have been found in hypercholesterolemic rabbits to have an increased rate of turnover, relative to control groups. Based upon electron microscope studies of these cells, the megakaryocyte population of the marrow of hypercholesterolemic rabbits exists at a mean state of development which is less than fully mature (Dupont et al, 1987).

The change in fatty acid composition of platelet membranes in hypercholesterolemic rats is positively correlated with platelet immaturity, and with increased platelet sensitivity to thrombin-mediated aggregation, as measured by ADP luminescence aggregometry on isolated, washed platelets (Winocour et al, 1987). The higher sensitivity was correlated with an increased level of platelet aggregation on exposed subendothelial walls. This might be explained by the production of Factor Va by the vessel wall of hypercholesterolemic animals.

So now there have been at least two categories of circulatory components which have been identified as being altered in hypercholesterolemic animals. First, there is the vessel wall, in which nearly every cell type of its composition is affected. This change may first occur at the level of the endothelial lining of the vessel lumen, due to increased levels of cholesterol in cell membranes (Ross, 1986), or due to the toxic effects of oxidized LDL (Hennig & Chow, 1988). However, it is known that, later, the smooth muscle cells and arterial monocyte/macrophages play a role in the attraction of platelets and as the major constituents of atherosclerotic plaques. The mechanisms by which they may cause platelet aggregates to form involve at least two pathways; exposure of subendothelial collagen to the

blood following rupture of the endothelial lining, and by the increased activation of Factor X, resulting in the production of more thrombin.

A second circulatory component which is changed in hypercholesterolemic animals is the blood platelet. Its membrane fatty acid and cholesterol composition is changed, and its sensitivity to activation by thrombin is increased. The increased sensitivity to thrombin may be related to an enhanced thrombin-binding ability associated with hypercholesterolemia (Winocour et al, 1988).

However, the increased sensitivity to thrombin-mediated aggregation was measured using isolated, washed platelets. It has recently been found that in whole blood, platelets have a higher incidence of spontaneous aggregation than they do when in isolated platelet-rich suspensions. The increased aggregation is believed to be mediated by the release of ADP by red blood cells; ADP, which can activate the glycoprotein IIb-IIIa complex, induces platelet aggregation in the presence of fibrinogen. Red blood cells may also have some undiscovered role in the induction of platelet aggregation related to their physical contact with platelets (Saniabadi et al, 1987). Platelet aggregates have also been seen in electron microscope studies to

include physical contacts with polymorphonuclear blood cells and monocytes (Lehmann et al, 1986).

Of course, these cells are not the only components of the blood which could possibly exert some influence upon platelet actions. It is conceivable that, in whole blood, there are many undiscovered interactions between blood cells and blood-borne molecules which influence platelet aggregation. It would therefore be helpful to our understanding of atherogenesis if platelet sensitivity to aggregating agents in hypercholesterolemic and normal whole blood were investigated.

RESEARCH HYPOTHESIS:

Platelets from hypercholesterolemic animals, compared to normocholesteremic animals, demonstrate increased ability to aggregate in whole blood, and this increased ability to aggregate is related to an increase in sensitivity of these platelets to thrombin.

QUESTIONS ASKED BY THE STUDY:

Do platelets in the whole blood of hyper-cholesterolemic animals increase in their ability to aggregate? If so, is this change related to an increased sensitivity to thrombin?

MATERIALS AND METHODS

DIETARY GROUPS:

The test animals were male Sprague-Dawley rats
obtained from Harlan Sprague Dawley Inc. Each had an
initial weight of 275 to 299 grams, which correlates to
an age of 65 to 72 days. They were housed in individual
cages, 17 X 18 X 35 cm, and illuminated with artificial
light for 14 hours continually each day in the animal
room of Lappin Hall at Morehead State University.

A control group of 10 rats was fed a normal diet of
Purina 5001 Laboratory Rodent chow, which is cholesterol-
free. A high cholesterol diet group of 10 rats was fed
this diet for one week, then switched to a high
cholesterol form of the Purina 5001 diet, obtained from
Bio-Serve (Frenchtown, NJ). The high cholesterol chow
contained 5% cholesterol and 1% sodium taurocholate.
Rats are resistant to diet-induced rises in blood
cholesterol levels, and the addition of the bile salt
sodium taurocholate is used to facilitate the absorption
of cholesterol in the intestine (Winocour et al, 1988).

The high cholesterol diet was maintained for 8
weeks; Renaud et al, (1970) found that maintaining rats
on a lipid-enriched diet for 5, 7, or 10 weeks was
sufficient for inducement of hypercholesterolemia.

Platelets from rats eating these diets also showed an increased sensitivity to proaggregatory agents.

COLLECTION AND PREPARATION OF BLOOD SAMPLES:

The rats were anaesthetized with ether, and 4.5 ml of whole blood was drawn from their tail arteries, using 3 cc syringes with 22 guage, 1 inch needles without plungers. Their tails were submerged in warm water for two minutes prior to sampling in order to dilate the arteries. (Bober, 1988). The blood was mixed with 3.2% sodium citrate in a 9:1 ratio.

DETERMINATION OF PLASMA LIPID LEVELS:

Plasma was obtained by centrifugation in an Adams CT-3200 Analytical Centrifuge at 3200 rpm for 5 minutes. Total plasma cholesterol was measured using materials, according to Sigma Diagnostics Procedure no. 352, which is a modification of the procedure described by Allain et al (1974). Absorbances of the samples at 500 nm were measured in polypropylene cuvettes using an LKB Ultrospec Spectrophotometer (Model 4050). The sample absorbances were compared to a blank, a 50 mg/dl standard, a 250 mg/dl standard, and a control sample with an elevated level of lipids.

Plasma triglycerides were measured using the GPO-

Trinder method (Procedure no. 339 of Sigma Diagnostics), which is a modification of the method described by McGowan et al (1983). Absorbances of the samples were read at 540 nm, then compared to a blank, a 250 mg/dl standard, and an elevated-lipid control.

High-density lipoprotein levels were measured according to the HDL-Cholesterol Procedure no. 352-3 of Sigma Diagnostics, a procedure designed by Warnick et al (1982). This procedure precipitated all cholesterol fractions except HDL-cholesterol, and the supernatant was measured for cholesterol using the same procedure as was described for the total cholesterol assay.

The plasma LDL fraction was then calculated from the known total cholesterol, HDL, and triglyceride levels by the formula $C_{LDL} = T_{CHOL} - C_{HDL} - TG/5$, where C_{LDL} is the low-density-liprotein cholesterol level, T_{CHOL} is the total cholesterol level, C_{HDL} is the high-density-lipoprotein cholesterol level, and TG is the triglyceride level. This method has a 0.98 correlation coefficient with results obtained by the ultracentrifugation method of LDL-cholesterol measurement (Friedewald et al, 1972).

MEASUREMENT OF PLATELET AGGREGATION IN THE PRESENCE OF VARIOUS THROMBIN LEVELS:

Platelet counts were performed on platelet-rich plasma using a Coulter Counter, model Z_F. Platelet-rich

plasma was derived from whole blood by centrifugation in an Eppendorf 5415 centrifuge at 1100 rpm (200 x g) for 8 minutes. Of this plasma, 6.66 microliters was mixed with Isoton II diluent (Curtin Matheson Scientific, Inc.) in a 3000:1 dilution. The Coulter Counter was calibrated with latex particles having a diameter of 0.2 microns, and standardized with a Haem-PC platelet suspension obtained from Baker Instruments.

Measurement of platelet aggregation in whole blood was performed in a Chrono-Log Whole Blood Aggregometer, using the method described by Galvez, Badimon, Badimon, and Fuster (1986). Blood samples were prepared in a 1:1 mixture with calcium-free Tyrode solution, pH 7.4, in a modification of the method used by Russell-Smith, Flower, and Cardinal (1988).

The thrombin levels used were in the range of 0.1 to 0.6 units (0.1 to 0.6 micrograms) per ml of blood. Thrombin was obtained from the Chrono-Log Corporation.

ANALYSIS OF DATA:

The relative frequencies of each group's response to 0.15 and 0.20 units of thrombin were expressed as percentages within each group. The maximal impedance and maximal rate of impedance change were calculated for each rat following responses to 0.15 units of thrombin.

Within each group, these data were analyzed, and the mean
and standard deviation of the experimental group's
responses were compared to the responses of the control
group.   The null hypothesis was tested using the
student's t-test, and differences at the 0.05 level were
considered significant.

RESULTS AND DISCUSSION

Plasma lipid levels were altered by the addition of 5% cholesterol and 1% taurocholate to the diet of the rats (Table 1). All plasma lipid levels, except triglycerides, were altered significantly. Total cholesterol and LDL were elevated, while HDL was lowered.

TABLE 1: PLASMA LIPID LEVELS OF RATS FED FOR 8 WEEKS ON A NORMAL DIET OR A HIGH CHOLESTEROL DIET CONTAINING 5% CHOLESTEROL AND 1% SODIUM TAUROCHOLATE

Plasma Lipid	N Diet Rats		HC Diet Rats		t-test
	n	mg%	n	mg%	
Tc	10	10.5 +/- 13.8	7	199.2 +/- 24.8	S
LDL	10	6.2 +/- 10.0	7	152.0 +/- 30.4	S
HDL	10	58.2 +/- 8.2	7	38.2 +/- 8.2	S
TG	10	48.9 +/- 15.9	7	44.2 +/- 16.1	NS

mg% as mean, +/- standard deviation.
n = number of animals in diet group.
N = normal diet, HC = high cholesterol diet.
Tc = total cholesterol, LDL = LDL cholesterol.
HDL = HDL cholesterol, TG = triglycerides.
S = significant at $p < 0.05$
NS = not significant at $p < 0.05$

There was no significant difference in platelet counts of rats receiving the normal (N) diet and high cholesterol (HC) diet for 8 weeks (Table 2).

TABLE 2:   PLATELET COUNTS OF RATS FED FOR 8 WEEKS ON A
NORMAL DIET OR A HIGH CHOLESTEROL DIET CONTAINING 5%
CHOLESTEROL AND 1% SODIUM TAUROCHOLATE

Diet	n	Platelet Counts in Millions Per mL of Whole Blood	t-test
N	7	107.705 +/- 48.124	
			NS
HC	10	101.090 +/- 25.855	

N = normal diet, HC = high cholesterol diet.
n = number in diet group.
Platelet counts as mean +/- standard deviation.
NS = not significant at p < 0.05.

The minimum amount of thrombin which induced
aggregation in either group was 0.15 units (Figures 1 and
2).   Table 3 illustrates that the high cholesterol diet
group had a greater relative frequency (70%) of response
to the 0.15 units of thrombin compared to the relative
frequency (20%) of response by the normal diet group.
The relative frequency of thrombin-induced platelet
aggregation increased in the high cholesterol diet group
of animals when the thrombin was increased to 0.20 units
(Table 4).

Figures 1 and 2 illustrate typical thrombin-induced
aggregation responses in blood to 0.10, 0.15, 0.20, and
0.25 units of thrombin by the normal diet and high
cholesterol diet groups.   Aggregation responses were
measured by the maximal change in impedance (maximum

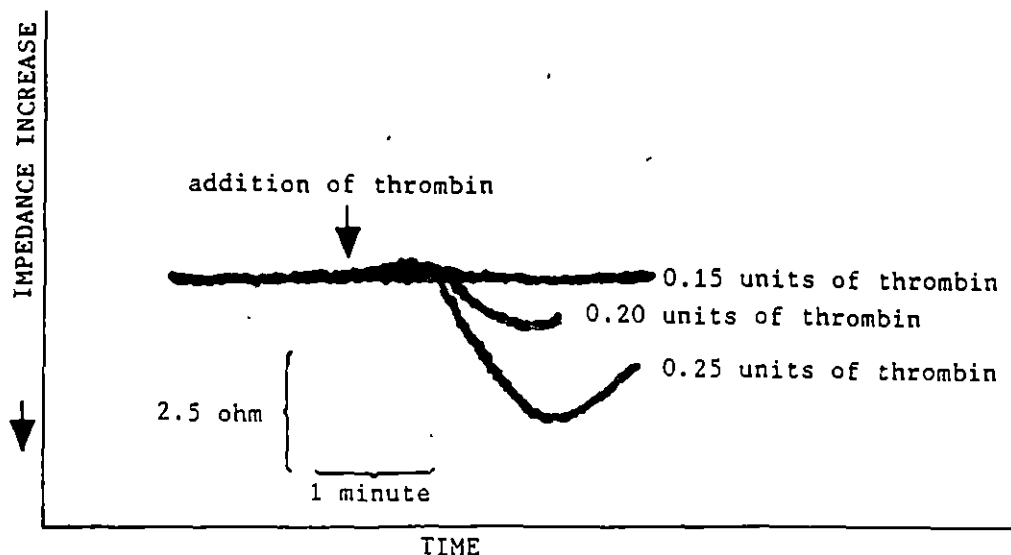Figure 1:   Aggregation responses of 1 mL blood (0.5 mL citrated blood:0.5 mL Tyrode calcium-free buffer, pH 7.4) from the normal diet group of animals to 0.15, 0.20, and 0.25 units of thrombin
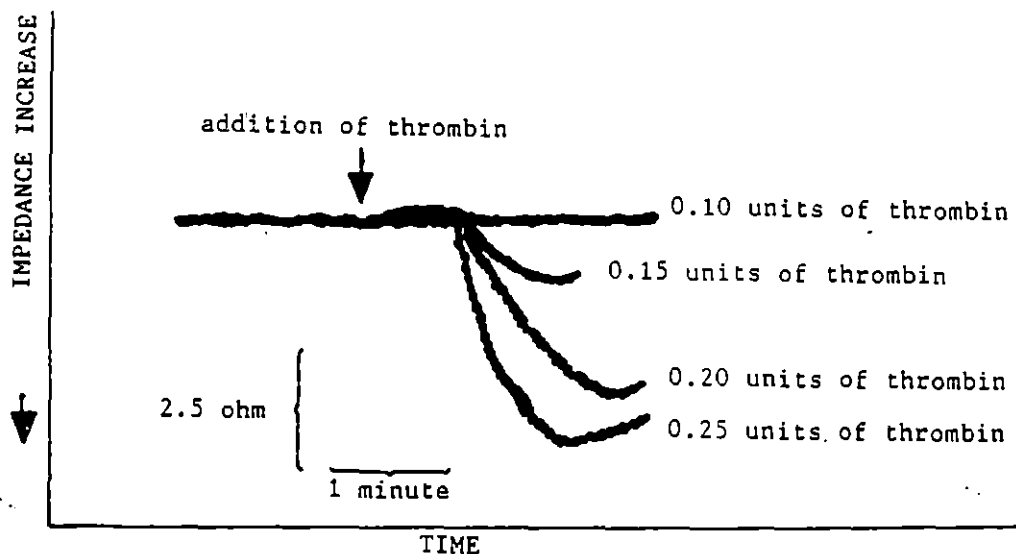

Figure 2:   Aggregation responses of 1 mL blood (0.5 mL citrated blood:0.5 mL Tyrode calcium-free buffer, pH 7.4) from the high cholesterol diet group of animals to 0.10, 0.15, 0.20, and 0.25 units of thrombin

TABLE 3:   RELATIVE FREQUENCIES OF PLATELET AGGREGATION
FOLLOWING ADDITION OF 0.15 UNITS OF THROMBIN TO BLOOD
FROM RATS FED FOR 8 WEEKS ON A NORMAL DIET OR A HIGH
CHOLESTEROL DIET CONTAINING 5% CHOLESTEROL AND 1% SODIUM
TAUROCHOLATE

Diet Group	n	Frequency of Aggregation	Percentage
N	10	2	20%
HC	10	7	70%

N = normal diet, HC = high cholesterol diet.
n = number in diet group.

TABLE 4:   RELATIVE FREQUENCIES OF PLATELET AGGREGATION
FOLLOWING ADDITION OF 0.20 UNITS OF THROMBIN TO BLOOD
FROM RATS FED FOR 8 WEEKS ON A NORMAL DIET OR HIGH
CHOLESTEROL DIET CONTAINING 5% CHOLESTEROL AND 1% SODIUM
TAUROCHOLATE

Diet Group	n	Frequency of Aggregation	Percentage
N	10	2	20%
HC	7	6	85.7%

N = normal diet, HC = high cholesterol diet.
n = number in diet group.

delta ohms), a time-indepedent parameter, and by the

maximal rate of impedance change (delta ohms per second),

which is a time-dependent parameter. When 0.15 units of

thrombin was tested on both groups, 8 out of 10 of the

normal diet group's blood samples had no response. The

other normal diet samples only had slight responses. In

the high cholesterol diet group, there were three blood

samples which showed no response, but the remaining 70%

of the high cholesterol diet group demonstrated a

significantly stronger response than any of those

elicited by the same amount of thrombin units added to

the normal group's blood (Table 5).

TABLE 5:   THROMBIN-INDUCED AGGREGATION FOLLOWING ADDITION
OF O.15 UNITS OF THROMBIN TO BLOOD FROM RATS FED FOR 8
WEEKS ON A NORMAL OR A HIGH CHOLESTEROL DIET CONTAINING
5% CHOLESTEROL AND 1% SODIUM TAUROCHOLATE

Diet Group	n	Delta Ohms/Sec	Maximum Delta Ohms
N	8	0.002 +/- 0.004	0.125 +/- 0.2673
HC	10	0.042 +/- 0.055	2.875 +/- 4.389
t-test		p < 0.025	p < 0.05

N = normal diet, HC = high cholesterol diet.
Maximum Delta Ohms/Sec as means +/- standard deviation.
Maximum Delta Ohms as means +/- standard deviation.

Following 8 weeks of the high cholesterol diet, the

high cholesterol diet group had significant increases in

both their total cholesterol (mean 199 mg%) and LDL

cholesterol (mean 152 mg%) plasma levels. The Lipid

Research Clinics Program (1984) defines

hypercholesterolemia as a total cholesterol level of over

240 mg% or an LDL cholesterol level of over 130 mg%.

Tyroler (1987) suggests that the level used as an

indicator of a risk of cardiovascular disease be lowered

to a plasma total cholesterol level of 160 mg%, based on

population statistics which show a steady increase in the

risk of disease as cholesterol levels above this point

occur.   It is very possible that even lower amounts of

plasma cholesterol than 240 mg% may produce

hypercholesterolemia-associated alterations in

circulatory physiology.

The high cholesterol diet group's mean HDL level

showed a significant decrease. The pattern of an

increase of LDL level and a decrease of HDL level is an

indication that the efficiency of the lipid transport

mechanism of the high cholesterol diet animals has become

reduced. This pattern normally occurs in

hypercholesterolemic animals and humans due to down-

regulation of hepatic LDL receptors (Havel, 1987).

This study has demonstrated that, associated with

these changes in blood lipid levels in the high

cholesterol diet group, there is an increase in the

aggregation response of platelets to thrombin in whole

blood. This certainly supports the discovery of Winocour

et al (1988), that isolated, washed, hypercholesterolemic

platelets have a stronger binding affinity for thrombin

than normocholesterolemic platelets.

The mechanism of thrombin activation of the adhesion

receptor for fibrinogen and vWF is proposed to occur
through an intracellular rise in Ca^{++}. This rise is
dependent upon the availability of extracellular Ca^{++}.
The anticoagulant used in this study (sodium citrate)
binds ionized calcium, preventing coagulation from
occurring. However, there must be a minimal Ca^{++}
concentration of 0.01 mM in the plasma for aggregation to
occur (Shattil & Brass, 1985). To determine if the 3.2%
citrate decreased the plasma level of Ca^{++} below 0.01 mM,
a sample of rat blood mixed in a 9:1 ratio with 3.2%
sodium citrate was analyzed in the clinical chemistry
laboratory at the University of Kentucky Medical Center.
The ionized calcium level was found to be at a
concentration of 0.01 mM in whole blood, a level
sufficient to permit aggregation to occur. The
measurement was performed using a calcium ion-selective
electrode (Betty Mulberry, personal communication, July,
1989).

When thrombin-induced aggregation is measured on
isolated, washed platelets, sensitivity to thrombin-
induced aggregation is increased; this increased
sensitivity is correlated with a change in the fatty acid
content of platelet membranes, and with a reduced mean
platelet age and survival time. Isolated, washed
platelets from hypercholesterolemic animals do not

demonstrate these changes after injection into normal rats, indicating that hypercholesterolemia-related changes in platelet physiology are not irreversible, but are dependent on high cholesterol levels (Winocour et al, 1987).

The study by Winocour et al (1988) utilized change in light transmission as the measurement of aggregation. Change in light transmission as a measurement of aggregation is not feasible in whole blood. The washed, isolated, hypercholesterolemic platelets, compared to normocholesterolemic platelets, had a threshold level of response to thrombin-induced aggregation at 60 ng (0.12 units) and 90 ng (0.18 units) of thrombin respectfully. The specific reactivity of the thrombin in the Winocour study was 2 units per microgram. The Chronolog thrombin used in this study had a specific activity of 1 unit per microgram. The threshold levels of response to thrombin-induced aggregation in this study for the high cholesterol diet group, compared to the normal diet group, was 0.15 units and 0.25 units of thrombin, respectively.

Since hypercholesterolemic animals' vessels may produce Factor Va, leading to the production of more thrombin in the blood (Rodgers et al, 1988), there is a higher likelihood of platelet aggregation at the vessel

wall of these animals If the platelet sensitivity to
thrombin is increased in hypercholesterolemia. This may
be one of the paths by which the risk of atherosclerosis
is increased by hypercholesterolemia.

SUMMARY

Blood platelets are thought to contribute to atherogenesis after they adhere and aggregate at sites of the vessel wall which have been either physically or biochemically injured. Once platelet aggregates form at the vessel wall, they release PDGF, a growth factor which stimulates the proliferation of smooth muscle cells in the vessel, resulting in a lesion which protrudes into the vessel lumen and reduces the flow of blood to the tissues downstream of the lesion. Thrombin is a protein which can stimulate the aggregation of platelets at the vessel wall injury sites, resulting in the production of PDGF at these sites.

Platelets from hypercholesterolemic animals, compared to normocholesterolemic animals, have been seen in this study been seen to have a higher sensitivity to thrombin-induced aggregation in whole blood. This supports the hypothesis that alterations in platelet aggregation associated with hypercholesterolemia are a part of the mechanism by which an increased incidence of cardiovascular disease occurs.

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