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ARACHIDONIC ACID METABOLISM IN THE PLATELETS AND NEUTROPHILS OF DIABETIC RABBIT AND HUMAN SUBJECTS

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ARACHIDONIC ACID METABOLISM IN THE PLATELETS AND NEUTROPHILS
OF DIABETIC RABBIT AND HUMAN SUBJECTS

DISSertation

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

By

Nicholas James Greco, B.S.

******

The Ohio State University
1985

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Department of Physiological Chemistry
To Joseph V. Greco
For unexpected generosity
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Abstracts


Journal Articles


N.J. Greco, S. Strauch, and R.V. Panganamala: "Alteration of Platelet Arachidonic Acid Metabolism in Alloxan-Induced Diabetic Rabbits". (Manuscript in preparation).

N.J. Greco and R.V. Panganamala: "Influence of Plasma on Platelet Arachidonic Acid Metabolism in Alloxan-Induced Diabetic Rabbits". (Manuscript in preparation).


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I. HISTORY

Numerous human and animal studies have shown platelet sensitivity is increased in diabetes mellitus (Hockaday, 1979, Halushka et al., 1977, Halushka et al., 1981, Greco et al., 1985). Atherosclerosis is the major cause of mortality in this group (Marks and Krall, 1971, Robertson and Strong, 1968). Vascular disease progresses more rapidly than in nondiabetics. Platelet physiology is included in theories about the pathogenesis of atherosclerosis as follows: 1.) endothelial injury, 2.) platelet adherence to injury sites, 3.) constituents of platelet release reactions, 4.) smooth muscle cell proliferation, 5.) accumulation of matrix material, lipids, and lipoproteins, and 6.) thrombosis. Results to be discussed suggest platelet abnormalities precedes macroscopic vascular lesions. Enhanced in vivo platelet aggregation has been demonstrated within 3 days after becoming hyperglycemic in studies with rats and rabbits made diabetic by treatment with alloxan or streptozotocin (Honour et al., 1977).

Animal models similar to human diabetes mellitus has been produced by a variety of methods including viral infections (Craighead, 1974) and chemical agents (Wellman et al., 1967). Alloxan cause specific pancreatic islet cell necrosis of the insulin-secreting β-cells (Dunn et al., 1943). Hockaday (1979) demonstrated altered platelet function in the alloxan-diabetic rabbit. The alloxan-induced diabetic rabbit was used as a model to demonstrate that maternal diabetes impairs the maturation of fetal
lung (Tsai et al., 1982). Decreased $[^{14}\text{C}]-\text{PGE}_2$ was synthesized from exogenous $[^{14}\text{C}]$ arachidonic acid.

Early history of prostaglandins is well known originating with the work of Kurzrok and Lieb (1930) who demonstrated the contraction of myometrium when in contact with semen. As analytical techniques were developed and refined, information pertaining to essential fatty acids and prostaglandins accumulated. Nonesterified arachidonic acid is the precursor of prostaglandins. A rate limiting step is the release of arachidonic acid from membrane phospholipids. Phospholipase A$_2$ or a concerted effort of phospholipase C and diglyceride lipase are important in releasing precursor substrate. Release of arachidonic acid can result in a variety of primary prostaglandins and hydroxylated compounds being produced. Sheep vesicular glands were demonstrated to synthesize PGE$_2$ from arachidonic acid by Van Dorp et al. (1964) and Bergstrom et al. (1964). In 1973 Hamberg and Samuelsson isolated the unstable intermediates, PGG$_2$ and PGH$_2$, which they demonstrated to have platelet aggregating ability and the ability to contract smooth muscle.

Formation and structure of the thromboxanes, TxA$_2$ and its inactive hydrolysis product TxB$_2$, were elucidated by Hamberg and Samuelsson (1974), Hamberg et al. (1975), and Svensson et al. (1976). Formation of TxA$_2$ is vital in regulating hemostatic mechanisms. TxA$_2$ is the most potent vasoconstrictor and aggregator of platelets known.
An unstable antiaggregatory metabolite of arachidonic acid was isolated by Moncada et al., (1976) and Moncada and Vane (1978) from arterial tissue. This compound inhibited platelet aggregation and has vasodilatory properties. Thus, PGI\(_2\) opposes the effects of TxA\(_2\). The inactive hydrolysis product of this compound (6 keto PGF\(_{1\alpha}\)) was described by Pace-Asciak (1976) from endoperoxide metabolism in rat stomach fundus. Subsequently Johnson et al., (1976) determined the structural precursor and renamed it prostacyclin (PGI\(_2\)). Theories of the pathogenesis of atherosclerosis include possible imbalances in the generation of PGI\(_2\) from aortic tissue and TxA\(_2\) from platelets (Figure 1).

TxA\(_2\) and PGI\(_2\) may work in concert to give physiologic hemostasis and allow vascular repair. With PGI\(_2\) levels or effects in excess of TxA\(_2\) levels or effects, a bleeding tendency may occur. If TxA\(_2\) effects surpassed those of PGI\(_2\), there could be an accentuation of platelet aggregation, endothelial injury, and thrombi formation. Resulting outcome of this imbalance could be enhanced smooth muscle cell proliferation which in the presence of elevated plasma lipids could lead to infiltration of lipid elements, and atherosclerotic lesion formation.

Another metabolite of arachidonic acid in platelets discovered by Hamberg and Samuelsson (1974) was 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). The initial lipoxygenase product is 12-L-hydroxyperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) which is reduced to 12-HETE by a selenium containing glutathione peroxidase enzyme.
Figure 1. Arachidonic acid cascade. Formation of PAF or AGEPC from phospholipid is shown.
These hydroperoxy intermediates can activate cyclooxygenase activity (Lands, 1979) while Siegal et al., (1979) has shown 12-HPETE inhibits cyclooxygenase activity and stimulates lipoxygenase activity. 12-HPETE can inhibit the production of PGI$_2$ and therefore may serve a role in the mechanisms of hemostasis (Bunting, et al., 1976). 12-HPETE can stimulate its own production by increasing lipoxygenase activity resulting in a positive feedback mechanism (Siegel et al., 1979). 12-HETE, the stable metabolite of the labile 12-hydroperoxide intermediate, is chemotactic for leukocytes and macrophages (Nugteren, 1975, Goetzli, et al., 1975) (Figure 1).

Initial observations demonstrated that stimulated leukocytes released a product which stimulates platelet function (Siraganian and Osler, 1971). Early work described the platelet-activating factor as 2-acyl-glycerol-3-phosphorylcholine (Benveniste et al., 1977). Subsequent work by Demopoulos et al., (1979) elucidated the structure as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC).

AGEPC is released from membranes after a deacylation / reacetylation reaction (Chilton et al., 1983a, Chilton et al., 1983b). AGEPC is synthesized in situ upon cellular stimulation. Various cells release AGEPC, basophils (Benveniste, 1974), macrophages (Vargaftig et al., 1980, Albert and Synder, 1983), neutrophils (Mueller et al., 1983, Clark et al., 1980), and platelets (Chignard et al., 1979) (Figure 1). Complement components C3a and C5a stimulate murine macrophages to release AGEPC (Mencia-Huerta and Benveniste, 1979). Neutrophils undergoing phagocytosis release AGEPC
Mueller et al., 1983, Clark et al., 1980). Low concentrations of AGEPC (10\(^{-9}\) - 10\(^{-12}\) M) stimulates platelet release and aggregation (Marcus et al., 1981, Chesney et al., 1982). Receptors for AGEPC on platelet membranes have been described (Valone et al., 1982, Chesney et al., 1984). AGEPC can be degraded to a lyso-AGEPC compound by neutrophils (Chilton et al., 1983) or by an acid-labile factor in human plasma (Farr et al., 1983). These mechanisms are essential for removal of AGEPC because of its potent action in causing platelet degranulation (Vargaftig et al., 1982), platelet aggregation, and increasing vascular permeability (Humphrey et al., 1982).

Feldberg and Kellaway (1938) observed the effects of spasmogenic substances on smooth-muscle preparations from the guinea pig jejunum produced when cobra venom was perfused into guinea pig lungs. Perfusion of the lungs of ovalbumin-immunized guinea pigs with antigen containing solutions caused the release of a spasmogenic substance (Kellaway and Trethewie, 1940). The compound, distinguished from histamine because the response was slow to develop, was named "slow-reacting, smooth-muscle stimulating substance" or SRS-A by Brocklehurst (1960). Rabbit polymorphonuclear leukocytes metabolize arachidonic acid to dihydroxy acids with absorption maxima at 259, 269, and 279 nm (Borgeat and Samuelsson, 1979a, Borgeat and Samuelsson, 1979b). These triplet peaks suggested three conjugated double bonds similar to those seen with the SRS-A compound. Subsequently the name leukotrienes was coined for molecules absorbing in the ultraviolet region. The
5,6-epoxy-eicosatetraenoic acid intermediate, LTA₄ was trapped and purified (Rådmark et al., 1980a, Rådmark et al., 1980b). Further work characterized the identity of LTD₄, LTE₄, and LTF₄. Initial liberation of arachidonic acid is acted on by a C-5 lipoxygenase to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Borgeat and Samuelsson, 1979). 5-HPETE serves as a precursor for 5-HETE, LTB₄, and the peptido-leukotrienes.

LTB₄ possesses potent degranulation, chemokinetic, and chemotactic properties towards polymorphonuclear leukocytes and macrophages both in vitro and in vivo (Ford-Hutchinson et al., 1980, Smith et al., 1980). LTB₄ has been proposed as a potential mediator of various cellular aspects of inflammation (Smith et al., 1980). Lysosomal enzyme release and superoxide generation can be mediated by LTB₄ (Lin et al., 1984). In the presence of a vasodilator as PGE₂, LTB₄ mediates vascular permeability. A significant potentiation of plasma exudation is produced by the intradermal injection of LTB₄ and bradykinin when compared to bradykinin alone. Concentrations producing these effects were in the nanogram range (Bray et al., 1981). Mediation of vascular permeability is believed to be essential for extravascular immune-complex deposition. LTB₄ could also act as AGEPC and vasoactive amines to increase vascular permeability (Virella et al., 1983).
Immunological and other stimuli release arachidonic acid which can be converted by lipoxygenation to unstable hydroperoxyeicosatetraenoic acid intermediates. Both 5-HETE and LTB₄ are produced. Concentrations which evoke chemotactic responses are LTB₄ (30 ng/ml), 5-HETE (1000 ng/ml), and 12-HETE (10,000-20,000 ng/ml) (Goetzl and Sun, 1979, Goetzl and Pickett, 1980). Increased levels of both 5-HETE and LTB₄ have been observed in patients with rheumatoid arthritis and spondyloarthritis (Klickstein et al., 1980).

Both 5-HETE and 5-HPETE potentiate the degranulation response of PMNL to AGEPC or LTB₄ although 5-HETE and 5-HPETE have only weak degranulation effects (O'Flaherty et al., 1983, O'Flaherty et al., 1984). 5-HETE and 5-HPETE do not stimulate superoxide production at concentrations to 1 x 10⁻⁶ M (Beckman et al., 1985). Metabolites of LTB₄ have been shown to be 1/10 to 1/30 as potent as LTB₄ in the ability to elicit a chemotactic response (Fitzpatrick et al., 1983).

The biological activities of LTB₄ are mediated by its ability to increase the intracellular levels of Ca²⁺. Serhan et al. (1982) demonstrated that LTB₄ translocated Ca²⁺ when added to liposomes. This observation supports the idea that products from endogenous lipid metabolism might act as endogenous Ca²⁺ ionophores. 6-trans-LTB₄, 5-HETE, and 5-HPETE did not translocate Ca²⁺. 20-hydroxy-LTB₄ has nearly the activity of LTB₄ in stimulating Ca²⁺ influx. The dicarboxylic acid metabolite is nearly devoid of
activity (Naccache et al., 1982). Nanomolar concentrations of f-Met-Leu-Phe, the C5a complement component, and LTB₄ all cause increases in the intracellular Ca²⁺ level in rabbit PMNL. Elevation in Ca²⁺ levels at these physiological levels would result from the release from internal stores and a net uptake from the extracellular medium (White et al., 1983). Synthetic N-formylmethionyl peptides as f-Met-Leu-Phe may be related to natural bacterial leukoattractants which induce leukocyte chemotaxis (Schiffman et al., 1975). Stereospecific receptors have been appears to be a direct correlation between the activities of various lipoxygenase products and their ability to increase the cytoplasmic levels of Ca²⁺ (Naccache et al., 1982).

Alterations of eicosanoids produced from platelets and leukotrienes produced from PMNL could influence the pathogenesis of atherosclerosis. Studies must determine if platelet and PMNL dysfunctions proceeds vascular complications or is a result of those complications. Products from both can influence other blood cells and vascular tissues and could theoretically play a role in the disease.
II. Statement of Problem

An alteration of arachidonic acid metabolism to prostaglandins and leukotrienes from platelets and polymorphonuclear leukocytes respectively is evident in subjects with diabetes mellitus. There is evidence of altered platelet/vascular wall interactions in diabetes mellitus and evidence that polymorphonuclear leukocytes influence the vascular walls. Theories on the pathogenesis of atherosclerosis include both blood cells.

The purpose of this dissertation is to 1.) investigate changes in platelet aggregation in human and rabbit diabetics, 2.) measure changes in TxB$_2$, PGE$_2$, and 12-HETE production, 3.) measure the release of granular serotonin, 4.) investigate the status of $\alpha$-toco-pherol (vitamin E) and cholesterol in platelets and plasma, 5.) determine the influence of insulin treatment on platelet aggregation and TxB$_2$ and 12-HETE production and, 6.) investigate the 5-lipoxygenase products (5-HETE, LTB$_4$, and LTB$_4$ isomers) produced from neutrophils of diabetic rabbits.
III. METHODOLOGY

The four following sections will use essentially the same materials. All methods will reviewed in the order that they will appear in the text.

MATERIALS

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

Dextran, bovine achilles-tendon collagen, bovine thrombin, allocan monohydrate, bovine albumin, Trizma base, gelatin, cholesterol (from porcine liver), acetylsalicylic acid, adenosine 5'-diphosphate, epinephrine bitartrate, imipramine hydrochloride creatine phosphate (hydrate, disodium salt), creatine phosphokinase (ATP: creatine N-phosphotransferase; EC 2.7.3.2 from rabbit muscle, 130-135 units / mg solid).

The following radiolabeled materials were purchased from New England Nuclear Corporation (NEN) Boston, MA or Amersham Corporation (AMC) Arlington, IL as indicated.

Thromboxane B₂ [5,6,8,9,11,13,14,15-³H (Ν )], 128 Ci / mmol, NEN, AMC

Prostaglandin E₂ [5,6,8,9,11,12,14,15-³H (Ν )], 160 Ci / mmol, NEN, AMC

12-L-hydroxy-5,8,10,14-eicosatetraenoic acid, [5,6,8,9,11,12,14,15-³H (Ν )], 40 Ci / mmol, NEN

5-hydroxytryptamine [2-¹⁴C (Ν )] creatine sulfate, 55 mCi / mmol, AMC
Antibodies to TxB2, 12-HETE, and PGE2 were provided by Dr. L. Levine, Brandis University, Waltham, MA. Unlabeled 12-HETE was provided by Dr. R. Bryant of George Washington University, Washington D.C. Unlabeled prostaglandins (TxB2, PGB1, PGE2) were provided by Dr. John Pike of Upjohn, Kalamazoo, MI. A standard of leukotriene B4 was provided by Dr. J. Rokach, Merck Frosst, Pointe Claire, Quebec, Canada.

Unopette platelet/leukocyte count (1:100 dilution) were purchased from Becton Dickinson (Rutherford, NJ). 8-acetyl-Y-0-alkyl-L-α-lecithin (platelet activating factor, PAF or AGEPC) and A23187 (Ca2+ ionophore) was purchased from Calbiochem, San Diego, CA. 5,8,11,14-eicosatetraynoic was a gift from Hoffmann-LaRoche, NJ, d-α-tocopherol was purchased from Eastman Kodak Co., Rochester, N.Y., 5-hydroxyeicosatetraenoic acid and 12-hydroxyeicosatetraenoic acid were purchased from Seragen, Boston, MA, HPLC-grade methanol, ethyl acetate, and hexane were purchased from MCB Reagents, Gibbstown, NJ, Innovar-Vet was purchased from Pitman-Moore, Inc., Washington Crossing, NJ, naloxone hydrochloride was purchased from Endo Pharmaceuticals, Inc., Manati, Puerto Rico, dextrose was purchased from Travenol Laboratories, Inc., Deerfield, IL, protamine zinc insulin was purchased from Eli Lilly and Co., Indianapolis, IN, B-D-LoDose syringes were purchased from Becton Dickinson, Rochelle Park, NJ, Nutri-Cal was purchased from Evsco Pharmaceuticals, Buena, NJ, CHEM STRIP5 was purchased from Bio-Dynamics, Indianapolis, In, arachidonic acid was purchased from NuChek Prep, Elysian, MN, aqueous counting scintillant (ACS II) was purchased from Amersham, Arlington
Heights, IL, new methylene blue and trypan blue were purchased from Aldrich Chemical Comp. Inc., Milwaukee, WS, Dulbecco's PBS (10x concentration, Ca²⁺ / Mg²⁺ free) was purchased from Gibco Laboratories, Gibco Division - The Dexter Corporation, Chagrin Falls, OH, a Zorbax ODS HPLC column (25 x 0.45 cm) was purchased from Dupont Instruments, Wilmington, DE, Ficoll-Hyapaque, dextran T-500, and Percoll (lot KL 38622 Code No. 17-0891-01 or lot KC 35310 Code No. 17-0891-01) were purchased from Pharmacia Fine Chemicals, Piscataway, NJ), SurfaSil was purchased from Pierce Chemical Company, Rockford, Ill.
Preparation of Materials

This section includes methods for the preparations for all materials used throughout this dissertation.

Arachidonic acid was stored at -70°C in aliquots of ethanol (approximate concentration 2.5 mg A.A./500μl ethanol) and prepared as the sodium salt for use in platelet rich plasma. Preparation is as follows; a 500μl aliquot of A.A. is evaporated under N₂ and 0.312 mg A.A. was dissolved in 1 μl of ethanol (i.e. 2.512 mg A.A. would be dissolved in 8.05 μl of ethanol). Na₂CO₃ (pH 11, 0.166M) would be added in a volume 10x the ethanol volume (i.e. in this case 80.51 μl). The solution would be capped under N₂, vortexed hard, then held at 37°C for 2 min. After this time a volume of distilled water (equivalent to the Na₂CO₃ volume) would be added, the solution capped under N₂ and again vortexed. This solution was diluted 1:10 with distilled water and aliquots were added to PRP. Bovine achilles-tendon collagen suspension was prepared according to modified procedure of Nakanishi et al. (1971) and standardized utilizing bovine serum albumin and the protein determination of Lowry et al. (1951). Modifications include using 2 g of collagen initially and increasing the homogenization time to 45 min. This allowed a more concentrated solution of collagen to be obtained. Serial dilutions are made of stock Aₐ₂₃₇₈7 (2 mM) in ethanol and added in 2.5 μl aliquots to produce the desired concentrations. Imipramine hydrochloride and adenosine-5′-diphosphate were prepared
in stock concentrations of 125 μM and 10 mM respectively. Imipramine was diluted in distilled deionized water and ADP was diluted in 0.9% NaCl and both stored at -20°C. A stock solution of ADP (10 ml) was adjusted to pH 7 with approximately 50 μl of 6N NaOH and stored in 100 μl aliquots. An 1:20 dilution of 10 mM ADP was prepared (0.5 mM) and 10 μl added to 500 μl PRP was 10 μM final concentration. Both creatine phosphate and creatine phosphokinase were diluted in 0.9% NaCl. Solutions of acetylsalicylic acid (ASA) were made by dissolving solid ASA in a minimal volume of 6N NaOH then adding the required volume of distilled water.

5,8,11,14-eicosatetraynoic acid (ETYA) was prepared as the ammonium salt (Goetz et al., 1976). Stock β-acetyl-γ-o-alkyl-L-α-lecithin or PAF (5.0 x 10⁻⁴ M) was stored at -20°C under N₂ in absolute ethanol. A working solution of PAF was prepared as follows: a 200 μl aliquot of PAF in alcohol was evaporated under N₂, and 200μl of Tris- Tyrode's albumin buffer (1.83 g Trizma base/liter, 8.0 g NaCl/liter, 0.195 g KCl/liter, 0.213 g MgCl₂·6H₂O/liter, 1.0 g D-glucose/liter, and 2.5 g bovine serum albumin/liter, pH 7.4) was added to dissolve PAF. The PAF solution was prepared fresh. Epinephrine bitartrate reagent was dissolved in distilled deionized water.

All glassware was siliconized using a 5% solution of SurfaSil in hexane. Glassware was immersed for 15 minutes, rinsed with hexane than rinsed with methanol after overnight drying.
Methods

Human subject selection. This research project was completed according to the principles of the declaration of Helsinki and approved by the Ohio State University Human Subjects Committee. Informed consent was obtained from all participating subjects. Type I diabetic subjects were selected from The Ohio State University Hospital outpatient diabetic clinic and inpatient Clinical Research Center. Eleven Type I diabetic subjects (7 men and 4 women) and eleven age and sex matched healthy controls were studied. The mean ages for the control and diabetic groups were 31 ± 3 years (25-50) and 33 ± 6 years (27-45, S.E.) respectively. All diabetic patients were taking insulin either as a subcutaneous injection or by an open-loop subcutaneous insulin pump. The average duration of diabetes mellitus was 21 ± 3 years (S.E.). Diabetic subjects participating in this study were selected based on the criteria that they were completely free of any medication except insulin for at least two weeks before the study and that they showed no evidence of background retinopathy, proliferative retinopathy, neuropathy, nephropathy, or advanced vascular lesions or occlusions.

Induction of Diabetes Mellitus. A total of 25 male New Zealand White rabbit litter mates weighing 1.8 ± 0.23 (S.D.) kilogram were randomly divided into two groups, control (C) and diabetic (D). Before treatment with alloxan all rabbits were fasted for 24 hours. Innovar-Vet (0.3 ml/kg, Fentanyl 0.4 mg/ml, droperidol 25 mg/ml) was injected intramuscularly (hind leg muscles) for anesthesia then the animal was allowed to be undisturbed for 15-20 minutes
Alloxan monohydrate was diluted in distilled water (final pH 3.2) and a single dose rapidly injected intravenously via a marginal ear vein at a concentration of 120 mg/kg (100 mg/ml, 10% solution). Control rabbits were treated as the diabetic group except 2.21% NaCl (adjusted to pH 3.2 with 1.0 N HCl), equivalent to the average volume of alloxan solution used, was injected. This was to simulate an osmotically active solution and the acidity of the alloxan solution. Naloxone hydrochloride (0.4 mg/ml, Narcan) was injected (0.3 ml/kg) into the marginal ear vein contralateral to the ear vein used for the alloxan injection. Dextrose (50%, approximately 20 ml) was administered either by a nasogastric route using a 30 cc syringe, 18 g needle, and 9' PE 190 (polypropylene) tubing or was injected (dextrose 5%) subcutaneously with an 18 g needle and a gravity assisted infusion set. Dextrose was injected at 3 hour intervals beginning approximately 2 hours post alloxan injection and continuing for 12 hours. Additional dextrose was given 24 hours post alloxan injection if the blood glucose level was < 150 mg/dl. This regimen was to counteract the acute and transient hypoglycemia induced by alloxan (Figure 2, line A). Note that alloxan injected rabbits were hyperglycemic 2-4 hours post injection with or without treatment with dextrose. Without treatment with dextrose, rabbits were acutely hypoglycemic 4-8 hours post injection (Figure 2, line A dotted) and the mortality rates sharply increased (as high as 75%) (Wellman et al., 1969). Treatment with dextrose limits the mortality rate to
< 20% by elevating blood glucose values (line B). All rabbits were maintained overnight with ad libitum water (with 5% dextrose; 50 ml of 50% dextrose plus 450 ml water), Nutri-Cal, and Purina rabbit chow. The same dietary regimen was continued for the duration of the study except for the 5% dextrose and Nutri-Cal. Efforts to restrict food consumption of the diabetic rabbits to levels similar to the controls is not necessary since diabetic rabbits consume approximately 170 g of Purina chow per day compared to approximately 140 g of Purina chow per day for control rabbits. Both animals were fed between 155-170 g per day. Initially, blood glucose levels in whole blood were determine hourly to characterize line A and B (Figure 2). Subsequent alloxan injections were followed less vigorously in terms of blood glucose levels. Fasting blood glucose values were determined 48 post injection to be control, 100 ± 2.80 and diabetic, 457 ± 27.0 mg/dl (mean ± S.D.). At 1 week post injection these values were control, 120 ± 4.00 and diabetic, 500 ± 19.0 mg/dl. These fasting blood glucose values were maintained for the duration of the experiments (2-3 months). Animals were maintained without treatment with insulin during the experiments except as otherwise noted.
Figure 2. Blood glucose values versus time after alloxan treatment: Influence of treatment with glucose (line B) versus without glucose (line A).
Initial criteria for the diabetic state was hyperglycemia. Polydipsia and polyurea were observed within the first several days post injection. Additional confirmation of the diabetic state was the determination of serum insulin levels and glycosuria (confirmed with CHEM STRIP5 during necropsy). Fasting insulin levels were determined by a $^{125}$I-radioimmunoassay with a porcine antiserum essentially 100% cross-reactive with rabbit insulin. Serum insulin was measured in the laboratory of Dr. Thomas O'Dorisio by Bob Meleca (Ohio State University Hospital). As expected insulin levels were lower in the diabetic rabbits. Insulin levels ranged from 13.3 to 28.4 $\mu$U/ml with an average of 24 ± 6.6 (SD) in control rabbit. Significantly lower levels of insulin (< 1-6.1 $\mu$U/ml) were found in diabetic rabbits. Both determinations involved an N = 12. Minimum sensitivity of the RIA is 1 $\mu$M / ml with a 100 $\mu$l aliquot of serum or plasma diluted 1:10.

Preparation of Platelet Rich Plasma. Rabbits under ethyl ether anesthesia had blood drawn by reversible percutaneous cardiac puncture. Human blood is drawn from the antecubital vein. Both are drawn directly into 3.8% trisodium citrate (9:1, vol/vol). All subjects fasted between 9-12 hours before obtaining the blood. The blood is transferred to 15ml centrifuge tubes, mixed and centrifuged at 1180 rpm (250 xg) for rabbits and 1580 rpm (400 xg) for humans in a Sorvall GLC-2 for 20 min. The upper platelet rich plasma (PRP) layer, above the packed erythrocytes, is removed and the remaining portion centrifuged at 3000 rpm (1500 xg) for 10 min. The upper
platelet poor plasma (PPP) layer is removed and mixed with the PRP layer. Platelets are counted on a phase contrast microscope after mixing in the Unopette platelet/leukocyte diluters. Platelet counts are adjusted to 200,000\(\mu\)l by centrifuging at 15,000 \(xg\) for 1 min. a portion of the PRP/PPP mixture. The calculation is as follows:

\[(\text{platelet count}) \times = (\text{total volume of PRP/PPP}) (200,000 \text{ PTs/\(\mu\)l})\]

Solving for \(x\) determines the volume of PRP/PPP that is actually needed for a count of 200,000 PTs/\(\mu\)l when diluted to the total volume. This calculated volume (\(x\)) is subtracted from the total volume to determine the volume of PRP/PPP which is spun. After centrifugation the supernatant is mixed with the calculated volume to adjust the total volume to 200,000 PTs/\(\mu\)l. With a 500\(\mu\)l aliquot, the total platelet number is \(1 \times 10^8\). Contamination of PRP preparations with other blood cells was also evaluated after staining aliquots of PRP with new methylene blue as described in the Cell count, purity, and viability of PMNL section. For seven PRP preparations the ratio of platelets to other blood cells was as follows; control, RBC (635:1 to none visible), PMNL (21,000:1 to none visible), lymphocytes/monocytes (\(> 9,800:1\)). Diabetic, RBC (1,100:1 to none visible), PMNL (11,000:1 to none visible) and lymphocytes/monocytes (\(> 15,000:1\)).
Platelet aggregation. All subjects (humans and rabbits) were fasted overnight. Insulin was withheld from the human diabetic subjects (except a continuously infused basal level with patients using the pump) until after the blood was drawn between 7:00-7:30 a.m.

Platelet aggregation was performed with PRP at 37°C on a Model PAP-3 aggregometer (Bio/Data Corp., Hatboro, PA). TxB$_2$, PGE$_2$, 12-HETE, and the release of $^{14}$C-5HT was determined subsequent to various aggregation times using single or duplicate determination experiments. PRP was preincubated at 37°C for one minute prior to the addition of different platelet agonist concentrations. Synergism of epinephrine with PAF was studied in the following order: Platelets were incubated with PAF (1.0 x 10$^{-7}$ M) for 2 minutes and the release of serotonin (5HT) was determined. In another experiment, platelets were incubated with epinephrine (2.5 x 10$^{-7}$ M) for 3 minutes and the release of 5HT determined. In a third series of experiments, platelets were preincubated for the first one minute with epinephrine (2.5 x 10$^{-7}$M) followed by challenge with PAF (1.0 x 10$^{-7}$M) for 2 minutes and the release of 5HT was determined. PRP was incubated at 37°C with ASA for 5 min. and creatine phosphate/creatine phosphokinase was preincubated for 2 min. prior to the addition of PRP agonist.

Preparation of Washed Rabbit Platelets. Blood is collected as discussed in the Preparation of Platelet Rich Plasma section. Collection of blood was into 77mM Na$_4$EDTA (pH 7.2, blood:Na$_4$EDTA, 9.2 : 0.8 v/v). Following centrifugation of the blood at 1,180 rpm, PRP is removed with a plastic tip pipette and transferred to a centrifuge tube. The PRP is spun at 4,000 rpm (1,980 xg) for 15
minutes at 4°C in a Sorvall RC2-B centrifuge. The platelet free plasma supernatant is decanted, and the platelet button resuspended gently in Tris-NaCl-Na₄EDTA buffer (Trizma base, 50 mM, NaCl, 1.6 mM, Na₄EDTA, 1.6 mM). The platelet suspension is recentrifuged at 3,000 rpm (1,400 xg) for 10 min. followed by two more cycles of centrifugations and resuspensions. Final resuspension of the platelet pellet is in 2-5 ml of Ca²⁺ free Krebs-Henseleit buffer (NaCl, 120 mM, KCl, 4.7 mM, MgSO₄·7H₂O, 1.2 mM, KH₂PO₄, 1.2 mM, dextrose, 1.0 mM, NaHCO₃, 2.5 mM). Final adjustment to 200,000 PTs/μl is as described in the Preparation of Platelet Rich Plasma section except dilution is made with oxygenated Ca²⁺ free Krebs-Henseleit buffer. Ca²⁺ free Krebs-Henseleit buffer is gassed with 95% O₂ / 5% CO₂ for 10-15 minutes while on ice. Incubations are slowly stirred with 9 mm teflon magnets at 37°C for the incubation periods. Incubation volumes are 0.5 ml containing 1 x 10⁸ platelets. 1 mM Ca²⁺ was added to those incubations which were stimulated with AGEPC. Preincubation was for 5 min at 0°C. Reactions are terminated with 100 μl of 1 N HCL and extracted one time with 3.5 ml diethyl ether.
Polymorphonuclear leukocyte (PMNL) isolation:

Isolation procedures are those modified and adapted from Böyum (1968) and from Phillips et al. (1983) in the isolation of human peripheral PMNL. Böyum's procedure involves the removal of mononuclear cells by centrifugation through Ficoll-Hyapaque followed by the removal of erythrocytes (RBC) by dextran sedimentation at 1 xg. Contaminating RBCs are removed by differential hypotonic lysis (Cullen et al., 1980). Criticism of the RBC lysis follow: such lysis has the possibility of adverse effects resulting in the divergence from physiological activity and membrane fragments and soluble proteins could bind to the PMNL and may not be removed with washing. The procedure of Phillips' involves isolation of the PMNL and RBC using Ficoll-Isopaque followed by dextran sedimentation. The supernatant above the settled RBC is placed in a centrifuge tube and underlayered with various densities (percentages) of Percoll. Use of Percoll will be described. Philips's procedure is designed to eliminate the hypotonic lysis step. Metabolic responses of cells isolated with this technique are comparable to those obtained with Böyum technique. Various modifications adapted from these procedures include changing the Percoll percentage to allow better separation of cells, the supernatant from the dextran sedimentation step was applied to the top of the Percoll gradient not underlayered with Percoll, and the pellet washed two times in one-half the volume of dextran supernatant applied to the top of the Percoll gradient.
Preparation of rabbit peripheral blood PMNL

Blood was drawn by an open-chest cardiac puncture technique from diethyl ether anesthetized rabbits into 77mM Na₄EDTA (pH 7.2, blood: Na₄EDTA, 9.2:0.8 v/v) using an 18 gauge needle. Blood volumes range from 60-105 ml. Blood will be transferred into a 100 ml siliconized graduated cylinder (23.8 cm height: 2.1 cm I.D.). EDTA-treated blood is mixed with Dextran T-500 (6.0% solution in 0.9% NaCl) in a 5:1 ratio (v/v, blood:dextran), inverted gently five times for uniform mixing, and allowed to stand (1 xg) at room temperature for 45-60 min during which time the majority of the RBC will settle. The dextran solution is used at room temperature. Because of their large numbers, RBC interact with other RBC through the dextran particles, undergo rouleau formation and settle under the force of gravity. Both control and diabetic blood separated at the same rate. The percent increase in the appearance of the dextran supernatant (DS) paralleled each other i.e. control (n=10, mean ± S.D.) 47 ± 17 and diabetic (n=6, mean ± S.D.) 57 ± 4 at 45 minutes. Initial whole blood cell ratio was approximately 1:1200 (PMNL to RBC). Greater than 99% of the RBC are eliminated by the dextran sedimentation step. The DS is gently delivered to the top of the Percoll gradient.

Percoll is the medium utilized for the isopycnic density gradient centrifugation of PMNL. Percoll is colloidal silica coated with polyvinylpyrrolidone rendering the material non-toxic. Because
of the osmolality (< 20 mOsmoles/kg H2O) precise adjustments to physiological conditions can be made without significant interference from Percoll. Percoll does not adhere to biological membranes and is not phagositized by PMNL. Percoll is supplied at a density of 1.130 ± 0.005 g/ml and a pH of 8.9. Percoll used in these studies were all from Lot KL 38622 Code No. 17-0891-01 or Lot KC 35310 Code No. 17-0891-01. Percoll solutions are diluted with Dulbecco's phosphate buffered saline (PBS) which contains; NaCl 137mM, KCl 2.68 mM, Na2HPO4 8.1 mM, and KH2PO4 1.47 mM. Ca2+ and Mg2+ ions are omitted. Calculated osmolality is 308 mOsm/kg H2O. Actual osmolality, checked by freezing point depression, was PBS (282 mOsm), 100% (326 mOsm), and 65% (320 mOsm). Dulbecco's PBS, purchased from Gibco, pH 7.3) at a 10x concentration solution, is used to dilute stock Percoll and is used to make isotonic PBS solutions. Working PBS is made by diluting 10x concentrated PBS 1 to 10 with distilled water i.e. 100 ml to 900 ml water for 1 liter. Percoll solutions are made as follows: 100% Percoll solution = 9 parts Percoll supplied from Pharmacia + 1 part 10x concentrated PBS i.e. 180 ml Percoll + 20 ml 10x PBS; 65% Percoll solution = 6.5 parts 100% Percoll + 3.5 parts isotonic (working) PBS i.e. 130 ml Percoll + 70 ml isotonic PBS. All 15 or 50 ml centrifugations tubes are made of polypropylene. Preliminary isolation was obtained using the discontinuous Percoll gradient composed of three percentages, 55%, 65% and 80% used for human PMNL isolation. Calculated densities of the solutions are
Platelets remain on top of the 55% layer and mononuclear cells, PMNL, and RBC are observed at the 55%/65% interface, 65%/80% interface, and in the pellet respectively. No studies exist demonstrating isolation of peripheral rabbit PMNL with Percoll. From the literature PMNL isolated from rabbits are elicited and peritoneal and are separated by using a different centrifugation medium, Ficoll-Paque. To compare arachidonic acid metabolism studies in the rabbit to the human, it was desired to use peripheral blood and Percoll as a centrifugation medium. Experiments were already being conducted using human peripheral PMNL separated by Percoll.

Isolation of rabbit PMNL resulted in a slightly altered sedimentation pattern when the 3 percoll gradient system was used. With three discontinuous gradient layers, both platelets and mononuclear cells of rabbit were found as in the human isolation, but the PMNL and RBC were distributed at the 65%/80% interface as well as in the pellet at a 1:3-7 ratio (PMNL to RBC respectively). This observation possibly results due to the probable overlap of cell densities. Eliminating the 80% Percoll layer allows both the RBC and PMNL to pellet together using 2150 rpm/600 xg/15 min in a GLC 2 (swinging bucket rotor). Using Percoll in this manner is similar to using Ficoll-Paque, an isolation medium for lymphocytes/monocytes (Smith et al., 1982, Lafuze et al., 1984). With a Ficoll-Paque gradient (single density) lymphocytes/monocytes are retained on top of the medium while RBC and PMNL migrate through the medium and pellet upon centrifugation. Yield and purity were similar between
the experiments using Percoll and Ficoll-Paque. Percoll was chosen because of reports of lower toxicity to cells and because established methods of human PMNL utilized Percoll.

Experiments were also conducted modifying the volume of Percoll gradients (1 ml to 4 ml), changing the percent Percoll 82% and 86% of the bottom gradients, and also varying the centrifugation speed (2150 rpm to 3000 rpm) to attempt to better separate the RBC and PMNL. These experiments did not resolve the dilemma and the RBC and PMNL were still recovered in mixed form.

Since the PMNL and RBC were recovered in an admixture, it was decided to lyse the RBC, not the initial desire, but one consistent with the handling of PMNL and RBC mixtures from the literature. PMNL/RBC pellets obtained after the 600 xg spin were suspended gently in ice cold distilled water for 30-45 seconds to cause hypotonic lysis of the RBC. The volume of water is 1/10 of the volume of DS initially layered on top of the 65% layer. NaCl (3.507%, 0.6M) is immediately added to the PMNL and lysed RBC suspension at 1/3 of the volume of distilled water used for lysing. This volume ratio brings the mixture to isotonicity. Duration of lysis longer than this time are not necessary since essentially all RBC have lysed and decreased viability is noted with longer times.
After lysis a volume of PBS equivalent to one-half of the volume of DS initially layered on top of the 65% Percoll layer is added to the cell suspension. Residue dextran and particles of Percoll will be removed from the PMNL preparation by diluting with PBS and centrifugation to collect the cells. The pH of the resuspending solution was determined (after first wash pH 7.75, after second wash pH 7.3) to note how readily Percoll (pH 8.9) would be removed and/or buffered by PBS (pH 7.3) used to resuspend the pellets.

The diluted cell suspension is centrifuged at 400xg (1580 rpm, 7.5 min) and the resulting PMNL pellet free of RBC washed two times with PBS (each time the pellet is gently resuspended in one-half of the volume of DS initially layered on the Percoll and recentrifuged at the above speed and time). After washing the PMNL, they are resuspended in 0.5-1.0 ml of PBS (without Ca\(^{2+}\) or Mg\(^{2+}\)) and diluted further in PBS to the required cell number (typically 1-15 x 10\(^6\) PMNL/0.5 ml). Figure 3 demonstrates the isolation of rabbit peripheral PMNL as described above.

**Cell Count, Purity, and Viability of PMNL:**

A 1:20 dilution in PBS of the final PMNL resuspension was made to check for viability, to determine the number of PMNL, and to note preparation purity. Ninety \(\mu\)l of PBS was added to 10 \(\mu\)l of the PMNL suspension in a siliconized 13 x 100 mm test tube. After gently mixing, 50 \(\mu\)l was mixed with 50 \(\mu\)l of 0.1% new methylene blue and the remaining 50 \(\mu\)l mixed with 50 \(\mu\)l of 0.5% trypan blue.
Mixed blood and Na\textsubscript{4}EDTA in siliconized graduated cylinder

1) Add volume of 6% Dextran, mix and wait 45 min.

2) Dextran Supernatant (20-60 ml)

Red blood cells

3) Transfer supernatant to 50ml centrifuge tube

10-15% ml 65% Percoll 1.077 gm/ml

Spin at 600 x g for 15 minutes

Mononuclear cells/platelets

Red blood cells/neutrophil pellet

Remove supernatant to pellet, lyse RBC, wash 2X with PBS, cells spun free of Percoll at 400 x g for 7.5 minutes.

Final resuspension 0.5 - 1.0 ml PBS, Adjust to desired cell number.

Figure 3. Rabbit neutrophil isolation by dextran sedimentation of RBC followed by a single gradient of Percoll.
A differential cell count will be obtained after cell chromatin have stained in 10 min. Uptake of trypan blue (permeability check for viability (Ford, 1978)) will be obtained after 3 min.

In several samples, viability of PMNL incubations were assessed after the addition of PMNL stimulators and compared to viabilities obtained before the PMNL challenge. Incubation conditions tested were those for 2 minutes containing 1 mM Ca$^{2+}$ / 10 μM A23187 / 10 x 10$^6$ PMNL with AA concentrations of 10, 50, and 100 μM. After the 2 minutes, 10 μl of cell suspension was removed and the remainder of cells mixed with 100 μl of 2N formic acid. Ten microliters of trypan blue was mixed with the 10 μl of cell suspension in a siliconized 13 x 100 mm test tube and applied to a hemocytometer. After 2-3 minutes both viable and nonviable cells were counted and compared to counts obtained before the incubations. Viability was maintained > 85% in both C and D incubations (N = 4 pairs at the three levels of AA). No difference was noted between C and D preparations. Before the incubations, cells were round to irregular in shape with multilobed nuclei. After the incubation, cells were very round and swollen in appearance with contracted nuclei.

Typically 95 ± 15 x 10$^6$ (S.D.) PMNL can be recovered from 70 ml of control rabbit peripheral blood (40-45% yield, N = 11).

Yield of PMNL from the diabetic rabbit is lower than the control (20-27%). For 8 diabetic rabbits the blood volume was 96 ± 10
ml (S.D.) and the recovered PMNL was $57 \pm 15 \times 10^6$ (S.D.). This decreased yield was not a result from an initial lowered PMNL count in whole blood. Initial counts showed no significant different in the initial count. The lowered yield could be accounted for by examining the mononuclear cell layer. A substantial amount of diabetic PMNL remained in this layer when compared to control PMNL (20-25%). Similar observations have been noted by Segal et al. (1980) using Percoll gradients and Ferrante et al. (1982) in abnormal cell banding. Patients with chronic granulomous disease and rheumatoid arthritis failed to separate in distinct bonds. Because of the diabetic condition, changes in vivo may produce slight changes in the buoyant density of some PMNL, i.e. PMNL which have been in circulation longer may have been altered by plasma components in the diabetic. These alterations may result in these cells being preferentially lost during the Percoll isolation procedure. Reasons for the lower yield of diabetic PMNL is not clear.

Viability is greater than 96% in PMNL preparations from controls while viability was $88 \pm 6$ (S.D.) in the diabetic PMNL preparations. Purified neutrophil preparations had neutrophil to platelet ratios consistently $> 20:1$, RBC $> 100:2$, lymphocytes and eosinophils $> 100:2$, and monocytes $> 100:1$. A limited amount of samples were stained with Wrights-Giesma stain to confirm differential counts.
Incubation and Extraction Procedure for Arachidonate Metabolites

Typical incubation procedures proceed as follows: PMNL (5 x 10^6 or 10 x 10^6), suspended in 0.5 ml PBS, are preincubated at 37°C for 2 min without stirring then exposed to Ca²⁺, A₂₃₁₈₇, and arachidonate, each added at 15 sec intervals. Volumes of addition are Ca²⁺ (5 µl), A₂₃₁₈₇ (2.5 µl), and arachidonic acid (5 µl). After the arachidonate addition, the suspension is manually mixed to insure complete mixing. The reaction mixture is incubated for the designated times while stirring at a low speed. In some experiments one or two agonists are omitted i.e. Ca²⁺ may be omitted to observe if A₂₃₁₈₇ could release enough Ca²⁺ from endogenous stores to stimulate 5-lipoxygenase activity or arachidonate may be omitted to note the arachidonate releasing potential of A₂₃₁₈₇ and the resulting metabolite formation. Release of arachidonic acid in PMNLs exposed to chemoattractants is predominately released by a Ca²⁺-dependent phospholipase A₂ (Rubin et al., 1979, Walsh et al., 1981. At the end of the incubation period, the reaction is terminated with 100 µl of 2N formic acid (pH 2.3) which is sufficient to lower the pH of the aqueous layer to 2.6. Titration of PBS pH (initially 7.3) with 2N formate volume is as follows; 12.5 µl formate (pH 3.25), 25 µl (pH 3), 50 µl (pH 2.75), and 100 µl (pH 2.6). From preliminary experiments with human neutrophils, arachidonic acid metabolism was not observed when 100 µl of 2N formic acid was added to a PMNL suspension containing 15 x 10^6 cells, followed by the addition of 1 mM Ca²⁺, 2.5 µM A₂₃₁₈₇, 10 µM arachidonic acid for a 5 min.
incubation. Addition of formic acid eliminated the neutrophil response to metabolize arachidonic acid under these incubation conditions where significant levels of both LTB₄ and 5-HETE would be expected. Initial extraction were with diethyl ether (three extractions with three ml). Extraction with diethyl ether led to observation of two large impurity peaks migrating sufficiently close to the 5-HETE peak (on HPLC) to re-evaluate extraction with diethyl ether. Further extractions were with ethyl acetate. Impurity peaks near 5-HETE were not present on the chromatograph. Extractability also increased from 30-50% over that obtained with diethyl ether for compounds of interest. This was determined by treating two identical samples to the different organic extracting agents and comparing the areas of integration from HPLC.

After formate addition, samples are injected with 10 μl (100 ng) of an internal standard solution of PGB₁ (Sun and McGuire, 1984) (stock concentration of 10 ng/μl methanol) to adjust for variabilities in the extraction procedure. One hundred nanograms of PGB₁ is in the nanogram range of products analysed by HPLC. Ultraviolet absorbance of a stock solution of PGB₁ (10 μg/ml), demonstrated an intense absorbance at 277 nm. Under the HPLC conditions, a symmetric, narrow peak was observed. Area determinations by the integrator for samples which were extracted for PGB₁ seldom varied by more than 10% from the area observed by injecting PGB₁ directly on column.

Washing the ethyl acetate extract to remove dissolved formate (water dissolves slightly in ethyl acetate) was not necessary and
determined as follows; 100 μl of 2N formate was added to 500 μl of PBS to a siliconized 13 x 100 mm tube with a 9 mm teflon magnet. The aqueous phase was extracted three times with three ml of ethyl acetate each time (1 min vortex per extraction). With siliconized 5 3/4 in. Pasteur pipettes, the ethyl acetate layer (upper) was transferred to a siliconized 1 dram vial, evaporated under N₂, and diluted with 500 μl of water (pH 5.65). The pH of the water was 5.7 using pH indicator paper indicating that residual acidity was not present.

The ethyl acetate extract is concentrated under N₂ and the residue resuspended in HPLC grade methanol (500 μl). Most extraction methods employed for the isolation of leukotrienes (peptido and LTB₄) use a combination of sequential XAD resin columns and silicic acid open-column chromatography (Metz et al., 1982). Since peptido-leukotrienes are not made in extensive amounts from PMNL, hydroxylated arachidonic acid metabolites alone can be efficiently extracted into ethyl acetate eliminating extensive sample preparation. Prior to injection on HPLC, this volume is evaporated under N₂ and 50 μl methanol readded. One-half (25 μl) of the sample is injected on column.
Analysis of Platelet Free Plasma Samples for TxB₂, PGE₂ and 12-HETE. After the aggregation period, platelet reactions were terminated either by 2N formic acid (100 μl, pH 2.3) or addition of a solution (50 μl) containing 0.01 mol/L Na₄EDTA and 0.1 mmol/L ASA. Samples were allowed to stand at room temperature for 10 min., then centrifuged at 15,000 x g for 1 min. The supernatant was removed, frozen in liquid N₂ and subsequently stored at -20°C under N₂. Aliquots of up to 20 μl from the supernatant were removed for the direct radioimmunoassay for TxB₂ and PGE₂. Cross-reactivity of the TxB₂ antibody was: PGF₂α 0.26%, PGE₂ < 0.021%, PGD₂ < 1.6%, cross-reactivity of the PGE₂ antibody was: PGF₁α 0.76%, PGF₂α 0.31%, PGD₂ 0.051%, 6 Keto- PGF₁α 0.04%, TxB₂ 0.0078%, AA 0.0004%. Cross-reactivity is defined as the concentration of the compound to be measured that displaces 50% of the label from the antibody multiplied by 100% then divided by the concentration of cross reacting compound that displaces 50% of the label from the antibody.

Development of the analysis of prostaglandins from PFP samples was by David J. Duffy. Verification was by David J. Duffy and Nicholas J. Greco. All nonlipemic PFP used was from fasted animals. Initially, aliquots of PFP (0-200μl) from both C and D subjects were added to RIA tubes containing only ³H-TxB₂ and the TxB₂ antibody. Binding of ³H-TxB₂ to the antibody decreased with increasing PFP volumes as shown in Figure 4. Decreased binding could result from endogenous TxB₂ generated upon drawing the blood or a nonspecific interfering factor. In subsequent RIAs, volumes were
Figure 4. Effect of increasing volumes of PFP on the binding of $^3$H-TxB$_2$ antibody.
limited to 20 μl and below. C and D PFP exhibited similar profiles. Standard curves were also ran spiked with 20 μl of C and D PFP and compared to standard curves containing only RIA buffer. No significant differences were observed. Results were similar with 3H-PGE₂. Figure 5 represents the results of a recovery experiment where known quantities of unlabeled TxB₂ is added to PFP samples, then aliquots are subjected to RIA. Detection of TxB₂ in C and D PFP samples without the addition of unlabeled TxB₂ were below the sensitivity of the assay (< 16 picograms / tube). Actual picograms of TxB₂ added and the measured amounts in PFP samples is shown in Table 1.

The data reported in this study are the means of single determination experiments of different subjects. Quantitation of TxB₂ and 12-HETE production and 5HT release is highly reproducible. Intra-assay variation (mean ± standard error of the mean of four aggregation determinations on the PRP from one human diabetic subject challenged with 5.0 x 10⁻⁶ M PAF) was as follows: % aggregation at 4 min, 69 ± 0.5; TxB₂, 12 ± 0.5 pmoles / 10⁸ platelets; HETE, 24 ± 3 pmoles / 10⁸ platelets; and % 5HT release, 39 ± 2.

Table 1. Recovery of unlabeled TxB₂ added to PFP.

<table>
<thead>
<tr>
<th>Picograms TxB₂ added</th>
<th>55</th>
<th>106</th>
<th>195</th>
</tr>
</thead>
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<td>Picograms TxB₂ measured in control PFP</td>
<td>58</td>
<td>110</td>
<td>187</td>
</tr>
<tr>
<td>Recovery (percentage of added TxB₂)</td>
<td>105%</td>
<td>104%</td>
<td>96%</td>
</tr>
<tr>
<td>Picograms TxB₂ measured in diabetic PFP</td>
<td>65</td>
<td>117</td>
<td>205</td>
</tr>
<tr>
<td>Recovery (percentage of added TxB₂)</td>
<td>118%</td>
<td>110%</td>
<td>105%</td>
</tr>
</tbody>
</table>
Figure 5. Recovery of unlabeled TxB$_2$ from control and diabetic platelet free plasma.
The values shown in Table 1 are the averages from a N of 4 for both C and D PFP samples determined in triplicate. Diabetic PFP consistently demonstrated an increased recovery of TxB$_2$ from 6 to 13% in the range tested. This was not of significance since stimulated D PRP samples produced approximately two times greater levels of TxB$_2$ as measured by RIA. Values of TxB$_2$ measured by the RIA from stimulated PRP samples ranged from 24-1200 pmoles. The range of picomoles of TxB$_2$ added in Figure 5 were from 150-530. For radioimmunoassay of iTxB$_2$ (immunoreactive TxB$_2$) over the range of 150-530 pmoles / tube, the recovery of added authentic TxB$_2$ was quantitative for control PFP ($y = 0.915x + 9.74$, $r = .999$) and slightly greater than quantitative for diabetic PFP ($y = 0.999x + 10.5$, $r = .999$). The minimal detectable amount of TxB$_2$ in 500 µl of PFP was approximately 40 picograms. Interassay variability, evaluated using three pooled human platelet controls, was 15.2%, 6.2%, and 7.0% (CV) for the low control (approximately 80% binding, 30.9 ± 4.7 pg / 200 µl (∆ SD)), medium control (approximately 50% binding, 158 ± 9.9 pg / 200 µl), and high control (approximately 20% binding, 329 ± 23 pg / 200 µl) respectively. Results are compiled from 12 standard curves.

Unlabeled PGE$_2$ was added to C and D PFP samples then subjected to radioimmunoassay as described above. Recovery, as a percentage of added PGE$_2$, averaged 95 ± 4 over a range of 100 - 2000 picograms. Recoveries were similar using C and D PFP. Interassay variability, evaluated using three pooled human platelet controls, was 17% (158 ± 27 pg / 200 µl), 11.1% (285 ± 31.7 pg / 20 µl), and 4.7%
(621 ± 29 pg / 200 μl) for the low, medium, and high controls respectively.

Development of Analysis of PFP for 12-HETE

Arachidonic acid and other free fatty acids bind to plasma proteins especially albumin. 12-HETE has a similar affinity. Initially unlabeled 12-HETE (1500 pg) was added to 500 μl PFP samples of C and D subjects. Protein in platelet free plasma (PFP) was precipitated with 1.2 ml ethanol. Following ethanol addition, vortexing for 1 min, and centrifugation (3000 rpm, 1500 xg / 10 min), a portion of the ethanol supernatant (1.0 ml) was removed and evaporated under N2. A RIA for 12-HETE was completed after the addition of RIA buffer to the evaporated sample. Recoveries were low, 20 ± 7% (SD) of the quantities that were added. 12-HETE seemed to have a greater affinity for a ethanol soluble factor which reduced the apparent detection or a factor interfered with the antigen-antibody. 3H-HETE (approximately 13,500 dpms) was subjected to the same procedure and 93 ± 2% of the added label was detected in the ethanol extract. The next modification was to add acid (600 μl of 1 N HCl) to the ethanol extract to attempt to free "bound" 12-HETE. 12-HETE was back-extracted into 3.5 ml ethyl ether, shown previous to quantitatively extract 12-HETE from washed platelet suspensions (Karpen et al., 1985). Recovery of 12-HETE in the ethyl ether residue was 99 ± 3%. Similar results were obtained with 600 μl of 0.1 M citric acid (99 ± 4%). If the acid (1 N HCl) was added first, followed by addition of ethanol recovery was approximately 31%. Various normalities of HCl were used to determine an optimal
concentration. Results (normality and recovery of 12-HETE) are as follows: 2.0 N, 45%, 1.0 N, 99%, 0.5 N, 100%, 0.25 N, 99%, and 0.125 N, 70%.

Interassay variability of the 12-HETE RIA, evaluated using three pooled human platelet controls, was 10.2% (608 ± 62 pg / 200 µl), 6.7% (1233 ± 82 pg / 200 µl), and 11.0% (2954 ± 320 pg / 200 µl) for the low, medium, and high controls respectively.

**Recovery of ³H-HETE from Platelet Free Plasma After Ethanol Precipitation.** The first step in the determination of 12-HETE levels in PFP involves mixing 50 µl of PFP with 1200 µl of ice cold 100% ethanol. Experiments were conducted to determine how efficiently ³H-HETE would be dissolved in ethanol and removed from the protein matrix of PFP. Two levels of 12-HETE, which approximated observed values for PFP samples, were tested. These levels were 1000 pmoles (318 ng) and 100 pmoles (31.8 ng). Briefly an aliquot of ³H-12-HETE was added to control or diabetic PFP, lightly vortexed, then a 50µl portion mixed with 1200 µl of ice cold ethanol for 1 min. Following centrifugation (3000 rpm/ min), 1 ml of the ethanol supernatant was removed, evaporated under N₂, and counted after the addition of 5.5 ml ACS cocktail.

For 1000 pmoles, 18.27 µl of the ³H-12-HETE and for 100 pmoles 1.83µl of ³H-HETE was used. Theoretically, 5293 cpm should be observed in the evaporated sample. The average cpm observed of a N=4 in triplicate for control PFP was recovered with an efficiency of 103.7 ± 3.25% with a CV of 3.13%. Results were similar with diabetic PFP.
Measurement of TxB$_2$, PGE$_2$, and 12-HETE from Washed Platelet Suspensions. After the incubations, platelet reactions were terminated with 100 µl of 1.0 N HCl. Reaction products were extracted into diethyl ether, followed by evaporation under N$_2$ and dissolving the residue in Trizma Base (50 mM)-gelatin (0.1%) RIA buffer (pH 7.8). Greater than 90% of the formed eicosanoids (TxB$_2$ and PGE$_2$) were extractable with one 3.5 ml ether extraction (Karpen et al., 1982). Recovery of 12-HETE with this method is 88 ± 0.7% (mean ± S.D. (Karpen et al., 1985). Final dilution of the samples ranged from 1-6 ml depending on the agonist. Final values are expressed as pmoles eicosanoid/10$^8$ PTs. All incubations determinants were performed in duplicate or triplicate.

Plasma Exchange Experiments. Several studies have examined the effect of human diabetic platelet poor plasma on normal platelet aggregation (Kwaan et al., 1972, Leone et al., 1977). These studies involved mixing diabetic plasma and control PRP in ratios of 1:8 and 1:6 (v/v) respectively. Observations in this laboratory demonstrated that a platelet pellet could be isolated from citrated platelet rich plasma by centrifugation at 15 x 10$^3$ xg. Subsequently, it was demonstrated that a platelet pellet could also be recovered with a 4000 rpm (1980 xg) spin in a RC2-B. Speeds lower than this allowed more platelets to remain in the supernatant and speeds greater than 4000 rpm did not recover additional platelets.
Preliminary experiments demonstrated that when platelets from PRP samples were isolated by centrifugation then gently resuspended back into the platelet poor plasma supernatant activity with respect to both aggregation, TxB₂, and 12-HETE production was retained. Loss of platelet activity was never greater than 10% either in terms of aggregation or prostaglandin production using arachidonic acid or collagen as agonists. Both C and D plasma were similar in this manner. The objective of the plasma exchange experiments was as the experiments described in the literature using mixtures of plasmas. These experiments could potentially allow one to observe if components in the plasma would effect platelet function during short term incubations. The procedure for exchanging plasma is shown in Figure 6. Functions of the three parts are discussed as follows; part A is used to compare and validate that C PRP and D PRP are responding as shown in the PRP Results Section, Part B is used to examine any deviations in behavior due to the centrifugations when comparing responses to part A, and part C is used to observe potential effects that the plasma would have on the other subject's platelets.

Serum and plasma chemistry - All serum and plasma values shown in this dissertation were completed by the Clinical Chemistry (Pathology) department of The Ohio State University using established analytical techniques.
Blood (9 parts) + Anticoagulant (1 part) Na$_3$ citrate

1180 rpm, Sorvall GLC-2, 20 min.

Remove PRP and divide into three portions A, B, C
One portion (A) is held at room temperature.

portions B and C
4000 rpm, 1980 xg,
Sorvall RC2-B, 20 min.

Remove supernatant and save the platelet pellet on ice under N$_2$. The supernatant is centrifuged further

Portion B

Portion C

The resulting supernatant is used to resuspend the platelet pellet obtained from the same subject.
The resulting supernatant is used to resuspend the platelet pellet obtained from the other subject.

Figure 6. Procedure for exchanging plasma between platelet suspensions.
Measurement of $[^{14}C]$-5HT Release. PRP was adjusted to 200,000 platelets/μl with autologous PPP. PRP was incubated at 20° to 23° C for 30 minutes with $^{14}$C-5HT at a final concentration of 5 x $10^{-7}$ μmol/L (Mills et al., 1967, Smith et al., 1974). PRP (0.5 ml) was preincubated (Valdorf-Hansen et al., 1971) with imipramine (2.5 x $10^{-6}$ mol/L) for 1 minute at 37° C before challenge. Reactions were terminated at 2 or 4 minutes by adding 50 μl 0.1 mol/L Na$_4$EDTA. Samples were centrifuged at 15,000 x g for 1 minute. Aliquots (100 μl) of the supernatant were removed and $^{14}$C was counted by a liquid scintillation counter (Ardlie and Han, 1974). The same procedure was used for humans and rabbits.

Determination of the purity of AGEPC by straight-phase HPLC. Various reports have defined analytical systems for the determining the purity of AGEPC (Chilton et al., 1980, Patton et al., 1982, Alam et al., 1982). A straight phase, gradient elution system developed and ran by Laura Dugan was utilized. Mobile phase characteristics are solvent A (hexane:isopropanol, 3:2) and solvent B (hexane:isopropanol, 3:3:2 plus 5.5% water). Column temperature was thermostated to 34° C. Sample runs were for 35 minutes. Solvent ratios were changed as follows: initially 50% A : 50% B, t = 11.5 min (a linear gradient of 50% B to 100% B over 5 min), t = 21 min (a linear gradient of 100% to 50% B over 3 min), 50% A : 50% B to the completion of the run. Mobile phase flow rate was 1.5 ml/min. AGEPC was monitored at 205 nm. The column was a silicic acid, polar column. Injection volume (25 μl) contained 100 nM of AGEPC.
Approximate retention times of authentic standards are: neutral lipids 2.5 min, cerebrosides / sulfatides 3.5 - 6.5 min, phosphatidylethanolamine 8.5 min, phosphatidylethanolamine plasmalogen 9.5 min, phosphatidylinositol 16.5 min, phosphatidylserine 24.5 min, phosphatidylcholine 29.5 min, phosphatidylcholine plasmalogen 30.5 min, and sphingomyelin 31.5 min. AGEPC elutes at 32 min in this system. An additional impurity peak was noted at 33.5 min. Phosphorus analysis indicated a 8:2 distribution of area. Both peaks were collected, HPLC elutent evaporated, and the residue dissolved in ethanol. Only the larger peak at 32 min stimulated TxB\(_2\) production from a rabbit washed suspension.

**Measurement of \(\alpha\)-tocopherol (Vitamin E) and Cholesterol by HPLC.** Analysis of \(\alpha\)-tocopherol and cholesterol in samples of rabbit plasma and platelets was performed using a modification of the described HPLC procedure of Hatam and Kayden (1979). Initial modification of the procedure of Hatam and Kayden to include quantitation of cholesterol was by Dr. Hisayuki Kaseki. Modifications include; samples are extracted 2 times to quantitatively remove cholesterol from the sample and the ultraviolet wavelength is changed during the run. HPLC equipment is identical to that described for the measurement of metabolites of arachidonic acid by HPLC. Mobile flow rate was 1.2 ml/min (1800 psi Pump A, water, 1500 psi Pump B, methanol). The isocratic mobile phase consisted of 95% HPLC grade methanol: 5% HPLC grade water. This mixture was degassed as previously described in the HPLC section. The column
Effluent was monitored at 254 nm (0-7.5 min), 294 nm (7.5-10.5 min), and 200 nm (10.5-17.5 min). Detector range changes occurred depending on the sample type. For plasma samples the range was 0.05 at 254 and 294 nm. For platelet samples the range was 0.02 at 254 nm and 294 nm. The range was 0.5 at 200 nm for both samples. Attenuation was set at 1 on the Altex C-RIA integrator.

Procedure for Saponification and Extraction. Plasma samples (0.5 ml) and platelet suspensions (0.5-2 x 10^9/ml Tris (50 mM) NaCl (1.6 mM) EDTA (1.6 mM)) in 16 x 125 mm test tubes were mixed with 2.0 ml of 1.0% L-ascorbic acid in 100% ethanol. Samples were lightly vortexed. Tubes were heated at 70°C for 2 min, followed by the addition of 0.3 ml of a saturated KOH solution (in 100% ethanol) and light vortexing. Stopped samples were heated for 30 min at 70°C in a water bath, then cooled on ice (approximately 2 min). One ml of distilled water was added (vortex lightly) followed by the addition of HPLC grade hexane (4 ml). The nonsaponifiable portion was extracted into hexane (2 x 4 ml). After vortexing 2 minutes and centrifugation (3000 rpm/5 min). Two 3.7 ml aliquots (of 4 ml used to extract) were pooled, evaporated under N₂, and the residue dissolved in 500μl HPLC grade methanol. Final calculated values were for 8 ml total hexane. Aliquots (50 μl) were injected on the HPLC column after concentrating the 500 μl under N₂ and readding 100 μl methanol. With the platelet number (0.5-2 x 10^9) and plasma volume (0.5 ml) designated, integrated areas for both α-tocopherol and cholesterol would lie in the linear region of each standard curve (injecting 1/2 of the initial sample).
Figure 7. HPLC of α-tocopherol quinone, α-tocopherol, and cholesterol standards. A indicates α-tocopherol quinone, B indicates α-tocopherol, and C indicates cholesterol. Wavelength changes are indicated under the chromatographs. The same designations hold for Figures 8 and 9.
Figure 8. HPLC of α-tocopherol and cholesterol extracted from a platelet suspension.
Figure 9. HPLC of \( \alpha \)-tocopherol and cholesterol extracted from a plasma sample.
Cholesterol and d-α-tocopherol were injected in ethanol from 46-1250 ng (α-tocopherol) and 1.23-33.3 μg (cholesterol) to provide linear concentration versus integrated area standard curves. Concentrations greater than 33.3 μg cholesterol demonstrate a nonlinear curve. Restriction to the platelet number and plasma volume allowed both a maintenance of sensitivity and the integrated areas would lie within the linear portion of the standard curves. Minimal amounts of α-tocopherol and cholesterol that could be detected was approximately 10 ng and 300 ng respectively.

Retention times for the following compounds in this HPLC system are as follows; α-tocopherol quinone, 6.34 ± 0.280 min, γ-tocopherol 8.04 ± 0.0989 min, α-tocopherol, 9.20 ± 0.070 min, and cholesterol 15.3 ± 0.150 (mean ± S.D., n=34, Figure 7, γ-tocopherol not shown). Coefficients of variations are α-tocopherol quinone 4.41%, γ-tocopherol 1.23%, α-tocopherol 0.76%, and cholesterol 0.98%. β- and γ-tocopherol elute as one peak in this system.

Extractability for both α-tocopherol and cholesterol increased when the extraction procedure was performed on platelet suspensions with two freeze/thaw cycles (in liquid N2) versus extractions of platelet pellets. Percent recovery was increased as follows; α-tocopherol 205% and cholesterol 12.8%. Extractability of α-tocopherol and cholesterol was determined by successive HPLC runs of individual extractions from both platelet and plasma samples. Samples were extracted 3 times with 4 ml of hexane (Figures 8 and 9). The total volume of hexane was removed and treated as individual HPLC samples. α-tocopherol was recovered completely (100%) with one hexane
extractions. Cholesterol required 3 extractions for complete recovery, first extraction 85.8%, second extraction 13.0%, and the third extraction 1.18%. Approximately 98.8% was recovered in the first two extractions. Therefore two extractions of 4 ml hexane would sufficiently recover both α-tocopherol and cholesterol. During the actual procedure only 3.7 ml was removed during each extraction and values calculated for 4 ml. The objective for this was to 1) accurately know the volume of hexane removed and 2) eliminate contamination of the organic phase with the aqueous phase. Removing close to 4 ml allowed one to maintain sensitivity of the method. Figures 10 and 11 show the dose-response for the assay of α-tocopherol and cholesterol by HPLC. The standard curve for α-tocopherol demonstrated linearity to 1250 ng (r = .999) while linearity was observed to 33.3 µg in the cholesterol standard curve.
Figure 10. Dose-response relationship for assay of $\alpha$-tocopherol (vitamin E) by HPLC.
Figure 11. Dose-response relationship for assay of cholesterol by HPLC.
Analysis of Arachidonate Metabolites by Reverse-Phase HPLC. Table 2 shows the extraction of biologically formed LTB₄ isomers, LTB₄, and 5-HETE as assessed by peak area versus extraction number. Results are derived from HPLC chromatographs from individually injected extractions. The percentages are based on the total area from all three extractions. Greater than 99% of the extractable PGB₁, LTB₄ isomers, and LTB₄ was extracted in two 3 ml ethyl acetate extractions. Approximately 95% of the produced 5-HETE was extracted in two extractions and an additional four percent in the third extraction. These percentages may be slightly elevated since a fourth extraction was not completed. Three extractions of 3 ml was used throughout the PMNL experiments. Similar extraction data was obtained when 500 µl volumes of PBS was spiked with 100 ng PGB₁, 75 ng LTB₄, 200 ng 5-HETE, and 500 ng AA.

Stock LTB₄ was stored in methanol at -70°C until diluted for HPLC. A 1:100 dilution in methanol was used to run the ultraviolet spectrum for the concentration calculation.

Initially, the modified HPLC procedure of Peters et al. (1983) was utilized. This procedure separates primary prostaglandins, peptido-leukotrienes and LTB₄, monoHETEs, and arachidonic acid in one HPLC run. Modifications by Dr. Hisayuki Kaseki (unpublished) include using 1.0% (v/v) aqueous phosphoric acid, pH 2.2 (solvent A) instead of 0.01% (v/v) aqueous acetate pH 3.7. This chromatograph procedure was not used extensively because of several considerations.
Table 2

Extraction of arachidonic metabolites from 10 x 10⁶ rabbit PMNL stimulated with 1 mM Ca²⁺, 10 μM A₂₃₁₈₇, 10 μM arachidonic acid for 2 minutes (includes PGB₁).

<table>
<thead>
<tr>
<th>Extraction Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGB₁</td>
<td>97.6%</td>
<td>2.4%</td>
<td>N.O.</td>
</tr>
<tr>
<td>Isomers of LTB₄</td>
<td>96.8%</td>
<td>3.3%</td>
<td>N.O.</td>
</tr>
<tr>
<td>LTB₄</td>
<td>96.8%</td>
<td>3.2%</td>
<td>N.O.</td>
</tr>
<tr>
<td>5-HETE</td>
<td>81.7%</td>
<td>14.0%</td>
<td>4.3%</td>
</tr>
<tr>
<td>A.A.</td>
<td>86.4%</td>
<td>10.1%</td>
<td>3.5%</td>
</tr>
</tbody>
</table>

Percent extraction for 5-HETE and arachidonic acid are determined by the areas appearing in three extractions.

N.O. = not observed.
If PMNL preparation were devoid of platelets primary prostaglandins as TxB$_2$ and PGE$_2$ probably would not be observed since their concentrations would be subnanogram (minimal detection limits are approximately 500 ng for both) and their low extinction coefficients do not allow appreciable ultraviolet absorbance. Platelet contamination could be determined by radioimmunoassay for TxB$_2$ and/or PGE$_2$. Purified PMNL preparations devoid of platelets have been described to also make primary prostaglandins (Goldstein et al., 1978, Zurier and Sayadoff, 1975). The HPLC run could then be shortened by starting at a greater acetonitrile percentage. This would elute compounds faster. By decreasing the elution time for LTB$_4$ to between 7.5 and 15 min, the two LTB$_4$ isomers would comigrate with LTB$_4$. Retaining LTB$_4$ on column for 32 min. still does not clearly resolve LTB$_4$ isomers and LTB$_4$ (Peters et al., 1983). Owing to the appreciable similarity in structure, ACN could not separate them because of a lack of selectivity. Because of the latter consideration and the feasibility of a shorter HPLC run, ACN was changed to methanol.

Running conditions of the following HPLC procedure was that of Dr. Michael M. Milks (unpublished method). Published methods used as additional references were those of Metz et al. (1982) and Sun and McGuire (1984). The advantage of this HPLC procedure was to obtain close to baseline separation of the two isomers and LTB$_4$. Conversion of LTA$_4$ to LTB$_4$ could be monitored since under certain incubation conditions LTB$_4$ production becomes maximal and the isomers derived from LTA$_4$ could be expected to increase. One could
then theoretically monitor when enzymatic reactions halted and nonenzymatic conditions played a bigger role.

A binary solvent system of 1.0% (v/v) aqueous phosphoric acid and methanol was utilized. Ionization of fatty acid carboxyl groups was suppressed with the acidic environment. Migration is then based on lipophilicity. Separation of metabolites differ in carbon number or in the number, position or geometry of double bonds is possible with the HPLC procedure. Chromatography will be carried out using a 5 µm Zorbax ODS column (25 x .46 cm). The HPLC system includes; a Beckman 421 controller, dual Beckman Model 110A pumps, Altex 210A injector (with 50 µl loop), Beckman 164 variable wavelength detector, and an Altex Model C-R1A integrator. Elution of metabolites will utilize a gradient, reverse-phase mode of chromatography. Mobile phase characteristics will vary during the chromatography run:

initial 72.5% methanol (pump B) 27.5% water pH 2.2 with H₃PO₄ (1.0%) (pump A), duration 10 min \( \lambda = 270 \) nm, \( R = .04 \); t=10 min linear increase of methanol from 72.5% to 82.5% over 10 min, t=20 min.

linear increase of methanol from 82.5% to 94% over 20 min, t=23 min, \( \lambda = 234, R = 0.2; \) wavelength change to 210 nm, \( R = 0.4 \) at 36 min.

Two potential drawbacks exist with this procedure. With the initial starting point of the run at 72.5% methanol, the peptido-leukotrienes i.e. LTC₄ and LTD₄, would elute with the solvent front. These compounds can be kept on column if desired by decreasing the initial methanol concentration. Under the incubation conditions (no supplementation with reduced glutathione or L-cysteine) and extraction procedure (extraction at pH 2.6 rather than at pH 5-6) the
peptido-leukotrienes would not be expected to be synthesized or extracted quantitatively. As shown by Sun and McGuire (1984), under similar incubation conditions with human PMNL, subnanogram levels of LTC₄ (order of 100 pgs) are measurable by RIA. These levels would not be observed with HPLC. Compounds after LTB₄ can be eluted more rapidly (by increasing the methonal concentration faster) but the baseline slope increases too rapidly for accurate and consistent integration. Decreasing the range during the HPLC run serves two functions; 1) as the wavelength is decreased and the methanol concentration increases, methanol alone begins to absorb more strongly and the baseline drifts upward. Decreasing the range (detector sensitivity) blunts the absorption of methanol and the subsequent baseline shift 2) the quantities of the peaks of interest increase in the order of LTB₄ < 5-HETE < arachidonic acid. Retention times also increase in the same order. Therefore, less sensitivity is needed to quantitate these compounds as the HPLC run continues and the range can be decreased. Equilibration to the starting mobile phase characteristics from 100% method is achieved in 30 min (100% to 72.5% over 10 min). Decreasing the methanol concentration too rapidly from 100% (over a shorter time) prolongs the re-equilibration time.

This HPLC format gave reproducible results: Coefficient of variations with respect to the retention time are PGB₁, 4.06, 5S, 12R-all-trans-LTB₄, 5.79; 5S, 12S all-trans-LTB₄, 6.26; LTB₄, 6.14; 15-HETE, 2.14; 12-HETE, 2.62; 5-HETE, 2.56; and arachidonic acid, 3.47. Retention times (minutes) are as follows in order of
elution (N=10-15 chromatographs) PGB\textsubscript{1} $8.87 \pm 0.36$ (mean ± S.D.); 5S, 12R-all-trans LTB\textsubscript{4}, 10.7 ± 0.62; 5S, 12S all-trans-LTB\textsubscript{4}, 11.5 ± 0.72; LTB\textsubscript{4}, 12.7 ± 0.78; 15-HETE, 24.7 ± 0.53; 12-HETE, 26.0 ± 0.68; 5-HETE, 27.7 ± 0.71; and arachidonic acid, 37.2 ± 1.30 (Figure 12).

Samples were initially injected in 50μl. This was subsequently changed to 25μl to achieve better separation between the two LTB\textsubscript{4} isomers and LTB\textsubscript{4}. This allows greater consistency in integration from run to run and also allows for peak isolation from the HPLC effluent. A large, unidentified peak eluted at approximately 17-18 min and was subsequently shown to be correlated with RBC contamination (increasing in area with RBC contamination). It was not determined if this peak was an arachidonic acid metabolite.

To confirm that PMNLs could synthesize LTB\textsubscript{4} and 5-HETE under the incubation conditions and that these compounds would be observed by HPLC, the retention times of authentic standards were compared to peaks observed upon injecting a biological sample onto the HPLC. LTB\textsubscript{4} and primarily 5-HETE would be expected from the literature to be the dominant peaks observed at 270 nm and 234 nm respectively. Retention times of the authentic standards eluted as the dominant peaks suggesting the identity of the unknown peaks. A further check was to spike biological samples with authentic standards and compare the HPLC tracing with a HPLC tracing without the addition of standards. Samples prepared in this manner demonstrated that the unknown peaks comigrated with authentic standards again suggesting their identity. From literature reports, levels of LTB\textsubscript{4} (and its
Figure 12. HPLC chromatograph of standards. (LTB₄ and monoHETEs). The isomers of LTB₄ elute approximately 1 to 2 minutes prior to LTB₄ (standards unavailable). Peak identification: (1) prostaglandin B₁; (2) leukotriene B₄; (3) 5-HETE; (4) A₂₃₁₈₇; and (5) arachidonic acid. Monitored wavelength (nm) is as marked. X₁ indicates 270 → 234 nm and X₂ indicates 234 → 210 nm.
trans isomers) and 5-HETE can be controlled by substrate availability.

To confirm the tentative identification (LTB₄ isomers) of the two peaks eluting before LTB₄, HPLC eluent was collected and pooled from several biological samples. For an appropriate blank for recording a UV absorption spectrum, HPLC eluent was collected during the time when the isomers and LTB₄ would elute when only 25μl of methanol was injected on column. Both isomers have absorption maxima at 258, 268, and 280 nm (Bokoch, et al., 1981). Absorption maxima for authentic LTB₄ standard is 260, 270, and 281 nm. Absorption maxima from the pooled biological samples were near 260, 270, and 280 nm indicating the compounds contain a conjugated triene structure. Sufficient resolution is obtained using the usual injection volume of 25μl to obtain a "heart-cut" from the peak of interests.

Contamination of PMNL preparations by platelets was evaluated by determining TxB₂ levels by radioimmunoassay. One-half of the sample was injected on the HPLC while one-half was retained for the RIA. Samples in methanol were evaporated under N₂ followed by the addition of 500μl of Tris-gelation buffer (composition as described under Preparation of Washed Platelet Suspensions). Aliquots were removed for RIA. Nanogram levels of TxB₂ ranged from < 0.2 to a maximum of 4.8. At TxB₄ levels of 5 ng, this represents 5% of the levels of LTB₄ isomers and LTB₄ and 0.9% of the levels of 5-HETE under incubation conditions of 1mM Ca⁺², 1μM A₂₃₁₈₇, 10μM arachidonic acid, and 5x10⁶ PMNL for 2 minutes. Cross-reactivity of
the TxB₂ antisera with compounds within the incubation mixture are as follows; 5S, 12R-dihydroxy-6,8,10-trans-14 cis-eicosatetraenoic acid (isomer 1 of LTB₄) unknown, 5S, 12S-dihydroxy-6,8,10-trans-14 cis-eicosatetraenoic acid (isomer 2 of LTB₄) unknown, 5S, 12R-dihydroxy-6,14 cis-8,10-trans-eicosatetraenoic acid (leukotriene B₄ or LTB₄) <0.003 %, 5S- hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) unknown, 5S-hydroxy- 6,8,11,14-eicosatetraenoic acid (5-HETE) <0.001 %, 15S-(hydroxy or hydroperoxy)-5,8,11,13-eicosatetraenoic acid unknown, A23187 0.04 %, and arachidonic acid < 0.001%. A similar approach could be taken for the C-12 lipoxygenase product produced from platelets, 12-HETE. RIA for 12-HETE was not completed since interpretation of the results could be complicated by the known cross-reactivity of the antisera for metabolites of arachidonic acid from neutrophils i.e. 5-HETE < 2.0%. Since 5-HETE levels are consistently > 15 times the 12-HETE levels, "12-HETE" as noted from the RIA would likely be really 5-HETE.

Platelets were incubated as PMNL preparations to determine quantities of 12-HETE that could be formed. Incubations included 1 mM Ca²⁺, 10 µM A23187, 10 µM arachidonic acid and two levels of platelets, 230 x 10⁶ and 100 x 10⁶. Incubations were for 2 minutes. These platelet numbers represent platelet/PMNL ratios of 23:1 and 10:1 if incubations would contain 10 x 10⁶ PMNL. Within 2 min., 866 ng and 379 ng of 12-HETE were observed from 230 x 10⁶ and 100 x 10⁶ platelets, respectively. With neutrophil to platelet ratios typically > 20:1, production of 12-HETE would be approximately
2 ng or less in incubations with 10 x 10^6 PMNL. Under these incubation conditions, 10 x 10^6 PMNLs produce approximately 190 ng LTB₄ and 800 ng 5-HETE. Production of 12-HETE from platelets would be insignificant compared to quantities of arachidonic metabolites produced from PMNLs.

LTA₄ that is formed can be converted to two other non-enzymatically formed metabolites, 5(S), 6(S.R.)-dihydroxy-7,9,11,14-eicosatetraenoic acid. These compounds can be monitored at 270 nm. From the literature (Bokach et al., 1981), under similar HPLC conditions have shown that these compounds elute after LTB₄ and before 5-HETE. Peaks in the area following LTB₄, monitored for an additional 10 minutes of run time did not increase when the concentration of arachidonic acid was increased from 10 μM to 100 μM. If these compounds eluted within 10 min after LTB₄ elution, the data does not support the conversion of LTA₄ to these metabolites.

Quantitation of Arachidonic Acid Metabolites. For quantitation of arachidonic acid metabolites, standard curves were constructed from HPLC runs of authentic compounds. Figures 13,14, 15 and 16 show the area of integration versus the nanograms of authentic standards. Production of LTB₄, 12-HETE, and 5-HETE from biological
Figure 13. Dose-response relationship for assay of leukotriene LTB₄ by HPLC.
Figure 14. Dose-response relationship for assay of 12-HETE by HPLC.
Figure 15. Dose-response relationship for assay of 5-HETE by HPLC.
Figure 16. Dose-response relationship for assay of arachidonic acid by HPLC.
samples ranged from 20 ng to 220 ng, 10 ng to 30 ng, and 80 ng to 1800 ng respectively. Quantitation of LTB₄ isomers utilized the standard curve for LTB₄. The equation of the line for the LTB₄ standard curve was \( y = (5.56 \times 10^{-4}) x + 4.79 \) with a correlation coefficient (r) of .999. A linear line was observed between 20 and 150 ng of LTB₄. Similar standard curves were observed for 12-HETE, 5-HETE, and A.A. and were also linear from 15 to 500 ng (12-HETE), 200 to 1000 ng (5-HETE), and 200 to 4000 ng (A.A). Equations for the standard curves were \( y = (3.30 \times 10^{-3}) x - 0.680 \), \( y = (5.47 \times 10^{-3}) x - 33.3 \), and \( y = (101) x + 1937 \) respectively. Correlation coefficients were 0.999 for all of the standard curves. Standard curves points were averaged from 2 to 5 chromatographs.

Quantitation of the LTB₄ isomers, LTB₄ and 5-HETE was done by 1.) Adjustment of observed area with a factor relating the integration area of PGB₁ (internal standard) during the HPLC run with the area of integration of the same PGB₁ concentration injected directly on column, i.e. the area of integration of PGB₁ injected on column was 55,000 area units. The area of integration of PGB₁ during a sample run was normalized to this value and the areas of the peaks of interest were either multiplied or divided by this factor. 2.) Then the area of integration was compared with a standard curve derived by injecting known quantities and plotting area versus ng. Approximate quantitation of isomers of LTB₄ followed the same procedure as quantitation of LTB₄ since no authentic standards are available. The isomers have the same molecular weight and absorption properties.
Results are expressed in nanograms (ng) per number of cells obtained by the use of standard curves. Recovery of PGB₁ was consistently high (> 99%) in three extractions.
Treatments with Protamine Zinc Insulin. Effects of treatment with protamine zinc insulin (PZI U-100/cc) was evaluated for two reasons; 1) insulin should decrease the hyperglycemia observed if it results from an insulin deficiency and 2) insulin may in part correct the increased sensitivity of the diabetic platelets. Figure 17 shows the blood glucose values (mg/dl) versus time in four diabetic animals receiving PZI. PZI (1-4 units, 100 units/ml) was administered intramuscularly (B-D. LoDose Insulin syringe), 28 gauge needle, semitendinosous muscle) two times per day for up to 21 days. Limited portions of food (150 g/day) given with the PZI assisted in control of the hyperglycemia. This level of food did not effect the weight gain of the diabetic animal. The dotted lines indicate a range of 75-150 mg glucose/dl which approximates values observed for control rabbits (literature values). Eighty percent of the mean values full within the desired range. Values are mean ± S.D. Without PZI administration blood glucose values for diabetic rabbits was 407 ± 77 mg/dl whereas in control rabbits this value was 116 ± 6 mg/dl. These values were accumulated over the same period of time when other rabbits were receiving insulin.

Necropsy of Animals. All animals were examined for signs of infections, arterial lesions, fatty livers, cardiac and pericardium trauma (due to percutaneous cardiac puncture), renal fat, muscle structure, and any other overt physical changes. Urine, obtained by bladder puncture, was evaluated for pH, protein, glucose (glycosuria), ketones, and blood using CHEM STRIP5. Changes noted in the diabetic animals were losses of renal fat and muscle mass.
Figure 17. Blood glucose values versus time for rabbits receiving insulin. Four rabbits received protamine zinc insulin for 15 days. Only one rabbit received insulin for 21 days. Values shown are mean blood glucose values determined two times per day. Values are mean ± S.D..
particularly in the abdominal wall and limbs. Arterosclerotic plaque formation was not observed in the diabetic animals up to 8 months post alloxan injection. Values that were not different between C and D included urine pH (8), protein (negative to trace), and blood negative to 5-10 Ery/μl. Values that were different were ketones (negative to +) in D urine and glucose (+++ or > 1 g/dl) in D urine.

Weight changes were monitored for 4 weeks after the injection of alloxan as shown in Table 3. Initial weights were 4 ± 0.5 lbs. Weight gain in the diabetic animals lagged behind but paralleled the gain by control rabbits. No significant differences were observed for periods up to 6 months.

Table 3.

<table>
<thead>
<tr>
<th></th>
<th>initial</th>
<th>1 wk</th>
<th>2 wks</th>
<th>3 wks</th>
<th>4 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4 ± 0.5</td>
<td>4 ± 0.5</td>
<td>4.65 ± 0.58</td>
<td>5 ± 0.8</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>D</td>
<td>4 ± 0.5</td>
<td>4 ± 0.4</td>
<td>4.40 ± 0.6</td>
<td>4.7 ± 0.6</td>
<td>5.2 ± 0.8</td>
</tr>
</tbody>
</table>

Statistical analysis. Differences between means were evaluated using the Student's two tailed t test. Data is presented as mean ± standard error of the mean (S.E.M.) or mean ± standard deviation (S.D.). Significance was considered to be a p < 0.05 or less. Mean values not significantly different are indicated by N.S.
IV. Action of Platelet Activating Factor on Type I Diabetic Human Platelets

Introduction

Platelet activating factor (PAF), identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine, has been shown to induce platelet aggregation, cause platelet degranulation, (Vargaftig et al., 1982) and stimulate thromboxane A\textsubscript{2} (TXA\textsubscript{2}) synthesis (Shaw et al., 1981). PAF has been shown to increase vascular permeability (Humphrey et al., 1982, Gimbrone, 1982). A number of investigators have shown that PAF is released from a variety of tissues and from blood cells (Pinckard et al., 1979, O'Flaherty et al., 1981, Albert and Synder, 1983, Chignard et al., 1980).

Platelets from diabetic patients are characteristically hyperaggregable and demonstrate increased thromboxane production when induced by different stimuli (Halushka et al., 1977, Colwell et al., 1979). In view of the above findings, the present study was designed to investigate the effects of PAF on platelets from Type I diabetic subjects.
Platelets from Type I human diabetics exhibited more sensitivity to aggregation, when compared to platelets from non-diabetic control subjects after challenging with platelet activating factor (PAF). The production of TxB$_2$ and 12-HETE and release of 5-hydroxytryptamine (5HT) was increased when challenged by PAF (5.0 x 10$^{-6}$ M and 1.0 x 10$^{-6}$ M). The production of TxB$_2$ and 12-HETE and the release of 5HT were related to the irreversible biphasic aggregation profiles observed with the diabetic subjects. Inhibition of TxA$_2$ production by acetylsalicylic acid abolished the secondary wave of aggregation of platelets from diabetics, changing an irreversible aggregation to a reversible one. Inhibition of both TxA$_2$ and 12-HETE production by eicosatetraynoic acid did not contribute further to the inhibition caused by acetylsalicylic acid alone indicating that 12-HETE was not involved in the secondary wave of aggregation. These data show that the increased aggregation observed with the platelets from the diabetic group in response to PAF results in part from their higher production of TxA$_2$ and release of 5HT.
Results

Serum values. No statistically significant differences were observed between the diabetic and control subjects for serum cholesterol as shown in Table 4. Significant differences between diabetic and control subjects were found for serum triglycerides levels and fasting glucose concentrations.

Table 4.
Serum values for diabetic and control human subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>167 ± 3</td>
<td>69 ± 9</td>
<td>85 ± 4</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td>182 ± 7</td>
<td>137 ± 11</td>
<td>269 ± 23</td>
</tr>
</tbody>
</table>

Number of subjects = 11.
Numbers represent mean ± SEM for the control and diabetic subjects.
N.S. = not significant.
Platelet aggregation. Fig. 18 demonstrates typical concentration dependent PAF induced aggregations observed for both diabetic and control subjects. When challenged with PAF, platelets from diabetic subjects exhibited irreversible biphasic aggregations whereas platelets from control subjects exhibited reversible monophasic aggregations. Table 5 shows the comparison of aggregations between control and diabetic subjects for three concentrations of PAF over a 4 min aggregation time. The most significant differences were noted with $1.0 \times 10^{-6}$ M PAF at all incubation times. Upon increasing the PAF concentration to $1.0 \times 10^{-5}$ M, biphasic aggregation profiles of platelets from diabetic subjects changed to monophasic profiles without a further increase in aggregation. Monophasic irreversible aggregations were also observed at this concentration with platelets from control subjects although the extent of maximum aggregation was lower than for the diabetic subjects.

Release of $^{14}$C-5HT. PRP from both subject groups were incubated with $^{14}$C-5HT as described in the methods. Percent uptake of $^{14}$C-5HT into the platelets was not significantly different between the control and diabetic subjects ($80 \pm 2$ vs. $82 \pm 3$, n=7 respectively). Percent release of 5HT in response to $1.0 \times 10^{-7}$ M PAF was not different between the two groups. However, as PAF concentration was increased to $5.0 \times 10^{-6}$ M and $1.0 \times 10^{-6}$ M, platelets from the diabetic subjects exhibited significantly greater percent release at 2 min. The differences were more pronounced when the aggregations were continued for 4 min (Table 6). The release
Figure 18. Typical aggregation profiles for platelets from control (C) and diabetic (D) subjects. Primary or first wave aggregation is maximal at 1 min for both subject groups. Prolonging the incubation time resulted in reversible aggregations for the control subjects and biphasic, irreversible aggregations for the diabetic subjects. Challenge with $1.0 \times 10^{-7}$ M PAF demonstrated weak, irreversible aggregation profiles for both subject groups (profile not shown).
**Table 5.**

Aggregation response of platelets from control and diabetic subjects to different concentrations of PAF with time

<table>
<thead>
<tr>
<th>Subject</th>
<th>1 min.</th>
<th>2 min.</th>
<th>3 min.</th>
<th>4 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41 ± 4*</td>
<td>35 ± 4</td>
<td>32 ± 4</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>5.0 x 10^-6*</td>
<td>N.S.**</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td>48 ± 2</td>
<td>51 ± 2</td>
<td>55 ± 2</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>Control</td>
<td>33 ± 4</td>
<td>21 ± 4</td>
<td>15 ± 4</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>1.0 x 10^-6*</td>
<td>p &lt; 0.025</td>
<td>p &lt; p.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td>46 ± 3</td>
<td>46 ± 3</td>
<td>53 ± 4</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>Control</td>
<td>19 ± 3</td>
<td>7 ± 2</td>
<td>5 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>1.0 x 10^-7*</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Diabetic</td>
<td>27 ± 2</td>
<td>22 ± 4</td>
<td>23 ± 4</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

*Platelet activating factor concentration [M].
Number of subjects = 8 for 5.0 x 10^-6 and 1.0 x 10^-6 M PAF.
Number of subjects = 7 for 1.0 x 10^-7 M PAF.
** Significance shown for each comparison of control and diabetic subjects at each time interval.
*Mean aggregation percentage ± SEM.
N.S. = not significant.
Table 6.

Release of 5HT from platelets of control and diabetic subjects in response to different concentrations of PAF with time

<table>
<thead>
<tr>
<th>PAF [M]</th>
<th>5.0 x 10^{-6}</th>
<th>1.0 x 10^{-6}</th>
<th>1.0 x 10^{-7}</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2 min</td>
<td>4 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 ± 2</td>
<td>12 ± 2</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>p&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 ± 3</td>
<td>35 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of subjects = 7 for 5.0 x 10^{-6} M PAF.
Number of subjects = 6 for 1.0 x 10^{-6} and 1.0 x 10^{-7} M PAF.
N.S. = not significant.
N.D. = not determined.
of 5HT from platelets of diabetic subjects coincided with the degree of biphasic irreversible aggregation profile (increased release coincided with increased aggregation). There were no significant differences for the aggregation profiles between the PRP incubated with and without 5HT for both groups.

Synergism between epinephrine and PAF. Both control and diabetic subjects exhibited reversible aggregation profiles when platelets were incubated with 1.0 x 10^-7 M PAF. There was no significant difference in the percent release of 5HT (controls 3 ± 1, diabetics 2 ± 1) with this concentration of PAF. Incubation of platelets with epinephrine for 3 minutes exhibited weak aggregations for both controls and diabetics with no difference in the release of 5HT (controls 3 ± 0.2, diabetics 3 ± 1). However, reversible aggregations noted for both control and diabetic groups with PAF alone were changed to irreversible monophasic profiles and irreversible biphasic profiles for the control and diabetic groups respectively when both agonists were used. This synergism enhanced the percent release of 5HT (controls 22 ± 3 and diabetics 48 ± 5, p < 0.005, n=7) to a greater extent in the diabetic group than in the control group. The percent aggregation for either epinephrine or PAF alone or in combination did not differ significantly between the control and diabetic subjects.
Production of TxB₂ and 12-HETE. PAF at 5.0 x 10⁻⁶ M and 1.0 x 10⁻⁶ M stimulated production of greater amounts of TxB₂ and 12-HETE from platelets of diabetics as compared to platelets from controls (Table 7). For example, 5.0 x 10⁻⁶ M PAF stimulated an 11-fold greater production of TxB₂ and a 14-fold greater production of 12-HETE from platelets of the diabetic subjects when compared to the amounts formed from the control subjects. Synthesis of TxB₂ and 12-HETE coincides with the degree of irreversible biphasic aggregations shown by the diabetic subjects with 5.0 x 10⁻⁶ M and 1.0 x 10⁻⁶ M PAF. The greater the aggregation and the expression of the biphasic wave, the greater was the production of TxB₂ and 12-HETE. TxB₂ and 12-HETE were not synthesized at the detection level of the assay in either subject group with 1.0 x 10⁻⁷ M PAF. Only reversible aggregations were observed for both groups of subjects at this concentration. Platelet TxB₂ production but not 12-HETE production demonstrated a significant positive linear correlation with plasma glucose levels (r = 0.73, p < 0.02, n = 8 with 5.0 x 10⁻⁶ M PAF at 4 minutes incubation).
<table>
<thead>
<tr>
<th>Subject</th>
<th>PAF [M]</th>
<th>TxB₂</th>
<th>12-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmoles/10^8 platelets in 4 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.0 x 10^-6</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td>11 ± 1</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 x 10^-6</td>
<td>0.8 ± 0.4</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.005</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Diabetic</td>
<td></td>
<td>6 ± 2</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Table 7.
Effect of PAF on the production of TxB₂ and 12-HETE from platelets of control and diabetic subjects (N = 7)
Platelet aggregation induced by PAF was carried out in the presence of a cyclooxygenase inhibitor ASA (10^{-4} M) and ETYA (10^{-3} M), a dual inhibitor for lipoxygenase and cyclooxygenase (Miller et al., 1982, Colwell et al., 1982) (Fig. 19). Preincubation of platelets at 37°C from diabetics with ASA (5 min) or ETYA (2 min) abolished the second wave of aggregation to PAF but not the initial primary aggregation wave. Incubation of PRP from the diabetic subjects with either ASA or ETYA demonstrated similar inhibition of second wave aggregation. Since only reversible aggregation profiles were obtained in the presence of 5.0 x 10^{-6} M PAF, aggregation of platelets from non-diabetics were unchanged by either ASA or ETYA preincubation. Both ASA and ETYA completely inhibited TXB₂ production from platelets of diabetics while ETYA also inhibited 12-HETE production. 12-HETE levels remained the same or were slightly elevated with the preincubation of ASA.

Discussion

Previous studies have shown that platelets obtained from diabetic subjects exhibit enhanced in vitro aggregation when stimulated by a variety of agents (Halushka et al., 1977, Colwell et al., 1979). Enhanced phospholipase activity has been observed in platelets from diabetic subjects as evidenced by increased TxB₂ synthesis (Takeda et al., 1981). To date, no published studies have examined the effects of PAF on platelets obtained from diabetic subjects. PAF has been shown to cause platelet aggregation with normal human platelets (Colwell et al., 1975, Marcus et al., 1981). The involvement of TxA₂ in PAF induced aggregation has been a subject of controversy (Marcus et al., 1981, McIntyre et al., 1983,
Figure 19. Effect of ASA and ETYA on 5.0 x 10^{-6} M PAF-induced aggregation. Open circle/solid line indicates aggregation mean ± SEM for the diabetic subjects without ASA or ETYA. Solid circle/narrow dashed line indicates aggregation mean ± SEM for the same diabetic subjects when the PRP was preincubated with ASA or ETYA. Statistics demonstrate significant inhibition of secondary aggregation. Open circle/wide dashed line indicates aggregation mean ± SEM for the control group with and without ASA or ETYA. PRP was incubated at 37°C with ASA (5 min) or ETYA (2 min) before addition of PAF. Number of subjects = 5.
AGGREGATION %
(Light Transmission)

TIME (minutes)

N.S.  
P < 0.05

P < 0.01

P < 0.001

(D)  (D)  (C)
Vargaftig et al., 1981). PAF induces deacylation and release of arachidonic acid and subsequent TxB2 synthesis in rabbit platelets. This increase in deacylation was demonstrated to be related to an increase in phospholipase activity (Shaw et al., 1981). In this report, we have examined the effects of PAF on platelet aggregation, production of TxB2 and HETE, and release of 5HT from platelets of Type I human diabetics. The present report demonstrates that platelets from subjects with Type I diabetes are more sensitive to the action of PAF.

At a high PAF concentration (5.0 X 10^{-6} M), the initial wave of platelet aggregation at 1 min does not differ between the control and diabetic subjects. With increasing incubation time, the percent aggregation of the control subjects is significantly reduced compared to the diabetic subjects. On the other hand with a lower PAF concentration (1.0 X 10^{-6} M) highly significant differences in aggregation were noted at all incubation times. PAF (1.0 X 10^{-7} M) caused reversible platelet aggregations in both the control and diabetic subjects. The extent of reversibility was lower in diabetic subjects as compared to the control subjects. These differences are attributed to the apparent increased sensitivity of the platelets from diabetics to PAF.

Studies involving the action of PAF on normal platelets have shown that PAF stimulates the release of 5HT from dense granules (Vargaftig et al., 1982, Marcus et al., 1981). 5HT has been shown to have a weak proaggregatory property (Gordon et al., 1978). Increased vascular permeability is also associated with 5HT upon release from platelets (Humphrey et al., 1982). Platelets from diabetic subjects
show a significantly increased release of 5HT in response to PAF when compared to the percent release of control subjects at 2 and 4 minutes. This increased release of 5HT coincides with the degree of biphasic aggregation.

Synergism between epinephrine and PAF during platelet aggregation has been previously described (Vargaftig et al., 1982, Tsien et al., 1982). Epinephrine increases the sensitivity of the platelets to the action of PAF (Rao and White, 1982). Synergism has also been shown to occur between 5HT and PAF in inducing heightened platelet aggregation (MacIntyre et al., 1983). Synergism between epinephrine and PAF in the present study results in an increased release of 5HT from platelets of diabetic subjects over the increased release observed with control subjects.

The extent of involvement of TxA2 in PAF induced aggregation in platelets from normal subjects has been questioned. Proaggregatory and vasoconstrictive actions are associated with TxA2 (Hamberg et al., 1975). Irreversibility of PAF induced platelet aggregations has been suggested to be due to the formation of TxA2 (Ardlie and Ham, 1974). Formation of 12-HETE has been proposed to have a similar role in irreversible platelet aggregation (Dutilh et al., 1980). Stimulation of rabbit and human peritoneal polymorphonuclear leukocytes by PAF caused the release of arachidonic acid and subsequent production of bioactive mediators including the lipoxygenase products 5-HETE and 5,12-DHETE (Chilton et al., 1982). The ability of PAF to induce 12-HETE production in platelets has not been reported. The present study shows a direct relationship between the degree of irreversible biphasic platelet aggregations of the
diabetic subjects and the production of TxB₂ and 12-HETE. Production of TxB₂ and 12-HETE from platelets of diabetics was significantly increased over the levels produced by platelets from control subjects.

It has been shown that the second wave in a PAF-induced biphasic aggregation is cyclooxygenase dependent. For example, Chesney et al. (1982) have shown that both indomethacin and ASA inhibit the second wave of a PAF-induced biphasic aggregation such that a previously irreversible profile becomes reversible. Similarly, Miller et al. (1982) have shown that the second wave of aggregation induced by PAF is completely inhibited by indomethacin and 9,11-azoprost-5,13-dienoic acid, a thromboxane A₂ synthetase inhibitor. Our current study and studies from others suggest that PAF induced second wave aggregation coincides with TxA₂ production. We, therefore, have examined the effects of ASA and ETYA on the production of TxB₂ and 12-HETE, and on irreversible aggregation of platelets from diabetics (Fig. 19). The extent of reversibility of aggregation of platelets from diabetics was the same whether the platelets were incubated with ASA or ETYA. ASA inhibited only TxB₂ production whereas ETYA abolished both 12-HETE and TxB₂ production. We suggest that TxA₂, but not 12-HETE, appears to be directly responsible for the irreversible aggregation contrary to the findings of Dutilh et al. (1980) although Dutilh did not use PAF as an aggregation inducer. The irreversible aggregations noted in this study are associated not only with TxA₂ production but also with 5HT release. The aggregating effects due to TxA₂ and 5HT could not be separated since preincubation with either ASA or ETYA eliminated both
production of TxB2 and release of 5HT (release inhibited by 80% by both ETYA and ASA for both diabetic and control subjects).

Increased 5HT release and increased production of TxB2 in diabetics may be responsible for the observed irreversible biphasic platelet aggregation induced by PAF. Platelets from these diabetic subjects demonstrated an enhanced synergistic reaction when a combined challenge of epinephrine and PAF was used. Diabetics in this study have shown a characteristic accentuated second wave of platelet aggregation associated with increased release of TxA2 and 5HT. Since platelets possess binding receptors for PAF (Valone et al., 1982, Chesney et al., 1984) we speculate the increased response to PAF observed in diabetics may be due to an alteration of receptor binding capacity or binding affinity for PAF by alteration (i.e. glycosylation) of membrane receptors. We have reported that the platelets from Type I diabetics contain low vitamin E levels compared to the platelets from non-diabetics (Karpen et al., 1984). This may cause an increased deacylation of membrane phospholipids which could furnish a necessary substrate (2-lysophosphatidylcholine) for the reacetylation reaction needed in the synthesis of PAF and thereby further augment the sensitivity to PAF. Alternatively, there may be co-factors present in the circulating plasma of the diabetics that may be responsible for potentiation of the secondary aggregation (Triolo et al., 1984, Colwell et al., 1977). Further studies are required for exploring this possibility. The importance of PAF in the etiology of platelet induced vascular complications in diabetes mellitus (Colwell et al., 1979) at the present time is not known but merits further investigation.
CONCLUSIONS

1.) Diabetic platelets are more sensitive to PAF in inducing platelet aggregation.
2.) Production of TxB\(_2\) and 12-HETE is enhanced from diabetic platelets when stimulated with PAF.
3.) Granular release of serotonin is enhanced from diabetic platelets when stimulated with PAF.
4.) Treatment of platelets with ASA inhibits aggregation suggesting cyclooxygenase involvement.
V. Influence of Plasma on Platelet Arachidonic Acid Metabolism in the Alloxan-Induced Diabetic Rabbit

Introduction

Enhanced platelet activity was noted as early as 10 days after alloxan injection. Hyperglycemia (400-500 mg/dl) is apparent after 2-3 days. Results in this section indicate that platelet dysfunction in diabetic rabbits precedes macroscopic vascular lesions. This observation may support a theory for the early involvement of platelets in the pathogenesis of atherosclerosis.

Platelets from diabetic rabbits were more sensitive to lower concentrations of arachidonic acid, collagen, ADP, and A23187. AGEPC did not reveal differences (either with respect to percent aggregation, prostanoid production, or 14C-5HT release) between control and diabetic animals. Both cyclooxygenase and lipoxygenase metabolites were increased in diabetic platelets when stimulated by either exogenous arachidonic acid or receptor mediated agonists. Prostanoid production was not correlated with fasting blood glucose levels.

Comparing prostaglandin levels formed between arachidonic acid and collagen demonstrates that other mechanisms for stimulating platelet aggregation could be present.
The effect of the plasma environment was examined by studying the aggregation response of isolated platelets resuspended in either autologous or heterologous plasma. On incubating isolated platelets from normal subjects with diabetic derived plasma, platelet aggregation due to both arachidonic acid and collagen was substantially increased. After incubating platelets derived from diabetic subjects with plasma from normal subjects, a marked diminution in the aggregation response was observed. Eicosanoid production paralleled these changes. The latter observation indicates the reversibility of the platelet abnormality. Similar observations have been noted by Aviram and Brook (1982) where they incubated gel-filtered platelets with plasma from normolipidemic individuals and plasma from individuals with familial hypercholesterolemia (FH). Normal platelets demonstrated a heightened aggregation response to aggregating agents in the plasma from FH subjects. In their study cholesterol, but not triglyceride levels, were significantly different. In the presence neither cholesterol or triglyceride levels were different between the subjects.
Results

Data regarding the metabolic state of the fasted control and diabetic rabbits is shown in Table 8. Plasma glucose levels were significantly elevated in diabetic rabbits ($p < 0.001$). No significant differences were noted between total protein, albumin, triglycerides, cholesterol, and HDL-cholesterol. All groups of diabetic rabbits used in this study exhibited similar patterns of plasma and serum parameters. Fasting glucose levels ($236 \pm 132$ mg/dl) on the day of the experiment and nonfasting glucose levels ($454 \pm 101$ mg/dl) obtained the night before the experiment did not correlate with the percentage of aggregation or prostaglandin levels (either in platelet rich plasma or in platelet suspension system, Section VI). Similarly, fasting plasma levels of total cholesterol, high-density lipoprotein-cholesterol, triglycerides, total protein, fibrinogen, or albumin did not correlate with aggregation responses or prostaglandin levels.
Table 8
Plasma values for diabetic and control rabbit subjects

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>p value</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>120 ± 4</td>
<td>&lt;0.001</td>
<td>500 ± 19</td>
</tr>
<tr>
<td>Total protein (g/Liter)</td>
<td>40 ± 2</td>
<td>N.S.</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Albumin (g/Liter)</td>
<td>4.0 ± .2</td>
<td>N.S.</td>
<td>3.9 ± .15</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>63 ± 21</td>
<td>N.S.</td>
<td>63 ± 24</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>40 ± 13</td>
<td>N.S.</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>20 ± 7</td>
<td>N.S.</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>
Suspension of platelets in plasma (PRP) from both groups, control and diabetic, were incubated with various concentrations of stimulators for 4 min to determine if aggregation differences would be observed and at what concentrations such differences would be apparent. Figure 20 demonstrates the percent aggregation versus time with control and diabetic plasma stimulated with arachidonic acid. Concentrations of 75 and 100µM induced similar aggregation profiles. Concentrations of arachidonic acid of 50µM and below (to 37µM) again demonstrated similar aggregation profiles. A concentration of 55µM was chosen for future studies since maximal differences were observed at this concentration. Since arachidonic acid was added as a soap in a 10µl aliquot, a blank was ran with only ethanol, sodium bicarbonate, water, and acetic acid (to substitute for the acidity of arachidonic acid). No differences were observed between C and D PRP samples with respect to percent aggregation or TxB2 production during blank runs. Production of 12-HETE was approximately 1.5 times greater in comparing C versus D PRP. Background levels for both 12-HETE and TxB2 were subtracted from values obtained from stimulated samples.

Released 14C-5HT was also measured at the different levels of arachidonic acid in control PRP. The concentration with the corresponding release is as follows; 100µM, 25.3%, 75µM 22.4%, 65µM, 17.5, 55µM, 8.51%, 50µM, 8.3%, and 37µM 3.61%. Incorporation of labeled serotonin did not significantly interfere with the
Figure 20. Percent aggregation of platelet rich plasma samples of control and diabetic rabbits stimulated with various concentrations of arachidonic acid. The numbers designate arachidonic acid concentrations; 1-5 are 100, 75, 65, 55, and 50 \( \mu \text{M} \) respectively.
aggregation profiles when compared to aggregation profiles of PRP samples not incubated with $^{14}$C-5HT.

Similar concentration versus percent aggregation profiles were observed with collagen as the platelet stimulator (Figure 21). The optimal collagen concentration was 65 $\mu$g/ml and this was subsequently used in future experiments. As arachidonic acid, released $^{14}$C-5HT was also measured at the different levels of collagen in control PRP. The concentration with the corresponding release is as follows; 170 $\mu$g/ml, 62.8%, 85 $\mu$g/ml, 47.7%, 65 $\mu$g/ml, 28.1%, 45 $\mu$g/ml, 19.2%, and 20 $\mu$g/ml, 10.6%. Again incorporation of labeled serotonin did not significantly interfere with the aggregation profiles when compared to aggregation profiles of PRP samples not incubated with $^{14}$C-5HT.

Figure 22 shows the aggregation profile for $N = 15$ pairs of control and diabetic subjects at 55 $\mu$M arachidonic acid (mean ± S.E.M.). At 1 min intervals the significance was determined. The p values indicate significant differences obtained by the Student's two tailed t-test. At this concentration a rapid shape change occurs within 1 min followed by rapid aggregation with PRP of the D group. Only a slow shape change with minimal aggregation is observed with PRP from the D group. Increasing the incubation time to 10 minutes does not change the significance from that at 4 min. Challenge with exogenous arachidonic acid exhibits significant differences in the aggregation profile at all times.

Figure 23 shows the aggregation profile for $N = 15$ pairs of control and diabetic subjects at 65 $\mu$g/ml collagen (mean ± S.E.M.).
Figure 21. Percent aggregation of platelet rich plasma samples of control and diabetic rabbits stimulated with various concentrations of collagen. The numbers designate collagen concentrations; 1-4 are 195, 130, 65, and 32.5 μg/ml respectively.
AGGREGATION PERCENT
(Light Transmittance)

TIME (minutes)

CONTROL

DIABETIC

TIME (minutes)
Figure 22. Aggregation of platelet rich plasma versus time induced by 55 μM arachidonic acid. Comparison of control (C) and diabetic (D) samples.
Figure 23. Aggregation of platelet rich plasma versus time induced by 65 µg/ml collagen. Comparison of control (C) and diabetic (D) samples.
No significant difference is symbolized by N.S. Initiation of a shape change induced by collagen is apparent with diabetic PRP within 30 sec. This shape change, as with an arachidonic acid challenge, is followed with rapid and irreversible aggregation. At this concentration, platelets from control rabbits demonstrate the typical delayed onset of shape change followed by the beginning of aggregation. Increasing the incubation time to 10 min allows both aggregation profiles to obtain about 10% more aggregation.

Arachidonic acid-induced TxB₂ levels were also measured by a direct aliquot of PFP as described earlier. Diabetic PRP produced substantially more TxB₂ than its control counterpart. Figure 24 demonstrates this observation. The pmoles of TxB₂ produced per 1 x 10⁸ platelets (in a 0.5 ml incubate) is plotted versus two levels of AA. Statistical significance between the pairs is shown above the bar graphs. Both concentrations were used in all experiments to confirm that control PRP could aggregate completely at 100µM AA. Significance between C and D decreased as the AA concentration was increased, not unexpectedly since aggregation profiles were similar at 100µM AA. Interestingly, substantial TxB₂ is generated from 55µM AA with C PRP (Figure 24) even though platelets only undergo the shape change phenomenon shown in Figure 22. As will be shown, this level is 3-4 times greater than the level of TxB₂ generated from D PRP induced with collagen where greater than 60% aggregation was observed.
Figure 24. TxB₂ production by control and diabetic platelet rich plasma as a function of arachidonic acid concentration.
Production of TxB$_2$ at 50µM from D PRP was marginally significant ($p < 0.05$) when compared to C PRP. Levels of AA below 50µM still induced measurable TxB$_2$ (order of 50-200 pmoles/10$^8$ platelets) but no significant differences between C and D samples were noted. Aggregation profiles demonstrated either a gradual development of a shape change (gradual negative drift) over 4 minutes (to negative aggregation values of -5 to -15%) or no appreciable shift in the baseline. Generation of PGE$_2$ was similar to the pattern of TxB$_2$ observed although the levels were approximately 10 fold less. 12-HETE production parallels observations noted with TxB$_2$ (Figure 25). Overall production of 12-HETE compared to TxB$_2$ from control PRP is lower since the C-12 lipoxygenase enzyme kinetics are slower than the cyclooxygenase activity.

Collagen-stimulated platelets were studied since collagen could release AA from endogenous stores (Bills et al., 1976). Shown in Figure 26 are the levels of TxB$_2$ and 12-HETE produced from a 65 µg/ml collagen challenge. Both levels were significantly increased in D PRP. Generation of TxB$_2$ is approximately 3 times greater than the generation of 12-HETE from D PRP. This imbalance may result from the more rapid cyclooxygenase metabolism which converts low levels of AA more rapidly to cyclooxygenase products. Increased prostanoid production stimulated by collagen may or may not indicate increased phospholipase activity since any released AA would act as exogenous AA and be converted more rapidly to products in the D platelet.
Figure 25. 12-HETE production by control and diabetic platelet rich plasma as a function of arachidonic acid concentration.
Figure 26. TxB₂ and 12-HETE production by control and diabetic platelet rich plasma induced by 65 µg/ml collagen.
Aside from the arachidonic acid metabolites and the physical phenomenon of aggregation, another platelet release product, serotonin, was studied. \(^{14}\text{C}-5\text{HT}\), incorporated into platelet dense granules, is released by arachidonic acid and collagen. Figure 27 shows the release of \(^{14}\text{C}-5\text{HT}\) induced by 55 \(\mu\text{M}\) arachidonic acid and 65 \(\mu\text{g/ml}\) collagen. These agonist concentrations are the same used in Figures 22 and 23 respectively. Release of \(^{14}\text{C}-5\text{HT}\) is also significant increased in D PRP for both agonists.

Involvement of endogenous ADP in arachidonic acid induced aggregation was assessed by incubating PRP from both control and diabetic rabbits with creatine phosphate (CP)/creatine phosphokinase (CPK) (Chesney et al., 1982, Chignard et al., 1979). A combination of concentrations was chosen from incubating control PRP with 10 \(\mu\text{M}\) ADP and determining when aggregation would be inhibited or made reversible. The concentrations were 2 mM CP and 4 units/0.5 ml CPK. In this system ADP would react with CP and be converted to creatine and ATP by the action of CPK. Since receptor occupancy is needed for the continued response to ADP, exogenous or endogenously formed and released ADP would be eliminated. These concentrations were incubated with the arachidonic acid concentrations observed in Figure 20. At the lower concentrations (55 \(\mu\text{M}\)) aggregation was inhibited and only a shape change was apparent (as Figure 22). Increasing the concentration overcame the inhibition by CP/CPK such that at 100 \(\mu\text{M}\) only a slight delay in the shape change was apparent. Both control and diabetic PRP responded in a similar fashion.
Interesting differences were observed between PRP challenges with collagen and arachidonic acid. At 4 min, both agonists elicited approximately the same percent of aggregation for the D subjects (Figures 22 and 23). Values for the C subjects were slightly different. Arachidonic acid induced a 10-fold and a 28-fold increase in TxB2 and 12-HETE formation respectively with D PRP over a collagen challenge. Comparable increases with C PRP were 30-fold and 20-fold, respectively. Approximately, 15 pmoles TxB2/10⁸ platelets were observed with the collagen challenge (11% aggregation at 4 min.) whereas approximately 420 pmoles TxB2/10⁸ platelets were observed with an arachidonic acid challenge with only a -2% aggregation. For D PRP, increased TxB2 and 12-HETE levels did not increase the aggregation with an arachidonic acid challenge when compared to a collagen challenge. Less aggregation was observed with an arachidonic acid challenge in C PRP in comparison to one resulting from a collagen challenge even when appreciable TxB2 and 12-HETE production were observed. Levels of TxB2 and 12-HETE for D PRP stimulated by collagen (54% aggregation) are well below those observed in C PRP stimulated by AA (-2%). Collagen stimulated the release of ¹⁴C-5HT in greater amounts than by AA (C, 3-fold increase, D, 2-fold increase).
Figure 27. Comparison of $^{14}$C-5HT release between control and diabetic platelet rich plasma induced with 55 µM arachidonic acid and 65 µg/ml collagen.
In a separate series of experiments 6 control and diabetic pairs were used for plasma exchange experiments. Initially it was confirmed that C PRP and D PRP responded to AA and collagen as previously noted in Figures 22 and 23. Exchange of plasma (plasma refers to platelet free plasma described in Figure 6 containing < 5,000 platelet/μl) considerably altered previous aggregation profiles. Aggregation of control platelets in control plasma which demonstrated only a slow shape change (-3 ± 5%) was changed to a rapid shape change and a maximal aggregation response (65 ± 13%) in 4 min when control platelets were resuspended in diabetic plasma. This heightened aggregation closely parallels aggregation of diabetic platelets in autologous plasma (61 ± 16%). Observations of diabetic platelets suspended in control plasma were opposite to that above. When diabetic platelets were resuspended in control plasma only a slow change was visible, followed by minimal aggregation. These observations of aggregation profiles were noted at 55 μM AA at 4 min. of aggregation (Figure 28). In agreement with Lagarde et al., (1981) these observations of platelets isolated from diabetic plasma (previously hyperresponsive to AA) and resuspended in control plasma responded as control platelets. For example, after challenge with 55 μM AA D platelets showed an aggregation response of -5 ± 3% at 4 min. This closely parallels control values in autologous plasma.
Figure 28. Influence of plasma exchange experiments on arachidonic acid-induced control and diabetic platelet aggregation. The numbers designate the following; 1-4 are 100, 75, 65, and 55 μM with diabetic platelets/control plasma, 5 is 55 μM with control platelets/diabetic plasma. Open square/dashed line indicates diabetic PRP. Solid square/solid line indicates control PRP. Values for line 5 are mean ± S.D.
To confirm that platelets from diabetic subjects suspended in control plasma could aggregate higher levels of AA were added. Aggregation response increased with increasing AA concentration; 65μM, 25 ± 14%, 75μM 55 ± 6%, and 100μM 72 ± 4% (Figure 28). These values approximate the percent aggregation obtained with control platelets in control plasma (Figure 20). Responses of control platelets in diabetic plasma to 55 and 100μM AA were similar. These changes were noted as early as 30 min. after the mixing of platelets and plasma and retained this activity for as long as two hours. Experiments were completed by two hours after the preparation of PRP diluted to 200,000 PTs/μl. Experiments with collagen as the platelet stimulator paralleled the same observations seen with arachidonic acid.

Platelet free plasma was analysed for prostaglandin levels after aggregation. As shown in Table 9, levels of both TxB₂ and PGE₂ were significantly changed by the procedure. 12-HETE levels also increased or decreased, paralleling the shifts observed with TxB₂ and PGE₂. Initial differences in PRP were assessed and found to be significantly different. Arachidonic acid induced-TxB₂ and -PGE₂ levels increased when diabetic PFP was exchanged for control PFP. The increased TxB₂ production parallels the increased aggregation. No significant differences were noted between diabetic PRP and control PTs / diabetic PFP or control PRP and diabetic PTs / control PFP. Replacement of diabetic PFP with control PFP eliminated the hypersensitivity expressed by diabetic platelets in PRP. Parallel changes were observed between TxB₂ and PGE₂. TxB₂ and 12-HETE levels were similar to those observed in platelets in autologous plasma (Figures 24 and 25).
Table 9

Influence of plasma exchange on the platelet production of TxB$_2$
PGE$_2$ induced with 55 µM arachidonic acid

<table>
<thead>
<tr>
<th></th>
<th>TxB$_2$</th>
<th>PGE$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PRP</td>
<td>189 ± 42$^a$</td>
<td>38 ± 4$^a$</td>
</tr>
<tr>
<td>Diabetic PRP</td>
<td>724 ± 124$^b$</td>
<td>93 ± 23$^b$</td>
</tr>
<tr>
<td>Control PTs / Diabetic PFP</td>
<td>700 ± 109$^c$</td>
<td>100 ± 6$^c$</td>
</tr>
<tr>
<td>Diabetic PTs / Control PFP</td>
<td>173 ± 42$^d$</td>
<td>54 ± 22$^d$</td>
</tr>
</tbody>
</table>

Abbreviations are control PRP, control platelet rich plasma; diabetic PRP, diabetic platelet rich plasma; control PTs / diabetic PFP, control platelets usspended in diabetic platelet free plasma and; diabetic PTs / control PFP, diabetic platelets suspended in control platelet free plasma values (pmoles / $10^8$ platelets) are the mean ± S.D. of five experiments. Statistical comparison: $a$ vs. $b$, $a$ vs. $c$, and $b$ vs. $d$, $p$ < 0.001 for both TxB$_2$ and PGE$_2$. $b$ vs. $c$ and $a$ vs. $d$, N.S. for both TxB$_2$ and PGE$_2$. 

Factors influencing aggregation would seem to be present in the plasma since platelet isolation by centrifugation does not involve a washing procedure. Plasma factors, adsorbed to the platelet surface, would not be expected to be removed with this procedure. Platelet aggregation was not stimulated directly by mixing platelets and plasma since background levels of TxB2 remained undetectable and spontaneous aggregation did not occur.

The enhanced response of control platelets in diabetic plasma and the attenuation of diabetic platelets responses in control plasma was demonstrated to not be an artifact due to the isolation procedure by a number of experiments and observations. Part B of Figure 6 was carried through at the same time as the exchange experiments. These experiments demonstrated that the loss of platelet response was minimal. At no time did these experiments show complete loss of or marked stimulation of platelet responses. Therefore, the centrifugation procedure itself did not account for the extreme changes noted. Release of PGE1, and/or cyclic adenosine diphosphate, known inhibitors of platelet aggregation could explain responses observed with diabetic platelets in control plasma (lack of aggregation) but cannot explain responses observed with control platelets in diabetic plasma (enhancement of aggregation). Increasing the centrifugation time from 5 to 20 min. to eliminate a greater number of platelets did not influence the results described.

Lagarde et al. (1980) has demonstrated that the half-life of TxA2 is longer in human diabetic plasma and concludes that part of the hyperaggregability of diabetic PRP is due to this increase.
TxB2 levels were determined in samples obtained during the centrifugation procedure. Some samples were heated to 37°C for 10 min. to facilitate the conversion of TxA2 to TxB2. TxB2 levels in both instances were undetectable for both subjects.

Incubation of control PRP with exogenous glucose (10 μl additions) followed by stimulation with 55 μM arachidonic acid did not influence the percent aggregation or prostaglandin production. Final glucose concentrations were from 100 to 500 mg/dl. PRP was incubated at room temperature with glucose for periods up to one and a half hours or incubated at 37°C for 2 or 10 minutes. Aggregatory responses and prostanoid production were unchanged.

Erythrocytes (RBC) are a major endogenous source for ADP. Control PRP was incubated with various levels of diabetic RBC to determine if these RBC would release ADP and influence platelet aggregation. Platelet to RBC ratios were 625:1, 8:1, and 4:1. These ratios did not stimulate platelet aggregation when control PRP was stimulated with 55μM AA. In fact, at the lower ratios, platelet responses (platelet shape change) were reduced slightly.

Synergistic responses have been demonstrated between AA and ADP and collagen and ADP (Kinlough-Rathbone et al., 1977). ADP, at subthreshold levels for aggregation, was incubated with C PRP. The simultaneous addition of ADP (50-100 nM) and AA or preincubation with ADP for 10 or 30 minutes before challenge with AA did not heighten the aggregatory response from C PRP stimulated with AA alone. Therefore, release of ADP from RBC or the attempt to simulate endogenous levels of plasma ADP did not duplicate the observation with C platelets suspended in D plasma.
Both A23187 and ADP induce rapid platelet aggregation. A23187, at 10μM, stimulates approximately 200 pmoles TxB2/10^8 platelets while ADP at 50μM stimulates marginal levels of TxB2 (approximately 4 pmoles/10^8 platelets). Nevertheless significant platelet aggregation is observed even with low levels of TxB2 (especially with ADP). Aggregation is usually in excess of 50%. These observations suggested that TxA2 may or may not play a role in the enhanced aggregation of control platelets in diabetic plasma. Direct evidence that a cyclooxygenase product was involved was by preincubating C platelets in D plasma with 0.1 mM ASA for 3 min and observing that the aggregation response was completely eliminated. TxB2 levels were reduced to <10 pmoles/10^8 platelets. Incubation of C platelets in D plasma with CP/CPK (2 mM / 4 units / 0.5 ml) also eliminated the aggregation response suggesting the involvement of endogenously released ADP.

Rabbits (N=4) were treated with insulin for up to 21 days. Blood glucose values are shown in Figure 2. Levels of insulin returned to normal as assessed by radioimmunoassay. Experiments 7 days after treatment began demonstrated limited changes. No significant differences were noted between aggregation profiles obtained before treatment compared to post-treatment profiles for 100 μM and 55 μM arachidonic acid and 65 μg/ml collagen for both control and diabetic animals. However, at 55 μM arachidonic acid, the TxB2 levels were 31% lower than at the pretreatment time (determined at 4 minutes). This level was still significantly greater than the TxB2 produced from control PRP. 12-HETE levels were unchanged.
Animals maintained for 2 and 3 weeks exhibited similar data. Aggregation of diabetic PRP was reduced by 15% and 42% at 100 μM and 55 μl arachidonic acid respectively with insulin treatment. Even though aggregation was significantly decreased in the diabetic PRP, both eicosanoid and the percent aggregation were still significantly greater than control values.
Discussion

Honour and Hockaday (1976) demonstrated that the intra-arteriolar aggregation of platelets at injury sites was increased with the application of ADP in alloxan-induced diabetic rabbits. No other studies have appeared to further characterize a potential platelet dysfunction in this diabetic model. Consistent with literature reports in human, platelets from the alloxan-induced the diabetic rabbit also exhibits altered function. Critical concentrations of exogenous arachidonic acid, collagen, ADP, AGEPC and A23187 all elicit greater responses from platelets from diabetic subjects. This study demonstrates platelet dysfunction with respect to aggregation, alteration of arachidonic acid metabolism, and serotonin release. Discussion of platelet agonists will in large part be limited to arachidonic acid and collagen.

Arachidonic acid and collagen differ in their mechanism of inducing platelet aggregation. Arachidonic acid directly serves as a cyclooxygenase and lipoxygenase substrate eliminating the deacylation step. Using exogenous arachidonic acid, the phospholipase step is bypassed and the activities of the cyclooxygenase and lipoxygenase are observed directly. Collagen interacts with platelet receptors, in part releasing arachidonic acid for prostaglandin production and in part releasing ADP from platelet dense granules. Enhanced TxA2 synthesis may be due to increased loading of precursor molecules into phospholipids, increased deacylation of phospholipids, increased Ca2+ mobilization and internal flux, or increased cyclooxygenase and/or thromboxane synthetase activity. Increased arachidonic acid
incorporation and content has been observed in platelets of type 2 (non-insulin-dependent) diabetes (Morita et al., 1983, Takahashi et al., 1984). Increased deacylation of arachidonic acid from the platelet phospholipids of human diabetics has been examined by Taketa et al., (1981). Platelet aggregation, stimulated by arachidonic acid, is also less subject to inhibition by imidazole, a thromboxane synthetase inhibitor or 13-azaprostanoic acid, a thromboxane receptor antagonist (Halushka et al., 1981). In the present study, since exogenous arachidonic acid conversion to TxB$_2$ is greater with platelets from diabetic subjects, the increased production must result from a step after the deacylation of membrane phospholipids since exogenous arachidonic acid does not cause the release of precursor molecules. This observation contrasts with those in the streptozotocin-induced diabetic rat model (Karpen et al., 1982) and diabetic humans (Karpen et al., 1984). Diabetic platelets in this study released greater levels of TxB$_2$, PGE$_2$, and 12-HETE suggesting that overall the platelet metabolizes arachidonic acid to a larger extent. A study suggested an important role for albumin in modulating platelet aggregation in hypoalbuminemic dogs with nephrotic syndrome (Green et al., 1985). If alloxan caused renal damage, potentially albumin could be lost in the urine. Albumin also modulates the stimulation of platelet aggregation by binding platelet products (Bills et al., 1977, Jackson et al., 1982). Availability of these platelet products is inversely correlated with plasma albumin concentration. Humans with nephrotic syndromes causing hypoalbuminemia exhibit platelet hypersensitivity (Remuzzi et al.,
1979). Levels of plasma albumin was assessed and not found to be different between the subjects. Therefore the ability of arachidonic acid to interact with the platelet would not seem to be altered, in terms of albumin concentration. Increased production of 12-HETE is observed similar to TxB2 differences. From Figures 24, 25, and 26 it can be concluded that both enzyme activities are increased in the diabetic platelet. 12-HETE has been shown to be chemotactic for neutrophils (Goetzl et al., 1975) and 12-HPETE, the oxidized precursor of 12-HETE, also modulates cyclooxygenase activity (Siegal et al., 1979, Lands, 1979).

Significant aggregation differences noted with collagen may or may not imply functional differences in phospholipase activity since theoretically any released arachidonic acid would be metabolized more rapidly in the diabetic platelet with increased cyclooxygenase activity (shown by exogenous arachidonic acid data). One release product studied was serotonin (5HT). Serotonin increases vascular permeability and potentiates platelet aggregation with other agonists (MacIntyre et al., 1983). An increased release of serotonin is not unexpected since addition of TxA2 to platelet suspensions induced aggregation and secretion of serotonin (Parise et al., 1984). Since diabetic platelets produce more TxB2, increased release of 14C-5HT could be expected. Results with CP/CPK additions indicate endogenous release of ADP and the potential synergism with arachidonic acid appears to be similar in control and diabetic PRP. Addition of CP/CPK would also eliminate ADP that might have been released in obtaining the blood.
Contrasting observations were seen in comparing TxB2 and 12-HETE levels induced with arachidonic acid and collagen in the control rabbit. Even though arachidonic acid-induced TxB2 levels were approximately 28 times higher compared to collagen-induced TxB2 levels, the percent aggregation was similar. This data suggests that factors other than TxA2 formation may participate in platelet aggregation. Release of 14C-5HT induced by collagen is approximately 75% greater than that induced by arachidonic acid and may in part account for the aggregation independent of TxA2. Collagen-induced platelet aggregation is associated with granular release of ADP. TxA2 and released ADP act together, synergistically, to cause aggregation and release the contents of platelet granules (Kinlough-Rathbone et al., 1979). Fibrinogen binding sites appear on the platelet surface when stimulated with ADP (Peerschke, E.I., 1984). Aggregation could be caused by the dimeric fibrinogen molecular binding two platelets. Fibrinogen levels were assessed in plasma and no significant differences were observed. This suggested that increased collagen-induced aggregation cannot be attributed to altered plasma fibrinogen levels. Since fluid-phase calcium is necessary for aggregation (Zucker and Grant, 1978, Margnerie et al., 1979), plasma obtained after mixing blood with 3.8% trisodium citrate, was evaluated for ionized Ca2+ and the levels were not different between control and diabetic plasma.

Treatment of the diabetic rabbits with insulin demonstrates that 1.) mechanism which regulate glucose levels are still operative in the alloxan-induced diabetic rabbit as shown by the ability to reduce
the hyperglycemia and 2.) some reduction in platelet hypersensitivity can be demonstrated.

A complete reversal of the hyperreactive diabetic platelet to levels of control platelets probably would not be expected since human subjects receiving insulin still demonstrate platelet dysfunction. Platelets in rabbits turnover in 3-4 days. Therefore 2-5 platelet turnovers could be expected in periods up to 3 weeks. Insulin treatment for up to 3 weeks did result in some decreased sensitivity of the diabetic platelets.
CONCLUSIONS

1.) Arachidonic acid and collagen stimulate enhanced aggregation, and \( \text{TxB}_2 \) and 12-HETE production from diabetic platelets

2.) Granular release of serotonin is enhanced from diabetic platelets

3.) Control platelets exhibit enhanced sensitivity in diabetic plasma

4.) Diabetic platelets exhibit attenuated sensitivity in control plasma

5.) Arachidonic acid metabolism involvement demonstrated by eicosanoid measurements and ASA inhibition

6.) Identity of the enhancing factors not known

7.) Short term insulin treatment decreases the diabetic platelet hypersensitivity in terms of aggregation and arachidonic acid metabolism
VI. Alteration of Platelet Arachidonic Acid Metabolism in the Alloxan-Induced Diabetic Rabbit

Introduction

Eicosanoid production was investigated using washed platelet suspensions prepared from alloxan-induced diabetic rabbits. Production of platelet thromboxane B$_2$ (TXB$_2$), PGE$_2$, and 12-hydroxyeicosatetraenoic acid (12-HETE) induced by arachidonic acid, thrombin, acetyl-glycerol-ether-phosphatidylcholine (AGEPC), and a calcium ionophore A$_{23187}$, were measured using radioimmunoassay. Platelet α-tocopherol (vitamin E) and cholesterol and plasma α-tocopherol and cholesterol were also measured in the control and diabetic rabbits. TXB$_2$ and 12-HETE synthesis was significantly elevated in diabetic rabbit platelets compared to the control rabbit platelets when stimulated by all agonists. Similar reports are noted in the literature with platelets from human diabetic subjects (Karpen et al., 1984) using washed platelets and streptozotocin-induced diabetic rats (Karpen et al. 1982, Eldor et al., 1978, Gerrard et al., 1980).

The α-tocopherol content of diabetic rabbit platelets was significantly reduced while the plasma levels were significantly elevated compared to the control rabbits. Elevated platelet TXB$_2$ and HETE synthesis could, in part, be related to the low platelet 

133
α-tocopherol content. Studies have demonstrated the involvement of α-tocopherol in platelet aggregation in normal subjects (Lake et al., 1977, Steiner et al., 1978) diabetic rats with low cellular α-tocopherol (Karpen et al., 1982) and Type I diabetic humans (Karpen et al., 1984). Cholesterol quantities were not significantly different between the control and diabetic groups with respect to plasma and platelets. Reports have demonstrated that in mixtures of cholesterol-rich liposomes and platelets, platelet sensitivity is enhanced (Shattil and Cooper, 1975). Deposition of cholesterol in platelets, with the resultant hypersensitivity, may be involved in the pathogenesis of atherosclerosis. Studies in animals demonstrate that cholesterol feeding, and therefore cholesterol accumulation, is associated with endothelial damage (Ross and Harker, 1976, Pritchard et al., unpublished).

Incubation with 50 μM eicosatetrayenoic acid, a dual cyclooxygenase/lipoxygenase inhibitor, similarly inhibited 5 and 20 μM arachidonic acid-induced TxB2 and 12-HETE production by > 95% for both groups. Indomethacin (a cyclooxygenase inhibitor) at 50 μM inhibited TxB2 generation by > 98% with both control and diabetic platelets and allowed 12-HETE production to increase by 72% in the control and 101% in the diabetic. Exogenous arachidonic acid, not consumed by the cyclooxygenase enzyme, is shunted through the C-12 lipoxygenase for 12-HETE production. Decreased eicosanoid production with eicosatetrayenoic acid and indomethacin demonstrates that the enzyme systems in the diabetic platelet are still subject to inhibition patterns similar to control platelets.
Results

Biosynthesis of TxB₂ and 12-HETE

Figure 29 illustrates TxB₂ production versus time at 5 and 20 μM arachidonic acid. Reactions are rapid and essentially complete at 4 minutes for both subjects at both substrate levels. Significant differences are apparent as early as 0.5 minutes with 20 μM arachidonic acid. Significant differences are maintained to at least 10 minutes. Figure 30 demonstrates that 1) diabetic platelets produce elevated 12-HETE with apparent differences at 2 minutes to 10 minutes, 2) 12-HETE formation is rapid and essentially complete at 4 minutes with 5 μM arachidonic acid, and 3) an essentially linear production of 12-HETE between 0.5 and 10 minutes is observed with no apparent reaction completion as with 5 μM arachidonic acid.

Washed platelets from control and diabetic rabbits were incubated with arachidonic acid, thrombin, and acetyl-glycerol-ether-phosphatidylcholine (AGEPC) and TxB₂ synthesis was evaluated. Figure 31 shows the effects of different concentrations of arachidonic acid on its conversion to TxB₂ for a 2 minute incubation. At a low concentration of substrate, control platelet TxB₂ synthesis was maximal while at a concentration of substrate (20 μM), the synthesis of TxB₂ had decreased. Concentrations above 20 μM demonstrated a further decrease in TxB₂ levels. No significant differences were noted at 30 μM arachidonic acid between control and diabetic platelets. Incubations containing 2 μM for 2 min were marginally significant; control 385 ± 34 versus 494 ± 79 pmoles/10⁸ platelets, mean ± S.D., N=7, p < 0.01. Concentrations to 0.25 μM demonstrated the same significant difference. Control
Figure 29. Production of TxB$_2$ at 5 and 20 µM arachidonic acid: Comparison of control and diabetic subjects. Solid line indicates control, dashed line indicates diabetic at either 5 µM (5) or 20 µM (20) arachidonic acid.
Figure 30. Time dependent production of 12-HETE at 5 and 20 μM arachidonic acid: Comparison of control and diabetic subjects. See legend for Figure 29.
Figure 31. Production of TxB2 from washed control and diabetic platelets stimulated by 5,10, and 20 μM arachidonic acid (2 minutes).
TxB2 levels decreased and diabetic TxB2 levels increased with 10 μM arachidonic acid when compared to 5 μM arachidonic acid. TxB2 formation was significantly increased in diabetic platelets at all concentrations of arachidonic acid to 30 μM. In some platelet incubations, PGE2 was also measured at 5 and 20 μM arachidonic acid. PGE2 synthesis was also significantly increased similar to synthesis pattern for TxB2 except values were approximately ten-fold less. Addition of indomethacin (50 μM) or 5,8,11,14-eicosatetraynoic acid (ETYA) (50 μM) to the platelet incubations inhibited the TxB2 production by > 98% and 95% respectively. Increasing ETYA concentrations blocked the synthesis of TxB2 and 12-HETE to parallel degrees in both control and diabetic animals.

After a 10 minute incubation with arachidonic acid, 12-HETE levels are shown in Figure 32 at 5 and 20 μM. 12-HETE production was not different between the groups at 5 μM. Significant differences were noted with 20 μM arachidonic acid at 10 minutes.

Platelet agonists which interact with platelet receptors, thrombin and AGEPC, were evaluated since endogenous arachidonic acid would be released by phospholipase action. Thrombin-induced platelet TxB2 and 12-HETE synthesis was evaluated and is illustrated in Figure 33 at 0.5 and 3 U/ml. Significant differences in TxB2 production is noted at both 0.5 (p < 0.05) and 3.0 (p < 0.001) U/ml (Side A). As shown in Figure 33, side B, elevated 12-HETE synthesized from diabetic platelets is apparent at 3.0 U/ml (p < p.001) but is not significant when stimulated with 0.5 U/ml. All
Figure 32. Production of 12-HETE from washed control and diabetic platelets stimulated by 5 and 20 μM arachidonic acid (10 minutes).
Figure 33. Thrombin-induced TxB2 and 12-HETE production in 10 minutes at 3.0 and 0.5 U / ml.
incubations were for 10 minutes. Thrombin-induced TxB$_2$ formation was significantly elevated from the diabetic platelet to the lowest thrombin concentration tested, 0.2 U/ml.

AGEPC stimulated the release of arachidonic acid and the subsequent eicosanoid production with the range of concentrations examined (1 x 10^-6 to 1 x 10^-10 M). Production of TxB$_2$ and 12-HETE was not observed in incubations omitting Ca$^{2+}$. Table 10 illustrates the production of TxB$_2$ and 12-HETE generation with respect to time. TxB$_2$ differences are apparent at the earliest time tested (2 minutes) and were maintained as such to 10 minutes. Differences in 12-HETE production was generally more apparent at longer incubation times and higher AGEPC concentrations.

Table 10

Time dependency of AGEPC-induced TxB$_2$ and 12-HETE production at 1 x 10^-6 and 1 x 10^-8 M

<table>
<thead>
<tr>
<th>AGEPC [M]</th>
<th>Time (min)</th>
<th>TxB$_2$ Control</th>
<th>TxB$_2$ Diabetic</th>
<th>12-HETE Control</th>
<th>12-HETE Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^-6</td>
<td>10</td>
<td>138 ± 11</td>
<td>339 ± 17</td>
<td>367 ± 27</td>
<td>549 ± 27</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>99 ± 9</td>
<td>199 ± 11</td>
<td>176 ± 6</td>
<td>230 ± 17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62 ± 9</td>
<td>131 ± 18</td>
<td>87 ± 10</td>
<td>117 ± 18</td>
</tr>
<tr>
<td>1 x 10^-8</td>
<td>10</td>
<td>65 ± 5</td>
<td>122 ± 6</td>
<td>212 ± 14</td>
<td>301 ± 15</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>47 ± 7</td>
<td>78 ± 9</td>
<td>93 ± 11</td>
<td>139 ± 9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>37 ± 3</td>
<td>59 ± 5</td>
<td>56 ± 4</td>
<td>63 ± 4</td>
</tr>
</tbody>
</table>

Values are expressed as pmoles eicosanoid / 10^8 platelets. Values are means ± S.D. (N = 5-7 experiments with duplicates determinants).
Fig 34 illustrated AGEPC-induced TxB₂ (side A) and 12-HETE (side B) synthesis at two concentrations. Incubation time was 10 minutes. All AGEPC incubations contained 1 mM Ca²⁺. Results are comparable to thrombin-induced TxB₂ and 12-HETE synthesis (Fig 33). At the two lowest AGEPC concentrations examined (1 x 10⁻⁹ and 1 x 10⁻¹⁰ M) only TxB₂ production was significantly different (p < 0.05) between the groups at 10 minutes. For example, at 1 x 10⁻⁹ M AGEPC, TxB₂ levels were 20 ± 1 and 41 ± 3 pmoles/10⁸ platelets while 12-HETE levels were 151 ± 8 and 172 ± 5 pmoles/10⁸ platelets (control and diabetic groups respectively). No concentration difference was observed between 1 x 10⁻⁹ and 1 x 10⁻¹⁰ M AGEPC. Table 10 also illustrates that platelets from the diabetic group exhibit a greater increase in eicosanoid production in comparing incubations of 2 versus 10 minutes. At 1 x 10⁻⁸ M AGEPC, TxB₂ production increased 76% versus 107% for the control and diabetic groups when comparing 2 minute levels data to 10 minute levels. Similarly 12-HETE production increases 279% and 378% under the same incubation conditions.

TxB₂ and 12-HETE production, stimulated by collagen was evaluated in platelets challenged with 22-160 μg/ml for incubation times to 10 minutes. Responses from control and diabetic platelets were equivalent under all incubation conditions with collagen.
Figure 34. AGEPC-induced TxB₂ and 12-HETE production in 10 minutes at 1 x 10⁻⁶ and 1 x 10⁻⁷ M.
Table 11
Platelet and plasma levels of α-tocopherol and cholesterol from control and diabetic rabbits

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th></th>
<th>diabetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-tocopherol:</strong></td>
<td></td>
<td>plasma</td>
<td>plasma</td>
<td></td>
</tr>
<tr>
<td>control plasma</td>
<td>15 ± 3</td>
<td>ug/.5 ml</td>
<td>25 ± 5</td>
<td>ug/.5 ml</td>
</tr>
<tr>
<td>control platelets</td>
<td>135 ± 40</td>
<td>ng/10⁹</td>
<td>75 ± 12</td>
<td>ng/10⁹</td>
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<tr>
<td>diabetic platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol:</strong></td>
<td></td>
<td>plasma</td>
<td>plasma</td>
<td></td>
</tr>
<tr>
<td>control plasma</td>
<td>221 ± 8</td>
<td>ug/.5 ml</td>
<td>233 ± 16</td>
<td>ug/.5 ml</td>
</tr>
<tr>
<td>control platelets</td>
<td>58 ± 11</td>
<td>ug/10⁹</td>
<td>54 ± 11</td>
<td>ug/10⁹</td>
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<tr>
<td>diabetic platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values (means ± S.D., N = 6) were determined by the HPLC procedure defined in the text. Statistical analysis for α-tocopherol, plasma; p < 0.005 (C vs. D), platelets, p < 0.01. Statistical analysis for cholesterol, plasma; N.S., platelets; N.S.

Alpha-tocopherol levels were significantly elevated in diabetic plasma (p < 0.005) and significantly reduced in diabetic platelets (p < 0.01) when compared to matched controls (Table 11). No significant differences were observed either in the cholesterol content of plasma...
or in the cholesterol content of the platelets. Correlation of α-tocopherol levels was not observed probably reflecting the sample number and narrow data scatter. Results are consistent with literature data wherein platelets and plasma show reduced and elevated α-tocopherol levels respectively. These platelets demonstrate hypersensitivity, correlated to the α-tocopherol levels (Karpen et al., 1985).

Inhibition of cyclooxygenase activity by indomethacin and inhibition of both cyclooxygenase and lipoxygenase activities with ETYA with platelets from control rabbits is illustrated in Table 12. Indomethacin at 50 μM inhibited TxB₂ production by > 98% and enhanced 12-HETE production by 72% (1.7 fold increase). 12-HETE production was maximally enhanced at 50 μM indomethacin. Production of TxB₂ and 12-HETE is inhibited by 95% and > 99%, respectively with 50 μM ETYA. Inhibition profiles for both compounds is concentration dependent. Approximately twice the concentration levels were required for an equivalent inhibition when inhibitors were added to platelet suspension at 0°C, three minutes before the addition of arachidonic acid.

Platelets of both control and diabetic subjects were incubated with 50 μM indomethacin as described in Table 12. 12-HETE production, from diabetic platelets, was significantly enhanced when compared to the levels generated from control platelets (p < 0.02). Levels increased from 2226 to 3830 pmoles generated from 10⁸ control platelets and from 3200 to 6420 pmoles generated from 10⁸ diabetic platelets. This concentration of indomethacin also inhibited TxB₂ production by greater than 98% in diabetic platelets.
Table 12

Effect of indomethacin and eicosatetrayenoic acid concentration on 5 µM arachidonic acid-induced TxB₂ and 12-HETE production

<table>
<thead>
<tr>
<th>µM</th>
<th>Indo</th>
<th>ETYA</th>
<th>TxB₂ pmoles/10⁸ platelets</th>
<th>12-HETE pmoles/10⁸ platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>746</td>
<td>2226</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>331</td>
<td>3925</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>119</td>
<td>3547</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>78</td>
<td>3642</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>3830</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2887</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td></td>
<td>473</td>
<td>944</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td></td>
<td>282</td>
<td>48</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td></td>
<td>192</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td></td>
<td>37</td>
<td>9</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td></td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations are indomethacin (Indo) and eicosatetrayenoic acid (ETYA). Data is representative of 3 experiments where all 10 minute incubations are in duplicate. Inhibitors were preincubated for 1 minute at 37°C prior to the addition of arachidonic acid.
Since insulin treatment partially reversed the heightened aggregation demonstrated by diabetic PRP, a similar study was completed noting the possible effects of a 3 week treatment with insulin on washed diabetic platelets. With an arachidonic acid concentration of 2 μM or less (to 0.25 μM) TxB₂ production at 2 minutes was no longer different between control and diabetic subjects. TxB₂ production at 5, 10, and 20 μM arachidonic acid was reduced by 42%, 31%, and 15% respectively from diabetic platelets. TxB₂ levels were comparable to those in Figure 31. Production of 12-HETE at 5 and 20 μM arachidonic acid in 10 minutes was reduced by 27% and 17% respectively. 12-HETE levels were comparable to those in Figure 32. After insulin treatment, thrombin-induced TxB₂ and 12-HETE production at 3 U/ml and 0.5 U/ml was not significantly different between control and diabetic platelets. This observation contrasts with thrombin-induced TxB₂ and 12-HETE (Karpen et al., 1985) production in human diabetic platelets where significant differences are noted even though these patients are receiving insulin. Insulin treatment was discontinued and platelet activity was reassessed in one month. Enhanced prostanoid production was again demonstrated similar to that observed in Figures 31, 32, and 33.
**Discussion**

The present study has demonstrated an increased production of TxB$_2$ and 12-HETE from the platelets of alloxan-induced diabetic rabbits. Studies examining platelet arachidonic acid metabolism in the alloxan-induced diabetic rabbit have not been reported.

In contrast to previous studies in the streptozotocin-induced diabetic rat (Gerrard et al., 1980, Karpen et al., 1982) and Type I diabetic human (Karpen et al., 1984), greater amounts of exogenous arachidonic acid is converted to TxB$_2$ and 12-HETE in the platelets from the diabetics when compared to non-diabetics. From Figures 29 and 30, it is evident that diabetic platelets more rapidly metabolize exogenous arachidonic acid. Overall levels of TxB$_2$ and 12-HETE are elevated from diabetic platelets even when synthetic reactions have reached maximal levels. The apparent discrepancy in the production of TxB$_2$ (Figure 31) and 12-HETE (Figure 32) at 5 μM arachidonic acid may be explained by a preferential early consumption of substrate by the cyclooxygenase enzyme thereby limiting substrate availability to the C-12 lipoxygenase. This is supported by the observation that significant differences are noted for TxB$_2$ production at arachidonic acid levels to 0.25 μM whereas 12-HETE is not different. Increased cyclooxygenase activity is further supported by increased synthesis of PGE$_2$ in diabetic rabbit platelet.

Maximal production of TxB$_2$ at 2 minutes is reached between 5 and 10 μM arachidonic acid. Initial production of endoperoxides and hydroperoxide intermediates may be so rapid with 20 μM arachidonic
acid that levels could accumulate and demonstrate feedback inhibition on further synthesis.

Increased platelet lipoxygenase activity demonstrated with and without indomethacin would be expected to increase both 12-hydroperoxyeicosatetraenoic acid levels (12-HPETE), the hydroperoxy precursor of 12-HETE, as well as the observed 12-HETE level. Indomethacin inhibited the production of TxB₂ and PGE₂ but not 12-HETE. The increased 12-HETE production in diabetics is further stimulated by indomethacin (p < 0.02). Reports have demonstrated extraplatelet effects by 12-HPETE. Observations include stimulation of C-5 lipoxygenase activity (Maclouf et al., 1982) in neutrophils, inhibition of cyclooxygenase activity, and inhibition of endothelial prostacyclin synthetase activity by lipid hydroperoxides (Moncada et al., 1976, Han et al., 1979). Lipid peroxides are increased in the plasma of streptozotocin-induced diabetic rats (Karpen et al., 1982). Theories of the development of atherosclerosis include decreases in prostacyclin production.

Previous studies have presented evidence for increased deacylation and increased TxB₂ and 12-HETE production in diabetic human and diabetic rat platelets (Eldor et al., 1978, Karpen et al., 1985). Thrombin is a potent physiologic stimulus. This data, showing an elevated production of TxB₂ and 12-HETE from a receptor mediated agonist as thrombin, implies both the activity of the phospholipase and cyclooxygenase enzymes might be increased. Platelets obtained from the diabetic rabbits possess increased activity of the prostaglandin synthetase system and this characteristic may be related to the increased platelet aggregation
associated with the disease. Release of endogenous arachidonic acid, and conversion to 12-HETE is more significant at 3.0 U/ml than at 0.5 U/ml. The delay in the consumption of released arachidonic acid and subsequent 12-HETE production is apparent at 0.5 U/ml where a lower release of arachidonic acid would be expected. Besides changes in phospholipase activity, platelet receptor number or affinity may be altered in the diabetic state. Increased thrombin-receptor interactions may result in an enhanced release of endogenous arachidonic acid. AGEPC is not involved in thrombin-induced secretion (Marcus et al., 1981).

Effects of AGEPC on the production of TxB_2 has been a subject of debate (Shaw et al., 1981, Cazenave et al., 1979). AGEPC has not been shown in the literature to stimulate increased prostaglandin production from the isolated platelet of the diabetic subject. AGEPC has been shown to stimulate enhanced TxB_2 production and serotonin release in platelet rich plasma of human Type I diabetics (Greco et al., 1985).

AGEPC has been shown to be catabolized by an acid-labile serum enzyme (Farr et al., 1983) and be deacetylated by isolated platelets (Kramer et al., 1984) and rabbit neutrophils (Chilton et al., 1983). Continued receptor occupancy of ADP has been shown to be essential to maintain availability of fibrinogen binding sites. Receptor affinity for AGEPC may be altered by the in vivo environment of diabetic blood. Decreased catabolism of AGEPC or changes in receptor binding affinity could alter platelet responses. Data shown for AGEPC (Figure 33) is similar to thrombin, both which interact with platelet receptors.
Enhanced phospholipase activity has been demonstrated for diabetic subjects (Takeda et al., 1981). This could in part explain enhanced eicosanoid production. Endogenous platelet arachidonic acid levels may be increased therefore a larger source of potential substrate could be available. Increased levels of arachidonic acid have been demonstrated in platelets from type II diabetic subjects (Morita et al., 1983, Takahashi et al., 1984) and an increased uptake of labeled arachidonic acid into platelet phospholipids has been noted in type II diabetics (Jones et al., 1983). Decreased activities of desaturase enzymes have been noted in streptozotocin-induced diabetic rats (Dang et al., 1984). This would theoretically decrease available stores of arachidonic acid. This could be balanced by an increased reacylation of available dietary arachidonic acid into membrane phospholipids. Such a mechanism has been demonstrated in normal human platelets by Majerus et al. (1983).

No differences in collagen-induced aggregation in washed platelets of streptozotocin diabetic rats described by Eldor et al., (1978) agrees with the present study where there is no significant difference in TxB₂ and HETE synthesis between control and diabetic groups.

Increased prostaglandin synthesis may reflect the decreased half-life of the diabetic platelet in vivo with a higher activity of the prostaglandin synthetase (Ferguson et al., 1975). Some workers suggest that reduced platelet survival in diabetes only occurs when there is obvious vascular disease (Jones et al., 1981) while others report vascular disease is not necessary (Dassin et al., 1978,
Ferguson et al., 1975). Extensive atherosclerosis is not found in streptozotocin-induced diabetic rats although platelet survival is found to be reduced (Winocour et al., 1984, Johnson et al., 1979). Previous studies have shown an increased number of megathrombocytes (immature platelets) in patients with diabetes mellitus which demonstrate increased response to ADP, increased metabolic activity, and an enhanced release reaction (Garg et al., 1972, Colwell et al., 1976).

Various reports have correlated changes in eicosanoid levels upon platelet stimulation to α-tocopherol levels (Karpen et al., 1982, Karpen et al., 1984, Karpen et al., 1985). Alpha-tocopherol also inhibits platelet aggregation (Panganamala et al., 1977). The present work shows similar observations as the animal and human studies in the α-tocopherol content in plasma and platelets of diabetic subjects. Actual correlations of prostanoid production with α-tocopherol levels are difficult because of the narrow scatter of data points. Accurate evaluations of a possible relationship would require a more dispersed range of values. Elevated plasma and depressed platelet α-tocopherol levels from diabetic subjects agrees with literature reports though. Enhanced activity of diabetic platelets does not seem to be related to plasma or platelet cholesterol levels since no differences were noted between the control and diabetic subjects. Incorporation of cholesterol into membranes could alter fluidity and hence cellular reactivity.
Treatment with insulin partially corrected the excessive prostanoid production from diabetic platelets. This data is comparable to the data observed with PRP. Treatment may have to be prolonged for further reductions to occur. Patients on insulin still exhibit significantly greater levels of prostanoids indicating that insulin alone may be insufficient to counteract the platelet dysfunction. Insulin \textit{(in vivo)} can in part modulate platelet activity. Before treatment diabetic platelets were more sensitive to platelet stimulators. After 3 weeks of insulin treatment, sensitivity had significantly decreased indicating that insulin does effect the platelet status, whether directly or indirectly. After discontinuing insulin treatment, pre-insulin observations were again demonstrated. This modulation of sensitivity demonstrates that treatment with insulin does in part reduce platelet reactivity and that insulin does play some role.
CONCLUSIONS

1.) Isolated diabetic platelets are more sensitive to receptor mediated agonists, thrombin and AGEPC, in stimulating enhanced TxB₂ and 12-HETE production
2.) Arachidonic acid stimulates enhanced TxB₂ and 12-HETE production from diabetic platelets
3.) Clear demonstration of increased lipase activity not shown
4.) Cholesterol does not appear to account for enhanced sensitivity
5.) A decrease of α-tocopherol in diabetic platelets could account for enhanced sensitivity
6.) Short term insulin treatment decreased the production of TxB₂ and 12-HETE produced from the diabetic platelet after exposure to arachidonic acid and thrombin
VII. Decreased LTB₄ and 5-HETE Synthesized from Neutrophils of the Alloxan-Induced Diabetic Rabbits.

Introduction

Leukocyte dysfunction is apparent in PMNLs from diabetes at several levels. Diabetic PMNLs demonstrate defects in several antimicrobial functions thereby potentially lending diabetics to be more prone to infection. Metabolites of arachidonic acid are important as chemotactic agents in inflammation and infections. Reduction in their synthesis could effectively decrease mobilization of other cell types important in suppressing infections.

Washed PMNL suspensions have been used extensively to examine the metabolism of arachidonic acid to hydroxylated metabolites. This section demonstrates differences in the ability of control and diabetic PMNLs to metabolize arachidonic acid to products. Nanograms amounts are visualized and quantitated by an HPLC technique. Overall metabolism of arachidonic acid to LTB₄ isomers, LTB₄, and 5-HETE is depressed when diabetic PMNLs are stimulated and compared to control PMNLs. Incubations with and without arachidonic lead to the same observations indicating that substrate availability probably is not a limiting factor. Since both 5-HETE and LTB₄ levels are depressed, production of 5-HPETE may be the limiting factor. Jubiz
et al. (1984) has shown that patients with diabetic mellitus produce less LTB4 than control subjects.

Diabetic PMNL do not seem overly sensitive to Ca\textsuperscript{2+} or A\textsubscript{23187}. Reduction of Ca\textsuperscript{2+} concentrations to 20 μM or A\textsubscript{23187} concentration to 100 μM continues to display depressed LTB4 and 5-HETE levels. Depressed levels do not seem attributable to increased catabolism of LTB4 or reesterification of 5-HETE. The disappearance of exogenous 5-HETE and LTB4 added to diabetic PMNL suspensions parallels that seen with control PMNL suspensions. Time-course experiments demonstrate a similar phenomenon in that disappearance of synthesized products parallels that seen in control PMNLs.
Results

From the literature preliminary incubation conditions were chosen from human PMNL experiments. Reports describing arachidonic acid metabolism in rabbit peripheral neutrophils is lacking. Incubations describing LTB4 production involves, typically 30-100 x 10^6 PMNLs derived from the peritoneal cavity of the rabbit (Borgeat et al., 1976). Conversion of arachidonic acid into 5-HETE and LTB4 was respectively 2 to 10% and 0.5 to 2%. Formation was maximal at 80 μM arachidonic acid. Clearly shown from preliminary human experiments is that arachidonic acid metabolites can be visualized by HPLC using lower cell numbers. As mentioned, terminal cardiac puncture procedures in control rabbits yield approximately 95 x 10^6 PMNL. Yields for diabetic rabbits are lower. A more complete picture of arachidonic acid metabolism involving C and D PMNLs is obtained using more incubations with reduced cell numbers. Experiments devoid of Ca^{2+} or A23187 did not produce either LTB4 or 5-HETE which could be observed by HPLC. Similar results were obtained with C and D incubations. Without the addition of A23187, arachidonic acid is incorporated into phospholipids and neutrolipids. No lipoxygenase products are formed (Sun and McGuire, 1984). This indicates that the critical step for oxygenation is Ca^{2+} / A23187 dependent. In incubations minus exogenous arachidonic acid, LTB4 isomers, LTB4, and 5-HETE are synthesized from endogenous arachidonic acid released by phospholipase action.

Figure 35 shows the production of 5-HETE and LTB4 from 2 minute incubations varying in PMNL number (control) under conditions
Figure 35. Nanograms of 5-HETE and LTB4 generated as a function of PMNL number. The line designation in Figure 35 as well as in Figures 36, 37, 39, and 40 is as follows; open circle, LTB4 isomers, open square, LTB4, solid square, 5-HETE, and dot, arachidonic acid. Solid lines indicates control, dashed lines indicate diabetic.
of 1 mM Ca\(^{2+}\), 1.0 \(\mu\)M A23187, 10 \(\mu\)M arachidonic acid. Production of metabolites is approximately linear with respect to PMNL number. Incubation with PMNL numbers below 1 \(\times\) 10\(^6\) and greater than 15 \(\times\) 10\(^6\) were not completed (restriction of incubations with cell numbers > 15 \(\times\) 10\(^6\) is due to yield limitations). Both levels of PMNL, 5 \(\times\) 10\(^6\) and 10 \(\times\) 10\(^6\), afforded detectable levels of metabolites by HPLC. Future experiments were limited to these cell numbers unless otherwise noted. Production of 5-HETE, under any incubation condition, always exceeded both LTB\(_4\) and LTB\(_4\) isomers levels by approximately 2-3 times.

Incubation conditions for PMNL are more complex than platelet incubations since five parameters can be changed. As noted, incubations lacking Ca\(^{2+}\) or A23187 did not produce metabolites. Therefore, all incubations contained these two elements. As will be shown though, wide variations in the concentrations for both of these agents did little in influencing metabolite formation from control PMNLs. Metabolite formation from D PMNL was more altered with changing concentrations of Ca\(^{2+}\) and A23187. Three parameters influenced overall metabolite formation was arachidonic acid concentration, PMNL number, and incubation time.

Assay of products from PMNLs incubated with changing levels of arachidate, by reverse-phase HPLC, showed a concentration-dependent stimulation of LTB4 isomers and LTB4 production. These observations were under conditions of 1 mM Ca\(^{2+}\), 10 \(\mu\)M A23187, 10 \(\times\) 10\(^6\) PMNL with a 2 min. incubation (Figure 36). Formation of LTB4 was rapid when comparing incubations without exogenous
Figure 36. Nanograms of LTB4 isomers and LTB4 as a function of arachidonic acid concentration. Comparison of control and diabetic PMNL. Lines designed as Figure 35.
arachidonic acid and incubations with 2.5 μM arachidonic acid. A 5.6 fold increase was observed when comparing these incubation conditions. An additional increase of arachidonic acid concentration (4 times) results in an additional 1.5 fold increase in LTB$_4$ production to the maximal quantity produced at 10 μM. Decreased production of LTB$_4$ is observed when the arachidonic acid concentration is increased to 50 and 100 μM. A decrease of 18% is observed when comparing the LTB$_4$ level at 100 μM arachidonic acid to that at 10 μM. Similar observations are observed with human PMNLs where arachidonic acid concentrations (250 μM) in excess of 100 μM aggregate PMNLs (Sun and McGuire, 1984). Production of both isomers paralleled increases in LTB$_4$ synthesis. The ratio of LTB$_4$ to LTB$_4$ isomers was greatest at 2.5 μM arachidonic acid (3.7 : 1). This ratio decreased with increasing AA concentration. As LTB$_4$ levels became maximal then started to decrease, levels of the LTB$_4$ isomers continued to increase. At 100 μM arachidonic acid this ratio was close to unity. The data suggests that without the exogenous addition of arachidonic acid (arachidonic acid only released by A$_{23187}$ from endogenous sources) or with low levels of arachidonic acid, produced LTA$_4$ is converted primarily to LTB$_4$. As the exogenous concentration of arachidonic acid increases, metabolism is shifted to favor isomer production.

Production of LTB$_4$ and isomers of LTB$_4$ from diabetic PMNL mirrors that produced from control PMNL except levels are reduced. A decreased level of LTB$_4$ isomers between 50 and 100 μM arachidonic acid, compared to control incubations at these concentrations, suggests that availability of LTA$_4$ is limiting. Ratios of LTB$_4$
to LTB₄ isomers for D PMNL over the arachidonic acid concentration range is approximately the same as the ratios from C PMNL except with the addition of 100 μM arachidonic acid.

Under the same conditions as Figure 36, 5-HETE production and the unmetabolized arachidonic acid were followed (Figure 37). As with LTB₄ production, C levels exceed yet paralleled D levels. Between 10 and 50 μM, 5-HETE sharply increase, especially in the C subject. Between 10 and 50 μM arachidonic acid it was noted (Figure 36) that LTB₄ production had leveled off. Control PMNL produce approximately 69 and 72% more 5-HETE at 10 μM and 50 μM arachidonic acid respectively then D PMNL. A23187 stimulated PMNL (without the addition of AA) demonstrate the largest difference in 5-HETE production, control PMNL producing nearly four times the 5-HETE levels produced from D PMNL.

Levels of arachidonic acid remaining after the incubation, observed from the HPLC chromatographs, were quantitated (Figure 37). A23187 at 10 μM did not appear to release more AA then could be metabolized unless quantitites of arachidonic acid were subnanogram. An arachidonic acid peak was not observed with A23187-stimulated PMNLs. When 2.5 μM arachidonic acid was added, arachidonic acid quantities, detected by HPLC, were unchanged between C and D even though LTB₄ and 5-HETE levels were elevated from C PMNL. Unmetabolized arachidonic acid was elevated in HPLC traces of D PMNL at > 10μM.

Table 13 shows the compiled data from both Figures 36 and 37. Only data for 10, 50 and 100 μM arachidonic acid is shown. The data is representative of 3 to 8 separate experiments performed in single
Figure 37. Nanograms of 5-HETE and unmetabolized arachidonic acid as a function of arachidonic acid concentration. Comparison of control and diabetic PMNL. Lines designated as Figure 35.
Table 13

Effect of A.A. concentration on C-5 lipoxygenase products. Cells were incubated with 1 mM Ca\(^{2+}\), 10 \(\mu\)M A23187, and different levels of arachidonic acid for 2 min.

<table>
<thead>
<tr>
<th>Arachidonic acid [(\mu)M]</th>
<th>Control</th>
<th>p value</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB4 isomers</td>
<td>114 ± 29</td>
<td>&lt;0.05</td>
<td>80 ± 31</td>
</tr>
<tr>
<td>LTB4</td>
<td>186 ± 52</td>
<td>&lt;0.05</td>
<td>138 ± 16</td>
</tr>
<tr>
<td>5-HETE</td>
<td>802 ± 146</td>
<td>&lt;0.01</td>
<td>590 ± 118</td>
</tr>
<tr>
<td>A.A.</td>
<td>624 ± 126</td>
<td>&lt;0.01</td>
<td>1027 ± 355</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB4 isomers</td>
<td>144 ± 15</td>
<td>&lt;0.05</td>
<td>118 ± 19</td>
</tr>
<tr>
<td>LTB4</td>
<td>174 ± 28</td>
<td>N.S.</td>
<td>142 ± 22</td>
</tr>
<tr>
<td>5-HETE</td>
<td>2052 ± 130</td>
<td>&lt;0.001</td>
<td>1194 ± 160</td>
</tr>
<tr>
<td>A.A.</td>
<td>3755 ± 537</td>
<td>N.S.</td>
<td>4353 ± 832</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB4 isomers</td>
<td>148 ± 45</td>
<td>N.S.</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>LTB4</td>
<td>152 ± 28</td>
<td>N.S.</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>5-HETE</td>
<td>1578 ± 106</td>
<td>N.S.</td>
<td>1096 ± 394</td>
</tr>
<tr>
<td>A.A.</td>
<td>6912 ± 1026</td>
<td>N.S.</td>
<td>7455 ± 541</td>
</tr>
</tbody>
</table>

Metabolites were extracted and analyzed as described under "Methods". Arachidonic acid is abbreviated as A.A. Significance between control and diabetic values is shown in the p values column.
determinations. Values indicate mean ± standard deviation. At all concentrations of arachidonic acid, D PMNL produce reduced levels of all metabolites. Levels of unmetabolized arachidonic acid are increased in D PMNL incubations. Similar differences were observed at 1.0 μM A23187 and 5 × 10⁶ PMNL, although the nanogram quantities were only about one-half of those observed in Table 13. Figure 38 demonstrates the comparison of HPLC chromatographs between control and diabetic subjects. Incubation conditions were 0.1 mM Ca²⁺, 1.0 μM A23187, 10 μM arachidonic acid, 5 × 10⁶ PMNL, for 2 minutes.

Dependence of 5-lipoxygenase activity on levels of exogenous Ca²⁺ was evaluated in control PMNL incubations containing varying levels of Ca²⁺ and constant levels of A23187 (10 μM), arachidonic acid (10 μM), cell number (10 × 10⁶), and incubation time (2 min.). Final concentrations of Ca²⁺ that were added was 0.5, 1.0, and 2.0 mM. Metabolite formation from control PMNL was essentially unchanged over the Ca²⁺ concentration range as follows: LTB₄ isomers, 139 ± 22 ng (mean ± S.D.), LTB₄, 196 ± 19 ng, and 5-HETE, 790 ± 11 ng. A significant difference were noted at 1.0 mM between C and D PMNL preparation as noted in Figure 36.

Since PMNL activity appeared to be independent of the levels of Ca²⁺ used above, other experiments were conducted with a lower Ca²⁺ concentration (0.1 mM) and A23187 concentration (1.0 μM). Since reduced metabolite formation from D PMNL was easily detectable using 10 × 10⁶ PMNL, similar observations could be expected by lowering the cell numbers to 5 × 10⁶ (Figure 35). Since LTB₄ levels were lower than 5-HETE levels, incubations with lower PMNL
Figure 38. High performance liquid chromatograph of leukotrienes-control versus diabetic. The data shows representative chromatographs of five sets of experimental results under the incubation condition discussed in the text. Retention times and wavelength changes are as in Figure 12. Identities of compounds not shown in Figure 12 are (1) 5S,12R-\(\Delta^6\)trans-leukotriene B\(_4\) and (2) 5S,12S-\(\Delta^6\)trans-leukotriene B\(_4\).
TIME (minutes)
numbers would have to consider this observation especially with D PMNL where production of LTB4 is lower than controls. Experiments with $5 \times 10^6$ D PMNL were also detectable by HPLC.

Experiments with $0.1 \, \text{mM} \, \text{Ca}^{2+}$, $1.0 \, \mu\text{M} \, \text{A}_2\text{3187}$, $10 \, \mu\text{M}$ arachidonic acid, $5 \times 10^6$ PMNL with a 2 min. incubation demonstrated that D PMNL were more influenced by the changing Ca$^{2+}$ concentration. A comparison was made between using $1.0 \, \text{mM} \, \text{Ca}^{2+}$ versus $0.1 \, \text{mM} \, \text{Ca}^{2+}$. Production of LTB4 isomers, LTB4, and 5-HETE were unchanged between the two Ca$^{2+}$ levels when C PMNL were stimulated. Production from D PMNL was reduced as follows in comparing levels at $0.1 \, \text{mM}$ to $1.0 \, \text{mM} \, \text{Ca}^{2+}$: LTB4 isomers and LTB4, 36% and 5-HETE, 16%.

These experiments were extended by examining a reduction in the ionophore concentration to $0.5 \, \mu\text{M}$. Incubation mixtures contained $0.1 \, \text{mM} \, \text{Ca}^{2+}$, $0.5 \, \mu\text{M} \, \text{A}_2\text{3187}$, $10 \, \mu\text{M}$ arachidonic acid, $5 \times 10^6$ PMNL with a 2 min. incubation. In Figure 39 metabolite levels are compared between $1.0 \, \mu\text{M} \, \text{A}_2\text{3187}$ and $0.5 \, \mu\text{M} \, \text{A}_2\text{3187}$. With this change C PMNL begin to show decreased metabolite levels (especially 5-HETE) at the lower concentration. Production of 5-HETE from D PMNL was more influenced by this change. A decrease in 5-HETE production exceeds the decreased LTB4 production from both C and D PMNLs. Greater levels of apparently unmetabolized arachidonic acid is observed with both C and D PMNLs at $0.5 \, \mu\text{M} \, \text{A}_2\text{3187}$. Levels of unmetabolized arachidonic acid from the D PMNL exceed the reduction of arachidonic acid metabolites at both $\text{A}_2\text{3187}$ concentrations.
Figure 39. Nanograms of LTB₄ isomers, LTB₄, 5-HETE, and arachidonic acid as a function of A₂₃₁₈₇ concentration. Comparison of control and diabetic PMNL. Lines designated as Figure 35.
Various investigators have shown that LTB\textsubscript{4} can be converted to 20-hydroxy and 20-carboxyl metabolites with human PMNLs (Naccache et al., 1982, Sun and McGuire, 1984). Catabolism can be noted by 1.) adding authentic LTB\textsubscript{4} to PMNL suspensions and observing the disappearance of LTB\textsubscript{4} or 2.) comparing HPLC profiles of incubations run over various times i.e. comparing 2 minute versus 15 minute incubations. The more polar metabolites would elute before LTB\textsubscript{4} on the reverse-phase HPLC procedure that is used. Comparable elution would be expected in comparing this procedure with that of Sun and McGuire (1984). Suspensions of 10 x 10\textsuperscript{6} PMNLs were incubated for 15 minutes at 37°C with 75 ng LTB\textsubscript{4} and 200 ng 5-HETE. Both C and D suspensions were treated in an identical manner. Sample areas corresponding to LTB\textsubscript{4} and 5-HETE were compared to direct on column injection of 75 ng of LTB\textsubscript{4} and 200 ng 5-HETE. Areas for the standards were divided by two since only one-half of the biological sample was injected on column. A substantial decrease in the observable areas corresponding to LTB\textsubscript{4} and 5-HETE was similarly exhibited by both C and D suspensions. Less than 7\% of the added unlabeled LTB\textsubscript{4} was observed in HPLC chromatographs. In contrast essentially 100\% of LTB\textsubscript{4} is recovered intact after incubation at 37°C for 15 min. in PBS alone. A split peak was observed to increase in samples with added LTB\textsubscript{4} near the solvent front. Similar observations were noted by (Sun and McGuire, 1984). Exogenous LTB\textsubscript{4} did not stimulate metabolite formation from the PMNLs or arachidonic acid release in the nanogram range.
Less than 16% of exogenous 5-HETE was observed in sample incubates after 15 minutes. Similar to LTB4, 5-HETE did not stimulate metabolite formation from the PMNLs or arachidonic acid release. No other unknown peaks were observed in PMNL suspensions incubated with LTB4 and 5-HETE. The HPLC procedure was exactly as described in the section titled Analysis of Arachidonic Metabolites by Reverse-Phase HPLC. Arachidonic acid also decreased in comparing 2 min. incubations with 15 min. incubations.

From this data, decreased 5-HETE and LTB4 levels from D PMNLs may not be attributable to increased reesterification of 5-HETE and increased catabolism of LTB4 respectively. Equivalent reduction of added 5-HETE and LTB4 are noted when incubated with control or diabetic PMNLs. Actual increased catabolism and reesterification reactions have be considered with more time points to observe if differences exist in the rates of catabolism or incorporation. Low cells yields limit these experiments.

Incubations for 1, 2, 5, and 15 minutes were compared with respect to the appearance of C-5 lipoxygenase products (Figure 40). Reaction mixtures contained 0.1 mM Ca\(^2+\), 1.0 \(\mu\)M A23187, 10 \(\mu\)M arachidonic acid, and 5x10\(^6\) PMNL. Reactions were terminated and extracted as noted in the HPLC section. At all time points, products of control PMNL exceed those produced from diabetic PMNL. The data is representative of three sets of experimental results. Maximal production of both LTB4 and 5-HETE was between 2 and 5 minutes. The appearance of the LTB4 isomers follows that of LTB4 (not shown). Data from control and diabetic PMNL parallel each other
over the incubation time. Appearance of unmetabolized arachidonic acid decreased between 1 and 15 minutes similar to 5-HETE levels. Levels remaining in diabetic PMNL incubations, exceeded those in control incubations by approximately 1.5 to 2 fold over the range of incubation times.
Figure 40. Nanograms of LTB4 and 5-HETE produced at different incubation times. Products were produced from ionophore-stimulated cells with exogenous arachidonic acid. Lines designated as Figure 35.
Discussion

Jubiz et al. (1984) described the decreased LTB4 synthesis by PMNL from male patients with diabetes mellitus, at levels of 407 ng/30 x 10^6 cells/10 min for diabetic subjects versus 709 ng/30 x 10^6 cells/10 min for control subjects. The data presented agrees with these findings over wide concentration ranges of Ca^{2+}, A23187 and arachidonate. For instance, at 1 mM Ca^{2+}, 10 μM A23187, 10 μM arachidonic acid, 10 x 10^6 PMNL with a 2 min incubation, PMNLs from control rabbits produced 186 ± 52 ng LTB4 versus 138 ± 16 from the PMNL of diabetic rabbits, a 26% decrease. Important data lacking in the paper of Jubiz was the synthesis of the two LTB4 isomers, 5-HETE, and the changes in arachidonic acid levels. This study extends the observation of the decreased LTB4 synthesis and also considers the LTB4 isomers, 5-HETE, and arachidonic acid levels. As with LTB4, both LTB4 isomers and 5-HETE levels are depressed (30% and 20% respectively at the above incubation conditions) when diabetic PMNL are challenged.

In these studies, differences between control and diabetic PMNL persisted whether or not arachidonic acid was added. Arachidonic acid is added to the incubation to increase the mass of material that is formed. Addition of arachidonic acid and the persistence of the depressed production suggests that it is not substrate availability which explains lower LTB4 and 5-HETE levels. Sun and McGuire (1984) have reported that with concentrations of exogenous arachidonic acid less than 10 μM, greater than 90% of the arachidonic acid metabolites are from arachidonic acid released from endogenous stores in human PMNL. Elevated glucose levels could affect the
glutathione system (i.e. glutathione peroxidase) by maintaining reduced glutathione levels through the hexose monophosphate shunt. Available substrate could preferentially be shunted from 5-HPETE to 5-HETE formation thereby effectively decreasing LTB₄ levels. From the data presented this does not seem to be the case since both 5-HETE and LTB₄ levels are depressed.

Metz (1983) notes that with fasting plasma glucose levels greater than 250-300 mg/dl, decreased formation of arachidonic acid metabolites in PMNL could be due to deficiencies of substrate synthesis and release. PMNL have insulin receptors and low insulin levels could decrease overall glucose metabolism. But it is unlikely low levels play a major role since insulin is not required for glucose transport (Leroux et al., 1975). As shown by Leroux et al. (1975) chronic but not acute insulin treatment in vivo partly reverses some metabolite defects. Insulin treatment in vitro is not effective as the in vivo situation. Severe insulin deficiency and glucagon excess inhibits desaturation of precursor fatty acids to arachidonic acid.

Various studies (Bagdade et al., 1978; Jones, et al., 1983) have shown arachidonic acid deficiencies in PMNL of human diabetics and a similar observation is observed in streptozotocin-induced diabetes in the rat (Holmann et al., 1983). Decreased arachidonic acid levels in human diabetics has been shown (Jones, et al., 1983) to be inversely correlated to glycosylated hemoglobin. A similar correlation to fasting plasma levels was not found and suggests that acute changes in hyperglycemia is less important to a reduction in arachidonic acid availability then chronic, long term control.
Potential Consequences of Contamination of PMNL Preparations

Contamination of PMNL preparations with platelets and/or RBC can affect the availability of arachidonic acid to 5-lipoxygenase. Platelet derived arachidonic acid can be utilized by the PMNL for leukotriene synthesis (Marcus et al., 1982). Coincubation of platelets with PMNL results in the activation of 5-lipoxygenase and increases the formation of 5-HETE and LTB4 (Maclouf, et al., 1982). The stimulator responsible was determined to be 12-HPETE since 1.) eicosatetrayeonic acid, an inhibitor of C-12 lipoxygenase, suppressed the formation of 5-HETE and LTB4 and 2.) stimulation of 5-HETE and LTB4 production was observed upon the direct addition of 12-HPETE to PMNL suspensions. Effects of 12-HPETE resembles that of A23187. ETYA lacks an inhibitory effect on the C-5 lipoxygenase with rabbit peritoneal PMNL (Borgeat, et al., 1977 and Vanderhoek, et al., 1980). The C-15 hydroperoxy component of platelet-derived PGG2 might also contribute to the platelet-induced stimulation of arachidonic acid metabolism in the PMNL. 12-HETE, metabolized through the C-5 lipoxygenase, can produce 5S,12S-DHETE. This compound comigrates with LTB4 in the described HPLC chromatography. Conceivably, if such reactions did occur, 5-HETE levels would be lowered and the "LTB4" peak would increase. If the PMNL from diabetic subjects synthesizes lower LTB4 and 5-HETE levels, contamination with platelets would shift the appearance of LTB4 and 5-HETE in the chromatography. LTB4 levels would be greater and 5-HETE levels would be lower than the actual case.
Retention time corresponding to the 12-HETE peak was monitored during each run and was found to be consistently less than 2% of the area for the 5-HETE peak for both control and diabetic incubations. Conceivably, any synthesized 12-HETE could react to form the 5,12-DHETE compound and artifactually increase the LTB4 peak. Observation of 12-HETE could also be missed since Stenson et al. (1979) has shown 12-HETE is rapidly incorporated into neutrophil membrane lipids. Incorporation is rapid, approximately 10% is incorporated into the cells within 1 min and approximately 40% is incorporated within 5 min.

Various investigators have disputed the cyclooxygenase capacity of PMNL. Clear demarcation between platelet-derived eicosanoids versus PMNL derived eicosanoids is lacking. Higgs et al. (1976) demonstrated that microsomes of rabbit PMNL incubated with PGG2 or PGH2 while undergoing phagocytosis produce thromboxane A2-like activity. The present study also demonstrates that immunoreactive thromboxane B2 is measured. Diabetic PMNL synthesize approximately 1.8-6.8 times more immunoreactive thromboxane B2. Levels for both are from 237-3500 pg/incubate or 0.2-3.5 ng/incubate. Decreased levels of LTB4 and 5-HETE from diabetic PMNL cannot be accounted for by increased TxB2 synthesis since LTB4 levels are in the 100-200 ng range and 5-HETE levels are in the 400-2000 ng range. The absolute origin of the immuno-reactive TxB2 is still questionable since picogram levels could be produced from residual platelets adhering to PMNL. Isolated diabetic platelets alone differences
were observed between the control and diabetic PMNL preparations and no differences were observed in reticulocyte numbers. Since RBC contamination is negligible, reticulocyte contamination would be even more negligible. HPLC tracing were monitored for 15-HETE (Rf of 24.7 min.) and peaks were always less than 2% of the major 5-HETE peak at 234 nm. Incubation of 10 x 10^6 RBCs from a control rabbit with 1mM Ca^{2+}, 10μM A23187, 10μM arachidonic acid for 2 minutes produce approximately 90 ng of 15-HETE. This number of RBC, mixed with a usual PMNL preparations of 10 x 10^6, would be a 1:1 ratio. Typically this ratio is greater than 100 to 2, PMNL to RBC respectively. Therefore, calculated 15-HETE production would be less than 5 ng in a PMNL preparation. This amount is insignificant compared to the 400 to 2000 ng of 5-HETE usually observed. 15-HETE is also a potent inhibitor of C-5 lipoxygenase (Vanderhoek, et al., 1980). Products from the C-15 lipoxygenase can interact with the C-5 lipoxygenase to produce 5S, 15S-dihydroxy 6,8,11,13-eicosatetraenoate (Vanderhoek et al., 1980). This reaction could decrease the observed 5-HETE. HPLC migration for this compound is not known. As discussed though, actual production of 5,15-DHETE would not be likely in PMNL preparations devoid of reticulocytes.

Various reports have shown both 5-HETE, LTB4, and peptido leukotrienes produced from PMNL (Borgeat and Samuelsson, 1979, Aehringhaus et al., 1982). Viable PMNL suspensions convert exogenously added LTA4 into various leukotrienes (Rådmark et al., 1980a, Rådmark et al., 1980b). Verhagen et al., (1980) reported that LTC4 "formed" from PMNL suspensions was observed to be
dependent on the relative amounts of eosinophils. Subsequently, Verhagen demonstrated that purified eosinophil preparations produce almost exclusively LTC\textsubscript{4} while PMNL preparations produce almost exclusively LTB\textsubscript{4}, two all trans LTB\textsubscript{4} isomers, 20 hydroxy-LTB\textsubscript{4}, and 6-trans-LTC\textsubscript{4}.

Stimulated control PMNL synthesize greater levels of 5-HETE than LTB\textsubscript{4} (Figure 35). As the cell number increases, the ratio of 5-HETE to the combined quantities of LTB\textsubscript{4} and LTB\textsubscript{4} isomers increases as follows: 1.9 (5 x 10\textsuperscript{6}), 2.2 (10 x 10\textsuperscript{6}), and 6.4 (15 x 10\textsuperscript{6}). Synthesis of LTB\textsubscript{4} and 5-HETE from PMNL is similar to the production of TxB\textsubscript{4} and 12-HETE from platelets. Combining the quantities of LTB\textsubscript{4} and the two LTB\textsubscript{4} isomers allows a comparison of the amount of LTA\textsubscript{4} that is formed compared to the 5-HETE that is formed. As cell number increases, 5-HPETE is preferentially reduced to 5-HETE. The sharp increase in 5-HETE formation could come from the greater availability of 5-HPETE plus enzyme rate limitations in the formation of LTA\textsubscript{4} and LTB\textsubscript{4}. 5-HETE and LTA\textsubscript{4} that are synthesized cannot act as substrates for the LTA\textsubscript{4} hydrolase and 5-HPETE glutathione peroxidase enzyme systems respectively. Similar ratios are observed from levels of 5-HETE and LTB\textsubscript{4} from D PMNLs. Levels of both have been shown to be reduced. This supports the suggestion that 5-HETE and LTB\textsubscript{4} formation is not due to substrate availability (from a quantitative point of view) but rather results from low C-5 lipoxygenase activity.

Conversion of exogenous substrate by the C-5 lipoxygenase also requires the auxilliary stimulation by A\textsubscript{23187} presumably by elevating intraleukocyte Ca\textsuperscript{2+} levels enough for enzyme activation.
Similar observations were noted in macrophages stimulated with A23187 and soluble agonists (Tripp et al., 1985). Thus mobilization of intracellular Ca\(^{2+}\) could be a rate limiting step for PMNL activation. Low intracellular Ca\(^{2+}\) levels in D PMNL could theoretically limit C-5 lipoxygenase activation and hence the production of 5-HPETE. Control PMNL require the entrance of extracellular Ca\(^{2+}\) for optimal neutrophil function as assessed by arachidonic acid metabolite levels. A saturating level of Ca\(^{2+}\) (0.5-1.0 mM) would not seem to be a limit step in activating PMNL. It would seem that low C-5 lipoxygenase activity is not a result of low Ca\(^{2+}\) levels in D PMNL since intraleukocyte Ca\(^{2+}\) levels should be elevated sufficiently by the Ca\(^{2+}\) influx prompted by A23187.

A23187 mimics many of the effects of physiological stimuli as serum-coated zymosan particles. Both affect arachidonic acid metabolism, activate the respiratory burst, produce increases in \(O_2\) consumption, and stimulate hexose monophosphate shunt activity.

As arachidonic acid concentration increases, greater levels of LTB\(_4\) isomers are formed compared to LTB\(_4\) indicating that the rate-limiting step is the cytosolic LTA\(_4\) hydrolase (Jakschik et al., 1983). This controlled system allows a finite quantity of LTB\(_4\) to be produced. Intermediate substrates as 5-HPETE or LTA\(_4\) must accumulate and be processed along alternate enzymatic and nonenzymatic pathways or be released. Conversion of LTA\(_4\) to LTB\(_4\) has been shown to occur in cell-free mammalian plasma independent of neutrophils (Fitzpatrick, et al., 1983). The appearance of 5-HETE and LTB\(_4\) decreases over time. Sun and McGuire (1984) have shown
that LTB₄ isomers and 20-hydroxy-LTB₄ increase with time as LTB₄ decreases when human PMNL are stimulated. It appears that only LTB₄ and 5,12-DHETE (Shak et al., 1984) are subject to ω-oxidation. This pathway is similar to the microsomal cytochrome P450 system which catalyses the ω-oxidation of prostaglandins. Carbon monoxide, a potent inhibitor of the cytochrome P450 system, also inhibits the ω-oxidation of LTB₄. Rapid catabolism of LTB₄ also reduces its effective concentration. Catabolism of a potent physiological agent as LTB₄ is one mechanism whereby inflammatory reactions can be modulated.

Besides catabolic pathways, the rapid disappearance of LTB₄ suggests that LTB₄ synthesis may be terminated perhaps by enzyme inhibition by a hydroperoxide intermediate. Both soybean lipoxygenase and sheep vesicular gland cyclooxygenase have previously been shown to undergo self-inactivation (Lands et al., 1972). A similar analogy may apply to the LTA₄ synthetase enzyme.

Catabolism of exogenous LTB₄ by human PMNL is rapid, specific, temperature dependent, varies with cell number, and with the initial substrate concentration (Shak et al., 1984a, Shak et al., 1984b). Stereospecific binding to neutrophils and catabolism of LTB₄ in PMNL are coupled processes (Lin et al., 1984). Similar information is not seen in the literature for rabbit PMNL. Omega-hydroxy and omega-carboxy derivatives of LTB₄ have been prepared by incubating human PMNL (30 x 10⁶ cells/ml) with synthetic LTB₄ at a concentration of 3000 ng/ml for 15 min. at 37°C (Naccache et al., 1982). Other investigators have used 1.0 µM LTB₄ and 20 x 10⁶
PMNL/ml (Shak et al., 1984b). This study used 150 ng LTB₄/ml incubated with 15 x 10⁶ PMNL. Results indicate that the exogenous LTB₄ underwent ω-oxidation since less than 7% of added LTB₄ was observed and an early eluting split peak, tentatively identified from literature reports as the ω-oxidation products, increased in area. The area of this peak though can only account for approximately 41% of the added LTB₄. Approximately 60% of the added LTB₄ cannot be accounted for. Previous reports demonstrate that LTB₄ is not found in the phospholipids (Sun and McGuire, 1984). The disappearance of LTB₄ cannot be completely explained.

5-HETE levels decreased when incubations were extended from 2 to 15 min. with both C and D PMNL (Figure 40). Both 5-HETE and arachidonic acid are esterified into phospholipids (Sun and McGuire, 1984). Esterification reactions could account for the disappearance of added 5-HETE. Incorporation of 5-HETE into phospholipids could be missed on HPLC chromatographs since they would elute near the solvent front or in the void volume before the solvent front because of their polarity.

From the data presented it would appear that the C-5 lipoxygenase of diabetic PMNL lacks the ability to metabolize arachidonic acid to 5-HPETE. Levels of both 5-HETE and LTB₄ are depressed when compared to levels generated from control PMNL. Depressed levels cannot be attributed to an increased catabolism (LTB₄) or reesterification (5-HETE) since (1.) the appearance of endogenous LTB₄ and 5-HETE as shown by Figure 40 parallels control levels and (2.) addition of LTB₄ and 5-HETE to PMNL suspensions results in similar observations between control and diabetic incubations.
CONCLUSIONS

1.) Diabetic PMNL produce less 5-HETE, LTB₄, and LTB₄ isomers over wide ranges of Ca²⁺, A₂₃₁₈₇, and arachidonic acid

2.) Unmetabolized arachidonic acid balances in part for the decreased synthesis

3.) Decreased synthesis not explained by increased catabolism of LTB₄ or reesterification of 5-HETE

4.) Decreased synthesis not explained by increased TxB₂ or PGE₂ levels since leukotriene levels are in the nanogram range and prostaglandin levels are in the picogram range

5.) Viability of diabetic PMNL remain similar to control levels throughout wide ranges of Ca²⁺, A₂₃₁₈₇, and arachidonic acid
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