THE REGULATION OF CELL-CELL ADHESION BY MONOHYDROXY LIPOXYGENASE METABOLITES

by

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ABSTRACT

Cell-cell adhesion is an important and an initiating event in thrombosis, inflammation and metastasis. Recent data suggest that these adhesion events are mediated by cell adhesion receptors and/or ligands present on blood cells, the endothelium and the basement membrane underlying the endothelium. This thesis hypothesizes that endogenous lipoxygenase metabolites regulate blood cell-vessel wall interactions. The following data, as presented in this thesis, support this hypothesis. First, the intracellular level of 13-HODE in endothelial cells, inversely correlated with platelet, tumor cell and leukocyte adhesion. Second, intracellular 13-HODE levels and/or 13-HODE:HETE ratios in blood cells and in tumor cells also inversely correlated with cell adhesivity. Third, the level of 13-HODE in basement membranes and in artificial grafts, inversely correlated with platelet adhesion. Fourth, 13-HODE co-localized with the vitronectin receptor (VnR) in resting endothelial cells. Following stimulation, 13-HODE and the VnR dissociated, and the VnR relocated to the periphery surface of the endothelial cell. Fifth, the increased peripheral expression of the VnR on stimulated endothelial cells mediated platelet adhesion. Finally, modulation of the lipoxygenase pathway markedly influenced experimentally-induced thrombosis.

In summary, there is an abundance of data presented in

this thesis which suggest that monohydroxy lipoxygenase metabolites regulate blood cell-vessel wall adhesion. This regulation appears to be a result of lipoxygenase metabolites influencing adhesion receptor/ligand recognition. Therefore, it is a reasonable expectation that agents which modulate the lipoxygenase pathway may also be useful in the prevention and treatment of thrombosis, inflammation, and metastasis.

CHAPTER 1:

INTRODUCTION

The endothelium, or the endothelial cells lining the lumen of blood vessels, synthesizes and/or secretes a number of constituents. These constituents influence endothelial cell reactivity and the reactivity of its underlying basement membrane to circulating blood cells. The endothelium also synthesizes and secretes other constituents into the blood which, in turn, influence blood coagulation, platelet function and thrombolysis. Thus, the endothelium contributes to the biocompatibility of the vessel wall with circulating blood components (Anderson, 1987; Gimbrone, 1987; Mustard et al, 1987; Preissner, 1988; Schleef and Loskutoff, 1988; Jaffe, 1989).

The mechanisms by which the endothelial cell constituents achieve these effects will be discussed in relationship to vessel wall structure and to components of both the endothelium and its underlying basement membrane. The structure of the vessel wall will be discussed in relation to the current dogma concerning how the endothelium is thought to provide a continuous biocompatible barrier between blood and the vessel wall. In addition, the concept of cell-cell adhesion as a necessary step not only for thrombosis, but also for inflammation and metastasis will be discussed. This will direct the reader's attention to the importance of a family of cell receptors and ligands which are expressed on both the surface of the endothelium and blood cells. Recently, there

has been a major increase in the number of studies determining which stimuli influence the expression of these receptors/ligands and how they facilitate cell-cell adhesion in thrombosis, inflammation and metastasis. However, little focus has been given to the identity of the intracellular regulators which mediate the expression of these receptors/ligands. The major focuses of this thesis are identifying one possible regulator of these receptors/ligands, and the possible alternative avenues for the treatment and prevention of thrombosis, inflammation and metastasis.

VESSEL WALL STRUCTURE

The vessel wall is composed of 3 distinct layers separated by 2 elastic laminae: the tunica intima; the tunica media; and the tunica adventitia (Figure 1). The thickness and basic structure of the 3 layers varies according to the location and type of blood vessel. For example, the outer layer of large vessels, such as the aorta (i.e. >29 smooth muscle cell layers in the media), are perfused by small arterioles, or vasa vasorum (Wheater et al, 1987). The vasa vasorum are absent in smaller arteries and veins, which receive their metabolic needs directly by diffusion from the blood. In general, most arteries have a thicker tunica media, rich in smooth muscle cells with little extracellular matrix

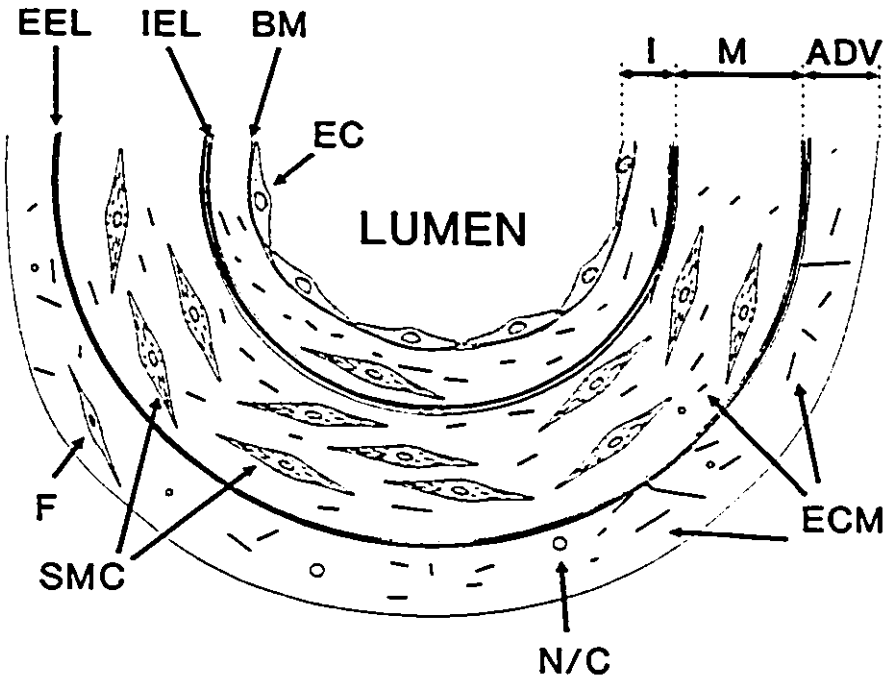


FIGURE 1. Cross-section of the vessel wall. Abbreviations used are: ADV, Adventitia; BM, Basement membrane; EC, Endothelial cell; ECM, Extracellular matrix; EEL, External elastic lamina; F, Fibroblast; I, Intima; IEL, Internal elastic lamina; M, Media; N/C, Nerve or Capillary.

(ECM), while veins have a thin media containing fewer smooth muscle cell layers and a large amounts of ECM (Stary, 1989). Capillaries on the other hand, lack both the tunica media and tunica adventitia, consisting only of the tunica intima.

Tunica Intima:

The innermost cellular layer, or tunica intima, is composed of an endothelium, an underlying basement membrane and an ECM.

The endothelium is a single layer of endothelial cells which lines the lumen of all blood vessels. Endothelial cells are relatively flat, approximately 20 x 50 μm^2, covering a total surface area of more than 1000 m^2 (Jaffe, 1989). They synthesize and/or secrete a variety of basement membrane components and plasma proteins. They also express a variety of cell-cell and cell-matrix receptors, the significance and function of which are discussed in more detail later.

The intimal ECM is derived from both endothelial cells and the occasional smooth muscle cell which has migrated from the tunica media (Stary, 1989). The presence of smooth muscle cells in the intima is characteristic of age and/or previous injury. Unchecked invasion of smooth muscle cells into the intima leads to vessel wall thickening, lumenal narrowing and subsequent atherosclerotic plaque formation (Stary, 1989; Clarkson et al, 1987). Hence, the characteristics of the ECM are always changing with age and disease.

The ECM is predominantly amorphous, diffuse and contains endothelial cell-derived elastase, collagens, proteoglycans, vitronectin and fibronectin. The area of the ECM located immediately below the endothelial cell is classified as the endothelial cell-derived basement membrane. This area of the ECM is more diffuse and is thought to have properties specific to the transport of plasma proteins and the regulation of blood cell-vessel wall interactions. The basement membrane is also the first ECM produced during embryology since it is required for morphogenesis (Leivo, 1983). Generally, basement membranes are thought to provide physical stability to maintain tissue structure and integrity.

Various components of the basement membrane and ECM influence cell adhesion, migration, extravasation, growth and differentiation. These components are members of a family of matrix molecules including collagen, fibronectin, vitronectin, laminin, von Willebrand factor and thrombospondin.

There are at least six different collagens present in the basement membrane, namely types I, III, IV, V, VI and VIII (Sage and Bornstein, 1982; Mayne, 1986). In vitro studies have demonstrated that endothelial cells are able to produce all six types (Mayne, 1986). The relative amounts and types of collagens synthesized by and deposited into the matrix by cultured endothelial cells depends on the source of the cells, the passage number, the substrate on which the cells are grown and the nutrients in the culture medium (Madri et al, 1980).

Whether all 6 types of collagens are synthesized by the vessel wall endothelial cells in situ, is not yet clear.

The use of specific antibodies directed against the various collagen types has allowed for the extracellular localization of the collagens in the vessel walls. Most of collagen types I and III in the intima appear to be located in the deeper part of this layer, i.e. adjacent to the internal elastic lamina and any smooth muscle cell that has migrated into the intima. Collagens types I and III are co-localized, and are present as fibres of approximately 400 Å in diameter, intermingled with branching elastin (Bierring and Kobayasi, 1963; Mayne, 1986). In contrast, collagen type IV is concentrated in the basement membrane in a region adjacent to the endothelial cells (Madri et al, 1980; Mayne, 1986). Small amounts of collagen type IV are also found in the basal lamina surrounding smooth muscle cells (Liau and Chan, 1989). Collagen type IV does not form fibres like types I and III, but forms a network which is thought to be the major structural scaffolding of the basement membrane (Mayne, 1986).

Types I, III and IV constitute the bulk of the collagens in the ECM, but other collagens such as V and VI have been described (Mayne, 1986). It is unclear whether these collagens are synthesized by smooth muscle cells or by endothelial cells (Sage and Bornstein, 1982; Madri et al, 1980; Mayne, 1986). Collagen type V appears to form part of the type I and III fibres, whereas collagen type VI appears to

form a secondary fibrillar network (Mayne, 1986).

The difference in location of these collagen structures has important consequences on blood cell-vessel wall interactions following vessel wall injury. The non-fibrillar collagens (types IV and V), which are closer to the lumenal surface, do not induce platelet aggregation (Barnes and MacIntyre, 1979). In contrast, the fibrillar forms of collagen (types I and III), which are found deeper in the vessel wall, do induce platelet aggregation (Barnes and MacIntyre, 1979). This suggests that the collagen type plays an important role in thrombosis formation. In support of this hypothesis, there is only minimal platelet activation at the site of a superficial injury (when collagen type IV is only exposed), whereas there is marked platelet activation and thrombus formation at the site of a deeper injury (when types I and III are exposed).

The difference in platelet activation in response to the different collagen types has been attributed to differences in the fibrillar form of a given collagen rather than to the type of collagen. For example, when collagen type IV (which does not induce platelet aggregation) is polymerized into fibres, it also induces platelet aggregation, whereas when collagen type III (which induces platelet aggregation) is monomerized, it does not induce platelet aggregation (Barnes and MacIntyre, 1979). Thus, the tertiary structure of collagen influences its ability to induce platelet aggrega-

tion. The importance of this point will be elaborated on in the Discussion under 13-HODE AND BASEMENT MEMBRANE THROMBOGENECITY.

Fibronectin is another adhesive protein which is present in basement membranes, plasma, endothelial cells, fibroblasts, and the α-granules of platelets (Sage and Bornstein, 1982; Reinders et al, 1985; Ruoslahti, 1988). Fibronectin is concentrated in the reticular zone of the basement membrane, and in the lamina densa (Madri et al, 1980; Laurie et al, 1983). Endothelial cells are probably the major source of basement membrane fibronectin as they are capable of secreting fibronectin from both their lumenal and ablumenal membranes (Reinders et al, 1985).

Fibronectin binds to collagen IV, laminin, heparan sulphate proteoglycan, thrombospondin, plasminogen, and other receptors on the surface of endothelial cells, as well as receptors on platelets (Cheresh, 1987; Ruoslahti, 1988; Albelda et al, 1989; Dejana et al, 1989). This suggests that fibronectin is an important adhesive protein of the vessel wall. Consistent with this possibility, matrix-bound fibronectin facilitates hyperplasia by inducing smooth muscle cells to switch from their normal contractile non-proliferative phenotype to their synthetic proliferative phenotype (Rosen et al, 1986; Hedin et al, 1988).

Another important adhesive protein associated with the vessel wall and also present in plasma is vitronectin. Vitro-

nectin is a 70 kdalton polypeptide composed of 459 amino acid residues (Suzuki et al, 1985). It is identical with serum spreading factor (S-protein) found in plasma (Jenne and Stanley, 1985). The plasma concentration of vitronectin is 200-400 μg/ml (Collins et al, 1987; Ruoslahti et al, 1987). Whether vitronectin present in the vessel wall is synthesized by the vessel wall cells or is synthesized elsewhere and absorbed from plasma, is not known.

Vitronectin has a cell attachment site, a glycosaminoglycan binding site, and a thrombin-antithrombin III complex binding site (Suzuki et al, 1985; Collins et al, 1987; Ruoslahti et al, 1987). The cell attachment site for vitronectin is an arg-gly-asp (RGD) recognition sequence, similar to that found in other adhesive proteins such as fibronectin (Collins et al, 1987; Ruoslahti, 1988). Surface-bound vitronectin acts as an attachment site to which platelets adhere (Collins et al, 1987). Vitronectin also acts as an important adhesion ligand for endothelial cells, anchoring them to their basement membrane (Cheresh, 1987; Dejana et al, 1989). Thus, vitronectin functions as an important adhesion moiety influencing both vessel wall integrity, and the attachment of platelets to the injured vessel wall.

Laminin is another adhesive protein which is secreted by endothelial cells into the basement membrane (Martin and Timpl, 1987). Laminin is a large (900,000 daltons) multidomain glycoprotein which was first isolated from the basement

membrane of tumor cells (Timpl et al, 1979). Laminin is present in the basement membrane, associated with collagen type IV, heparan sulfate proteoglycans, and nidogen-entractin (Martin and Timpl, 1987). Laminin is thought to act as both a receptor and a ligand for the attachment and subsequent migration, growth and differentiation of a variety of cells, including endothelial cells (Graf et al, 1987; Martin and Timpl, 1987; Albelda et al, 1989; Panayotou et al, 1989).

Von Willebrand factor (vWF) is a large multimeric protein found in platelets, endothelial cells, plasma and the basement membrane. vWF is synthesized exclusively by megakaryocytes, the precursors of platelets, and by endothelial cells (Reinders et al, 1985; Ruggeri and Zimmerman, 1987). vWF is localized in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells (Wagner et al, 1982; Wencel-Drake et al, 1985; Reinders et al, 1985). The majority of both plasma and basement membrane-associated vWF is derived from endothelial cells (Wagner et al, 1982; Reinders et al, 1985; Reinders et al, 1988).

vWF is thought to be an important cofactor for platelet adhesion to the injured vessel wall, particularly in areas of high shear (Meyer et al, 1987; Ruggeri and Zimmerman, 1987; Sixma et al, 1987). The extent to which vWF facilitates platelet-vessel wall interactions depends, in part, upon the release of vWF from the platelet α-granules and upon the abundance of vWF in the basement membrane. It should also be

noted that the interaction of vWF with receptors on the endothelial cell may be dependent upon with which fatty acids the receptor is interacting (Conforti et al, 1990).

Thrombospondin is a 420,000 dalton glycoprotein of 3 polypeptide chains containing 4 multi-functional domains (Lawler et al, 1985). Thrombospondin is synthesized by endothelial cells, fibroblasts and smooth muscle cells (Hunter et al, 1984; Reinders et al, 1985; Silverstein et al, 1986). Thrombospondin is also found in platelet alpha granules (Wencel-Drake et al, 1985). Thrombospondin promotes platelet aggregation, and modulates focal adhesion of endothelial cells to its basement membrane (Silverstein et al, 1986; Tuszynski et al, 1988; Murphy-Ullrich and Hook, 1989). Thrombospondin also binds to blood and basement membrane proteins such as fibrinogen, fibronectin, plasminogen, thrombin, and collagen type IV (Silverstein et al, 1986). The presence of thrombospondin in the basement membrane therefore, has the potential of contributing to ECM thrombogenecity.

Thus, there are an abundance of glycoproteins in the basement membrane of the vessel wall which contribute to platelet-vessel wall adhesion. The extent to which platelets interact with the vessel wall appears to depend not only on the presence of these adhesion proteins, but also upon their tertiary structure. It will become evident that the tertiary structure of these glycoproteins can be modulated by monohydroxy fatty acids, thereby altering their adhesivity.

Tunica Media:

The mid layer of the vessel wall, or tunica media, is located between the internal and external elastic laminae. It is composed primarily of smooth muscle cells and their ECM. The major collagen types of the medial ECM are types I and III, suggesting that smooth muscle cells are their major source (Mayne, 1986). These 2 collagens account for approximately 80% of the total collagen present in the vessel wall. Collagens types I and III are co-localized and are present as fibres interlaced with branching elastin (Bierring and Kobayasi, 1963; Mayne, 1986). This fibrous network provides the vessel wall with both strength and flexibility. As mentioned previously, these fibres induce marked platelet aggregation and may contribute to the massive activation of platelets when they are exposed following vessel wall injury (Barnes and MacIntyre, 1979).

Tunica Adventitia:

The outermost layer, or tunica adventitia, is composed of fibroblasts and connective tissue. This layer is interspersed with various nerves and capillaries (Matthews and Gardner, 1966; Wheater et al, 1987). The connective tissue, which is composed of various glycosaminoglycans and proteins, anchors the vessel wall to its surrounding tissues. The primary proteins are collagen types I and III, synthesized by the fibroblasts (Madri et al, 1980; Mayne, 1986).

In summary, the blood vessel wall is composed of 3 major cell types: endothelial cells, smooth muscle cells and fibroblasts. These cells synthesize and/or secrete a number of proteins which function as an extracellular fibrillar/fibre support system to maintain vessel wall calibre, structure, and strength. The ECM proteins also facilitate cell adhesion. Thus, the type of protein exposed and the extent of injury, determines the extent to which blood cells interact with the vessel wall (i.e. platelet-vessel wall interactions during thrombosis, leukocyte-vessel wall interactions during inflammation, and tumor cell-vessel wall interactions during metastasis).

The endogenous mechanisms which modulate the abilities of these receptors to recognize their ligands and thereby influence cell adhesion, have not been well elucidated. It is interesting to note, that the reactivity of some of these proteins is mediated, in part, by their tertiary structure, such as collagen types III and IV. This suggests that altering the tertiary structure of these proteins may be a useful strategy to modulate cell-vessel wall responses following vessel wall injury.

REGULATION OF THROMBOSIS BY ENDOTHELIAL CELLS

The endothelium plays a major role in haemostasis, thrombosis and fibrinolysis by influencing coagulation, fibrinolysis and cell adhesion (Gimbrone, 1987; Preissner, 1988; van Hinsbergh, 1988; Schleef and Loskutoff, 1988).

Coagulation:

Procoagulant Components: As previously discussed, the endothelium synthesizes and secretes fibronectin, thrombospondin and vWF. These proteins are also secreted by platelets. vWF forms the major component of factor VIII necessary for the amplification of the coagulation cascade. VWF, fibronectin and thrombospondin facilitate platelet adhesion and aggregation. The activated platelets in turn, assemble the prothrombinase complex on their surface, thereby facilitating prothrombin conversion into thrombin.

Endothelial cells also express tissue factor on their apical surface following stimulation (Schorer and Moldow, 1988). Tissue factor, (also known as thromboplastin, tissue thromboplastin, or coagulation factor III) is a transmembrane protein of approximately 29,000 daltons. It functions as a receptor for factor VII, accelerating its conversion into factor VIIa. The tissue factor-factor VIIa complex, in turn, activates factor IX and X. Thus, by expressing tissue factor

on its surface, the endothelium contributes to the activation of both the intrinsic and the extrinsic pathways of thrombin formation (Jackson and Nemerson, 1980).

The stimuli which induce the expression of tissue factor on endothelial cells include thrombin, interleukin 1 (IL1), tumor necrosis factor (TNF) and endotoxin (Bevilacqua et al, 1985; Schorer and Moldow, 1988). Interestingly, IL1 also stimulates further IL1 synthesis by endothelial cells (Bevilacqua et al, 1985). In addition, TNF stimulates IL1 release. Thus, during the course of thrombosis, inflammation and metastasis, there is a common response of enhanced tissue factor expression on the surface of the endothelium which leads to enhanced coagulation. If this response remains unchecked, thrombosis may occur. It is not surprising therefore, that the endothelium has an additional role in down-regulating coagulation.

Anticoagulant Components: The endothelium synthesizes and/or secretes a number of constituents which catalyze the inactivation of thrombin. These constituents include protein S, thrombomodulin and various glycosaminoglycans (Preissner, 1988; Stern et al, 1988).

When the endothelium is stimulated and coagulation is activated, thrombin binds to the endothelium. Approximately 50% of the thrombin binding sites on the endothelium are thrombomodulin. When thrombin is bound to thrombomodulin,

thrombin undergoes a conformational change which allows it to markedly accelerate the activation of protein C, instead of activating prothrombin (Thompson and Salem, 1987; Preissner, 1988). Activated protein C, in the presence of protein S, inactivates factors Va and VIIIa, thereby decreasing the coagulation. Protein S is also synthesized by the endothelium (Preissner, 1988; Stern et al, 1988).

The endothelium also expresses a variety of glycosaminoglycans on its apical surface, some of which catalyze thrombin inhibition. Heparin catalyzes the rate of thrombin inactivation by ATIII by a 1000 fold (Barrowcliffe and Thomas, 1987; Wight, 1989). ATIII is a circulating glycoprotein (MW 58,000 daltons), which is synthesized by the liver and has a circulating half-life of approximately 3 days. ATIII not only inhibits thrombin, but also inhibits other proteases in the intrinsic coagulation pathway, including factor Xa, kallikrein, plasmin and complement C1s. The rates of their inactivation by ATIII are also catalyzed by heparan sulfate (Rosenberg and Rosenberg, 1984; Barrowcliffe and Thomas, 1987; Bikfalvi and Beress, 1987; Wight, 1989).

Dermatan sulfate is another glycosaminoglycan which is synthesized by endothelial cells, as well as by smooth muscle cells (Wight, 1989). Dermatan sulfate comprises greater than 50% of the glycosaminoglycans present in the ECM of large arteries (Wight, 1989). Dermatan sulfate catalyzes the inhibition of thrombin by heparan cofactor II (HCII) (Tollefsen et

al, 1982; Tollefsen et al, 1983; Bikfalvi and Beress, 1987). HCII is a glycoprotein (MW 65,600 daltons) which exists in plasma at a concentration of 1-1.3 μM and has a similar half-life as ATIII (Preissner, 1988). Recent studies indicate that dermatan sulfate inhibits thrombin more effectively than other glycosaminoglycans when the thrombin is bound to an injured vessel wall or to a thrombus (Okwusidi et al, 1990). These observations suggest that dermatan sulfate plays a more important role in catalyzing the inactivation of any thrombin which binds to the vessel wall surface following injury.

Thus, a number of checks and balances exist to regulate coagulation, all of which are synthesized by the endothelium.

Fibrinolysis:

When thrombin is formed, it not only facilitates thrombus formation as described above, but it also stimulates the endothelium to synthesize and/or release constituents which lyze thrombi, specifically the 2 plasminogen activators (PAs), urokinase-type PA (u-PA) and tissue-type PA (t-PA) (van Hinsbergh, 1988). Endothelial cells are the primary source of t-PA, while renal cells are the primary source of u-PA. Both t-PA and u-PA cleave plasminogen to plasmin which, in turn, binds to and digests fibrin in the formed thrombus, thereby lyzing the thrombus.

To counterbalance this profibrinolytic activity of the

endothelium, endothelial cells also synthesize PA inhibitors (PAIs). These are released 4-6 hours after PA release. One and probably the major PAI is PAI-type 1, or PAI-1 (Schleef and Loskutoff, 1988). PAI-1 synthesis and release follows the same course as PA (Schleef and Loskutoff, 1988). Active PAI-1 is also present in the ECM, suggesting that PAI-1 is a primary local regulator of fibrinolysis following vessel wall injury (Schleef et al, 1990).

In summary, the endothelium actively participates in thrombosis and its resolution by synthesizing and releasing a number of constituents which either inhibit or enhance coagulation and fibrinolysis. It has been argued that these constituents contribute to the biocompatibility of the healthy intact vessel wall with blood. However, it should be noted that the synthesis and release of these constituents require endothelial cell stimulation and/or injury. This suggests that other factors present in the resting endothelium play a more important role in regulating the biocompatibility of the endothelium surface under resting conditions. The focus of the rest of this thesis will be aimed at better understanding the mechanism which influences the interaction of circulating blood components with specific cell adhesion receptors.

ROLE OF RECEPTORS IN CELL-CELL ADHESION

Glycoproteins present on platelets, endothelial cells and the basement membrane, such as fibronectin, fibrinogen, and vWF, play a major role in mediating cell-cell adhesion. It is becoming increasingly clear that adhesive glycoproteins are all ligands for a variety of receptors derived from a super family of glycoproteins called the integrins (Tamkun et al, 1986; Buck, 1987; Hynes, 1987). The integrins share a similar characteristic, namely they recognize and bind to their ligands through a RGD tripeptide (Tamkun et al, 1986; Buck, 1987; Hynes, 1987). This peptide is the recognition site for various ligands of the fibronectin receptor, vitronectin receptor (VnR), glycoprotein IIb/IIIa (GPIIb/IIIa), $\alpha_v\beta_1$, $\alpha_v\beta_5$, macrophage antigen-1 (Mac-1) and lymphocyte function-associated antigen-1 (LFA-1) (Hynes, 1987; Ginsberg et al, 1988; Ruoslahti, 1988).

The receptors of the integrin family are composed of 2 glycoproteins subunits, the α and β chains, which exist as non-covalent 1:1 complexes (Table 1). The majority of these receptors share a common β subunit (the smaller disulfide-rich subunit) but have a distinct α subunits. The function and identity of these receptors in platelets, leukocytes and tumor cells, as they relate to blood cell-vessel wall adhesion will be discussed.

Platelet Adhesion: Platelet adhesion to the damaged vessel wall is an initial event in haemostasis. Adherent platelets provide a cohesive surface for the build-up of the haemostatic plug. The receptors which are thought to be necessary for the initial platelet-vessel wall adhesive event are GPIa, GPIb/GPIX, GPIIb/IIIa and the VnR (Berndt and Caen, 1984; Lam et al, 1989).

Platelet GPIa is necessary for platelet adhesion to collagens which are exposed following vessel wall injury (Coller, 1987; Sakariassen et al, 1987). GPIb is also required for vWF-dependent platelet adhesion to the ECM (Coller, 1987; Sakariassen et al, 1987). Recently, GPIb has been co-localized with GPIX on the surface of platelets forming a 1:1 heterodimer complex (Berndt et al, 1985).

GPIIb/IIIa is also necessary for vWF-dependent platelet adhesion to the vessel wall, particularly at high shear rates (Berndt and Caen, 1984; Girma et al, 1986; Sakariassen et al, 1987). This receptor complex facilitates platelet spreading on the subendothelium through either fibrinogen or vitronectin, and mediates thrombin-induced platelet-platelet adhesion (Berndt and Caen, 1984; Weiss et al, 1986; Asch and Podack, 1990). The RGD binding domain has been localized to amino acids 109-170 of GPIIIa (D'Souza et al, 1988). In addition, it has been demonstrated that calcium is required for the stability of the GPIIb/IIIa receptor complex (Jennings and Phillips, 1982).

TABLE 1. Members of the Integrin Family.

Receptor	Subunit Composition	Ligands	Function
Chicken integrin complex	$\alpha_0\beta_1$ $\alpha_3\beta_1$	collagen, FN, LM, VN	Cell adhesion and migration Cytoskeletal connections
VLA-1	$\alpha_1\beta_1$	-	-
Collagen receptor	$\alpha_2\beta_1$	collagen	-
VLA-4	$\alpha_4\beta_1$	-	-
FN receptor	$\alpha_5\beta_1$	FN	Adhesion to FN
LM receptor	$\alpha_6\beta_1$	LM	-
	$\alpha_v\beta_1$	FN	-
LFA-1	$\alpha_L\beta_2$	ICAM-1	Leukocyte adhesion and immune function
Mac-1	$\alpha_M\beta_2$	C3bi, FB, Factor X	C3b receptor; monocyte and PMN adhesion
GPIIb/IIIa	$\alpha_{IIb}\beta_3$	FB, FN, VN, vWF	Platelet adhesion and aggregation
VN receptor	$\alpha_v\beta_3$	VN, FB, vWF	Adhesion to ECM

Abbreviations used are: FB, fibrinogen; FN, fibronectin; ICAM-1, intracellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; LM, laminin; Mac-1, Macrophage antigen-1; PMN, polymorphonuclear leukocyte; VN, vitronectin; vWF, von Willebrand Factor.

Recent studies have demonstrated that GPIIb/IIIa-like receptors also exist on other cell types, and of particular relevance to this thesis, on endothelial cells (Leeksma et al, 1986; Newman et al, 1986). The endothelial cell GPIIb/IIIa-like receptor has a β subunit (GPIIIa) identical to the β subunit of platelet GPIIb/IIIa (Fitzgerald et al, 1987a; Ginsberg et al, 1987), but has a novel α subunit (α_v) (Ginsberg et al, 1987; Suzuki et al, 1987). Unlike the platelet GPIIb/IIIa, the endothelial cell GPIIb/IIIa-like receptor preferentially binds vitronectin, and to lesser extent to vWF and fibrinogen (Pytela et al, 1985; Pytela et al, 1986; Conforti et al, 1990). Therefore, the endothelial cell receptor has been named the VnR (Pytela et al, 1985). The RGD binding domain of the VnR has been localized to amino acids 61-203 of the β subunit (Smith and Cheresh, 1988).

Recently, a VnR-like receptor also has been identified on platelets (Lam et al, 1989). The platelet VnR-like receptor is related to but distinct from both platelet GPIIb/IIIa and endothelial cell VnR, as it contains a distinct β subunit. The role of this receptor in platelet-vessel wall interactions has not as yet been determined.

Leukocyte Adhesion: Leukocyte adhesion to the endothelium is an initiating event in inflammation. Endothelial cells express a variety of receptors and/or integrins that facilitate leukocyte adhesion. Endothelial cell leukocyte

adhesion molecule-1 (ELAM-1), is expressed following endothelial cell stimulation with certain inflammatory cytokines such as IL1, TNF and endotoxin (Bevilacqua et al, 1987). ELAM-1 is not a member of the integrin family.

Endothelial cells also express intracellular adhesion molecule-1 (ICAM-1, Dustin et al, 1986). ICAM-1 facilitates the adhesion of PMNs, lymphocytes, and possibly monocytes to the injured endothelium (Dustin et al, 1986; Springer et al, 1987; Jurgensen et al, 1990). ICAM-1 is detectable on resting endothelial cells, but its expression is markedly and rapidly increased by IL1 or TNF, and only increased slowly by gamma-interferon (γ-IFN) stimulation (Pober et al, 1986a; Jurgensen et al, 1990). Some studies suggest that ICAM-1 is the ligand for lymphocyte function-associated antigen 1 (LFA-1), an integrin expressed on a variety of leukocytes (Springer et al, 1987). If so, the LFA-1/ICAM-1 receptor/counter-receptor complex may be the complex by which most leukocytes bind to the endothelium.

Another leukocyte receptor related to LFA-1 is Mac-1 (CD11b/CD18). Mac-1 mediates non-specific adhesion of granulocytes and monocytes to endothelial cells and functions as the complement receptor type 3 (Law, 1988). However, Mac-1 alone is neither sufficient nor necessary for the enhanced adhesion of PMNs to the endothelium (Vedder and Harlan, 1988).

Leukocytes also have receptors for various components of the basement membrane. Human PMNs and monocytes contain

the laminin receptor, the fibronectin receptor and the VnR (Singer et al, 1989). All these receptors are co-localized with Mac-1 within specific vesicles named adhesomes. Activation of the leukocytes with fMLP or TNF induces fusion of the granules to the plasma membrane, facilitating in the expression of these receptors on the leukocyte surface. As a consequence, PMN adhesion to subendothelium is increased. More recently, a novel VnR-like receptor has been identified in monocytes (Krissansen et al, 1990). Thus, most circulating leukocytes and vascular endothelial cells express receptors and/or ligands which facilitate leukocyte-endothelial cell and leukocyte-basement membrane adhesion. The expression of these receptors is up-regulated in response to various inflammatory/ immune cytokines. The enhanced expression of these adhesion receptors is thought to facilitate the extravasation of leukocytes across the endothelium and subendothelium into the inflammatory tissues. If so, a better understanding of the mechanism(s) by which the expression of these receptors is regulated may provide a better understanding of the initiating events in inflammation and wound healing. However, it should be emphasized that these regulators of receptor expression have not been identified.

Tumor Cell Adhesion: The intravascular transport of tumor cells is an initial event in the development of secondary metastasis. Tumor cells must first escape their primary

tumor, transverse the vessel wall and enter the circulation. Once in the circulation, tumor cells must evade the blood-borne immune system and adhere to the endothelium and/or exposed basement membrane. Once adhered, they release various degradative enzymes, such as collagenases and heparitinases, which allow for their passage across the basement membrane and into the tissues (Liotta et al, 1986; Terranova et al, 1986; Albini et al, 1987).

While adhesion of the blood-borne malignant tumor cells to endothelial cells and the basement membrane is necessary for secondary metastasis, little is known about the intracellular mechanisms which regulate tumor cell adhesion to the vessel wall, particularly, to intact endothelial cells. Initial studies suggested that circulating tumor cells preferentially adhere to the exposed underlying ECM and not to the intact endothelium (Liotta, 1984; Weiss, 1985). However, more recent studies indicate that tumor cells also adhere to intact endothelial cells. Adherence to endothelial cells appears to be dependant upon the surface characteristics of both the endothelium and the tumor cells (Auerbach et al, 1987; Nicolson, 1988). Adhesion of tumor cells to endothelial cells and their basement membranes is also organ-specific (Lichtner et al, 1989). The latter observation suggests that organ-specific antigens on the endothelium are responsible for this specific tumor cell adhesion. Consistent with this possibility, other investigators found that adhesion receptors

and/or ligands, expressed following stimulation, participate in tumor cell/endothelial cell adhesion (Ruoslahti and Pierschbacher, 1986; Rice et al, 1988; Lauri et al, 1989).

More recently, Mortarini et al (1990) demonstrated that nine different human melanoma cell lines not only express HLA-class II antigens, but also express ICAM-1 and LFA-3. Furthermore, HLA-class II antigen, ICAM-1 and LFA-3 expression were enhanced by γ-IFN or TNF exposure. Cheresh and Spiro (1987) have shown that human melanoma cells also express the VnR, which enables the melanoma cells to adhere to basement membrane components such as vitronectin and vWF. Suzuki et al (1987) have reported that the VnR and GPIIb are also expressed in 2 leukaemic cell lines. Their expression is enhanced following phorbol ester stimulation, which results in enhanced tumor cell adhesion. Oesteosacroma cells also express the VnR (Pytela et al, 1985), and carcinoma cells express a novel VnR-like receptor ($\alpha_v\beta_x$) which facilitates their adhesion to vitronectin and fibronectin (Cheresh et al, 1989). Finally, Grossi et al (1988) reported that human cervical and colon carcinoma cell lines express platelet GPIb-like and GPIIb/IIIa-like complexes. Both glycoprotein complexes were found to be essential for the tumor cell-induced platelet aggregation, but only the GPIIb/IIIa-like complex was needed for tumor cell adherence to fibronectin. Thus, expression of integrin-like receptors on tumor cells is also important for tumor cell adhesion to the vessel wall.

REGULATION OF CELL ADHESION RECEPTOR EXPRESSION

The observations described above identify a commonality for all cell types in the circulatory system. Namely, that the expression of adhesion receptors related to the integrin family, is necessary for platelet, leukocyte and tumor cell adhesion to both the endothelium and its underlying basement membrane. Although many of these receptors have been identified, little is known about the identity of the intracellular mediators which regulate their expression. In the past, considerable attention has been focused on the role of fatty acid metabolites in regulating not only platelet-vessel wall interactions, but also in regulating leukocyte- and tumor cell-vessel wall interactions, and platelet-tumor cell interactions. The majority of these studies suggest that metabolites derived from arachidonic acid via either the cyclo-oxygenase or the lipoxygenase pathways, play a key role in these interactions.

Cyclo-oxygenase Pathway: In the 1970's, several investigators demonstrated that when platelets interact with various agents, such as subendothelium collagen, they release a variety of short-lived fatty acid metabolites which induce platelet aggregation (Marcus, 1978; Weeds, 1986). These short-lived substances were identified as the prostaglandin (PG) endoperoxides, PGG_2 and PGH_2, and their metabolite, thromboxane

A_2 (TXA_2) (Hamberg et al, 1974). TXA_2 has a half-life of approximately 30 seconds in plasma, being rapidly hydrolyzed into the biologically inactive metabolite, TXB_2 (Marcus, 1978). PGG_2 and PGH_2 have similar half lives.

All of these prostanoids are cyclo-oxygenase derivatives of arachidonic acid. Arachidonic acid is stored in the membrane phospholipids in resting cells. It is released from the phospholipids by several membrane phospholipases, primarily phospholipase A_2 and phospholipase C, following cell activation (Irvine, 1982). Once released, free arachidonic acid is rapidly oxygenated by cyclo-oxygenase. Cyclo-oxygenase is the rate-limiting, bifunctional, membrane-bound enzyme in the cyclo-oxygenase pathway (Ohki et al, 1979; Pagels et al, 1983). It is localized to the endoplasmic reticulum, the nuclear envelope and the plasma membrane (Smith, 1986; Dewitt and Smith, 1988; Merlie et al, 1988). Cyclo-oxygenase catalyzes both the bisoxygenation of arachidonic acid to PGG_2, and the peroxidative reduction of PGG_2 in PGG_2. The bisoxygenation activity of cyclo-oxygenase is inhibited by aspirin which acetylates cyclo-oxygenase at a single serine residue, serine 507 (Merlie et al, 1988; Yokoyama et al, 1988).

The oxygenation of arachidonic acid by cyclo-oxygenase is as follows. Free arachidonic acid is oxygenated by the bisoxygenase activity of cyclo-oxygenase at carbon positions C_{11} and C_{15}. The Δ^{12} double bond of arachidonic acid then

isomerizes, forming a new bond between C_8 and C_{12}, and in doing so, forms an oxygen radical at C_{11}. The oxygen radical then attacks C_9, forming an cyclic-peroxide between C_9 and C_{11}, resulting in the formation of PGG_2 (Figure 2). PGG_2 is reduced by the peroxidative reductase activity of cyclo-oxygenase into PGH_2 (Figure 2). PGH_2 is highly labile and is either degraded into 12-hydroperoxyheptanoic acid, or metabolized enzymatically into a number of biologically active end-products (Marcus, 1978; Moncada and Vane, 1979).

In platelets, PGH_2 is metabolized predominantly to TXA_2 by thromboxane synthetase (Figure 2) (Hamberg et al, 1974). In endothelial cells, PGH_2 is metabolized predominantly to PGI_2 (formerly prostacyclin) by prostacyclin synthetase (Moncada et al, 1976). PGI_2 has potent and biologically effects opposite to those of TXA_2 (Moncada and Vane, 1979). PGI_2 inhibits platelet aggregation and enhances vasodilation. Like TXA_2, PGI_2 is an unstable endoperoxide and rapidly hydrolyzes into biologically inactive 6-keto $PGF_{1\alpha}$ (Marcus, 1978). PGI_2 is thought to exert its effect by binding to specific receptors on the platelets and the vessel wall, and as a consequence, elevating intracellular cAMP levels (Siegl, 1982; Robertson, 1986; Marcus, 1978).

As both TXA_2 and PGI_2 have short half-lives and opposing biological activities, it was proposed that the relative amounts of these prostanoids mediated platelet-vessel wall interactions (Gryglewski et al, 1978; Moncada and Vane,

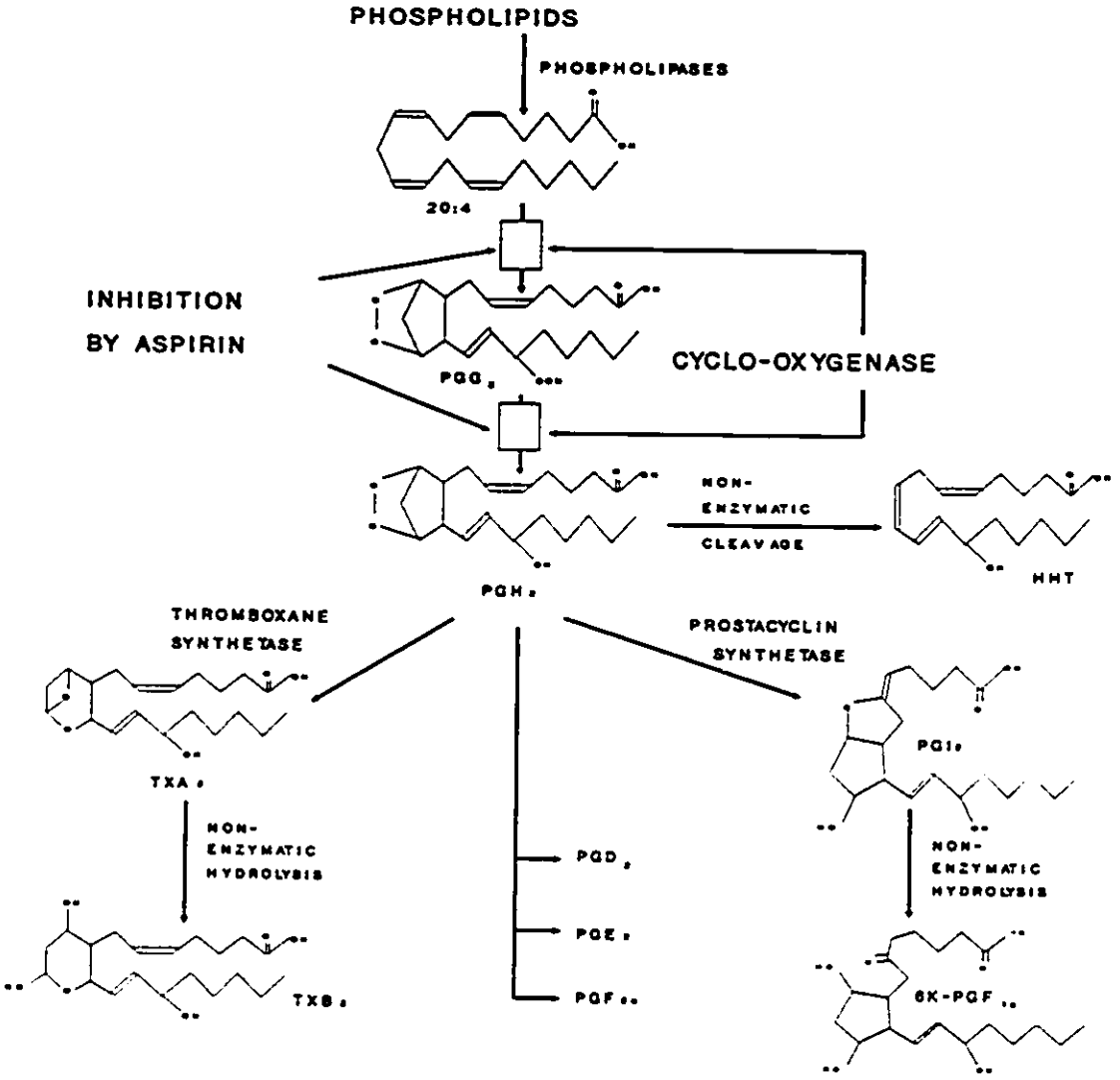


FIGURE 2. The cyclo-oxygenase pathway and its point of inhibition by aspirin. This pathway metabolizes arachidonic acid (20:4) into TXA_2 in platelets, PGI_2 in endothelial cells, and PGD_2, PGE_2 and $PGF_{2\alpha}$ in a variety of cell types.

1979; Bennett et al, 1981). In response to vessel wall injury, platelets become activated, and synthesize and secrete TXA_2. The released TXA_2 would induce vasoconstriction, and facilitate further platelet activation and platelet adhesion to both the endothelium and/or subendothelium, thereby initiating haemostasis. To keep this potential thrombogenic response in check, the injured endothelium would synthesize and secrete PGI_2. These investigators also suggested that healthy, intact endothelial cells synthesized basal levels of PGI_2, which contributed to the biocompatibility or "non-adhesivity" of the healthy vessel wall with the circulating blood cells.

Given that platelet/endothelial cell adhesion is known to be dependent upon the expression of adhesion receptors, one might postulate that the antithrombotic role of PGI_2 is, in part, to down-regulate platelet and/or endothelial cell integrin expression. If this is the case, non-steroidal anti-inflammatory drugs, such as aspirin, indomethacin and ibupfofen, which inhibit cyclo-oxygenase and subsequent PGI_2 synthesis, should enhance platelet and leukocyte adhesion to the subendothelium. This is not the case (Marcus, 1978; Buchanan et al, 1983; Buchanan et al, 1985). Furthermore, other investigators have demonstrated that neither treatment with nor inhibition of PGI_2 had any effect on experimentally-induced metastasis (Hilgard and Thormes, 1976; Karpatkin et al, 1984). If PGI_2 down-regulated integrin expression, one

would also expect that TXA_2 should increase platelet adhesion receptor expression. However, most specific TXA_2 synthetase inhibitors do not inhibit platelet adhesion.

Thus, while most evidence supports the concept that TXA_2 and PGI_2 are biologically important prostanoids in regulating platelet aggregation and vessel wall calibre following vessel wall injury and platelet activation, there is little evidence to support the concept that these prostanoids regulate blood cell and/or vessel wall adhesivity under homeostatic conditions. There is however, some recent evidence which suggests that the relative amounts of lipoxygenase metabolites derived from arachidonic and linoleic acid modulate adhesion receptor expression and consequently, cell-cell adhesion.

Lipoxygenase Pathway: When arachidonic acid is liberated from the phospholipids, it is also metabolized by cytosolic lipoxygenase enzymes into a number of hydroperoxyeicosatetraenoic acids (HPETE) (Spector et al, 1988). These HPETEs are reduced to their corresponding stable hydroxyeicosatetraenoic acids (HETEs). 5-, 12- and 15-HETE constitute the 3 major isomers of HETE (Figure 3). Other less predominant isomers which have been identified include 8-, 9-, 11-, 19- and 20-HETE.

The 3 major HETEs (5-, 12-, and 15-HETE) are synthesized by the action of stereospecific lipoxygenase enzymes which insert a single oxygen molecule into arachidonic acid at

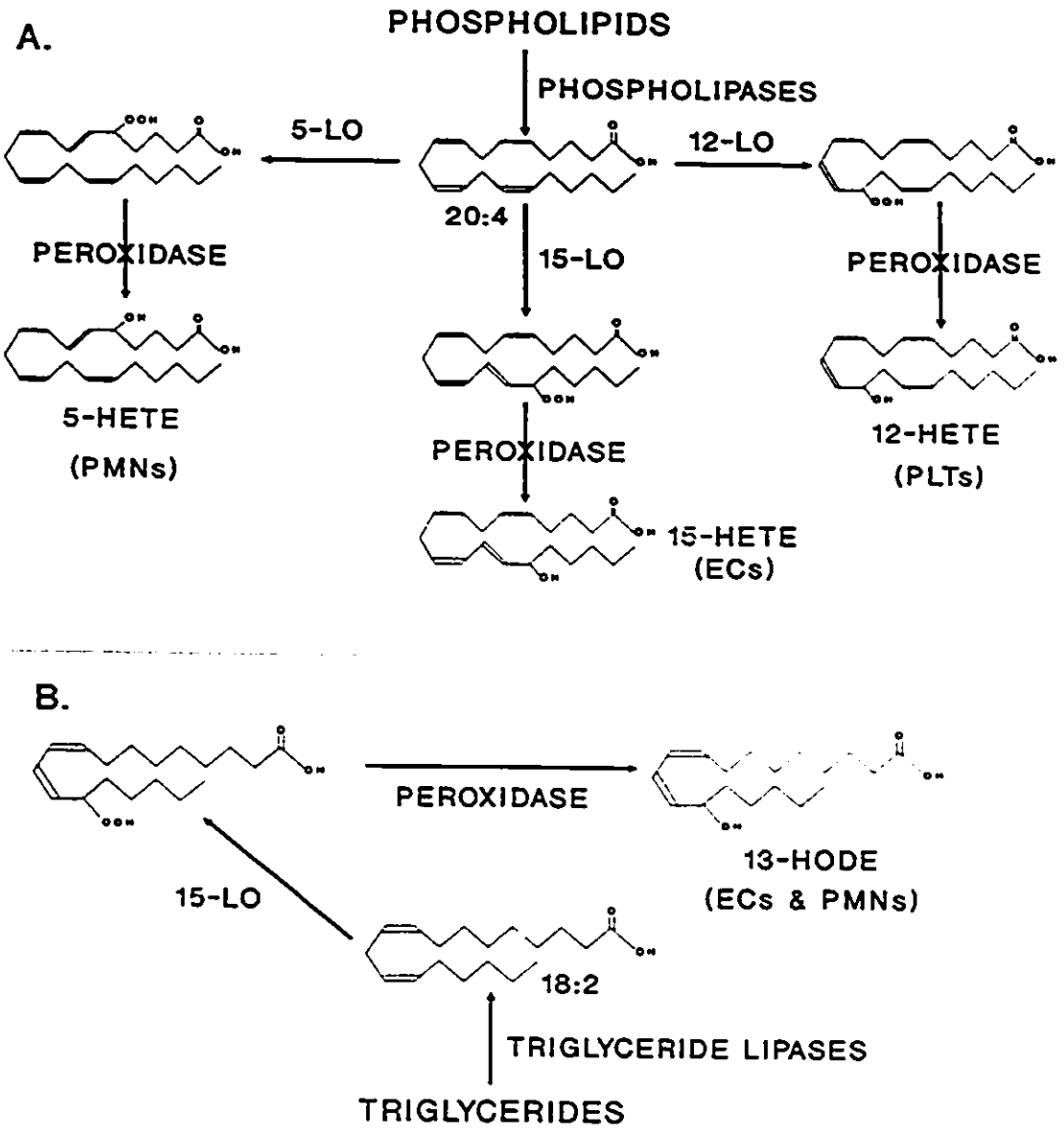


FIGURE 3. Monohydroxy lipoxygenase pathway. Lipoxygenase pathway of (A) stimulated PMNs, platelets (PLTs) and endothelial cells (ECs), derived from arachidonic acid (20:4); or (B) of resting ECs or PMNs derived from linoleic acid (18:2).

carbons C_5, C_{12}, and C_{15}, respectively (Figure 3). Each of these carbon positions is located at position 1 of a 1,4-cis, cis-pentadiene structure, a stereo-specific conformation for all fatty acid substrates of lipoxygenases (Taylor and Morris, 1983). The intermediate by-products formed by the action of lipoxygenase, HPETEs, are then reduced by the action of peroxidases to their corresponding HETEs (Siegel et al, 1979; Spector et al, 1988). The formation and the biological activities of these HETEs in relationship to cell adhesion are discussed below.

5-HETE is synthesized by 5-lipoxygenase (Spector et al, 1988). 5-lipoxygenase is present in a variety of cell types including PMNs, monocytes, macrophages and mast cells (Spector et al, 1988). Recently, the full-length cDNA clone of 5-lipoxygenase has been isolated and its amino acid sequence deduced by nucleotide sequence analysis (Balcarek et al, 1988; Dixon et al, 1988; Matsumoto et al, 1988). Like cyclo-oxygenase, 5-lipoxygenase is a bifunctional enzyme. In addition to catalyzing the oxygenation of arachidonic acid to 5-HPETE, 5-lipoxygenase also catalyzes the subsequent dehydration of 5-HPETE to the labile epoxide, leukotriene (LT) A_4 (Spector et al, 1988). LTA_4 is the substrate for the synthesis of the biologically active LTs, LTB_4, and the peptido-leukotrienes, LTC_4, LTD_4, and LTE_4. These LTs are thought to play a major role in the inflammatory and allergic responses of asthma, rheumatoid arthritis and inflammatory

bowel disease (Spector et al, 1988). Despite the advances made in determining the biological potency of these LTs, little is known about the biological potency of 5-HETE. Data presented in this thesis suggests that 5-HETE up-regulates leukocyte adhesion.

12-HETE is synthesized by 12-lipoxygenase (Hamberg and Samuelsson, 1974). The complete amino acid sequence of human 12-lipoxygenase has also been deduced by nucleotide sequence analysis (Izumi et al, 1990; Yoshimoto et al, 1990). The major cellular source of 12-HETE is the platelet, but macrophages, neutrophils and vascular smooth muscle cells also synthesize 12-HETE (Spector et al, 1988). Studies which exploit the manipulation of endogenous 12-HETE suggest that 12-HETE is necessary for platelet adhesion to thrombogenic substrates and platelet-platelet adhesion (Dutilh et al, 1981; Aharony et al, 1982; Gimeno et al, 1983; Buchanan et al, 1986; Morita and Murota, 1987). 12-HETE is also thought to facilitate macrophage and tumor cell adhesion to the vessel wall (Spector et al, 1988; Honn et al, 1989). Some studies suggest that 12-HETE achieves this effect by inducing endothelial cell retraction, thereby exposing the more adhesive ECM. Other studies suggest that 12-HETE enhances tumor cell adhesion by regulating tumor cell integrin expression. Thus, Grossi et al (1989) demonstrated that tumor cell GPIIb/IIIa receptor dependent adhesion to endothelial cells and their ECM were enhanced in the presence of 12-HETE. However, the

mechanism by which 12-HETE enhances GPIIb/IIIa expression, has not been identified.

Greenwald et al (1979) were the first to provide evidence that the vessel wall was also capable of synthesizing a 12-HETE-like substance. Subsequently, other investigators demonstrated that endothelial cells were capable of synthesizing 15-HETE and not 12-HETE from exogenous arachidonic acid (Hopkins et al, 1984; Gorman et al, 1985), indicating that the lipoxygenase present in endothelial cells was 15-lipoxygenase and not 12-lipoxygenase. Subsequently, PMNs, macrophages, eosinophils, smooth muscle cells and fibroblasts have been identified as other cellular sources of 15-HETE (Spector et al, 1988). A number of studies suggest however, that 15-HETE is not the major endothelial cell-derived monohydroxy lipoxygenase metabolite derived from endogenous fatty acid stores. 15-HETE was only detected in endothelial cells when they were incubated with exogenous arachidonic acid (Hopkins et al, 1984; Gorman et al, 1985; Spector et al, 1988). In contrast, linoleic acid, not arachidonic acid, is continuously metabolized via the lipoxygenase pathway into the monohydroxide, 13-hydroxyoctadecadienoic acid (13-HODE, Figure 3, page 34) in resting endothelial cells (Buchanan et al, 1985a). When the cells are stimulated, 13-HODE synthesis decreased, arachidonic acid was liberated from the phospholipids, and PGI_2 was synthesized. There is however, no intracellular or extracellular 15-HETE detected under either condition.

Finally, we have reported that there is an inverse relationship between the amount of 13-HODE inside endothelial cells and their adhesivity for platelets (Buchanan et al, 1985). It is these latter observations, i.e. the inverse correlation between 13-HODE and platelet/endothelial cell adhesion, which is the basis for embarking on the studies described in this thesis. In particular, to test the hypothesis that 13-HODE down-regulates adhesion receptor expression and that HETEs up-regulate their expression.

DIET AND SUBSEQUENT ARACHIDONIC AND LINOLEIC ACID METABOLISM

The level of free arachidonic acid is low in most mammalian cells in comparison to other fatty acids, including linoleic acid, the substrate for 13-HODE synthesis (Irvine, 1982; Lagarde et al, 1984). The bulk of arachidonic acid found in mammalian cells is esterified, and found almost exclusively in the 2-acyl position of the membrane phospholipids. Similarly, the plasma level of free arachidonic acid is low in comparison to the plasma level of its esterified form. Furthermore, both cytosolic and plasma triglycerides (the other major lipid class besides the phospholipids), are not rich in arachidonic acid (Irvine, 1982). The level of free cytosolic linoleic acid is high in most mammalian cells, and both the phospholipid and triglyceride pools are rich in

linoleic acid (Lagarde et al, 1984). As humans can not synthesize linoleic acid de novo, linoleic acid is considered to be an essential fatty acid (Crawford, 1983; Hansen, 1986; Zoller, 1986; Lefkowith, 1990). The major dietary sources of linoleic acid are seed oils such as corn oil. Linoleic acid is the fatty acid from which arachidonic acid is derived. Arachidonic acid is synthesized from linoleic acid by the actions of desaturases and elongases (Crawford, 1983). First, linoleic acid ($18:2^{n-6}$) is desaturated to γ-linolenic acid ($18:3^{n-6}$) by delta-6-desaturase. Gamma-linolenic acid is then elongated to dihomo-γ-linolenic acid ($20:3^{n-6}$) by elongase, which in turn, is desaturated to arachidonic acid ($20:4^{n-6}$) by delta-5-desaturase. This series of fatty acids is referred to as the n-6 family, n-6 referring to the location of the first double bond closest to the methyl terminus of each fatty acid.

Another essential fatty acid derived from plants, α-linoleic acid ($18:3^{n-3}$), undergoes a similar desaturation-elongation process as linoleic acid, which leads to the formation of eicosapentaenoic acid (EPA, $20:5^{n-3}$) of the n-3 family. EPA is found in high levels in fish oil and is thought to be important in the prevention of atherosclerosis, vessel wall hyperplasia, inflammation and metastasis (Landymore et al, 1985; Prescott et al, 1985; Kondo et al, 1986; Reich et al, 1989). Both arachidonic acid and EPA can be further elongated and desaturated to other less abundant fatty acids. EPA is thought to exert its biological effect by

inhibiting both TXA_2 and 12-HETE synthesis by i) displacement of arachidonic acid from the membrane phospholipids, which reduces the availability of free arachidonic acid following stimulation; and ii) by competing with free arachidonic acid for both cyclo-oxygenase and lipoxygenase (Crawford, 1983; Powell and Funk, 1987).

OVERALL HYPOTHESIS

The relative amounts of the linoleic acid-derived lipoxygenase metabolite, 13-HODE, and the arachidonic acid-derived HETEs, modulate the expression of adhesion receptors on cell surfaces, thereby influencing cell-cell adhesion.

SPECIFIC OBJECTIVES

1. To develop an automated HPLC system which is cost effective in quantitating monohydroxy fatty acids from a variety of aqueous media.

2. To determine the intracellular source of linoleic acid from which 13-HODE is synthesized in endothelial cells, and to determine the mechanism by which this pathway is regulated.

3. To confirm that there is an inverse relationship between endogenous endothelial cell 13-HODE levels and endothelial cell adhesivity.

4. To determine whether exogenous 13-HODE or other exogenous monohydroxy lipoxygenase metabolites regulate endothelial cell adhesivity.

5. To determine the cellular localization of 13-HODE and the VnR in endothelial cells, under both resting and stimulated conditions.

6. To determine if 13-HODE is present in the basement membrane of endothelial cells, and if so, to determine if 13-HODE influences both endothelial cell-derived basement membrane adhesivity and artificial graft adhesivity, *in vitro*.

7. To determine whether drug or dietary manipulation of vessel wall 13-HODE synthesis influences vessel wall adhesivity for platelets, and whether manipulating platelet 12-HETE synthesis influences platelet adhesivity for the vessel wall, *in vivo*.

8. To determine if there is an inverse relationship between endogenous 13-HODE and HETEs in PMNs and tumor cells and PMN/tumor cell adhesivity.

The results of the experiments to achieve these objectives support the hypothesis that monohydroxy fatty acids regulate adhesion receptor expression both *in vitro* and *in vivo*. Therefore, additional experiments were performed to determine whether a similar relationship existed clinically.

9. To determine whether there was an inverse relationship between the level of 13-HODE in the vessel wall and vessel wall adhesivity in freshly isolated internal mammary arteries obtained from patients undergoing elective coronary artery bypass surgery.

CHAPTER 2:

MATERIALS

GENERAL CHEMICALS:

Non-radiolabelled free fatty acids, phospholipid standards, neutral lipid standards, prostaglandin B_2, N^6,2'-O-dibutyryl cAMP, essentially fatty acid-free bovine serum albumin (BSA), apyrase, indomethacin, dextran (MW ~ 220,000), gelatin, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and soyabean lipoxygenase type I (EC. 1,13,11,12, TYPE 1. 140,000 units/mg) were obtained from Sigma Chemical Company, St. Louis, MO. High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol, sodium borate (10-hydrate), sodium borahydride ($NaBH_4$), unlabelled adenine, silane A-174, sodium lauryl sulphate, toluene blue, monopotassium phosphate (KH_2PO_4), magnesium nitrate, malachite green (Gurr, Colour Index No. 42000), and ethylenediaminetetraacetic acid (EDTA) were obtained from BDH Chemicals Canada Limited, Mississauga, On. Butylatedhydroxytoluene, anhydrous sodium carbonate, chromerge, sodium potassium tartrate, cupric sulphate, 2 N phenol reagent solution (Folin & Ciocalteu), iodine, trisodium citrate, and trichloroacetic acid were obtained from Fisher Scientific, Mississauga, On. Dulbecco's phosphate-buffered saline (DPBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco, Burlington, On. Ficoll Lymphocyte Separation Medium (density = 1.077-1.08 g/ml) was obtained from Bionetics Laboratory Products, Kensington, MD. Triton X-100 and glycerine were obtained from Biorad Laboratories,

Mississauga, On. Paraformaldehyde was obtained from Can-en Chemical Distributors, Guelph, On. Glutaraldehyde, osmium tetroxide and other chemicals for tissue fixation, were obtained from Marivac, Halifax, NS or Merck, Drearmstadt, FRG. Molybdenum Blue reagent was purchased from Altech Applied Sciences, Deerfield, IL. Fluorescein and rhodamine were purchased from Dimension Laboratories Inc., Mississauga, On. Unless otherwise stated, all standard laboratory chemicals (i.e. reagent or chemical grade: $CaCl_2$, $MgCl_2$, NaCl, H_3PO_4, HCl, acetic acid, glucose) were obtained from BDH or Fisher Scientific.

SPECIALIZED GLASSWARE AND PLASTICS:

One ml conical vials were purchased from Mandel Scientific Company Ltd., Guelph, On. All specialized glassware used for either phosphorous, protein or glyceride analysis were obtained from Johns Scientific Inc., Toronto, On. Disposable polystyrene spectrometric cuvettes were purchased from Diamed Laboratory Supplies Inc., Mississauga, On.

RADIOACTIVE MATERIALS:

Tritiated adenine ([^{3}H]adenine, 1 mCi/ml), and [^{125}I]iodine (203.5 MBq, sodium salt) were obtained from ICN Biomedicals, Costa Mesa, CA. Tritiated 5-HETE ([^{3}H]5-HETE, 183 Ci/mmol), [^{3}H]12-HETE (130 Ci/mmol), [^{3}H]15-HETE (285 Ci/mmol), [^{3}H]LTC_4 (51 Ci/mmol), all ^{14}carbon (^{14}C)-labelled

free fatty acids, ACS liquid scintillation fluid and NCS tissue solubilizer were obtained from Amersham Oakville, On. Tritiated arachidonic acid (^{3}H-20:4, 190 Ci/mmol) and linoleic acid (^{3}H-18:2, 47 Ci/mmol, purchased by special order) were purchased from New England Nuclear, Dorval, PQ.

ENDOTHELIAL CELL STIMULI:

Human recombinant-interleukin 1α (IL1) was donated by Dr. P. LoMedico, Hoffman La Roche Inc., Nutley, NJ. Purified human thrombin was donated by Dr. J. Fenton, Albany, NY. γ-Interferon (γIFN) and tumor necrosis factor (TNF) were donated by Dr. E. Bastida, University of Barcelona, Barcelona, Spain. The chemotactic peptide N-formyl-methionyl-leucyl-phenyl-alanine (fMLP) was obtained from Sigma.

LIPOXYGENASE METABOLITES:

5-HETE, LTB_4, LTC_4, LTD_4, and the diHETEs were obtained from Merck Frosst Canada, Dorval, PQ. 12-HETE, 15-HETE and 13-HODE were prepared as described under Methods.

ANTISERA AND ANTIBODIES:

Rabbit polyclonal human vWF immunoglobulins (IgGs), rabbit anti-mouse IgGs and swine anti-rabbit IgGs were obtained from Dimension Laboratories Inc., Mississauga, On. Mouse monoclonal IgGs to the α-chain (LM609) and the RGD-recognition site on the β-chain (LM142) of the VnR, were gifts

from Dr. D.A. Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA.

HPLC MATERIALS:

HPLC grade acetonitrile and methanol were obtained from BDH. All other solvents and phosphoric acid (HPLC grade) were purchased from Fisher Scientific. HPLC grade water (see 'Water' under Materials) was prepared using a Milli-Q water purification system (Millipore, Mississauga, On.). One ml limited volume inserts (8 x 43 mm Kimble insert), purchased from Mandel, were silanized prior to use. All reversed-phase HPLC equipment, columns and 300 µl limited volume inserts were purchased from Waters Scientific. The HPLC instrumentation was comprised of: 2 M510 pumps, a RCM 100 cartridge module, a Wisp™ automatic injector, a M490 programmable detector, an M720 system controller, 810 integration software and a Waters automated valve selector (WAVS). The columns and pre-columns routinely used for the separation of the monohydroxy fatty acid were Nova-Pak cartridges (3 µm, 100 mm x 5.0 mm I.D.) and µBondapak C_{18} pre-columns, respectively.

GAS CHROMATOGRAPHY:

All gas chromatographic (GC) analyses were performed using a CARLO-ERBA model 5160 Mega Series Gas Chromatograph (Milan, Italy) equipped with an automatic cold on-column injector, and a tailor made capillary column. The chromato-

grams were recorded using a Spectra Physic 4270 integrator (San Jose, CA). The standards for GC analysis were obtained from Nuchek Prep, Elysian, MN.

TISSUE CULTURE MATERIALS:

All tissue culture media and supplements were purchased from GIBCO, Burlington, On. T25, T75 culture flasks, and 15 mm Thermanox™ tissue culture discs were obtained from Miles Scientific, Naperville, IL. Costar 24-well plates were obtained from Lux Scientific, Newbury Park, CA., or Nunc, Kamstrup, Denmark. Pooled human sera (type AB), obtained from the Hamilton Red Cross Blood Bank courtesy of Dr. M. Blajchman, were heat-inactivated at 56°C for 1/2 hour. Fibronectin (20 μg/ml) was prepared from outdated plasma supplied by the Hamilton Red Cross Blood Bank, as previously described (Enguall and Ruoslahti, 1977).

TUMOR CELL LINES:

U87MG (a human glioblastoma cell line), and A549 tumor cells (a human lung adenocarcinoma cell line), were obtained from Sloan-kettering Institute, Rye, NY. HL60 (a phorbol ester differentiated promyelocytic leukaemia cell line) and B16F10 tumor cells (a murine melanoma cell line) were obtained from American Type Culture Collection. FSL10 and FSNP tumor cells (a metastatic and non-metastatic fibrosarcoma cell line, respectively) were donated by Dr. F.P. Orr, McMaster

University.

U87MG and A549 cells were maintained in Dulbecco's modified Eagle media containing 1 Mm glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% and 15% (v/v) fetal calf sera, respectively. HL60 cells were maintained in Roswell Park Memorial Institute media, supplemented with 1 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, 25 mg/ml amphotericin B, and 15% (v/v) pooled fetal calf sera. The B16F10, FSL10 and FSNP cells were maintained in Eagles minimum essential medium supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin, 0.25 μg/ml fungisone and 10% (v/v) fetal calf sera.

All tumor cells were labelled with either ^{111}In-oxine according to the methods of (Thakur et al, 1977) or with 0.5 μCi/ml of ^{125}Iododeoxyuridine for 24 hours, for the adhesion assays. All tumor cells were pelleted by centrifugation and resuspended in the appropriate medium. Cell viability was >95% as assessed by trypan blue exclusion assay.

ANAESTHETICS:

Sodium pentobarbital (6.5 gm, MTC Pharmaceuticals, Hamilton, Ontario) was dissolved in 80 ml water. The pH was adjusted to 7.35 with 1 N NaOH and the volume increased to 100 ml with H_2O. The rabbit anaesthetic dose used was 30 mg/kg, administered intravenously via the marginal ear vein. The mouse anaesthetic dose was 50 mg/kg, administered intra-

peritoneally.

A mixture of Ketamine (200 mg) and Atravet (25 mg), obtained from central animal facilities, was also used as a rabbit anaesthetic, administered intramuscularly.

ANTICOAGULANTS:

1) Acid citrate dextrose (ACD, 0.08 M trisodium citrate, 0.07 M citric acid and 0.11 M dextrose) was prepared according to previous methods (Aster and Jandi, 1964). ACD was used as the anticoagulant (0.35% final concentration, v/v) for both human and rabbit blood when the blood was collected for the preparation of [^{3}H]adenine-labelled platelets.

2) 3.2% trisodium citrate (w/v) was used as the anticoagulant when platelets were prepared for *in vivo* adhesion studies and for the thrombocythemia studies (1 part trisodium citrate: 9 parts whole blood).

3) A third anticoagulant (BTG anticoagulant) was used when blood, medium from cells, or other aqueous samples (9 parts sample: 1 part anticoagulant) were collected for prostanoid analysis. The BTG anticoagulant consisted of 1 mM EDTA, 13 μM 2-chloroadenosine, 0.9 mM theophylline and 360 μg/ml indomethacin, all of which were obtained from Sigma. It has been shown previously that this anticoagulant effectively blocks plasma protein activation and platelet activation, thereby preventing generation of prostanoids *in vitro* (Buchanan et al, 1982).

4) Ethyleneglycol bis(β-aminoethylether)-tetraacetic acid (EGTA, K. K. Laboratories, New York, NY), was used at a final concentration of 0.2% (w/v) to chelate calcium in various preparations of platelets suspension.

5) Unfractionated heparin (150 anti-Xa and 150 anti-IIa units/mg, Third International Standard) was obtained from Diosynth BV, Oss, The Netherlands.

MISCELLANEOUS EQUIPMENT:

A Microplan Imager Analyzer (Nikon Canada, Toronto, On.) was used to assess the surface area of various vessel wall segments. A Zeiss ICM 405 Invertoscope microscope (Carl Zeiss Canada Ltd., Don Mills, On.) was used for routine tissue culture work. A Nikon Diaphot T.M.D. inverted microscope equipped with a Nikon Microflex UFX-II and a FX-35A camera (Nikon Canada, Toronto, On.) was used for the histoimmunofluorescence studies. A Beckman LS7000 beta scintillation spectrometer, with a ^{137}Cesium external standard, was used to count all ^{3}H- and ^{14}C-labelled samples.

ANIMALS:

New Zealand white rabbits were used for all rabbit studies. Males only were used in the chronic diet studies while both sexes were used in the acute studies. Eight to 10 week old rabbits used for diet studies weighed approximately 2 kg at the start of the 4 week diets. All other rabbits were

approximately 12 weeks of age and weighed 2.5-3.3 kg at the onset of the studies. All rabbits were housed in metal cages in a temperature- and light-controlled room and fed the standard rabbit chow diet (Ralston Purina, Canada Inc.). The rabbits used for the diet studies were fed a standard diet for 1 week before being randomized into specific diet-fed groups. Rabbits were fed fresh diets daily and allowed free access to water. The rabbit weight gains were monitored weekly.

DIETS:

Four semi-purified diets (BCO, WO, Lard and EPA) were prepared by Nestec Ltd., Lausanne, Switzerland. Each diet was soyabean-based and supplemented with minerals, vitamins and different fatty constituents to be nutritionally adequate. The basic composition of each diet is summarized in Table 2.

To achieve the fatty acid composition of each diet, the diets were supplemented with oil derived from: black currant seed oil (BCO diet) obtained from F.I.S. St. Denis, Switzerland; walnut oil (WO diet) obtained Du Baron, France; lard supplemented with grape seed oil (Lard diet:9 parts lard and 1 part grape seed oil); lard supplemented with eicosapentaenoic acid (EPA Diet: 7 parts lard, 1 part WO and 2 parts fish oil) obtained from Jahres Fabrikker, Norway; and a control diet, regular rabbit chow obtained from Ralston Purina, St. Louis, MO.

TABLE 2. Composition of rabbit diets.

Ingredients	g/100 g
Soya Isolate	22.00
Corn Starch	30.00
Sucrose	12.00
Glucose	5.00
Cellulose	16.00
Mineral Mix	3.50
Vitamins	1.00
Choline bitartrate	0.20
DL-Methionine	0.15
NaCl	15.00
Total Fat*	10.00

* Fatty acid content described in Table 3.

Soya protein isolate (supro 710K) was obtained from the Ralston Purina Company, St. Louis, MO. Corn starch was obtained from Siegfried, AG, Zofinque, Switzerland. Sucrose, table-grade, was obtained from Sucrerie Aarberg, Aarberg, Switzerland. Cellulose (Solka Floc^{Rx}), derived from wood pulp, was obtained from Brown Company, NH. Chemical grade glucose, choline, methionine and NaCl were obtained from Merck, Zurich, Switzerland. Vitamin and mineral supplements were obtained from ICN Biochemicals, Cost Mesa, CA. The fatty acid composition of the diets are shown in Table 3.

TABLE 3. Fatty acid content of semi-purified diets.

FATTY ACID*	BCO % mol	WO % mol	LARD % mol	FO % mol
14:0	0.00	0.00	1.53	3.08
$14:1^{n-5}$	0.00	0.00	0.00	0.00
16:0	7.48	7.59	21.75	24.86
$16:1^{n-7}$	0.07	0.08	16.77	3.18
17.0	0.05	0.00	0.25	0.48
18:0	1.55	2.27	11.15	11.56
$18:1^{n-9}$	14.87	18.55	32.88	33.30
$18:2^{n-6}$	43.31	59.02	13.90	14.19
$18:3^{n-3}$	13.75	12.50	0.94	2.19
$18:3^{n-6}$	15.72	0.00	0.00	0.04
$18:4^{n-3}$	2.83	0.00	0.15	0.47
20:0	0.18	0.00	0.14	0.15
$20:2^{n-6}$	0.19	0.00	0.21	0.22
$20:3^{n-3}$	0.00	0.00	0.05	0.05
$20:3^{n-6}$	0.00	0.00	0.00	0.06
$20:4^{n-3}$	0.00	0.00	0.00	0.14
$20:4^{n-6}$	0.00	0.00	0.06	0.21
$20:5^{n-3}$	0.00	0.00	0.00	2.87
22:0	0.00	0.00	0.07	0.07
$22:1^{n-9}$	0.00	0.00	0.00	0.23
$22:5^{n-3}$	0.00	0.00	0.07	0.40
$22:5^{n-6}$	0.00	0.00	0.00	0.05
$22:6^{n-3}$	0.00	0.00	0.09	2.18
SUM^{n-6}	59.22	59.02	14.17	14.77
SUM^{n-3}	16.58	12.50	1.22	7.91

Data are expressed as mol % of diet. Each fatty acid formulation is present as the # of carbon atoms: # of double bonds and the position of the last double bond. For example, $18:2^{n-6}$ = a fatty acid with 18 carbon atoms, 2 double bonds, with the last double starting 6 carbon atoms away from the methyl terminal carbon. Fatty Acids*: 13:0, 14:1, 17:1, $20:1^{n-9}$, $20:3^{n-9}$, $22:4^{n-6}$, and 24:1 were not detected in any of the diets. Abbreviations are: BCO, black currant seed oil; WO, walnut oil; FO, fish oil.

The first 4 diets were prepared in bulk by Nestec Ltd., Lausanne, Switzerland. Each diet was pelleted, dried for 18 hours at 40°C, and packaged in 5 kg lots in heat-sealed, nitrogen-flushed plastic bags. The bags were then packed in reinforced barrels and shipped to McMaster University. Upon arrival, all diets were refrigerated at 4°C until used. A fresh 5 kg diet package was opened daily.

NOTE: Abbreviations and the composition of routinely used buffers, extraction solvents, stock solutions and reagents are described in Appendix 1.

CHAPTER 3:

METHODS

PURIFICATION OF WATER:

Deionized water was further purified using a Milli-Q 4 bowl water purifier (Millipore, Mississauga On.) as follows. First, the deionized water was cycled through 4 cartridges placed in the following order: charcoal, ion exchange, charcoal, and an Organex-Q cartridge. A 0.2 micron filter was used at the collection spout to remove all bacteria and particulates. This system produced water with a resistance of 18 MΩ, which was suitable for the preparation of tissue culture media, HPLC solvents, and various solutions.

SILANIZATION OF GLASSWARE:

All glassware to be silanized was first acid-washed for 12 hours in chromerge, rinsed with purified water, and then air-dried. The glassware was submersed in a large glass beaker containing 2.5% silane solution. [2.5% silane solution was prepared by mixing 25 ml silane A-174 (BDH) with 975 ml toluene.] After 4 hours, the silane was poured off. The glassware was rinsed twice with methanol, rinsed and then soaked in purified water for 1 hour. The water was poured off and the glassware inverted in a rack until dry.

PURIFICATION OF FREE FATTY ACIDS:

Each free fatty acid was suspended in 50 µl 80% acetonitrile:water and purified by HPLC, using a C18 µBondapak

(10 μm, 8 mm x 10 cm) column and a C18 μBondapak pre-column. The fatty acid was eluted off the column using a solvent composed of acetonitrile:water:trifluoroacetic acid (80:20:0.01, v/v) at an isocratic flow rate of 2.0 ml/minute. The M490 UV spectrophotometer was set to 205 nm to monitor the elution of the fatty acids. The retention times of linoleic and arachidonic acid were 10.4 ± 0.1 and 9.5 ± 0.1 minutes, respectively. The solvent eluted off with each fatty acid was removed by evaporation under a stream of nitrogen and the remaining fatty acid was resuspended in 500 μl of ethanol.

The purified fatty acid was then quantified by HPLC using the same conditions as described above except that a Nova Pak (3 μm, 5 mm x 10 cm) column, a flow rate of 1.0 ml/minute, and phosphoric acid (instead of trifluoroacetic acid) were used. The amount of free fatty acid purified was determined by comparing its HPLC peak area against the peak area of a known amount of ^{14}C-fatty acid standard. The retention times of purified linoleic acid and arachidonic acid were 11.4 ± 0.1 and 10.0 ± 0.1 minutes, respectively.

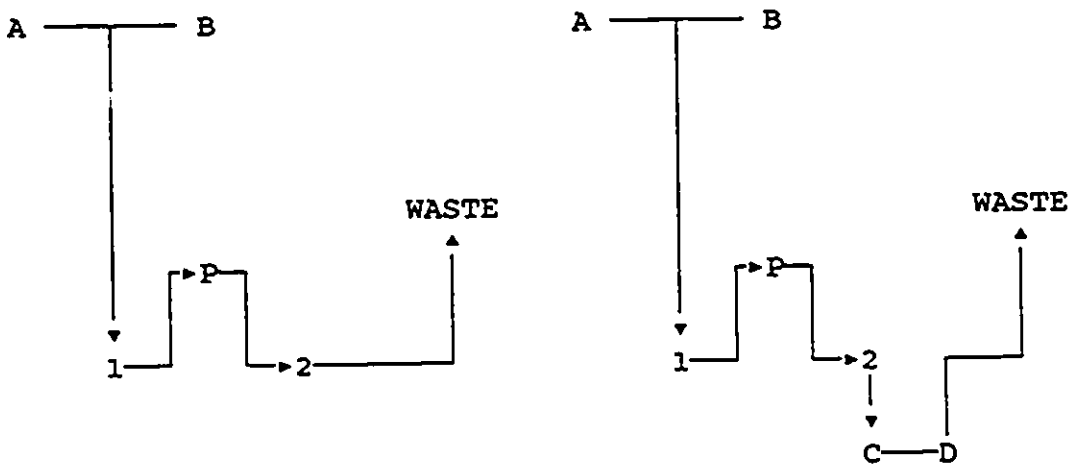
AUTOMATED HPLC EXTRACTION PROCEDURE:

Samples to be analyzed for lipoxygenase metabolites were extracted, using an automated HPLC extraction and quantification method. This method was made possible by incorporating a Waters automated solvent controller (WAVS) into the HPLC system. The WAVS was used to direct the solvent

eluting from the injector, through the pre-column, to either the analytical column or to waste.

The automated extraction procedure is referred to as the pre-column method and was performed as follows: First, the pre-column and the analytical column were equilibrated with an initial solvent (the loading solvent) for 2 to 2.5 minutes (depending on the sample volume) before all standards and/or samples were injected. Once the pre-column and analytical column had been equilibrated, the solvent flow eluting from the pre-column was diverted from the analytical column to waste (Figure 4a). The standard or test sample was injected into the solvent stream and eluted onto the pre-column. The pre-column was washed for 3 minutes with the loading solvent to remove unwanted material (i.e. protein). The solvent eluting from the pre-column was then redirected from waste back to the analytical column and detector (Figure 4b). All retained material (i.e. lipoxygenase metabolites) was then eluted off the pre-column and separated on the analytical column, using an acetonitrile gradient.

To calculate the recovery of lipoxygenase metabolites using the pre-column method, the 100% recovery method was developed. This method was identical to the pre-column method except that the solvent eluting from the pre-column was not diverted to waste but continuously flowed through the analytical column. Confirmation that the 100% recovery method gave complete recovery of all lipoxygenase metabolites, was



A) Loading pre-column.

B) Elution and analysis of sample.

FIGURE 4. The solvent pathway during the extraction and analysis of a sample. (A) Solvent flow for first 3 min. (B) Solvent flow after 3 min. Abbreviations used are: A, pump A; B, pump B; C, column; D, detector; P, pre-column; W, Wisp injector; 1 (and 2), flow selector 1 (and 2) of WAVS.

performed using radiolabelled metabolites and collecting the HPLC eluent at the appropriate retention times.

The lipoxygenase metabolites were quantitated by UV spectrophotometry at either 234 nm (monohydroxy derivatives), 270 nm (dihydroxy derivatives) or 280 nm (LTs). They were monitored simultaneously at 250 nm for quality control.

The solvents routinely used for monohydroxy fatty acid analysis were: solvent A (loading solvent), acetonitrile: water:phosphoric acid (15:85:0.01, v/v/v); solvent B, acetonitrile:water:phosphoric acid (80:20:0.01); and solvent C, acetonitrile:water:phosphoric acid (40:60:0:03, pH 6.0). Solvent C was also used for LT analysis.

PREPARATION OF 13-HODE AND 15-HETE:

Linoleic acid (dissolved in ethanol) was added to a glass beaker, blown to dryness under nitrogen, and resuspended in borate buffer to a final concentration of 200 μM. The linoleic acid suspension was stirred constantly and flushed with O_2. After 5 minutes, 10 μl of dilute S-stock (i.e. soyabean lipoxygenase type I) was added per ml fatty acid suspension. The enzymatic reaction was allowed to proceed at 20°C, under O_2. After 5 minutes the reaction was terminated by the addition of 0.1 volumes of ethanol and by acidification to pH 4.5, using acetic acid.

The suspension was extracted 3 times with 3 volumes of diethyl ether. If the amount of ether extract collected was

<50 ml, the ether was evaporated under a stream of nitrogen in a 20°C water-bath. If >50 ml of ether extract was collected, the extract was first washed with 10 ml of 0.1% acetic acid (v/v, to remove excess borate), and a rotary evaporator was used to evaporate the ether. The remaining 13-hydroperoxy-linoleic acid was resuspended in 5 ml of ice-cold dry-methanol and incubated at 4°C with a 10 molar excess of $NaBH_4$. After 1 hour at 4°C, the sample was placed at -70°C for 18 hours to reduce the hydroperoxy fatty acid to its hydroxyl form, 13-HODE. The sample was warmed to 20°C and blown down to near dryness. 13-HODE was extracted from the sample using 3 times 3 ml of ether. The ether was evaporated under a stream of nitrogen, and the remaining 13-HODE was resuspended in acetonitrile. 13-HODE was then purified and quantitated by HPLC as described under "PURIFICATION OF MONOHYDROXY FATTY ACIDS". Greater than 90% of the linoleic acid starting material was recovered as 13-HODE. A similar efficiency in the synthesis of [^{3}H]13-HODE, [^{14}C]13-HODE, [^{14}C]15-HETE, 13-hydroxyoctadecatrienoic acid (13-HOTE) and 15-hydroxy-eicosapentaenoic acid (15-HEPE) were achieved when using the appropriate fatty acid.

PREPARATION OF 12-HETE:

Whole blood (43 ml) was collected into 7 ml ACD. Platelet-rich plasma (PRP) was prepared by differential centrifugation (180 g for 12 minutes at 37°C). The platelets

were then pelleted by centrifugation at 1700 g for 10 minutes at 37°C. The platelet pellet was resuspended in 10 ml of Ca^{++}-free Tyrodes (pH 7.35, 37°C), containing 100 µl apyrase and 500 U of heparin. (The apyrase was added to degrade any ADP released from the platelets, thereby preventing ADP-induced platelet aggregation. Heparin was added to prevent thrombin-induced platelet aggregation). The platelets were then repelleted and resuspended in 5 ml Ca^{++}-free Tyrodes containing 100 µl apyrase and 10 µM indomethacin. (Indomethacin was added to inhibit TXA_2 formation, thereby preventing TXA_2-induced platelet aggregation). The platelet suspension was gently stirred at 37°C. Five minutes later, arachidonic acid dissolved in 10% ethanol, was added to a final concentration of 100 µM. Thirty minutes later, 30 ml of ice-cold methanol was added to stop the reaction. The platelet suspension was cooled to -10°C. All platelet protein precipitates were then pelleted by centrifuged at 1700 g for 1 hour at -10°C. The supernatant was transferred to a 50 ml centrifuge tube, warmed to 22°C and evaporated under a stream of nitrogen. The residue was resuspended in 500 µl of 15% aqueous acetonitrile and purified by HPLC as described below.

12-HEPE was also prepared using this method, by substituting eicosapentaenoic acid for arachidonic acid.

PURIFICATION OF MONOHYDROXY FATTY ACIDS:

Each monohydroxy fatty acid was dissolved in 500 µl

15% acetonitrile and injected onto a NOVA-Pak C18 (3 μm, 5 mm x 10 cm) column. The fatty acid was then purified using an acetonitrile gradient (15% to 80%), and detected by UV absorbance at 234 nm, similar to the method described for free fatty acid purification. For example, the solvent eluted with the monohydroxy fatty acid was evaporated under nitrogen. The remaining monohydroxy fatty acid was resuspended in 1 ml of either 10% ethanol or 50% acetonitrile and transferred to a 1 ml quartz cuvette. The amount of metabolite present was quantified spectrometrically by measuring its absorbance at 230-240 nm in a Gilford 250 spectrophotometer (path length = 1 cm, slit width = 0.1 mm). Each monohydroxy fatty acid was quantitated at its lambda maximum using an extinction coefficient of 2.95×10^4 $M^{-1}cm^{-1}$.

ENDOTHELIAL CELL CULTURE:

Human umbilical vein endothelial cells were cultured according to previously established methods (Jaffe et al, 1973), with the following modifications. Endothelial cells were harvested from umbilical cords with collagenase. The cells were plated onto fibronectin-coated T25 flasks and grown in M199 supplemented with 20% pooled human sera, 25 mM Hepes (pH 7.4), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml endothelial cell growth supplement, in an atmosphere of 95% air, 5% CO_2. The medium was replaced with fresh supplemented medium every 2-3 days until confluency. Confluent

primary endothelial cell cultures were then passaged (1:3 split) onto either T25 flasks or fibronectin-coated Thermanox™ discs, grown to confluency, and used in the first passage.

ENDOTHELIAL CELL 13-HODE ANALYSIS:

The media covering the confluent endothelial cell monolayer was removed, and the monolayer was covered with 2 ml of ice-cold methanol:water (1:1, v/v). The endothelial cell monolayer was then scraped into the aqueous methanol, and transferred to a silanized glass tube containing another 2 ml of ice-cold methanol (final methanol concentration 75%, v/v). The tube was vortexed for 30 seconds and incubated 30 minutes in a -20°C freezer. The unsolubilized particulates were pelleted by centrifugation (-10°C, 1700 g, 1 hour). The remaining supernatant was transferred to a conical silanized glass tube and the supernatant evaporated under a stream of nitrogen. The residual was resuspended in 800 µl of acetonitrile:water (15:85, v/v), and the amount of 13-HODE present was quantitated using the automated HPLC extraction procedure.

PREPARATION OF 6-KETO-$PGF_{1\alpha}$, TXB_2, AND 13-HODE ANTISERA:

Briefly, 6-keto-$PGF_{1\alpha}$ and TXB_2 were conjugated to BSA in the presence of EDC. Each conjugate (250 µg) was mixed with Freund's adjuvant and injected intramuscularly into rabbits at multiple sites. Booster shots were given at 4 week intervals. Test bleeds were performed monthly to assay the

antiserum titre. When a high titre (> than 1/3000) was obtained, usually by 6 months, the rabbits were exsanguinated, and the blood was allowed to clot. Antiserum was then prepared by differential centrifugation. Rabbit polyclonal 13-HODE antiserum was prepared in a similar manner as described above. Its specificity and titre are described in the Results.

RIA TECHNIQUE:

Iodinated 6-keto-$PGF_{1\alpha}$ was prepared by conjugating 6-keto $PGF_{1\alpha}$ to [^{125}I]histamine using EDC. The 6-keto-$PGF_{1\alpha}$ RIA procedure consisted of incubating duplicates of either 100 μl of the 6-keto-$PGF_{1\alpha}$ stock solutions (standard curve from 0 to 2000 pg/ml), the sample or the buffer (to correct for non-specific binding), with 100 μl of 6-keto-$PGF_{1\alpha}$ antiserum and 100 μl of iodinated 6-keto-$PGF_{1\alpha}$ in plastic centrifuge tubes. The antiserum was titrated to give 40% binding of the iodinated 6-keto-$PGF_{1\alpha}$ added in the absence of unlabelled 6-keto-$PGF_{1\alpha}$. After 12 hours at 4°C, 100 μl of 2% (v/v) normal pooled rabbit sera: 1.86% (w/v) disodium EDTA in PBS, 100 μl of 10% (w/v) polyethylene glycol in PBS, and 100 μl of 7% (w/v) goat anti-rabbit IgGs in PBS, were added to each sample. The sample was vortexed and placed on ice for 15 minutes. The 6-keto-$PGF_{1\alpha}$ which bound with the antiserum was then pelleted by centrifugation at 1700 g, 4°C for 30 minutes. The supernatant was decanted and the radioactivity of the remaining

pellet was determined, using a gamma counter. The amount of 6-keto-$PGF_{1\alpha}$ present in each test sample was extrapolated from an optimized scatchard plot of known amounts of standard. All samples that contained an amount of 6-keto-$PGF_{1\alpha}$ that was above the linear region of the standard plot, were diluted in PBS and reassayed.

The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antiserum used in these studies is shown in Table 4. The cross-reactivity and sensitivity of the TXB_2 antiserum was similar to that of the 6-keto-$PGF_{1\alpha}$ antiserum (Buchanan et al, 1982). The sensitivity of the 6-keto-$PGF_{1\alpha}$ RIA was 50 pg with a maximum range of 2000 pg. The TXA_2 RIA was performed in a similar manner as described above for 6-keto-$PGF_{1\alpha}$.

HISTOIMMUNOFLUORESCENCE TECHNIQUES:

Detection of endothelial cell metabolites and adhesion molecules: Glass discs (12 mm, Bellco Glass Inc., Vineland, NJ) were placed in Costar 24-well plates, washed with 70% ethanol for 10 minutes, rinsed 3 times with HBSS, and then incubated with 1% gelatin (w/v) at 22°C. Thirty minutes later, the discs were rinsed 3 times in PBS and incubated with 0.5% glutaraldehyde (w/v) for 20 minutes at 22°C, to cross-link the gelatin. The glutaraldehyde was removed and the discs rinsed 3 times in PBS. Endothelial cells were then seeded onto the gelatin-coated discs and allowed to grow to confluency.

TABLE 4. Percent cross reactivity of 6-KETO-$PGF_{1\alpha}$ antisera at 50% B/Bo

6-keto-$PGF_{1\alpha}$	100.0
6-keto-PGE_1	4.0
15-keto-PGE_2	< 0.01
15-keto-PGF_2	< 0.001
PGB_2	< 0.01
PGD_1	< 0.01
PGE_1	0.016
PGE_2	< 0.01
PGF_1	< 0.01
PGF_2	< 0.001
TxB_2	< 0.001
13,14-Dihydro-6,15-Diketo-PGF_1	< 0.001
13,14-Dihydro-15-keto-PGE_2	< 0.01
13,14-Dihydro-15-keto PGF_1	7.8
6,15-Diketo-PGF_1	0.800

To determine if 13-HODE and the VnR were located on the surface of endothelial cells, the medium was removed from the endothelial cell monolayers. The monolayers were rinsed 3 times with HBSS. The monolayers were then fixed with 1% paraformaldehyde in PBS (pH 7.2) for 18 hours at 4°C. The paraformaldehyde was removed and the monolayers covered with 0.1% glycine (v/v) in buffer (1.68% $NaH_2PO_4.H_2O$, 0.385% NaOH, 0.54% glucose, w/v, pH 7.2). After 30 minutes, the monolayers were rinsed 3 times with PBS.

To determine where 13-HODE and the VnR were located inside the endothelial cell, the paraformaldehyde-fixed endothelial cell monolayers (described above) were incubated with 1 ml 2% Triton X-100 (v/v) for 3 minutes at 22°C. The Triton X-100 was removed and the permeabilized cells were rinsed 4 times with PBS for 5 minutes.

The fixed and [fixed and permeabilized] endothelial cell monolayers were incubated at 22°C with 100 μl of the first antiserum. Each antiserum was diluted 1/100 (either, the rabbit polyclonal 13-HODE antiserum, the rabbit polyclonal human vWF IgG, or 1 of the 2 mouse monoclonal antisera to the VnR, LM609 and LM142). Ninety minutes later, the monolayers were washed 4 times for 5 minutes with PBS to remove any unbound antiserum. The monolayers were then incubated in the dark with 100 μl of a second antibody, conjugated with either fluorescein or rhodamine. The second antibody, which was also diluted 1/100, was either swine IgG, directed against rabbit

or human IgG, or rabbit IgG, directed against mouse IgG. After 1 hour at 22°C, the endothelial cells were again washed 3 times for 5 minutes in the dark with PBS and then once with purified water to remove any unbound IgG. The washed endothelial cells were covered with 1 ml of glycerine (glycerine: PBS, 70:30, v/v), wrapped in tinfoil and placed in a 4°C refrigerator for 18 hours.

Endothelial cell fluorescence was detected using a Nikon Diaphot T.M.D. inverted microscope equipped with a Nikon Microflex UFX-II and a FX-35A camera. The excitation and cut-off wavelengths used were ~ 480 nm and 520 nm for fluorescein, and 546 nm and 590 nm for rhodamine, respectively.

MONOHYDROXY FATTY ACIDS BINDING TO ENDOTHELIAL CELL MONOLAYERS:

The medium covering the confluent endothelial cell monolayers was removed. The monolayers were rinsed 3 times in 3 consecutive beakers of HBSS+ and transferred to a Costar 24 wells. Each monolayer-covered disc was then incubated with 1 ml Tyrodes in a 37°C shaking water-bath. All ^{14}C- or ^{3}H-labelled fatty acids (i.e. monohydroxy lipoxygenase metabolites, linoleic and arachidonic acid), dissolved in 10 µl of 10% ethanol, were added to the monolayers in a final concentration, ranging from 1 nM to 10 µM. One hour later, the monolayers were removed from the wells and rinsed 3 times in HBSS+ and transferred to liquid scintillation vials containing

1 ml of distilled water to lyze the cells. Thirty minutes later, 10 ml of ACS scintillation fluid was added to each vial and its radioactivity determined.

ENDOTHELIAL CELL INCORPORATION OF ^{3}H-18:2 AND ^{3}H-20:4:

Confluent monolayers of endothelial cells were washed with supplemented medium (20% human sera) and then incubated with fresh supplemented medium containing 1 nM of either ^{3}H-18:2 or ^{3}H-20:4 for 18 hours at 37°C, in a 5% CO_2 atmosphere. The ^{3}H-fatty acids were used as markers of the incorporation of unlabelled 18:2 and 20:4 from the medium. The endothelial cells were then washed with Tyrodes (2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.35), to remove any unbound ^{3}H-fatty acid.

To determine the % fatty acid uptake by endothelial cell monolayers, fatty acid-labelled monolayers were placed into liquid scintillation vials containing 1 ml of distilled water to lyze the cells. Thirty minutes later, 10 ml of ACS scintillation fluid was added to each vial and its radioactivity was determined. % fatty acid uptake was expressed as a percent of total ^{3}H-fatty acid added.

To determine the distribution of the fatty acids between the phospholipid and triglyceride pools, the ^{3}H-fatty acid-labelled monolayers, were rinsed 3 times in 3 consecutive 50 ml beakers of HBSS+ and scraped into 1 ml ice-cold methanol:water (1:1, v/v containing 50 μM butylatedhydroxy-toluene). The cell extracts were transferred to a 15 ml

silanized centrifuge tube and placed on ice. The discs were scraped in the presence of an additional 1 ml of aqueous methanol to ensure complete transfer of the monolayers. Total lipids were then extracted into chloroform:methanol according to the methods of Bligh and Dyer (1959), with the following modifications. First, 2.75 ml of chloroform: methanol (5:6, v/v) was added to each sample. The sample was vortexed for 30 seconds and placed back on ice. After 10 minutes, 1.25 ml chloroform was added. Each sample was vortexed and 1.25 ml 0.9% NaCl (w/v) in water was then added. The sample was again vortexed and centrifuged at 1700 g, -10°C, for 5 minutes. The top aqueous phase was removed and re-extracted with another 2.25 ml of chloroform. The 2 organic layers were combined and blown to dryness under nitrogen at 20°C in a 1 ml conical vial. The residual was dissolved in 20 μl chloroform:methanol (1:1, v/v) containing 50 μM butylatedhydroxytoluene.

To separate the phospholipid and triglyceride pools, the lipid extracts were transferred to heat-activated Merck silica gel 60 TLC plate (20 x 20 cm x 250 μm, Fisher Scientific), 2.5 cm above the bottom. Phospholipid, neutral lipid, and free fatty acid standards were spotted in the centre and 1 cm in from each side of each TLC plate. The total phospholipid and triglyceride pools were separated from the free fatty acids and fatty acid esters by placing the TLC plate into TLC tanks saturated with a solvent system, comprising of hexane:diethylether:ethanol:acetic acid (80:19.8 :0.2:1, v/v).

A glass lid was immediately placed on top of the TLC tanks and the plate was developed until the solvent front was 1 cm from the top of the plate. The plate was removed from the tank and air-dried in a fume hood. Iodine was used to visualize the entire TLC plate, and Molybdenum Blue reagent was used to visualize the lipid standards.

To determine the relative distribution of ^{3}H-fatty acids in the phospholipid and triglyceride pools, all lipid classes were scraped from the TLC plates and placed in scintillation vials containing 10 ml ACS scintillation fluid. Their radioactivities were determined in a Beckman LS7000 beta scintillation spectrometer. Greater than 95% of the total ^{3}H applied to the TLC plates was recovered with this method.

db-cAMP TREATMENT OF ENDOTHELIAL CELLS:

To assess the effects of endogenous cAMP on endothelial cell triglyceride turnover, and PGI_2 and 13-HODE synthesis, endothelial cell monolayers were incubated with exogenous db-cAMP (dissolved in M199 at a final concentrations of 10 or 100 μM). db-cAMP was added to the endothelial cells either concomitant with the ^{3}H-fatty acid (co-treatment), or 18 hours after adding the ^{3}H-fatty acid (post-treatment).

Endothelial cell monolayers were also incubated with 10 μM of dipyridamole (a phosphodiesterase inhibitor), added either immediately or 24 hours before adding the ^{3}H-fatty acids. These experiments were performed to increase the

endogenous levels of endotnelial cell cAMP, instead of adding exogenous db-cAMP.

ENDOTHELIAL CELL ENDOGENOUS cAMP ANALYSIS:

To determine the level of endogenous cAMP in endothelial cells, the medium was removed from the monolayers, which were then scraped into 2 ml of ice-cold 5% trichloroacetic acid (w/v). The cells were sonicated and the protein was precipitated by centrifugation at 10,000 g, -10°C for 10 minutes. The trichloroacetic acid was removed from the supernatant by diethyl ether extraction. Any remaining supernatant was evaporated under a stream of nitrogen at 50°C. The residue protein was dissolved in 0.2 ml acetate buffer (pH 5.4) and its cAMP content was determined, using a commercially available RIA kit (Amersham).

Cell count, phospholipid content, protein content and triglyceride content were also determined.

ENDOTHELIAL CELL PHOSPHOLIPID PHOSPHOROUS ANALYSIS:

To determine the phospholipid content of endothelial cell monolayers, the medium covering the monolayers was removed and the monolayers were rinsed 3 times with 1 ml HBSS+. The monolayers were covered with 1 ml of ice-cold methanol:water (1:1, v/v) containing 50 μM butylatedhydroxytoluene and scraped into the aqueous methanol. The cell extracts were transferred to a 15 ml silanized centrifuge tube

and placed on ice. The discs were scraped in another 1 ml of aqueous methanol, which was combined with the first scraping.

The total lipids were extracted into chloroform: methanol and washed free of non-lipid sources of phosphorous as described previously (Bligh and Dyer, 1959; Pollak and Munn, 1970). Briefly, 2.75 ml of chloroform:methanol (5:6, v/v) was added to each sample. Each sample was vortexed for 30 seconds and placed on ice. Ten minutes later, 1.25 ml chloroform was added and the sample vortexed. Next, 1.25 ml of 0.9% NaCl in water (v/v) was added, and the sample was vortexed and centrifuged (1700 g, -10°C, for 5 minutes). The top aqueous phase was removed and extracted with 2.25 ml of solvent Y. The sample was centrifuged and the resulting lower organic phase was combined with the first organic phase. The combined organic phases were washed with 10 ml of solvent X. The sample was vortexed and centrifuged, and the upper aqueous phase discarded. The remaining sample was transferred to a 15 x 125 mm acid-washed test tube and blown to dryness under nitrogen at 20°C. The residue was dissolved in 1 ml chloroform:methanol (1:1, v/v).

The phospholipid phosphorous was then digested according to the method of (Duck-Chong, 1979). Magnesium nitrate solution (30 µl of 10%, w/v) was added to each extract and the test tube vortexed. The extract was blown to dryness by a stream of nitrogen in a 95°C sand bath. Each test tube was heated at the top of a bunsen burner flame for 15 seconds

and then at the tip of the blue internal flame for 10 seconds. The test tube was placed back into the 95°C sand bath. Five minutes later, 1 ml 1 M HCL was added to each sample and an acid washed glass marble immediately was placed on top of the test tube to minimize evaporation. Fifteen minutes later, the test tube was removed from the sand bath, cooled in air for 10 minutes, and placed in a 22°C water-bath.

Standards containing 0-0.5 μg of inorganic phosphate-phosphorous (KH_2PO_4), dissolved in 1 ml 1 M HCL, were prepared in triplicate. Each standard was prepared in a 15 x 125 mm test tube, which was prepared as described above.

To quantitate the amount of phosphorous present in each sample, 30 μl of Reagent D and 2 ml of Reagent C were added. Each sample was immediately vortexed for 15 seconds and then incubated at 22°C for 20 minutes. The sample was transferred to 1.5 ml disposable polystyrene cuvettes and its optical density determined at 650 nm in a Gilford 250 spectrophotometer. The standard containing 0 μg phosphorus was used to zero the absorbance scale. Linear regression analysis (Minitab 5.0 software) of the optical densities of the standards was used to generate a standard curve, which was used to calculate the amount of phosphorous present in each test sample. A typical standard curve is shown in Figure 5.

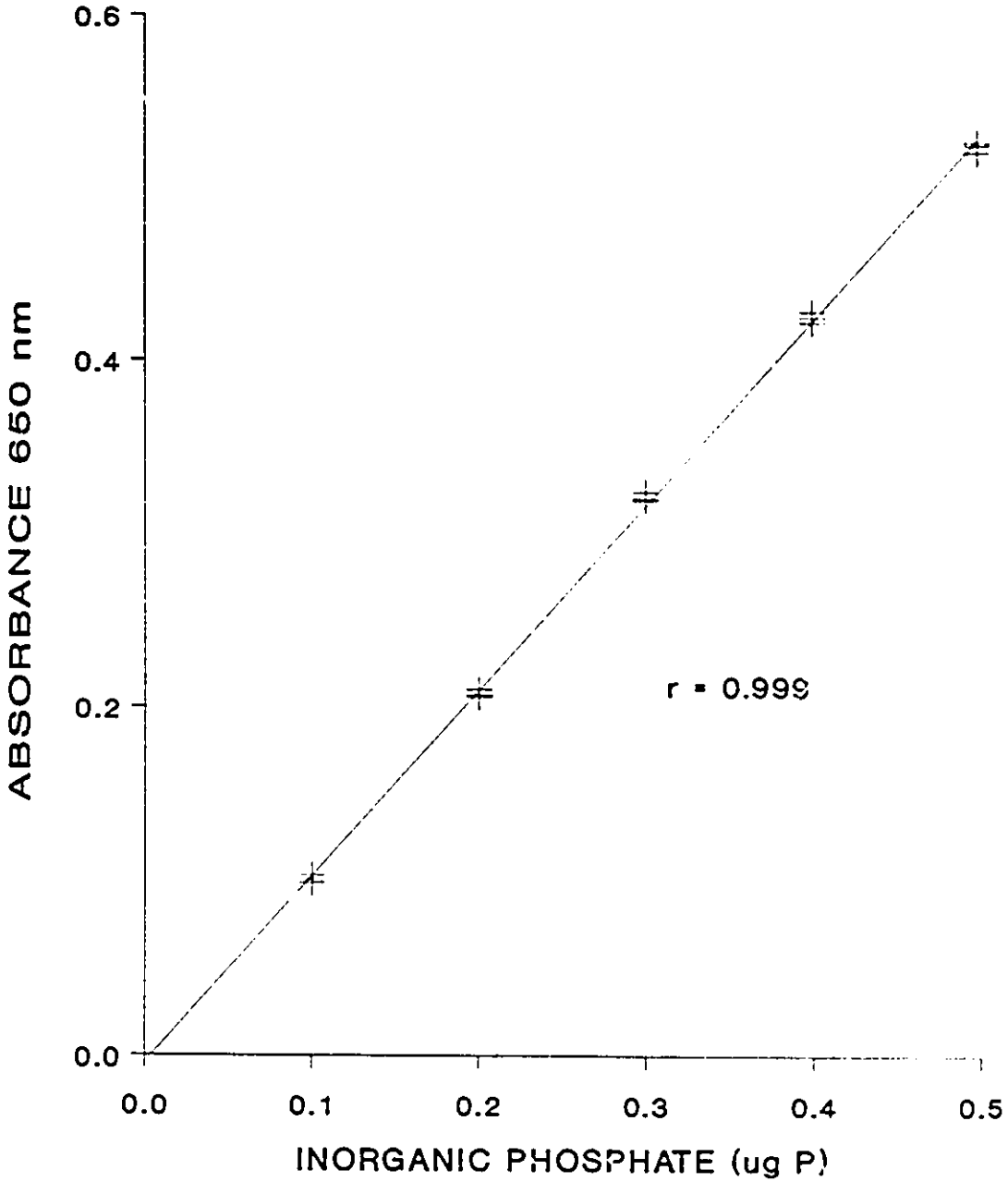


FIGURE 5. Standard phosphorous curve obtained using the Malachite green assay. ┼ = one data point.

ENDOTHELIAL CELL PROTEIN ANALYSIS:

The media was removed from each endothelial cell monolayer-covered disc. The monolayers were rinsed 3 times in 3 consecutive beakers of HBSS+ and transferred to a 17 mm glass test tube containing 600 µl of water. Each test tube was then vortexed for 30 seconds to lyze the cells. Two ml of Alkaline-Copper Reagent was added to each sample which was then vortexed for 30 seconds and incubated undisturbed at 22°C. Ten minutes later, 200 µl Folin Reagent was added to each sample. The sample was vortexed and incubated undisturbed for 1 hour at 22°C. Each sample was then carefully poured into a 4 ml disposable polystyrene cuvette. Its optical density was measured at 660 nm in a Gilford 250 spectrophotometer against a water blank (path length = 1 cm; slit width = 0.02 mm).

A standard curve was prepared by diluting the BSA stock solution 50 times in purified water to obtain a concentration of 100 µg/ml. This diluted BSA stock solution was then diluted further with water to obtain BSA standards ranging in concentrations from 100 to 0 µg/ml. The standards were then treated in the same manner as described above for the test samples. Linear regression analysis (Minitab 5.0 software) of optical densities of the standards was used to generated a standard curve, which was then used to calculate the amount of protein present in each test sample. A typical standard curve is shown in Figure 6.

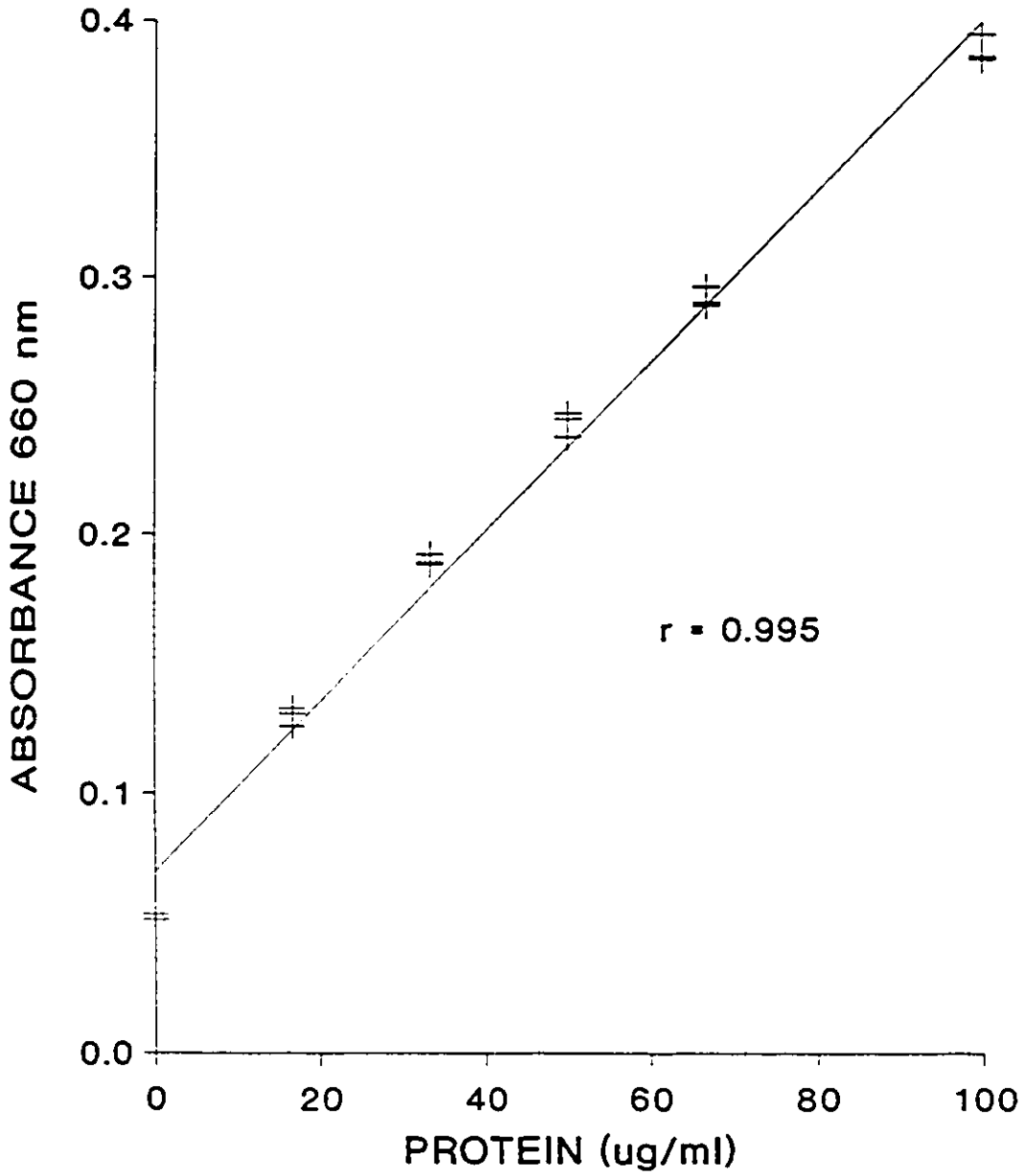


FIGURE 6. Standard protein curve obtained using the modified Lowry assay. + = one data point.

ENDOTHELIAL CELL GLYCERIDE ANALYSIS:

Endothelial cell monolayers, grown to confluency in T25 tissue flasks, were rinsed 3 times with 5 ml HBSS+ after the removal of the medium. Each monolayer was covered with 3 ml ice-cold ethanol and scraped with a teflon rack. The ethanol and cell particulates were then transferred to a 15 ml silanized centrifuge tube. The T25 flask was again scraped with another 1 ml ethanol. Both scrapings were combined and centrifuged at 1700 g, 4°C for 30 minutes. The supernatant was transferred to another centrifuge tube and blown to dryness under nitrogen in a 20°C water-bath. The residue was resuspended in 100 μl of HBSS.

Total cellular glycerides (mono-, di- and triglycerides) were then assayed, using the commercially available Behring Diagnostics Stat-Pack Enzymatic Triglyceride-Glycerol Test (Hoechst Canada Inc., Montreal, Quebec). An aliquot of a known amount of normal pooled human sera was assayed each time to confirm the reliability and reproducibility of the assay. The glyceride levels of the quality control sera and test samples were measured according to the method described in the triglyceride kit. UV absorbance at 340 nm was measured against a water blank in 4 ml disposable polystyrene cuvettes, using a Gilford 250 spectrophotometer (path length = 1 cm; slit width = 0.02 mm). Cellular triglyceride levels were corrected for cellular glycerol, dihydroxy-acetone and L-glyceraldehyde by omitting the lipase reagent.

PLATELET/ENDOTHELIAL CELL ADHESION ASSAY:

Confluent endothelial cell monolayers were rinsed 3 times with HBSS+ and incubated with 1 ml of Tyrodes for 15 minutes at 37°C. The Tyrodes was removed and 1 ml of a human ^{3}H-platelet suspension (described below) was added to each monolayer and incubated for 30 minutes in a 37°C shaking water-bath. The monolayer-covered discs were then removed and gently washed 3 times in 3 consecutive beakers of HBSS+ to remove any non-adherent platelets. The washed monolayers-covered discs were placed in scintillation via's and counted for radioactivity to assess the number of platelets adherent to the endothelial cell monolayers.

HUMAN ^{3}H-PLATELET PREPARATION:

Whole blood (43 ml) was collected into a 50 ml polypropylene centrifuge tube containing 7 ml of ACD, which had been previously warmed to 37°C. The blood was centrifuge at 180 g for 12 minutes at 37°C. The platelet-rich plasma (PRP) was transferred to another centrifuge tube and centrifuged at 1700 g for 10 minutes at 37°C. The platelet-poor plasma (PPP) was then aspirated off and the platelet pellet resuspended in 10 ml of Ca^{++}-free Tyrodes albumin (0.35%, pH 7.35, 37°C) containing 500 U of heparin and 100 μl of apyrase. (The amount of apyrase added was enough to degrade all ADP which may have been present). 150 μCi ^{3}H-adenine was added to the platelet suspension and incubate for 30 minutes at 37°C.

The platelets were pelleted at 1700 g for 10 minutes at 37°C, and the supernatant discarded. The platelets were resuspended in 15 ml of Ca^{++}-free Tyrodes albumin, containing 100 µl apyrase. After a 15 minute incubation at 37°C, the platelets were repelleted and resuspended in 20 ml Tyrodes albumin (pH 7.35, 0.35% albumin, 37°C) containing 3.5 µg/ml adenine. A 5 µl aliquot of each platelet suspension was then added to 15 ml Isoton II and the platelet concentration determined using a ZBI coulter counter (Coulter Electronics of Canada, Burlington, On.). The platelet count was adjusted to 200,000 platelets/mm^3 using Tyrodes albumin. Three 100 µl aliquots of the final platelet suspension and of its supernatant (obtained after centrifuging 1 ml platelet suspension at 17,000 g for 2 minutes) were then placed into scintillation vials containing 10 ml ACS scintillation fluid and counted for ^3H to assess platelet specific activity.

HUMAN ^{3}H-PMN PREPARATION:

PMNs were prepared according to a method of Borgeat et al (1984). Whole blood (43 ml) was collected from a human volunteer into a 50 ml syringe using a 19 gauge needle and quickly transferred to a 50 ml polypropylene centrifuge tube containing 7 ml ACD, prewarmed to 37°C. The blood was centrifuged at 180 g for 10 minutes at 37°C, and the PRP transferred to a separate centrifuge tube for the preparation of the platelet suspensions (see above). The remaining buffy coat

and erythrocytes were mixed with an equivolume of 6% dextran (MW 220,000 daltons, in DPBS), and incubated at 37°C. Forty minutes later, the PMN-rich supernatant was transferred to a separate polypropylene tube and the sedimented erythrocytes were discarded. The PMNs were pelleted at 1700 g for 10 minutes, washed with DPBS and lyzed in 10 ml isotonic ammonium chloride (9 parts 0.83% NH_4Cl (w/v) + 1 part 2.06% Tris base (w/v), pH 7.2). The erythrocyte-free PMN pellet was then resuspended in 5 ml of ice-cold DPBS and placed on top of 3 ml ice-cold Ficoll in a 15 ml polypropylene centrifuge tube. The PMNs were separated from contaminating platelets, monocytes, lymphocytes and erythrocyte ghosts by differential sedimentation through the ficol by centrifugation at 1200 g for 12 minutes at 4°C. The purified PMNs were incubated with ^3H-adenine for 30 minutes, washed twice with DPBS and resuspended in 0.35% Tyrodes albumin at a concentration of 5 x 10^6 PMN/ml. PMN preparations usually contained less than 1 platelet and 1 erythrocyte per 100 PMNs.

PREPARATION OF RABBIT PLATELETS FOR IN VIVO STUDIES:

Rabbit platelets suspensions were prepared by a modification of a previously published method (Ardlie et al, 1970). Three donor rabbits per 4 recipient rabbits were anaesthetized with sodium pentobarbital. A cannula was inserted into the left carotid artery and the rabbit exsanguinated. The blood was collected into ACD (6 parts blood:1 part

ACD) or Na Citrate and centrifuged at 150 g for 15 minutes to prepare PRP. The PRP was transferred to another tube and centrifuged at 1200 g for 10 minutes to separate the platelets from the plasma. The PPP was stored at 4°C until needed.

The platelet pellet was suspended in 5 ml of Ca^{++}- and albumin-free Tyrodes (pH 6.25, containing 0.02% EGTA and 100 μCi [^{3}H]adenine per 50 ml of whole blood initially collected. The platelets were incubated for 40 minutes at 22°C. The platelets were then pelleted by centrifugation at 1200 g for 10 minutes, and the supernatant discarded. The platelet pellet was resuspended in 10 ml Ca^{++}-free 0.35% Tyrodes albumin, and incubated for 5 minutes. This washing procedure was repeated to remove any free [^{3}H]adenine. The ^{3}H-adenine-labelled platelets (^{3}H-PLTs) were resuspended in PPP (1 part ACD-PPP, 9 parts citrated PPP). The platelet count of each suspensions was adjusted to 2.5×10^9 platelets/μl. Three ml of the final platelet suspension was then injected into the marginal ear vein of each recipient rabbit.

IN VIVO VESSEL WALL MODEL: MINIMAL INJURY:

The endothelial cells were selectively removed from the underlying basement membrane of rabbits carotid arteries, using an air-injury model (Fishman et al, 1975). Rabbits were anaesthetized with sodium pentobarbital and both carotid arteries were isolated by blunt dissection. A 2 cm segment of each artery was isolated and emptied of blood. The artery was

occluded first with a proximal suture and then with a distal suture. Each isolated segment was then perfused for 5 minutes with air at a flow rate of 250 ml/min, which was introduced through a 27 gauge needle inserted immediately proximal to the distal suture. The incoming air was allowed to escape from the segment through another opening produced by a 27 gauge needle, inserted in the vessel wall segment immediately distal to the proximal suture. Five minutes later, both needles and then both sutures were removed and blood flow was restored. The time taken for cessation of bleeding from the 2 needle hole puncture sites following blood flow restoration was recorded as an index of "bleeding time".

The injured segment of each carotid artery was then removed from the animals 1, 2, 4, 18 or 24 hours later (4 hours later in the diet studies), and processed for platelet accumulation assessed either morphologically or isotopically.

ASSESSMENT OF IN VIVO PLATELET ACCUMULATION:

Platelet accumulation was measured isotopically using a modification of previously described methods (Buchanan and Hirsh, 1984). Briefly, 3 ml of ^{3}H-platelet suspension were injected intravenously 1 hour prior to removal of the vessel wall segment. Immediately before removing the injured carotid artery segment, a 5 ml blood sample was collected from the rabbit into 3.2% sodium citrate to measure whole blood platelet specific activity. Each rabbit was then injected

with 200 U/kg of heparin to prevent post mortem thrombin-induced platelet adhesion. Two minutes later, the rabbits were killed with an overdose of sodium pentobarbital. A standard 1.5 cm length of each injured vessel segment was removed. Care was taken to avoid including the needle hole puncture sites. The segments were slit longitudinally and laid endothelial-side down in a liquid scintillation vial containing 1 ml of NCS tissue solubilizer for 1 hour. The segment was rinsed with 10 ml of ACS liquid scintillation fluid and removed from the vial. The number of platelets adherent/cm^2 surface area of vessel wall segment was calculated by measuring the radioactivity of the solubilized sample, the whole blood platelet-specific activity, and the calculated vessel wall surface area. The surface area of the segment was calculated by laying the segment flat and photocopied. The surface area of the photocopy of the vessel segment was determined using the image analyzer.

Platelet accumulation onto the injured and uninjured rabbit carotid artery segments was also assessed by scanning electron microscopy (SEM). Briefly, the arteries were fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. The arteries were dehydrated in a series of ethanol dilutions (50% to 95%), sputter-coated with gold (200 Å) and view in a Phillips scanning electron microscope as previously described (Buchanan et al, 1987).

ASSESSMENT OF IN VITRO RABBIT PLATELET ADHESION:

Blood was collected from each rabbit into sodium citrate (0.32% final concentration, w/v). The blood was centrifuged at 200 g for 12 minutes to prepare PRP. The PRP was transferred into a separate tube and the remaining blood was centrifuged at 1700 g for 30 minutes to obtain PPP which was used to dilute the PRP to a final concentration of 2.5 x 10^8 plt/ml. The PRP was incubated with 4 μCi ^{3}H-adenine/ml for 30 minutes at 22°C. Unlabelled adenine (0.12 mM final concentration: i.e. 100 fold excess of ^{3}H-adenine) was then added to block the uptake of any free ^{3}H-adenine onto the fibronectin-coated discs and to minimize the amount of ^{3}H-adenine released from the platelets during the adhesion assay. Thermanox™ plastic discs pre-coated with fibronectin were incubated with 500 μL ^{3}H-PRP for 30 minutes at 37°C in Costar 24-wells. The discs were removed from the ^{3}H-PRP, and rinsed 3 times in consecutive 50 ml beakers of HBSS+. The discs were placed into a liquid scintillation vial containing 10 ml ACS scintillation fluid, and their radioactivities were measured in a beta counter. The number of platelets adherent to each fibronectin-coated disc was then measured by determining by the ^{3}H-PRP specific activity and the disc radioactivity.

RABBIT VESSEL WALL/PLATELET FATTY ACID COMPOSITION:

The vessels walls segments were cleaned of their adventitia and placed in 4 ml of methanol. The segments were

ground in a mortar in the presence of liquid nitrogen, and then sonicated for 2 minutes in 10 ml of chloroform:methanol (2:1, v/v). Each sample was stored at -70°C and shipped to Nestec Research Centre, Switzerland for analysis. Platelets in citrate plasma (2.5 x 10^9/ml) were mixed with a equivolume of methanol and stored at -70°C. Total lipids were extracted according to the method of Christiansen (1975).

At Nestec, the lipids were then transesterified according to the method of Shehata et al (1970). They were dissolved in 1 ml of diethyl either: hexane (2:1, v:v), and diluted further with 1 ml of 2 M methanoic sodium methoxide. This mixture was sonicated for 10 minutes at 22°C and then washed with 1 ml distilled water. The fatty acid methyl esters were then extracted into hexane for gas chromatographic analysis.

Gas chromatography of the fatty acid methyl esters was performed by injecting the esters onto a fused silica pre-column (1 m x 0.53 mm) and column (27 m x 0.32 mm) coated with Carbowax 20 M under a hydrogen inlet pressure of 60 kPa. The oven temperature program was: 2 minutes at 80°C, an increase of 15°C/minute to 140°C, 140°C for 1 minute, and an increase of 4°C/minute to 220°C. The flame ionization detector was set to 320°C. Identification of peaks was made by comparison of retention times of samples with those of standards.

RABBIT VESSEL WALL 13-HODE ANALYSIS:

The levels of 13-HODE in the vessel wall were measured as follows. First, the vessel wall segments were placed into a silanized glass vial containing 2 ml of ice cold methanol: water (1:1) and diced into 1 mm^3 pieces. Thirty minutes later, the cell particulates and the aqueous methanol extract was transferred to a 15 ml silanized glass conical centrifuge tube and the sample processed for 13-HODE measurements as described under "ENDOTHELIAL CELL 13-HODE ANALYSIS".

RABBIT PLATELET 12-HETE ANALYSIS:

The levels of 12-HETE in rabbit platelets measured after the platelets were stimulated with 1 U/ml thrombin for 10 minutes at 37°C in silanized aggregometer tubes. An equal volume (1 ml) of ice-cold methanol was added to each platelet preparation and the sample was vortexed. The methanoic extract was transferred to a 4 ml silanized teflon-lined screw cap vial. The aggregometer tube was rinsed with another 2 ml of ice-cold methanol, which was combined with the first methanolic extract. The vial was sealed under nitrogen and stored at -70°C until analyzed as described under "ENDOTHELIAL CELL 13-HODE ANALYSIS'.

13-HODE COATING OF ARTIFICIAL GRAFTS:

Microvel Double Velour Hemashield grafts (6 mm I.D., Meadox Medicals, Inc., Oakland, N.J.) were cut into 300 mm^2

segments. The grafts were placed into Costar 24-well plates and covered with 1 ml 10% ethanol containing 0 to 100 μM [1-^{14}C]13-HODE ± an equimolar ratio of EDC. After a 0.5, 1, 4 or 18 hour incubation, the medium was removed and the grafts rinsed grafts 3 times with Tyrodes. The grafts were then incubated with 1 ml 0.35% Tyrodes albumin for 0 hours (surface bound 13-HODE) or for 1, 4 or 18 hours (non-surface bound 13-HODE) at 22°C. The Tyrodes albumin was removed and the grafts were rinsed with albumin-free Tyrodes, placed into scintillation vials, and hydrolysed for 12 hours at 22°C in 1 ml 0.1M NaOH. (In preliminary studies, we found that this hydrolysis step was necessary to release all bound fatty acids.) The hydrolyzed grafts were neutralized with 10 M H_3PO_4 and 10 ml ACS of scintillation fluid was then added to each vial. The amount of 13-HODE bound to the grafts was assessed by measuring ^{14}C radioactivity. In some experiments, radio-labelled linoleic acid was used in place of 13-HODE.

PLATELET ADHESION ONTO ARTIFICIAL GRAFTS:

Grafts, either uncoated or coated with [1-^{14}C]13-HODE (or linoleic acid), were rinsed with albumin-free Tyrodes and incubated with ^{3}H-platelets (200,000/μl) for 30 minutes at 37°C in a shaking water-bath. The grafts were removed and washed 3 times in 0.35% Tyrodes albumin and hydrolysed as described above. The number of adherent platelets was assessed by either counting their ^{3}H-radioactivity or by SEM.

PREPARATION OF ENDOTHELIAL CELL BASEMENT MEMBRANES:

The basement membrane underlying the endothelial cells was exposed according to one of the following methods as previously described (Aznar-Salatti et al, 1991):

1. Shear stress: Endothelial cell monolayer-coated discs were inserted into flat chambers and perfused with platelet-free reconstituted perfusate at a shear rate of either 1300 or 1600 sec^{-1} for 5 or 15 minutes. The discs were then removed from the chambers and washed with PBS.

2. Cellulose acetate stripping: Strips of cellulose acetate were laid flat on the endothelial cell monolayers for 2 seconds and then peeled off, thereby lifting off endothelial cells.

3. EDTA: Endothelial cell monolayer-coated discs were incubated with 1 ml 2% EDTA for 30 minutes at 37°C. The endothelial cells which lifted off were washed away with PBS.

4. NH_4OH: Endothelial cell monolayer-coated discs were incubated with 1 ml 0.1 N NH_4OH for 30 minutes at 37°C. The lyzed endothelial cells were washed away with PBS.

PERFUSION ADHESION ASSAY:

Plastic discs coated with the various basement membrane preparations were inserted into flat chambers according to Sakariassen et al (1987). Anticoagulated blood samples were then recirculated for 5 minutes at 37°C. Flow was maintained by a peristaltic pump and adjusted to obtain a

shear rate of 1300 sec^{-1}, similar to the shear rate in the microvasculature.

ASSESSMENT OF ENDOTHELIAL CELL BASEMENT MEMBRANE 13-HODE AND vWF LEVELS AND THEIR ADHESIVITY FOR PLATELETS:

Basement membranes were prepared from endothelial cell-coated discs using one of the methods described above. 13-HODE and vWF were measured in both the intact endothelial cell monolayer and in the various basement membrane preparations by either HPLC analysis, histoimmunoflourescence or an ELISA. Platelet adhesion onto either the intact endothelial cell monolayers or the different basement membrane preparations, was measured under both static and flow conditions (1300 sec^{-1}) (for methods see PLATELET/ENDOTHELIAL CELL ADHESION ASSAY and PERFUSION ADHESION ASSAY, respectively).

MORPHOMETRIC EVALUATION OF ENDOTHELIAL CELL BASEMENT MEMBRANE AND PLATELET INTERACTIONS:

The morphology of the adhered platelets and the % of exposed basement membrane was evaluated by 2 methods (Aznar-Salatti et al, 1990).

1. En face: After perfusion, the basement membrane-coated discs were removed from the flat chambers, rinsed in PBS, and then fixed in 0.5% glutaraldehyde in PBS for 2 hours at 4°C. The discs were then dehydrated with a graded series

of ethanol and stained with May-Grunwald and Giemsa. The percentage of exposed basement membrane and the degree of platelet interactions with the basement membrane were evaluated by means of a semi-automated method. The exposed basement membrane surface was expressed as the % of the total disc area devoid of endothelial cells. Profiles of single or grouped platelets were projected with an electromagnetic pen to an optical analysis system which integrated the areas and expressed them as a % of total basement membrane screened.

2. Cross-section: The second method involved embedding the dehydrated basement membrane-coated discs in JB-4 solution and allowing the solution to polymerize (Aznar-Salatti et al, 1990 and 1991). After polymerization, the basement membranes were peeled from the JB-4 polymer, leaving the perfused side of the disc firmly embedded in the JB-4 polymer. Three micron thick sections of the polymer embedded with the basement membrane, were stained with 1% Methylene Blue. The morphology of the adhered platelets was evaluated according to the criteria described by Baumgartner et al (1976). Platelets were classified as i) contact platelets (C), those that were attached but had not spread; ii) adhered platelets (Adh), those that had attached and spread on the basement membranes forming monolayers; and iii) aggregated platelets (Agg), those adherent platelets, forming multiple layers which exceeded 2.5 μm in height. Total surface area covered with platelets was calculated as (C) + (Adh) + (Agg).

ASSESSMENT OF INTERNAL MAMMARY ARTERY ADHESIVITY:

Segments of internal mammary arteries (IMAs) isolated from patients undergoing elective coronary artery bypass surgery, were obtained from Dr. S. Brister at the Hamilton General Hospital. Each IMA was cleaned of its adventitia, and then cut into 2 segments. One segment was processed for a 13-HODE measurement as described under "RABBIT VESSEL WALL 13-HODE ANALYSIS". The other segment was used to assess vessel wall adhesivity, according to the method of Buchanan et al, (1980, Figure 7A). Briefly, each segment was slit longitudinally and pinned onto a dissecting mat, endothelial-side up. A strip of cellulose acetate paper was laid flat on the endothelial cells, and then immediately removed, stripping the endothelial cells from the IMA. The de-endothelialized IMA was rinsed in HBSS+ and incubated at 37°C. IMA thrombogenecity was assessed by covering the lumenal surface of the de-endothelialized IMA with ~100 μl of a human ^{3}H-platelets for 30 minutes at 37°C (Figure 7B). The IMA segment was then washed free of any non-adherent platelets, by washing the IMAs in 3 consecutive 50 ml beakers of HBSS+. The washed IMA segment was pinned flat on a dissecting mat, and the adherent platelets were stripped from the vessel wall, using a piece of cellulose acetate in the same manner used to strip off the endothelial cells. The platelets bound to the cellulose acetate paper, were placed into a scintillation vial containing 1 ml NCS tissue solubilizer. After 1 hour, 10 ml

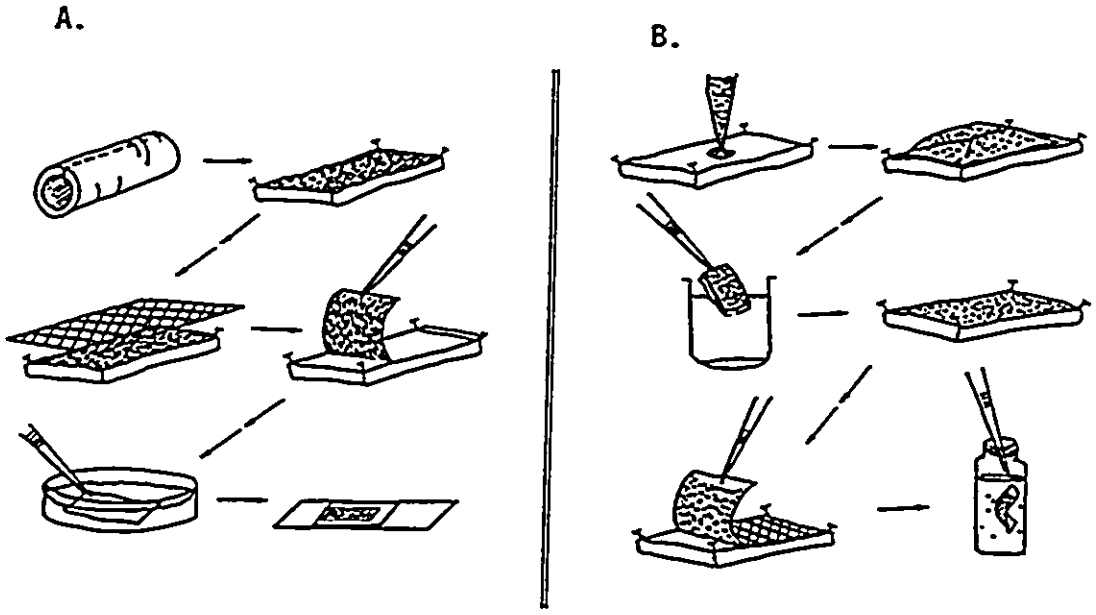


FIGURE 7. Internal mammary artery preparation. (A) Preparation of de-endothelialized internal mammary arteries and (B) assessment of internal mammary artery thrombogenecity.

ACS scintillation fluid was added and the sample counted to determine the amount of radioactivity.

The endothelial cells on the first cellulose acetate strip was made visible by incubating the cellulose acetate strip in 0.2% toluene blue for 5 minutes, and then rinsing the strip in HBSS. The surface area of the IMA segment could then be determined, using a image analyzer to measure the surface area of the stained endothelial cells.

ACKNOWLEDGEMENT OF TECHNICAL ASSISTANCE AND COLLABORATIONS:

The data obtained using these methods were obtained by myself in collaborations with a variety of doctors, graduate students and technicians and their efforts should be acknowledged. Unless otherwise stated, all these methods were performed at least once by myself. Endothelial cell and tumor cell cultures were routinely maintained by Mrs. L. Eltringham-Smith and Mrs. S. Gallo. 13-HODE measurements and maintenance of the HPLC were performed by Mrs. R. Nicholson. RIAs were performed by Mrs. L. Eltringham-Smith. Histoimmunofluorescence techniques were performed by Mrs. L. Eltringham-Smith and Mrs. C. Wale. Uptake of monohydroxy fatty acids by endothelial cells was performed by Drs. F. Hullin and K. Nakamura. Scanning electron microscopy was either performed by or under the supervision of Dr. M. Richardson. Rabbit vessel walls used in the basement membrane studies were isolated by Dr. E. Weber and Mrs. M. Falcone. Rabbit vessel

walls and platelet preparations used in the diet studies were prepared by Drs. M.C. Bertomeu and L. Cai. The fatty acid profile of rabbit vessel walls and platelets in the diet study were performed by Dr. G. Crozier and Ms. M. Fleith. Platelet adhesion to basement membranes under shear were performed exclusively by Drs. L. Almirall and E. Bastida. Static tumor cell adhesions were performed by Drs. L. Almirall, E. Bastida, M.C. Bertomeu and D. Lauri. Finally, IMA were exclusively isolated by Dr. S. Brister and their adhesivity for platelets and their 13-HODE levels were measured by Mrs. S. Gallo and Mrs. C. Wale, respectively.

EXPERIMENTAL DESIGN

1. To develop a cost effective automated HPLC extraction and quantification procedure for lipoxygenase metabolites, optimal solvent gradients, precolumns and analytical columns were assessed for their abilities to separate and/or extract the metabolites. Once the complete HPLC system was optimized, the efficiency of the entire automated HPLC extraction and quantification system was evaluated, using media with different protein content, sample volume, and pH.

2. To determine if intracellular linoleic acid was the source for 13-HODE synthesis, and if so, what was the source of the intracellular linoleic acid, the phospholipid and triglyceride pools of endothelial cells were labelled with ^{3}H-linoleic acid or ^{3}H-arachidonic acid. The turnover of the linoleic acid-rich triglycerides was measured in the absence and presence of exogenous db-cAMP. The db-cAMP was added to the endothelial cells either at the same time as the ^{3}H-fatty acids (co-treatment) or to endothelial cells previously labelled with the ^{3}H-fatty acids (post-treatment). Changes in the uptake and distribution of linoleic and arachidonic acid were measured, and related to 13-HODE and PGI_2 synthesis and endothelial cell adhesivity. Additional experiments

were also performed using dipyridamole to elevate endogenous cAMP levels without adding exogenous db-cAMP.

3. To determine if there is an inverse relationship between endogenous endothelial cell 13-HODE levels and endothelial cell adhesivity, endothelial cell monolayers were stimulated with a variety of agents for various lengths of time. Changes in endothelial cell endogenous 13-HODE and exogenous PGI_2 levels were correlated with changes in endothelial cell adhesivity for platelets, PMNs and tumor cells.

4. To determine whether exogenous 13-HODE or other monohydroxy lipoxygenase metabolites altered endothelial cell adhesivity, endothelial cell monolayers were incubated with varying concentrations of monohydroxy fatty acids (1 nM to 10 μM) for 1 hour. Half of the monolayers were used to assess monohydroxy uptake and the remaining monolayers were used to assess the effect of the monohydroxides on endothelial cell adhesivity for platelets, PMNs and tumor cells. Similar experiments were performed using stimulated endothelial cells, to determine whether the increased endothelial cell adhesivity observed following stimulation could be reduced with exogenous 13-HODE.

5. To determine if there is a physical relationship between 13-HODE and the VnR in endothelial cells under both resting and stimulated conditions, endothelial cell monolayers were incubated in the presence or absence of 10 ng/ml IL1 for 4 hours. The monolayers were fixed or fixed and permeabilized and the cellular localization of 13-HODE and the VnR in endothelial cells were visualized, using histoimmunofluorescence techniques.

6. To determine if 13-HODE is present in the basement membrane of endothelial cells, and if so, if 13-HODE influences both basement membrane and artificial graft adhesivity, 2 experimental approaches were taken. First, endothelial cell-derived basement membranes were prepared by different methods in vitro. The basement membranes were then either extracted for 13-HODE levels or characterized for their adhesivity towards platelets. Second, to determine if 13-HODE directly influence basement membrane adhesivity, the adhesivity of uncoated or [^{14}C]13-HODE-coated artificial grafts towards platelets were assessed.

7. To determine if 13-HODE is present in the basement membrane of rabbit vessel walls, and if so, if 13-HODE influences both basement membrane and artificial graft adhesivity, 2 experimental approaches were taken.

First, to determine whether drugs influenced vessel wall 13-HODE synthesis and basement membrane adhesivity, rabbit were given oral doses of either salicylate or dipyridamole for 1 week to 3 weeks. Rabbit carotid artery basement membranes were then exposed using an air-injury model and blood flow restored. Four hours later, segments of each carotid artery were then removed and processed for either 13-HODE levels or platelet accumulation.

Second, to determine whether diet influenced vessel wall and platelet lipoxygenase metabolites and adhesivity, rabbits were placed on defined fatty acid-supplemented diets. After 4 weeks, rabbit vessel wall fatty acid composition, 13-HODE and PGI_2 levels, and platelet fatty acid composition, and 12-HETE and TXA_2 levels were determined. In addition, rabbit vessel wall and platelet adhesivity were assessed individually.

8. To determine if there is an inverse relationship between endogenous PMN 13-HODE/HETE ratios and PMN adhesivity, PMNs were incubated in the presence and absence of exogenous 5-HETE and ETYA (a lipoxygenase inhibitor), and PMN adhesion to endothelial cells and endogenous PMN 13-HODE/5-HETE ratios were assessed.

   To determine if there is an inverse relationship between endogenous tumor cell 13-HODE/HETE ratios and tumor cell

adhesivity, tumor cell adhesivity and 13-HODE/HETE ratios of a number of tumor cell lines were assessed and correlated under resting and stimulated conditions. In addition, the levels of 13-HODE in A549 tumor cells, incubated with increasing amounts of dipyridamole, were assessed and correlated with A549 tumor cell adhesivity towards both endothelial cells and basement membranes.

9. To determine whether the inverse relationship between 13-HODE and vessel wall adhesivity was clinically relevant, de-endothelialized IMA 13-HODE levels and adhesivity towards platelets were measured and correlated in 6 patients undergoing coronary bypass surgery.

CHAPTER 4:

RESULTS

AUTOMATED HPLC EXTRACTION AND QUANTIFICATION PROCEDURE:

Preliminary studies were performed to determine what pre-columns, analytical columns and solvents were best suited to extract and quantitate the lipoxygenase metabolites. Using a C_{18} Sep-Pak extraction column, the recoveries of 500 ng of radio-labelled LTC_4 and 13-HODE from plasma were 79 ± 3% and 85 ± 2%, respectively. When 10% acetonitrile (v/v) was added to the plasma samples, their recoveries were increased by 9 ± 3% and 10 ± 2%, respectively. Acetonitrile also has a UV cutoff of 190 nm, having only minimal absorbance at 234 or 270 nm (the wavelengths used to detect the monohydroxides and the LTs, respectively). Therefore, pre-columns containing the same packing material as the Sep-Paks and HPLC gradients composed of acetonitrile were chosen for the development of an automated HPLC extraction system.

Separation of Lipoxygenase Metabolites: Analytical columns and pre-columns containing the same packing material as the Sep-Pak columns which were used for lipoxygenase analysis were the Nova-Pak analytical column and the μBondapak pre-column (Buchanan et al, 1985a). The resolve C_{18} analytical column and the resolve C_{18} pre-column were chosen for LT analysis since Borgeat et al (1984) found that these columns resulted in optimal retention and separation of the LTs.

Having chosen the columns, various acetonitrile gradients were then assessed for their ability to separate and detect the metabolites on the two analytical columns. When an acetonitrile gradient from 0 to 80% was used as the elution solvent, PGB_2 and the diHETEs eluted from the Nova-Pak column at ~40%, while the monohydroxides eluted off at ~60% acetonitrile. The level of sensitivity of detecting the metabolites was ~5 ng. This level of sensitivity was increased by 38 ± 3% by the addition of 2 mM H_3PO_4 (final concentration, pH 3.5). Thus, the solvents chosen for separating the monohydroxides were: Solvent A, acetonitrile: water:H_3PO_4 (15:85:0.01, v/v/v); Solvent B, acetonitrile:water: H_3PO_4 (80:20:0.01, v/v/v). The acetonitrile gradient selected for the separation of monohydroxy metabolites and diHETEs is shown in Table 5. A typical separation of 13-HODE, 15-HETE and 12-HETE is shown in Figure 8 (pg 108).

When the solvent pH was pH 3.5, none of the peptido-LTs (LTC_4, LTD_4, LTE_4) eluted off the resolve C_{18} analytical column. This was likely due to the retention of the positively charged LTs on the negatively charged column (Borgeat et al, 1984). Increasing the pH to 6.0 resulted in >90% recovery of the LTs. Thus, solvent C (acetonitrile: water:H_3PO_4, 40:60:0:03, pH 6.0) was used in the analysis of LTs. The solvent gradient selected for LT analysis is shown in Table 6.

TABLE 5. Solvent Gradient #1: Analysis of monohydroxy fatty acids, PGB_2, and diHETEs.

ANALYSIS TIME [min]	FLOW-RATE [ml/min]	SOLVENT %A	%B	RATE OF CHANGE
0.0 - 2.0	1.0	100	0	isocratic
2.0 - 2.5	1.5	50	50	linear
2.5 - 12.0	1.5	35	65	linear
12.0 - 18.0	1.5	35	65	isocratic
18.0 - 20.0	1.5	0	100	linear
20.0 - 30.0	2.5	0	100	convex
30.0 - 30.5	1.5	100	0	linear
30.5 - 35.0	1.0	100	0	delayed
40.0	Injection of the next sample.			

TABLE 6. Solvent Gradient #2: Analysis of LTs, PGB_2 and diHETEs.

ANALYSIS TIME [min]	FLOW-RATE [ml/min]	SOLVENT %A	%B	%C	RATE OF CHANGE
0.0 - 2.5	1.0	100	0	0	isocratic
2.5 - 3.0	2.0	50	50	0	linear
3.0 - 15.0	2.0	50	50	0	isocratic
15.0 - 15.5	4.0	0	100	0	linear
15.5 - 20.0	4.0	0	100	0	isocratic
20.0 - 20.5	2.0	100	0	0	linear
20.5 - 24.0	2.0	0	0	100	delayed
24.0 - 40.0	2.0	100	0	0	delayed
40.0 - 45.0	1.0	100	0	0	delayed
45.0	Injection of next sample.				

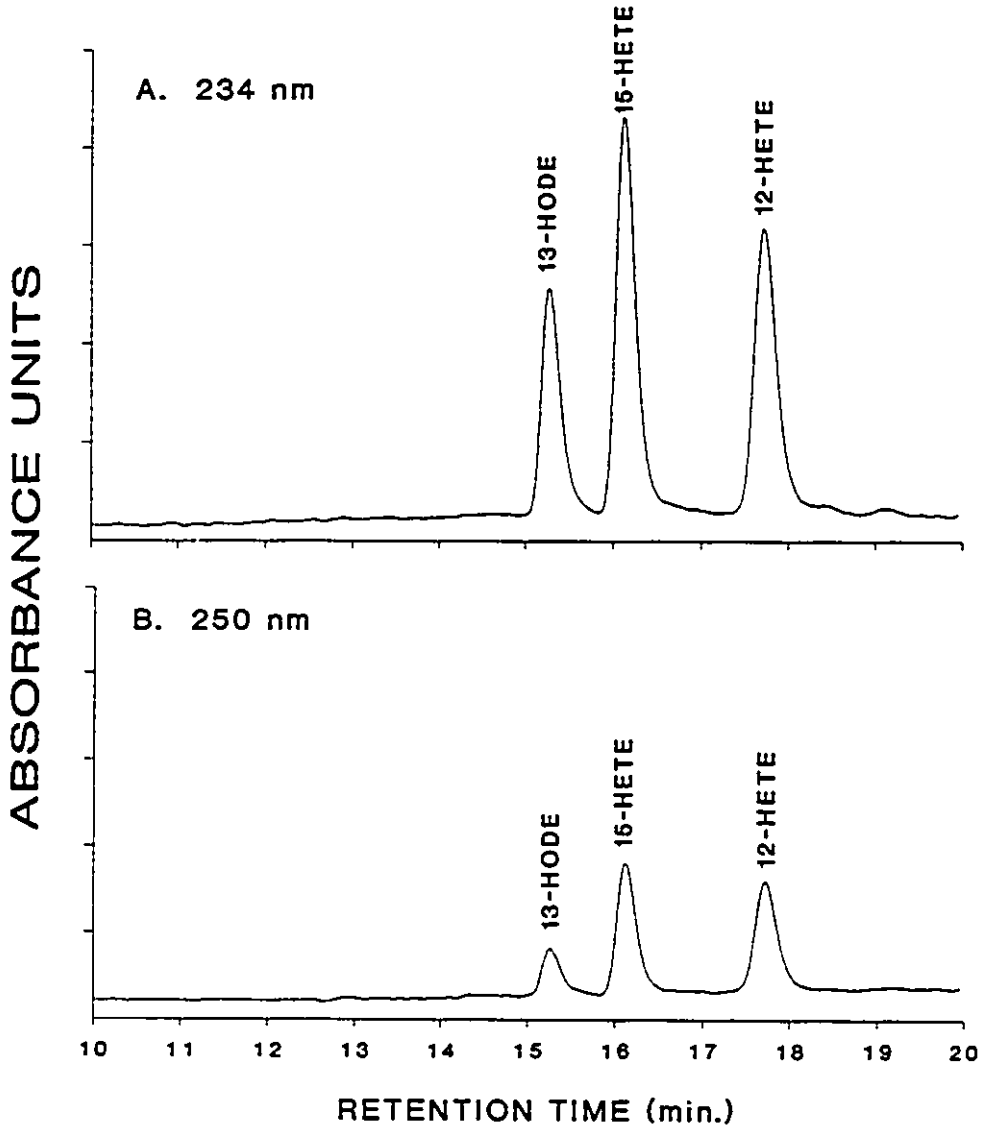


FIGURE 8. HPLC separation of 13-HODE, 15-HETE and 12-HETE, using a Nova-Pak C_{18} analytical column and solvent gradient #1. HPLC eluent was monitored simultaneously at (A) 234 nm and (B) 250 nm.

Effect of Acetonitrile Concentration on % Recovery: The efficiency of the automated extraction method was then evaluated by varying the acetonitrile concentrations in both the sample and the loading solvent. The recoveries of 13-HODE, 15-HETE and PGB_2 from HBSS, containing the same acetonitrile concentration as the loading solvent, are shown in Table 7.

TABLE 7. Effect of acetonitrile concentration on the recoveries of monohydroxy fatty acids and PGB_2.

Loading Acetonitrile Concentration (%)	n	Recovery*		
		13-HODE	15-HETE	PGB_2
40	6	96.5 ± 0.6	99.6 ± 0.7	< 0.1
20	3	100.4 ± 0.4	99.9 ± 0.3	95.9 ± 0.2
15	3	100.2 ± 0.1	99.5 ± 0.4	100.3 ± 0.1
5	3	104.1 ± 5.1	91.9 ± 3.0	109.8 ± 3.6

* Recovery is expressed as a percent of total fatty acid added to 500 µl HBSS. Data expressed as mean ± SEM, n = 3.

When a loading solvent of 40% acetonitrile was used, PGB_2 was not recovered (Table 7). When the acetonitrile concentration was lowered to 20% or 15%, there was a concentration-dependent increase in PGB_2 recovery (96% and 100%, respectively). The monohydroxy fatty acids were completely recovered at all these acetonitrile concentrations. However, lowering the acetonitrile concentration to 5%, resulted in poor qualitative recoveries of PGB_2 and the monohydroxides. Therefore, 15% acetonitrile was used as the loading solvent in all further studies.

Having established the columns, acetonitrile gradients and pH of the HPLC system, the extraction efficiency of the system was evaluated over a concentration range of monohydroxides (5 to 200 ng). As shown in Table 8, the recoveries of all metabolites exceeded 99% and were independent of the amount of metabolite present in each sample.

Effect of Protein Content, Sample Volume and pH on % Recovery: Having established that the HPLC system was efficient in extracting lipoxygenase metabolites from 500 µl of protein-free media, experiments were performed to determine whether the presence of protein or changing the injection volume influenced the recoveries of the lipoxygenase metabolites. Consistent with the previous data, >97% of each metabolite was recovered when 100 ng of a metabolite was dissolved in 2000 µl protein-free HBSS (Table 9, pg 112). In the presence of 0.35%

albumin, the recovery of each metabolites still exceeded 90%. Similar results to those obtained above were obtained when 100 ng of each metabolite was dissolved in either 500 or 1000 µl of media. For example, 99.3 ± 0.2% and 98.5 ± 0.5% of 13-HODE were recovered from 500 µl of HBSS in the absence and presence of albumin, respectively.

TABLE 8. Recovery of monohydroxy fatty acids from HBSS over a metabolite concentration range*.

	Recovery					
Metabolite	5 ng	10 ng	50 ng	100 ng	150 ng	200 ng
13-HOTE	99.7 ± 0.1	100.4 ± 0.3	100.1 ± 0.2	99.9 ± 0.2	100.2 ± 0.2	100.4 ± 0.3
13-HODE	99.5 ± 0.3	99.5 ± 0.4	100.0 ± 0.4	99.3 ± 0.5	99.9 ± 0.7	99.1 ± 0.2
12-HETE	99.1 ± 0.9	100.7 ± 1.1	101.5 ± 0.3	101.1 ± 1.0	101.7 ± 1.0	100.1 ± 0.6
15-HEPE	100.1 ± 0.6	100.7 ± 0.8	100.9 ± 1.3	101.0 ± 0.9	100.8 ± 0.7	100.1 ± 0.3
15-HETE	99.0 ± 0.7	100.4 ± 1.3	99.4 ± 1.2	98.9 ± 0.7	99.5 ± 1.1	99.6 ± 0.8
15-HETE:3	99.8 ± 0.5	99.5 ± 0.4	99.2 ± 0.5	99.9 ± 0.6	99.7 ± 0.6	99.6 ± 0.4
Mean	99.5 ± 0.2	100.2 ± 0.3	100.2 ± 0.3	100.0 ± 0.3	100.3 ± 0.2	99.8 ± 0.3

* 500 µl of HBSS containing 5 to 200 ng of each metabolite was injected. Data are expressed as mean ± SEM, n = 3.

TABLE 9. Effect of 0.35% albumin on the recoveries of lipoxygenase metabolites.

Metabolite*	Recovery	
0.35% BSA	-	+
13-HOTE[a]	99.4 ± 0.6	91.2 ± 0.9
13-HODE[a]	102.2 ± 0.9	95.6 ± 1.3
12-HETE[a]	97.8 ± 0.7	93.4 ± 0.8
PGB_2[a]	99.8 ± 0.6	89.2 ± 0.7
LTC_4[b]	99.4 ± 1.4	95.6 ± 0.6
LTD_4[b]	98.9 ± 1.8	100.2 ± 0.7
PGB_2[b]	99.8 ± 1.6	93.2 ± 0.4
diHETE[b]	99.1 ± 0.7	91.9 ± 0.4

* 100 ng of each metabolite was dissolved in 2000 µl HBSS, in the presence or absence of 0.35% albumin and injected into the HPLC. Data are expressed as mean ± SEM, n = 3. [a] Gradient #1 used; [b] Gradient #2 used.

As the LTs are highly labile, the influence of protein and pH on the recovery and stability of the LTs was also examined. At a pH of 7.35, there was ≤1% degradation of PGB_2, the diHETEs or LTD_4 in protein-free HBSS over 8 hours. Reducing the pH of the sample to 4.0, did not alter the stability of PGB_2 or of the diHETEs, but increased the degradation of LTD_4 to 5%. LTC_4 was labile at both pH values, degrading 5% at pH 7.35 and 20% at pH 4.0 over 8 hours. In contrast to the detrimental effect of acidification on the stability of LTC_4 and LTD_4, acidification of the medium resulted in the beneficial effects of increased peak sharpness (Figure 9).

When albumin was added to the samples at a final concentration of 0.35%, degradation of LTC_4 and LTD_4 at both pH values was accelerated by >15 ± 4%. There was no effect of albumin on the stability of PGB_2 or the diHETEs at pH 7.35. However, the stabilities and recoveries of PGB_2 and the diHETEs could not be assessed at pH 4.0, since acidification led to the retention of proteins which completely masked the diHETE region of the chromatogram (Figure 9B). These observations indicate that acidification should be avoided as it led to increased degradation of LTs. Finally, the retention of unwanted material did occur in unacidified albumin-containing medium. This only occurred after a total of 10-14 ml of medium had been injected, thereby allowing for the analysis of at least 12 samples before the pre-column had to be changed.

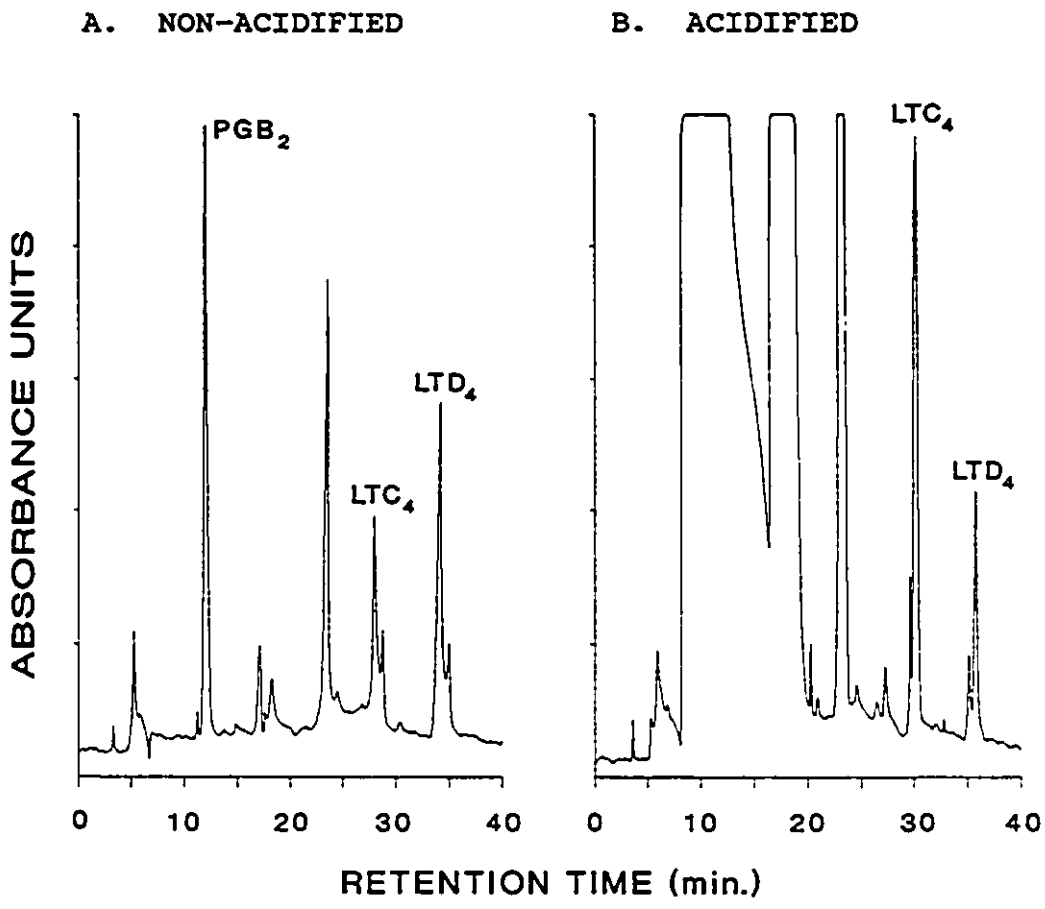


FIGURE 9. Automated HPLC extraction and separation of PGB_2, LTC_4 and LTD_4 using solvent gradient #2. Samples were suspended in HBSS (pH 7.35) containing 0.35% albumin and injected either (A) directly onto the HPLC or (B) injected after acidification. The UV absorbing material which eluted off the column between 7.5 and 19 minutes in 9B was determined to be albumin by SDS electrophoresis.

ENDOGENOUS SOURCE OF LINOLEIC ACID IN ENDOTHELIAL CELLS FOR 13-HODE SYNTHESIS:

Effect of Exogenous Linoleic Acid on Endothelial Cell Endogenous 13-HODE Synthesis: Experiments were performed to determine whether increasing the endogenous linoleic acid pool, which was readily exchangeable with exogenous linoleic acid, increased the intracellular levels of 13-HODE in endothelial cells. To do this, endothelial cell monolayers were incubated for 10 minutes with increasing concentrations of either linoleic or arachidonic acid (0 to 50 μM). The medium was then removed and both the endothelial cell monolayers and their medium were analyzed for 13-HODE and 15-HETE (Figure 10). Under resting conditions, endothelial cells produced 17.3 ± 0.2 ng 13-HODE/10^6 cell, all of which was cell-associated (Figure 10A and 10B, 0 μM). There was no 15-HETE detected either in the cells or the medium.

Increasing the concentration of exogenous linoleic acid to 10 μM resulted in decreased 13-HODE synthesis, while increasing the exogenous linoleic acid to 25 μM, resulted in increased cell-associated (intracellular) 13-HODE levels (Figure 10A). Again, there was no extracellular 13-HODE nor 15-HETE detected. When the endothelial cells were incubated with increasing concentrations of arachidonic acid, there was an initial increase followed by a marked decrease in endogenous 13-HODE levels (Figure 10A). The decreased

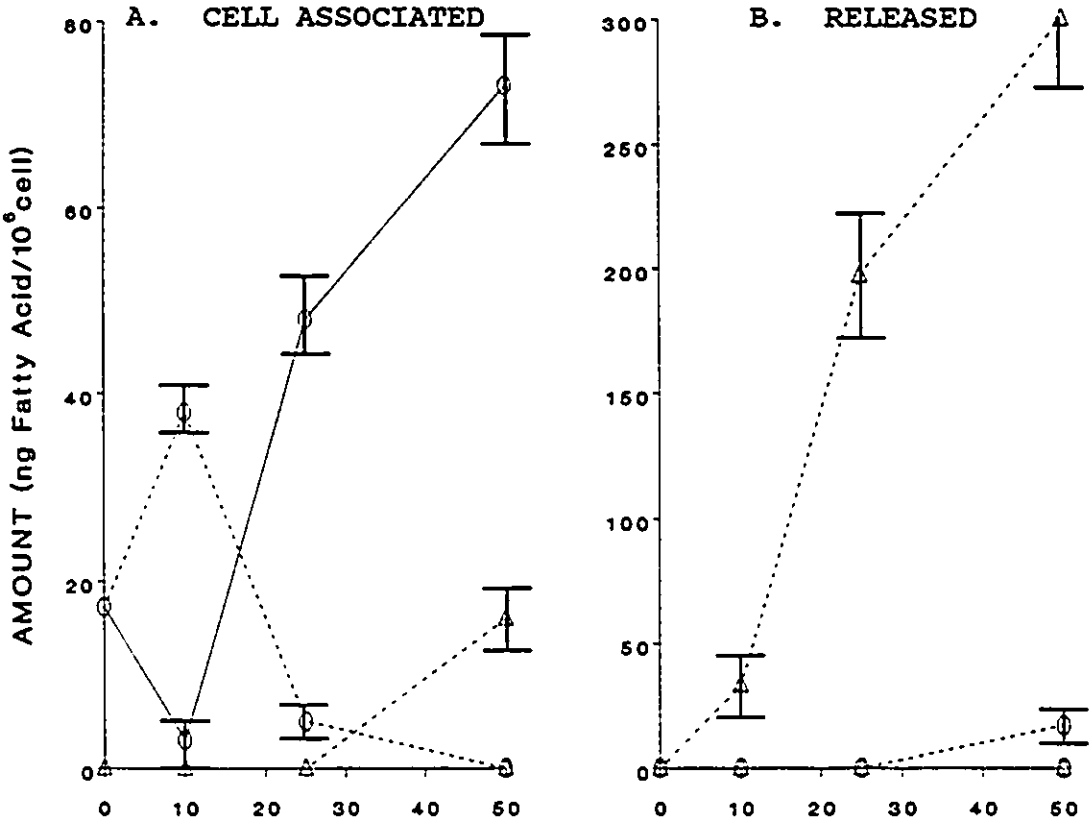


FIGURE 10. Production of 13-HODE (0) and 15-HETE (Δ) by endothelial cells after incubation with either linoleic (———) or arachidonic acid (- - - - -). Following the incubation, the media was removed and the (A) cell-associated and (B) released 13-HODE and 15-HETE levels were measured.

endogenous 13-HODE levels were associated with marked and a rapid increase in exogenous 15-HETE levels (Figure 10B). At 50 μM arachidonic acid, exogenous 13-HODE was detected for the first time.

These data indicate that i) endothelial cells synthesize both 13-HODE and 15-HETE, and ii) 13-HODE remains within the cell while 15-HETE is released from the cell. These results also suggest that 13-HODE and 15-HETE synthesis is dependent upon the relative amounts of free linoleic and arachidonic acid. Therefore, experiments were performed to determine whether varying the ratio of linoleate: arachidonate influenced 13-HODE synthesis.

Consistent with the previous study, resting endothelial cells produced 15 ng 13-HODE/10^6 cell and no 15-HETE (Table 10). When endothelial cells were incubated with 10 μM linoleic acid, 13-HODE levels decreased to 4 ng/10^6 cell. When the linoleate:arachidonate ratio was decreased to 5:5, the levels of 13-HODE increased to 75 ng/10^6 cell and 11 ng 15-HETE/10^6 cell was detected. Decreasing the linoleate: arachidonate ratio even further resulted in marked decreases in 13-HODE levels and marked increases in 15-HETE levels. In these experiments there was no extracellular 13-HODE or 15-HETE detected. Thus, these results demonstrate that endothelial cells preferentially metabolize linoleic acid by the lipoxygenase pathway at low fatty acid concentrations. However, as the levels of 13-HODE do not increase in a dose-

TABLE 10. Endothelial cell production of 13-HODE and 15-HETE in the presence of varying ratios of linoleic and arachidonic acid.

	LINOLEATE:ARACHIDONATE RATIO (μM:μM)						
	0	10.0	7.5	5.0	2.5	0	[18:2]
METABOLITE	0	0	2.5	5.0	7.5	10.0	[20:4]
15-HETE	0	0	0	11 ±2	43 ±6	66 ±5	
13-HODE	15 ±1	4 ±2	12 ±3	75 ±6	6 ±2	0	

All incubations were performed for 10 minutes at a final fatty acid concentration of 10 μM in the presence of 1 mM Ca^{++}. Data expressed as mean ng/10^6 endothelial cell, n = 3.

related manner, it seemed unlikely that the endogenous fatty acid pool that was readily exchangeable with exogenous linoleic acid was the fatty acid pool used for endogenous 13-HODE synthesis. Therefore, experiments were performed to determine what other fatty acid pool was the endogenous source of linoleic acid for 13-HODE synthesis.

Triglyceride Turnover and Endothelial Cell 13-HODE Synthesis: Experiments were performed to determine whether increasing the turnover of endothelial cell triglycerides increased the availability of free linoleic acid for 13-HODE synthesis. The basis for the rationale that the triglyceride pool was the endogenous source of linoleic acid for 13-HODE synthesis was i) endothelial cell triglycerides are rich in linoleic acid (Lagarde et al, 1984), and ii) the triglyceride pool in endothelial cells is rapidly turned over under resting conditions (Denning et al, 1983; Figard et al, 1986). The experiments were performed in the presence and absence of db-cAMP, as db-cAMP has been reported to increase vessel wall triglyceride turnover (Tertov et al, 1986a).

Complete uptake of arachidonic acid by endothelial cells from 20% human sera was achieved by 18 hours (Table 11). On the other hand, only 50% of linoleic acid was taken up within 18 hours. More arachidonic and linoleic acid were incorporated into the phospholipids than in the triglycerides. The majority of the remaining ^{3}H-fatty acids were associated with the free fatty acid pool, as less than 5% were associated with the neutral lipids (methyl and cholesterol esters). Increasing the incubation time from 18 to 36 hours resulted in > 95% of the linoleic acid being incorporated into the endothelial cells. However, the distribution of linoleic acid at 18 and 36 hours were essentially the same (data not shown). Since the distribution of linoleic acid in endothelial cells

at 18 hours was the same as at 36 hours and essentially all of the arachidonic acid was incorporated within the 18 hours, all further studies were performed with incubation times of 18 hours.

Incubating endothelial cell monolayers for 18 hours in the presence and absence of db-cAMP had was no effect on either the % uptake or the distribution of the fatty acids in

TABLE 11. Uptake and distribution of ^{3}H-18:2 and ^{3}H-20:4 in the endothelial cell over time.

	UPTAKE		DISTRIBUTION			
	% OF TOTAL		PHOSPHOLIPIDS		TRIGLYCERIDES	
TIME	18:2	20:4	18:2	20:4	18:2	20:4
30'	0.5±0.1	2.2±0.3	38.5±5.1	29.7±3.1	3.1±0.9	3.9±0.3
4 hr	2.7±0.2	7.7±0.5	36.5±4.2	64.4±2.8	3.3±1.2	9.9±1.6
8 hr	8.2±1.4	22.7±2.6	44.1±1.1	63.1±2.0	8.7±3.1	10.8±1.7
18 hr	46.9±2.8	97.2±5.5	49.3±1.6	63.8±0.4	12.4±4.9	12.6±4.4

Uptake is expressed as a % of total ^{3}H-fatty acid added which is associated with the cell. Distribution is expressed as a % of the total ^{3}H recovered from the TLC plate. Data are expressed as mean ± SEM, n = 3.

TABLE 12. Uptake and distribution of ^{3}H-18:2 and ^{3}H-20:4 in phospholipids and triglycerides of endothelial cells: Effect of co-treatment with db-cAMP.

TREATMENT	UPTAKE		% DISTRIBUTION			
db-cAMP	% OF TOTAL		PHOSPHOLIPIDS		TRIGLYCERIDES	
(μM)	18:2	20:4	18:2	20:4	18:2	20:4
0	45.9 ±1.9	95.7 ±1.4	50.9 ±1.1	64.9 ±0.5	14.4 ±1.2	14.5 ±0.8
10	46.1 ±1.2	94.2 ±2.0	50.3 ±3.2	57.8 ±2.5	10.6 ±1.1*	15.0 ±1.5
100	46.3 ±1.3	94.9 ±0.8	50.3 ±2.4	63.5 ±1.8	9.5 ±1.4*	14.2 ±0.8

Uptake is expressed as a % of total ^{3}H fatty acids added which was associated with the cell. Distribution is expressed as % total ^{3}H recovered from the TLC plate. Data are expressed as mean ± SEM, n = 8, *$p<0.01$.

the phospholipids, or the distribution of arachidonic acid in the triglycerides (Table 12). In contrast, there was a db-cAMP dose-dependent decrease in the amount of linoleic acid associated with the triglycerides (Table 12). This decrease could have been due to either i) a decrease in uptake, or ii) an increase in release, i.e. hydrolysis. To determine which explanation was more likely, the effect of db-cAMP on the distribution of ^{3}H-18:2 and ^{3}H-20:4 in the phospholipid and triglyceride pools were determined using endothelial cells which were pre-labelled with the ^{3}H-fatty acids.

When the ^{3}H-fatty acid pre-labelled endothelial cells were incubated with db-cAMP, again there was a reduction in the amount of linoleic acid associated with the triglycerides (Table 13). The amount of linoleic acid in the phospholipids and the amount of arachidonic acid in either fatty acid pool did not change under the same conditions. These data indicate that the decreased amount of linoleic acid associated with the triglycerides when the cAMP level was increased, was likely due to a specific increase in the hydrolysis of linoleic acid from the triglycerides (Table 13).

The increased linoleic acid turnover in the triglycerides in the presence of db-cAMP was paralleled by an increase in 13-HODE synthesis (Table 14). The correlation between linoleic acid triglyceride turnover and 13-HODE synthesis was $r = 0.968$, $p < 0.01$. PGI_2 production was not affected by db-cAMP treatment, consistent with the lack of any effect of db-cAMP on arachidonic acid turnover.

Pre-treatment of endothelial cells with db-cAMP for 18 hours also increased the thromboresistance of the endothelial cells as shown by the decrease in platelet adhesion (Table 14). This latter effect was significantly correlated with 13-HODE synthesis, $r = 0.941$, $p < 0.025$.

There was no correlation between platelet adhesion and PGI_2 production. Finally, when endothelial cells were treated with IL1 for 4 hours, 13-HODE levels markedly decreased and platelet adhesion markedly increased. This increase in

TABLE 13. ^{3}H-18:2 and ^{3}H-20:4 turnover in the phospholipid and triglyceride pools of endothelial cells. Effect of post-treatment with db-cAMP.

TREATMENT	% DISTRIBUTION[a]			
db-cAMP	PHOSPHOLIPIDS		TRIGLYCERIDES	
(μM)	18:2	20:4	18:2	20:4
0	51.5 ± 3.9	57.0 ± 4.9	12.6 ± 0.9	12.7 ± 0.7
100	47.5 ± 1.8	54.3 ± 6.6	8.3 ± 0.8[ú]	12.3 ± 1.2

[a]% Distributions are expressed as a % of the total ^{3}H recovered from the TLC plate, mean ± SEM, n = 4, [ú]$p < 0.01$.

TABLE 14. Effect of db-cAMP on endothelial cell 13-HODE and PGI_2 synthesis and endothelial cell adhesivity towards platelets.

Treatment	13-HODE (ng/10^6 ECs)	PGI_2 (ng/10^6 ECs/60')	Platelet Adhesion (#/cm^2 ECs)
nil	15 ± 2	4 ± 1	7.6 ± 0.5 x10^5
10 μM db-cAMP	132 ± 13	4 ± 1	1.0 ± 0.0 x10^5
100 μM db-cAMP	228 ± 13	3 ± 1	< 1000
IL1	ND (<1)	17 ± 8	29.1 ± 3.0 x10^5

EC = endothelial cell. 13-HODE α platelet adhesion, r = -0.9406, p< 0.025; PGI_2 α platelet adhesion, r= 0.6003, not significant. Data are expressed as mean ± SEM, n = 4.

platelet adhesion occurred at a time when PGI_2 production was elevated, suggesting that PGI_2 does not influence platelet adhesion to the endothelium (Table 14).

The concentrations of db-cAMP used in these experiments did not alter endothelial cell: morphology (as assessed by light microscopy), cell number, total protein content, total phospholipid content or total triglyceride content (Table 15). Thus, it was concluded from these experiments that under resting conditions, the triglycerides are the source of linoleic acid for endothelial cell 13-HODE synthesis, the turnover of which is dependent upon endothelial cell cAMP levels.

TABLE 15. Effect of db-cAMP treatment on endothelial cell total protein, phospholipid and triglyceride content, and on endothelial cell number.

	db-cAMP TREATMENT	
	0 μM	100 μM
CELL NUMBER (x10^5/disc)	1.25 ± 0.04	1.24 ± 0.03
PROTEIN (μg)	686 ± 12	727 ± 18
PHOSPHOLIPID (nmoles)	103 ± 6.5	105 ± 4.6
TRIGLYCERIDE* (nmoles)	13.5 ± 2.0	12.8 ± 1.4

Protein, phospholipid and triglyceride content are expressed per 10^6 endothelial cell. * Average molecular weight of a triglyceride was assumed to be 306 daltons (Lagarde et al, 1984). Data are expressed as mean ± SEM, n = 6.

Effect of Stimulation on Endothelial Cell 13-HODE Synthesis, Fatty Acid Distribution, and PGI_2 Production: Endothelial cell monolayers were pre-labelled with ^3H-18:2 and ^3H-20:4 for 18 hours. The medium was then removed and the monolayer was stimulated with either 0.1 U/ml thrombin or 10 μM A23187. Ten minutes later, the fatty acid distribution and 13-HODE and PGI_2 levels were measured.

Under resting conditions, the free fatty acid and the triglyceride pool was rich in linoleic acid, and the phospholipid pool was rich in arachidonic acid (Table 16). When the endothelial cells were stimulated for 10 minutes with either thrombin or A23187, the distribution of linoleic acid between the various lipid pools did not change. In contrast, there was a 2.4 fold increase ($p < 0.01$) in free cellular arachidonate in the stimulated endothelial cells. This increase was associated with decreased levels of arachidonate associated with the phospholipid pool (Table 16).

Endothelial cell stimulation was also associated with a marked decrease in 13-HODE levels and a marked increase in PGI_2 release, $p < 0.01$ (Table 16). The increase in PGI_2 synthesis can be explained, in part, by the release of arachidonic acid from the phospholipids which is quickly metabolized by cyclo-oxygenase and prostacyclin synthetase. However, it still remains unclear as to why the levels of 13-HODE decrease so rapidly following endothelial cell stimulation. It may represent an esterification of 13-HODE

into a fatty acid pool, or degradation of 13-HODE into either a ketone, an epoxy, or a dihydroxy fatty acid.

TABLE 16. Effect of thrombin and calcium ionophore A23187 stimulation of endothelial cells on the cellular distribution of linoleic and arachidonic acid, and on 13-HODE and PGI_2 synthesis.

	% DISTRIBUTION							
	LINOLEIC ACID[a]			ARACHIDONIC ACID			13-HODE	PGI_2
STIMULUS	PHO	FFA	TRI	PHO	FFA	TRI	ng/10^6 cells	ng/ml
Control	79.7 ±1.8	4.6 ±1.5	10.0 ±1.0	86.2 ±0.8	0.9 ±0.2	7.9 ±0.4	13.2 ± 2.1	239 ± 33
Thrombin 0.1 U/ml	77.7 ±0.4	4.4 ±0.9	11.5 ±1.2	84.3 ±1.9	2.2* ±0.3	8.5 ±1.4	2.2* ± 0.9	1331* ± 123
A23187 10 μM	80.2 ±1.4	3.4 ±0.4	10.2 ±1.8	83.8 ±2.8	2.2* ±0.2	8.2 ±1.9	2.1* ± 1.0	2461* ± 423

[a] Endothelial cells were pre-labelled with either linoleic or arachidonic acid, washed and stimulated either with or without 0.1 U/ml thrombin or 10 μM A23187 for 10 minutes. Fatty acid distribution is expressed as a % of total ^3H recovered. Endothelial cell endogenous 13-HODE levels were measured, while PGI_2 levels were measured in the Tyrodes. Data are expressed as mean ± SEM, n = 6; *$p < 0.01$ versus control. Abbreviations: phospholipids, PHO; free fatty acids, FFA; triglycerides, TRI.

EFFECT OF ENDOTHELIAL CELL STIMULATION ON 13-HODE LEVELS AND ADHESIVITY:

Effect of Endothelial Cell Stimulation on 13-HODE Synthesis and Platelet Adhesion: Under resting conditions, endothelial cell monolayers produced 12.1 ± 2.1 ng 13-HODE/10^6 cell and 7.9 ± 0.7 platelets adhered to their surface monolayer (Table 17). Stimulation of the endothelial cells with thrombin led to a 81 ± 7% decrease in endothelial cell 13-HODE levels and a 237 ± 16% increase in platelet adhesion, $p < 0.001$.

TABLE 17. Effect of thrombin and calcium ionophore A23187 stimulation on endothelial cell 13-HODE synthesis and subsequent platelet adhesion.

	13-HODE ng/10^6 cell	PLT. ADH. x 10^3/mm^2
Control	12.1 ± 2.1	7.9 ± 0.7
Thrombin (1 U/ml)	2.3 ± 0.8*	18.7 ± 1.3*
A23187 (10 μM)	1.9 ± 0.9*	19.0 ± 1.5*

Data are expressed as mean ± SEM, n = 4; *$p < 0.001$.

Similarly, stimulating endothelial cells with 10 μM calcium ionophore A23187 resulted in decreased endothelial cell 13-HODE levels and increased platelet adhesion to the endothelial cell monolayers. In these experiments, both cell-associated (endogenous) and cell-released (media) 13-HODE levels were measured. Consistent with previous results, no 13-HODE was detected in the medium.

When endothelial cells were stimulated with IL1 for either 10 minutes or 4 hours, the levels of 13-HODE decreased 22 ± 3% and a 55 ± 4%, respectively (Table 18). These decreases were associated with a time-related increase in endothelial cell adhesivity for platelets. Thus, platelet adhesion increased from 25.6 ± 13.3% to 72.1 ± 10.7% following a 10 minute to a 4 hour stimulation with IL1, $p < 0.001$ (Table 18). Endothelial cell adhesivity was still enhanced 24 hours later (149 ± 2%, $p < 0.001$).

The enhanced adhesion of platelets to the IL1 stimulated endothelial cells appeared to be due to an effect of IL1 on the endothelial cells and not on the platelets (Table 19). When platelets were stimulated with IL1 for increasing lengths of time and then incubated with the endothelial cells for 30 minutes (still in the presence of IL1), no further increased in platelet adhesion was observed (Table 19).

TABLE 18. Platelet adhesion to IL1-stimulated endothelial cell monolayers.

IL1 PRE-INCUBATION TIME (min.)	PLATELET ADHESION
0	100.0 ± 3.7
10	125.6 ± 13.3
30	126.3 ± 2.3
60	132.3 ± 7.7*
120	162.2 ± 7.3*
240	172.1 ± 10.7*

Endothelial cell monolayers were incubated with 10 ng/ml IL1 for various lengths of time. The medium was removed and the adhesivity of the endothelial cell monolayers for platelets was then measured over 30 minutes. Data are expressed as mean ± SEM, n = 6; *$p < 0.001$.

TABLE 19. Effect of IL1 stimulation of platelets on platelet adhesion to endothelial cells.

IL1 PRE-INCUBATION TIME (min.)	PLATELET ADHESION
0	100.0 ± 6.4
10	103.9 ± 5.6
30	102.1 ± 2.3
60	109.4 ± 3.5
120	104.4 ± 7.5

Platelets were stimulated with 10 ng/ml IL1 for various lengths of time and then incubated with endothelial cell monolayers for 30 minutes, still in the presence of IL1. Data are expressed as a mean % of control ± SEM, n = 3.

Effect of Endothelial Cell Stimulation on 13-HODE Synthesis and PMN Adhesion: Having determined that IL1 stimulation of endothelial cells resulted in decreased 13-HODE synthesis and increased platelet adhesion, additional IL1 stimulation experiments were performed to determine wether endothelial cell 13-HODE levels also correlated with PMN adhesion. In addition, other cytokines, such as γIFN and TNF, were also tested for their ability to effect both endothelial cell 13-HODE levels and PMN adhesion. Furthermore, to determine if cytokine stimulation of endothelial cells also effect PGI_2 production, the amount of PGI_2 released into the medium over 4 hours was determined by measuring the amount of 6-keto-$PGF_{1\alpha}$ present.

When the endothelial cell monolayers were incubated with γIFN for 4 hours, endothelial cell 13-HODE levels were not significantly affected (Table 20). In contrast, a 4 hour stimulation with either TNF or IL1 decreased endothelial cell 13-HODE levels (45.9 ± 2.1% and 32.7 ± 0.9% respectively, $p < 0.001$).

The decreases in 13-HODE levels were paralleled by corresponding increases in PGI_2 production, $r = -0.900$, $p < 0.025$ (Table 20). Furthermore, the decreases in endothelial cell 13-HODE levels following TNF or IL1 stimulation, were associated with increased endothelial cell adhesivity for PMNs, whereas γIFN had no effect (Table 20).

TABLE 20. Effect of γIFN, IL1 and TNF stimulation on endothelial cell 13-HODE and PGI_2 levels, and endothelial cell adhesivity for PMNs.

STIMULUS	13-HODE ng/10^6 cell	PGI_2 (pg/ml/60')	PMN ADHESION (10^3/mm^2)
Control	18.0 ± 0.5	606 ± 39	2.9 ± 0.3
γIFN	17.7 ± 0.8	513 ± 19	3.3 ± 0.3
TNF	10.5 ± 0.4*	910 ± 31*	4.0 ± 0.2*
IL1	11.2 ± 0.2*	1099 ± 50*	8.4 ± 0.3*

* Significantly different from the control, $p<0.01$. Data expressed as mean ± SEM, n = 6.

Effect of Endothelial Cell Stimulation on 13-HODE Synthesis and Tumor Cell Adhesion: Decreasing 13-HODE levels in endothelial cell was also associated with increased tumor cell adhesion. Thus, when endothelial cells produced 34 ± 4 ng 13-HODE/10^6 cells, 313 ± 39 A549 tumor cells adhered/mm^2 endothelial cell monolayer (Table 21). When the endothelial cells were stimulated with 0.1 μM fMLP for 10 minutes, endothelial cell 13-HODE levels significantly decreased by 61% (13 ± 4 ng/10^6 cells, $p < 0.01$), while increasing the fMLP

concentration to 1 μM, abolished 13-HODE synthesis (Table 21). The dose-related decrease in endothelial cell 13-HODE levels were associated with a dose-related increase in A549 tumor cell adhesion to the endothelial cells, $p < 0.01$ (Table 21).

Similarly, B16F10 tumor cell adhesion to endothelial cell monolayers was enhanced when 13-HODE levels were reduced following endothelial cell cytokine stimulation (Table 22). When ^{125}Iudr B16F10 tumor cells were incubated with resting endothelial cells, 1197 ± 114 B16F10 cells adhered/monolayer. When the endothelial cell monolayers were exposed to IL1 for 4 hours, B16F10 cell adhesion increased to 4184 ± 209 cells/monolayer, $p < 0.01$.

TABLE 21. Effect of fMLP stimulation on endothelial cell 13-HODE levels and subsequent A549 tumor cell adhesion.

	fMLP TREATMENT (μM)		
	0	0.1	1.0
13-HODE (ng/10^6 EC)	33 ± 4	13 ± 4*	< 1*
A549 Adhesion (cells/mm^2)	313 ± 39	432 ± 52*	759 ± 59*

Data are expressed as mean ± SEM, n = 6; *$p < 0.01$.

TABLE 22. Effects of IL1 on endothelial cell 13-HODE level and adhesivity for B16F10 cells ± GRGDS or GRGES peptide.

	TC/EC ADHESION[a] ($\times 10^3$/monolayer)		EC 13-HODE (ng/10^6 cell)	
	- IL1	+ IL1	- IL1	+ IL1
ECs	1.2 ± 0.1	4.2 ± 0.2**	15 ± 2	< 1**
EC + GRGDS	1.1 ± 0.3	1.4 ± 0.4*	-	-
EC + GRGES	1.4 ± 0.2	4.0 ± 0.6**	-	-

[a]5 x 10^4 B16F10 cells (TC) were added to each endothelial cell (EC) monolayer ± 10 ng/mL of IL1. Each peptide was added in a concentration of 300 μg/mL, 30 seconds before adding the B16F10 cells. Data are expressed as mean ± SEM, n = 5 experiments performed in triplicate. *$p < 0.01$ versus no peptide. **$p < 0.01$ versus IL1 stimulation. Peptide abbreviations used are GRGDS, gly-arg-gly-asp-ser; GRGES, gly-arg-gly-glu-ser.

To determine whether enhanced tumor cell adhesion was mediated by increased integrin receptor expression on the endothelial cell surface, endothelial cell monolayers were incubated with an RGD-containing peptide after IL1 stimulation, and 30 seconds before adding the tumor cells. Increased IL1-induced tumor cell adhesion to the endothelial

cell monolayers was blocked by the GRGDS peptide (gly-arg-gly-asp-ser), while baseline tumor cell adhesion was not (Table 22). In contrast, neither basal nor IL1-induced tumor cell/endothelial cell adhesion was blocked by the control peptide, GRGES (gly-arg-gly-glu-ser).

It should also be noted that in all of these experiments, the endothelial cell monolayers used were confluent both before and after stimulation. Furthermore, leukocyte and tumor cell adhesion was confirmed to be adhesion to the intact endothelial cells and not to any exposed ECM because of endothelial cell retraction, as assessed morphologically by light microscopy in every experiment and by scanning and transmission electron microscopy in random experiments.

EFFECTS OF EXOGENOUS MONOHYDROXY LIPOXYGENASE METABOLITES ON ENDOGENOUS ENDOTHELIAL CELL 13-HODE LEVELS AND ADHESIVITY:

It was concluded from the previous experiments that endogenous 13-HODE levels were inversely related to endothelial cell adhesivity. Therefore, additional experiments were performed to determine whether exogenous 13-HODE and/or other monohydroxy lipoxygenase metabolites altered endothelial cell adhesivity.

First, the specificity of their uptakes by endothelial cell monolayers was determined over a concentration range (10 nM to 10 μM), performed in both the presence and absence of

0.35% albumin and 2 mM calcium. The % uptakes of the fatty acids at a concentration of 1 μM are shown in Table 23.

In the absence of albumin, the % uptakes of the two fatty acid substrates, arachidonic and linoleic acid, were similar. In addition, the uptake of each was decreased by ~ 50% in the presence of albumin. In contrast, there were marked differences in the uptakes of the monohydroxy lipoxygenase metabolites. Both in the presence and absence of

TABLE 23. Uptake of exogenous lipoxygenase metabolites to endothelial cells in the absence and presence of albumin.

FATTY ACID (1 μM)*	0.35% ALBUMIN	% BOUND at pH 7.35		
	2mM Ca^{++}	−	+	−
		+	+	−
ARACHIDONIC ACID		26.9 ± 0.5	16.9 ± 0.8	20.4 ± 2.3
LINOLEIC ACID		21.3 ± 0.5	10.0 ± 0.4	17.5 ± 3.2
5-HETE		11.9 ± 0.4	4.8 ± 0.1	10.6 ± 1.2
12-HETE		0.3 ± 0.1	< 0.2	0.3 ± 0.1
15-HETE		C 5 ± 0.1	< 0.2	0.4 ± 0.2
13-HODE		0.6 ± 0.1	< 0.2	0.3 ± 0.2

* The uptakes were similar, using fatty acid concentrations ranging from 10 nM to 10 μM. Data are expressed as mean ± SEM, n = 9.

albumin, there was no uptake of either 12-HETE, 15-HETE or 13-HODE by the endothelial cell monolayers, while there was significant uptake of 5-HETE. Removal of Ca^{++} (and Mg^{++}) from the incubation medium had no significant effect on the uptake of the fatty acids.

Maximal uptake of the two substrates and 5-HETE was achieved at a concentration of 1 μM. Similar % uptakes were achieved at all other concentrations tested. For example, the % uptakes of 5-HETE at concentrations of 10, 1, 0.1, and 0.01 μM were 10.8 ± 1.3, 11.9 ± 0.4, 9.1 ± 1.5 and 8.0 ± 1.7%, respectively. The uptakes of higher fatty acid concentrations were not measured because cell retraction was noted when concentrations greater than 50 μM were added.

Finally, the two substrates and 5-HETE which were taken up by endothelial cell monolayers were also incorporated into their fatty acid pools. Thus, when endothelial cells were incubated for 1 hour with 100 nM of ^{3}H-20:4, ^{3}H-18:2 or ^{3}H-5-HETE, 73 ± 7% of the bound 5-HETE and 81 ± 8% of the bound arachidonic acid were esterified in the phospholipids, while 44 ± 4% of linoleic acid was esterified in the triglycerides. Furthermore, incubating the monolayers for 10 minutes with Tyrodes containing either 10 μM of unlabelled fatty acid or 0.35% albumin, resulted in 0 ± 0% displacement of the fatty acids. These data indicate that the uptakes were not readily reversible.

Interestingly, the three fatty acids which were taken

up by the endothelial cells did not affect endothelial cell PGI_2 production, while 12-HETE, 15-HETE and 13-HODE, which were not taken up by the endothelial cells, increased PGI_2 production (Table 24).

TABLE 24. Influence of exogenous lipoxygenase metabolites on PGI_2 productions*.

FATTY ACID	$6KPGF1_a$ (pg/10^6 ECs)	$6KPGE_1$
CONTROL	652 ± 83	< 10
ARACHIDONATE	712 ± 113	72 ± 11
LINOLEATE	639 ± 59	< 10
5-HETE	612 ± 95	< 10
12-HETE	1784 ± 90	22 ± 2
15-HETE	3575 ± 390	343 ± 28
13-HODE	3416 ± 546	169 ± 17

Endothelial cells were stimulated with 1 μM fatty acid for 1 hour. *Interesting, only those fatty acids which did <u>not</u> bind, stimulated PGI_2 production. Data are expressed as mean ± SEM, n = 6.

TABLE 25. Influence of exogenous fatty acids on adhesion of platelets, PMNs and A549 cells to endothelial cells.

FATTY ACID (1 μM)	PLATELETS	PMNs	A549
	(Cells/EC-coated disc, % of CONTROL)		
ARACHIDONIC ACID	99.0 ± 3.8	85.2 ± 4.0	-
LINOLEIC ACID	91.9 ± 3.5	86.8 ± 4.8	-
5-HETE	90.6 ± 2.4	104.1 ± 4.2	58.9 ± 7.4*
12-HETE	82.9 ± 3.1*	106.2 ± 4.0	127.2 ± 13.6
15-HETE	79.5 ± 3.2*	115.4 ± 6.6	104.1 ± 14.5
13-HODE	76.5 ± 2.9*	100.9 ± 3.5	97.3 ± 5.1

Data are expressed as mean ± SEM, n = 12; *$p<0.05$.

These differences in fatty acid uptake by the endothelial cells and on endothelial cell PGI_2 production, were associated with differences in endothelial cell adhesivity (Table 25). Under resting conditions, $31.0 \pm 2.4 \times 10^3$ platelets adhered/mm^2 of endothelial cell surface. When the endothelial cells were incubated with any of the three fatty acids which bound to the endothelial cells (arachidonate, linoleate and 5-HETE, Table 23), but which did not stimulate

PGI_2 production (Table 24), platelet adhesion was unaffected (Table 25). In contrast, when the endothelial cells were incubated with 12-HETE, 15-HETE or 13-HODE (the monohydroxides which were not taken up by endothelial cells but which stimulated endothelial cell PGI_2 production), platelet adhesion was decreased by 18.1%, 20.5% and 24.6% respectively, $p < 0.05$ (Table 25).

Adhesion of PMNs to resting endothelial cell monolayers was 3,906 ± 440 PMNs/mm^2. When monolayers were incubated with 1 μM of any of the fatty acids, PMN adhesion to the endothelial cells was unaffected (Table 25). Similarly, adhesion of A549 tumor cells to resting endothelial cell monolayers was 5,400 ± 450 tumor cells/mm^2. When the monolayers were incubated with any of the fatty acids (except 5-HETE), tumor cell adhesion was unaffected. However, incubation of the endothelial cell monolayers with 5-HETE did result in a dramatic decrease in tumor cell adhesion by 41.9 ± 7.4%, $p < 0.05$.

Effect of Stimulation on 13-HODE Uptake by and subsequent PMN Adhesion to Endothelial Cells: As exogenous 13-HODE did not bind to resting endothelial cells nor have any effect on endothelial cell adhesivity, experiments were performed to determine whether exogenous 13-HODE bound to stimulated endothelial cells, and, if so, whether 13-HODE reduced their enhanced adhesivity.

Again, there was no uptake of 13-HODE (0.76 ± 0.08%) either by resting endothelial cell monolayers (control) or by endothelial cells stimulated for 4 hours with either 1 nM γ-IFN, 10 U/ml TNF or 10 ng/ml IL1 (Table 26). Not surprisingly therefore, enhanced PMN adhesion to endothelial cells and enhanced endothelial cell PGI_2 production in response to TNF and IL1 stimulation, were not reduced by incubating the endothelial cell monolayers with exogenous 13-HODE (Table 27). In addition, when endothelial cells were incubated with γ-IFN (which did not reduce endogenous 13-HODE levels, Table 20) and then with exogenous 13-HODE, PMN adhesion and PGI_2 production were unaffected.

TABLE 26. Effect of stimulation on the uptake of 13-HODE by human endothelial cells.

STIMULUS	% UPTAKE OF 13-HODE*
CONTROL	0.76 ± 0.08
γ-IFN	0.57 ± 0.05
TNF	0.85 ± 0.06
IL1	0.61 ± 0.07

* % Uptake of 1 μM 13-HODE over 30 minutes by endothelial cells previously incubated with the suspending vehicle (control), 1 nM γIFN, 10 U/ml TNF, or 10 ng/ml IL1 for 4 hours. Uptakes are expressed as a % of total 13-HODE added, mean ± SEM; n = 5.

TABLE 27. Effect of 30 minute pre-treatment of endothelial cells with 13-HODE, on endothelial cell adhesivity towards PMNs and PGI_2 production following cytokine stimulation.

	PMN ADHESION ($10^3/mm^2$) 13-HODE		PGI_2 PRODUCTION (pg/ml) 13-HODE	
STIMULUS	0 μM	1 μM	0 μM	1 μM
Control	2.9 ± 0.3	2.6 ± 0.2	606 ± 39	723 ± 67
γ-IFN	3.3 ± 0.3	2.5 ± 0.2	513 ± 19	779 ± 56
TNF	4.0 ± 0.2*	4.4 ± 0.2*	910 ± 31*	1099 ± 19*
IL1	8.4 ± 0.3*	6.7 ± 0.4*	1099 ± 50*	1553 ± 109*

* Significantly different from the 0 μM 13-HODE control, $p<0.01$. Data are expressed as mean ± SEM, n = 6.

INTRACELLULAR LOCALIZATION OF 13-HODE AND ITS ASSOCIATION WITH THE VITRONECTIN RECEPTOR:

Specificity of 13-HODE Antibody: To determine the specificity and titre of the 13-HODE antiserum, 50 μg of 13-HODE, 15-HETE and 12-HETE were incubated with 1 ml of either normal pooled rabbit sera or normal pooled rabbit sera containing varying amounts of the 13-HODE antiserum. 13-HODE, 12-HETE and 15-HETE were then incubated in the sera for 1 hour at 22°C.

The immunoglobulins were then precipitated with PEG 8000 and removed by centrifugation. The remaining supernatant was then extracted and analyzed for monohydroxides.

Approximately 95% of the monohydroxides added to normal rabbit sera were recovered from the supernatant. Thus, the amount of 13-HODE, 15-HETE and 12-HETE recovered in supernatant of antiserum-spiked sera was compared against the amounts recovered in the normal rabbit sera. When increasing amounts of 13-HODE antiserum was added to normal sera, there was a dose-dependent decrease in the amount of 13-HODE recovered from the supernatant (Figure 11). When a total of 20 μl of antisera was added, no 13-HODE was recovered. In contrast, >98% of 15-HETE and 12-HETE were recovered at all dilutions of the antisera. These results are depicted both as % recoveries in the graph of Figure 11, and as changes in the area under the curve in the HPLC tracings shown in the insert of Figure 11. Thus, it was concluded that the 13-HODE antisera was of high specificity.

Localization of 13-HODE and the VnR in Endothelial Cells: Having established the specificity and the titre of the 13-HODE antiserum, localization of 13-HODE and the VnR were determined by histoimmunofluorescence. When the surface receptors of resting endothelial cells were fixed with paraformaldehyde and not permeabilized, neither 13-HODE nor the α or β chain of the VnR was detected (Figure 12C).

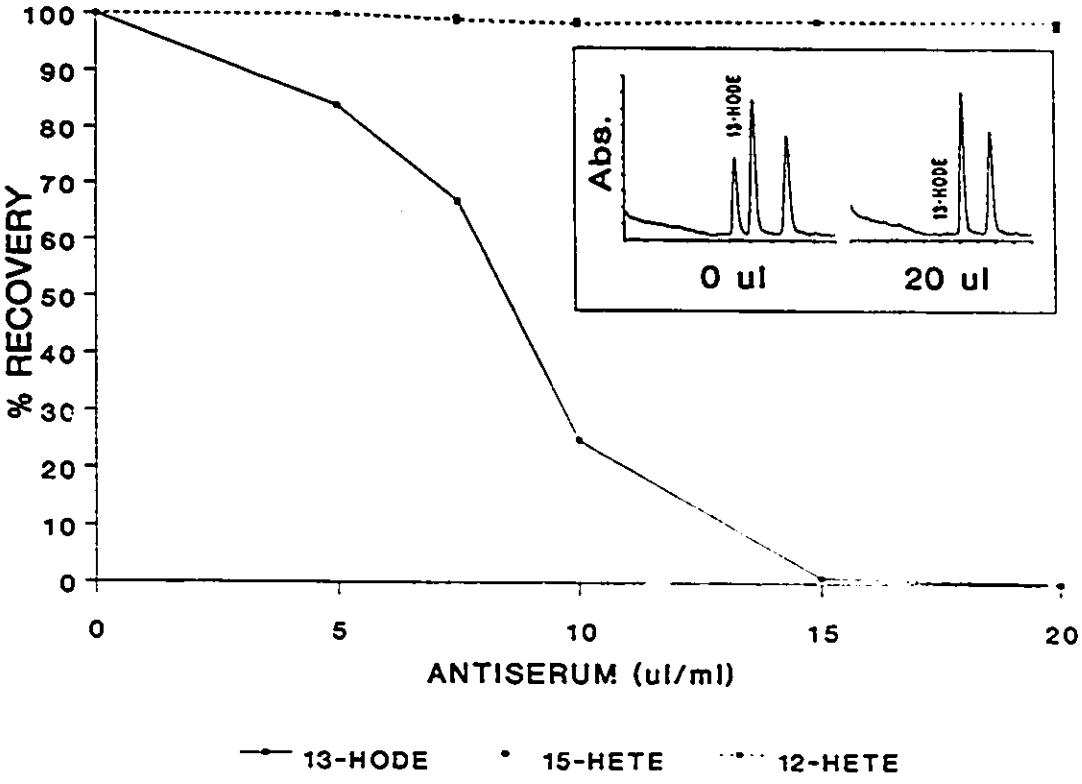


FIGURE 11. Determination of the specificity and titre of the 13-HODE antisera. Increasing amounts of 13-HODE antisera was added to normal rabbit sera spiked with 50 ng of 13-HODE, 12-HETE and 15-HETE. The samples were incubated and the supernatant which was obtained after IgG precipitation was analyzed for the monohydroxides. Insert: HPLC tracings of (0 μl) normal rabbit sera; and (20 μl) normal sera containing 20 μl of the 13-HODE antisera. The peaks in order are 13-HODE, 15-HETE and 12-HETE.

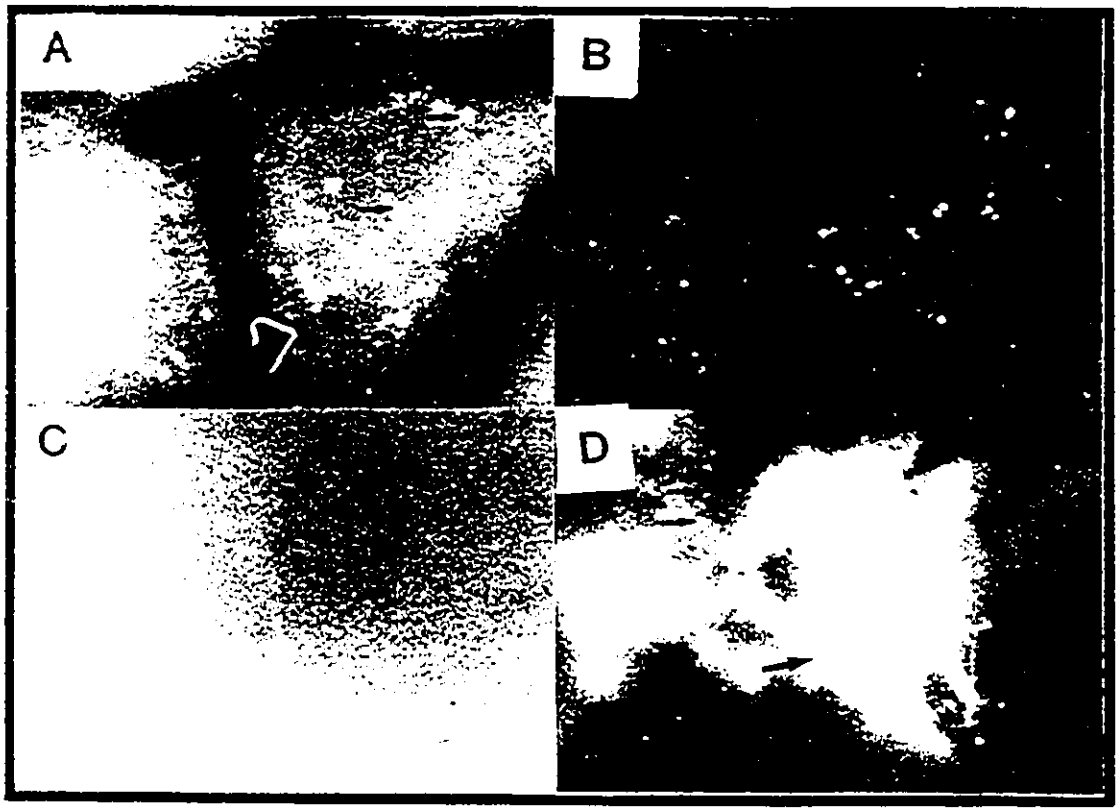


FIGURE 12. Co-localization of 13-HODE and the VnR in endothelial cells. Endothelial cells were permeabilized and then co-incubated with either (A) 13-HODE or (B) LM142 antisera. Non-permeabilized IL1 stimulated endothelial cells were incubated with either the (C) 13-HODE or (D) LM609 antisera. Figure C also represents non-permeabilized, resting endothelial cells incubated with any antisera, or permeabilized stimulated endothelial cells incubated with 13-HODE or LM142 antisera.

[Monoclonal antibody LM609 recognizes the RGD binding domain formed by the VnR α and β chains, while LM142 recognizes an epitope on the VnR α chain which is not involved in RGD binding]. In contrast, when the endothelial cell surface was first fixed, and then permeabilized with Triton X-100, 13-HODE was detected in vesicles, located immediately below the endothelial cell plasma membrane (Figure 12A).

When the same permeabilized endothelial cell monolayers were incubated with the monoclonal antiserum to the VnR α chain (LM142), the VnR α chain was also detected within the same vesicles as 13-HODE (Figure 12B). In contrast, the adhesive RGD recognizing epitope of VnR was not detected (using the LM609 antiserum) either on the endothelial cell surface or within the vesicles of the resting endothelial cells (represented by Figure 12C).

When IL1-stimulated endothelial cells were fixed and permeabilized, neither 13-HODE nor the VnR α chain were detected (same as Figure 12C). However, the RGD adhesive site of the VnR was now detected on the endothelial cell apical surface, concentrated at the periphery of the cells (Figure 12D).

The pattern of VnR expression on the surface of the endothelial cells was similar to the pattern of platelet adhesion to stimulated endothelial cells (Figure 13). These data suggest that the VnR is a major receptor for platelet adhesion to the endothelium.

FIGURE 13. Peripheral adhesion of platelets to IL1 stimulated endothelial cells.

13-HODE AND BASEMENT MEMBRANE THROMBOGENECITY:

Basement Membrane Thrombogenecity: In Vitro Studies: Experiments were also performed to determine whether endothelial cells release 13-HODE into their underlying basement membrane, and if so, whether the released 13-HODE influenced platelet adhesion to the basement membrane.

Removing the endothelial cells with cellulose acetate, exposed 68.9 ± 5.5% of the basement membrane. Exposing endothelial cells with 2% EGTA for 30 minutes, resulted in the exposure of 91.9 ± 1.4% of the basement membrane, while exposing the endothelial cells with 0.1 N NH_4OH resulted in a complete exposure of the basement membrane (99.1 ± 0.4%). Perfusion of the endothelial cells under shear stresses of 1300 and 1600 sec^{-1}, was not as efficient as the other methods in exposing the basement membrane (21.5 ± 2.6% and 35.7 ± 7.2%, respectively).

Both 13-HODE and vWF were detected by histoimmunofluorescence in both the intact endothelial cell monolayers and in the basement membranes prepared by shear stress. However, no 13-HODE was detected in basement membranes prepared with NH_4OH. The histoimmunofluorescence results were confirmed by HPLC analysis and an ELISA. Thus, resting endothelial cell monolayers contained 12.6 ± 1.0 ng 13-HODE/10^6 cell and 108.0 ± 12.6 ng vWF/10^6 cell (Table 28). NH_4OH basement membrane preparations contained less 13-HODE and vWF

(< 0.01 and 9.2 ± 1.1 ng/equivalent area of 10^6 cell, respectively), while both cellulose acetate and EGTA basement membrane preparations contained intermediate levels of both compounds.

TABLE 28. Platelet adhesion to various preparations of endothelial cell-derived basement membranes under static conditions.

	PLATELET ADHESION	13-HODE	vWF
ECs	4.7 ± 0.2	12.60 ± 1.0	108.0 ± 12.6
BMs- CA	5.9 ± 0.7	1.90 ± 1.0*	-
- EGTA	5.9 ± 1.1	0.05 ± 0.03*	28.7 ± 3.5*
- NH_4OH	17.9 ± 0.9*	< 0.01*	9.2 ± 1.1*

Platelet adhesion is expressed as the # of platelets x 10^3 adherent/mm^2 to either intact endothelial cell monolayers (ECs) or to the different basement membrane (BM) preparations, mean ± SEM, n = 7; *p < 0.01. These include BMs prepared by cellulose acetate (CA) stripping, 2% EGTA or 0.1 N NH_4OH. 13-HODE and vWF levels are expressed as ng/10^6 EC of an equivalent basement membrane surface area.

The level of 13-HODE and vWF present in the various basement membrane preparations were associated with changes in the adhesivity of the basement membranes for platelets, measured under both static and flow conditions (Tables 28 and 29). Thus, $4.7 \pm 0.2 \times 10^3$ platelets adhered/mm^2 of resting endothelial cell monolayers under static conditions (Table 28). Platelet adhesion to the basement membranes preparations containing 13-HODE (i.e. to either the cellulose acetate or the EGTA prepared basement membranes) increased slightly. In contrast, platelet adhesion increase four-fold onto the NH_4OH prepared basement membranes. These changes in platelet adhesion were inversely related to both basement membrane 13-HODE and vWF levels (Table 28).

Similarly the basement membranes prepared by shear stress or cellulose acetate (which contained the highest amounts of 13-HODE) were the least adhesive under flow conditions (Table 29). Total surface area covered with platelets onto basement membranes prepared by shear stress ranged from 8.7 to 14.5%, while only 22% of cellulose acetate prepared basement membranes were covered with platelets (Table 29). In contrast, the total surface area covered with platelets onto EGTA or NH_4OH prepared basement membranes was > 45%.

In agreement with EGTA and NH_4OH prepared basement membranes being the most adhesive (Tables 28 and 29), morphological evaluation reveal that the majority (> 58%) of these adhered platelets were spread and/or aggregated (Table 30).

In contrast, the majority of the platelets that interacted with the less adhesive basement membranes (prepared by shear stress or cellulose acetate), were only in contact (> 75%) and had not spread nor aggregated.

TABLE 29. Morphological evaluation of platelet/basement membrane interactions under flow conditions.

BASEMENT MEMBRANE PREPARATION	% OF SURFACE AREA COVERED
Shear Stress 1300 s^{-1}	8.7 ± 1.7
1600 s^{-1}	14.5 ± 2.3
Cellulose acetate	21.8 ± 2.3
EGTA	48.7 ± 3.7*
NH_4OH	45.5 ± 5.2*

Endothelial cell-derived basement membranes were prepared by shear stress (1300 and 1600 sec^{-1}), cellulose acetate stripping, 2% EGTA or 0.1 N NH_4OH. Platelet adhesion to the various basement membrane preparations were measured under flow conditions of 1300 sec^{-1} for 5 minutes at 37°C. Data are expressed as mean ± SEM, n = 7; *$p < 0.01$.

TABLE 30. Cross sectional morphological evaluation of platelet/basement membrane interactions under flow conditions.

BASEMENT MEMBRANE PREPARATION	MORPHOLOGY OF PLATELET INTERACTIONS		
	CONTACT	SPREAD	AGGREGATES
Shear stress:			
1300 s^{-1}	83.5 ± 6.6	10.7 ± 2.9	6.1 ± 3.8
1600 s^{-1}	86.5 ± 1.8	13.4 ± 1.8	0.2 ± 0.1
Cellulose acetate	75.6 ± 10.7	21.1 ± 8.6	3.4 ± 2.1
EGTA	21.2 ± 5.6**	67.1 ± 5.9**	11.6 ± 3.8*
NH_4OH	6.6 ± 1.3**	58.4 ± 6.2**	27.8 ± 9.2*

Morphological assessment of platelet/basement membrane interaction following a 5 minute exposure of platelets to the various basement membrane preparation, at a flow rate of 1300 sec^{-1}. Basement membranes prepared by shear stress were prepared by perfusion at flow rates of either 1300 sec^{-1} or 1600 sec^{-1} for 5 and 15 minutes, respectively. Data are expressed as percent of total surface area covered, mean ± SEM, n = 7: *$p < 0.05$, **$p < 0.01$.

Artificial Graft Thrombogenecity: To determine whether exogenous 13-HODE alters the thrombogenecity of basement membrane components bound to artificial surfaces, prosthetic grafts (Heamashield grafts embedded with collagen type IV), were coated with 0.1 μM 13-HODE for 1, 4, or 18 hours. To bind 13-HODE irreversibly to the graft surface, the grafts were incubated with 13-HODE in the presence and absence of EDC and 0.35% Tyrodes albumin.

There were similar time-dependent increases in the amount of either free or EDC conjugated 13-HODE bound to the grafts (Table 31). Washing the grafts with 0.35% Tyrodes albumin removed > 90% of the bound 13-HODE from either graft. Similar results were obtained when: 1) the concentrations of 13-HODE and EDC were increased 10 fold; 2) a 3.3 fold molar excess of EDC was used instead of an equal molar ratio; and 3) the concentration of Tyrodes albumin increased to 4%. These data indicate that either EDC failed to covalently bind 13-HODE to the surface of the grafts, or all of the 13-HODE/ EDC binding sites were occupied at the lower concentrations.

If it was the former, i.e. a lack of any effect of EDC on 13-HODE binding to the grafts, the lack of any effect was not due to the inability of EDC to couple 13-HODE (Figure 14). When an equal molar of a 3.3 fold molar excess of EDC was incubated with [^{14}C]13-HODE, > 50% of 13-HODE was coupled to EDC within 10 minutes, which increased to > 95% coupling within 2 hours (Figure 14). Therefore, it is more likely that

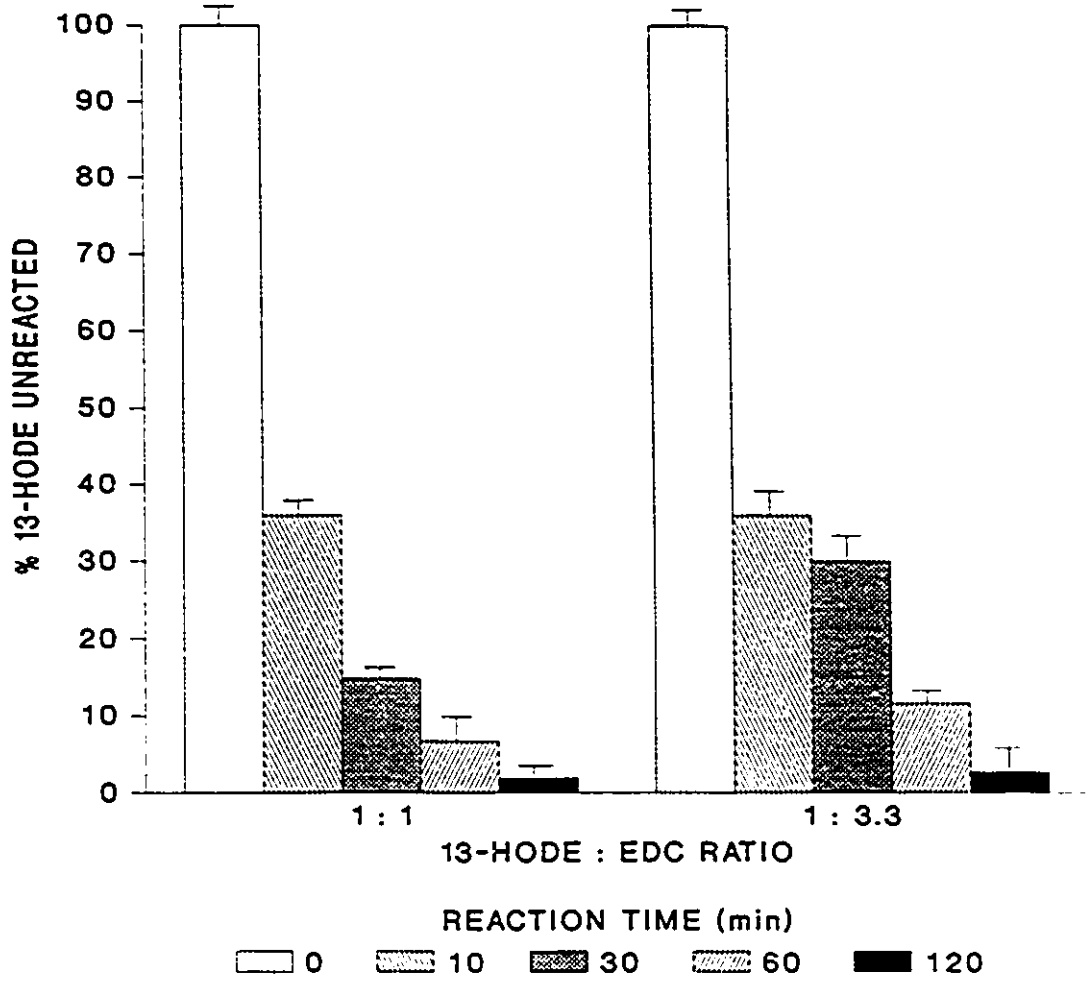


FIGURE 14. Percentage of 13-HODE remaining unconjugated to EDC after various lengths of time with either an equal molar ratio or a 3.3 fold excess of EDC. Data are expressed as mean ± SEM, n = 3.

all of the sites for 13-HODE binding were occupied. This is supported by the following study.

TABLE 31. Reversibility of 13-HODE binding to grafts: Effect of 13-HODE incubation time and EDC.

TYRODES INCUBATION TIME (hrs.)	0.1μM 13-HODE INCUBATION TIME					
	1 hr.		4 hrs.		18 hrs.	
	- EDC	+ EDC	- EDC	+ EDC	- EDC	+ EDC
0	8.77 ± 0.52	9.47 ± 0.65	18.34 ± 1.26	14.61 ± 0.42	24.95 ± 3.03	24.71 ± 2.86
1	1.32 ± 0.07	1.83 ± 0.33	2.16 ± 0.24	1.98 ± 0.15	2.78 ± 0.25	2.74 ± 0.16
4	0.63 ± 0.04	0.79 ± 0.06	1.40 ± 0.13	1.34 ± 0.06	1.82 ± 0.10	2.39 ± 0.06
18	0.50 ± 0.05	0.44 ± 0.04	0.81 ± 0.09	0.94 ± 0.04	1.91 ± 0.17	1.68 ± 0.14

13-HODE was bound to the grafts in either the absence (- EDC) or presence (+ EDC) of an equal molar ratio of EDC. To assess the reversibility of the binding, the grafts were rinsed in 0.35% Tyrodes albumin for either 0, 1, 4 or 18 hours. Data are expressed as pg of 13-HODE bound/mm^2 graft, mean ± SEM; n = 6.

Grafts were incubated with increasing concentrations of 13-HODE for 18 hours, washed and then incubated with or without 0.35% Tyrodes albumin for another 18 hours to remove surface bound 13-HODE. The adhesivity of the grafts was then assessed. When 2.5 ± 0.1 pg 13-HODE was bound/mm^2 graft, platelet adhesion deceased 20% (Table 32). When the amount of 13 HODE bound to the graft surface was increased to 21 pg/mm^2, platelet adhesion decreased 29%. Increasing the amount of 13-HODE bound, had no further effect. In contrast, non-surface bound 13-HODE had no effect on platelet adhesion (Table 32).

The decrease in platelet adhesion to grafts coated with 13-HODE was not a general fatty acid property, since platelet adhesion was not altered when linoleic acid (the substrate for 13-HODE) was used to coat the grafts (Table 33). When 18 or 274 pg of linoleic acid was bound/mm^2, platelet adhesion was unaffected. In contrast, 13-HODE decreased platelet adhesion to the grafts when similar amounts of fatty acid were bound (Table 33).

TABLE 32. Platelet adhesion to 13-HODE-coated grafts: Effect of Albumin.

	- ALBUMIN		+ ALBUMIN	
13-HODE [μM]	PLT ADH. % OF CONTROL	13-HODE pg/mm^2	PLT ADH. % OF CONTROL	13-HODE pg/mm^2
0	100.0 ± 3.0	-	100.0 ± 5.2	-
0.1	80.0 ± 6.1	2.5 ± 0.1	93.5 ± 4.0	0.9 ± 0.1
1.0	71.3 ± 4.7*	20.8 ± 1.9	100.3 ± 3.5	6.4 ± 0.3
10.0	67.7 ± 3.2*	304.9 ± 27.9	99.6 ± 3.5	60.5 ± 3.0
100.0	-	-	105.3 ± 5.0	450.4 ± 24.9

Grafts were incubated with 13-HODE for 18 hours, and then incubated with either albumin-free Tyrodes for 30 minutes (- albumin), or with 0.35% Tyrodes albumin for additional 18 hours (+ albumin). Platelet adhesions (PLT ADHs) were then performed. PLT ADH is expressed as a % of control (mean ± SEM), of the number of platelets adherent to non-13-HODE coated grafts (0 μM); n = 5 for - albumin; n = 15 for + albumin; *$p < 0.01$.

TABLE 33. Effects of surface bound 13-HODE and 18:2 on platelet adhesion to grafts.

FATTY ACID	[CONC.] (μM)	PLTs x 10^3 / mm^2	pg FA / mm^2
NONE	0	12.07 ± 0.36	-
13-HODE	1.0	8.61 ± 0.56*	20.8 ± 1.9
	10.0	8.17 ± 0.39*	304.9 ± 27.9
18:2	1.0	11.73 ± 1.66	18.2 ± 2.2
	10.0	13.12 ± 1.23	273.6 ± 26.0

To bind 13-HODE or linoleic acid (18:2) to the surface of the grafts, the grafts were incubated with 13-HODE or 18:2 for 30 minutes, rinsed and then platelet adhesion was measured. Data are expressed as mean ± SEM, n = 5; *$p < 0.01$.

These results were also confirmed by SEM (Figure 15). When 18 pg of linoleic acid/mm^2 was bound to the graft, platelet adhesion decreased only slightly (Figure 15B), while platelet adhesion was markedly decreased when 21 or 305 pg of 13-HODE/mm^2 was bound (Figure 15C and D).

These data are consistent with the endothelial cell basement membrane data, suggesting that 13-HODE must be bound to the surface of a vascular graft, presumably to basement membrane components, to inhibit platelet adhesion.

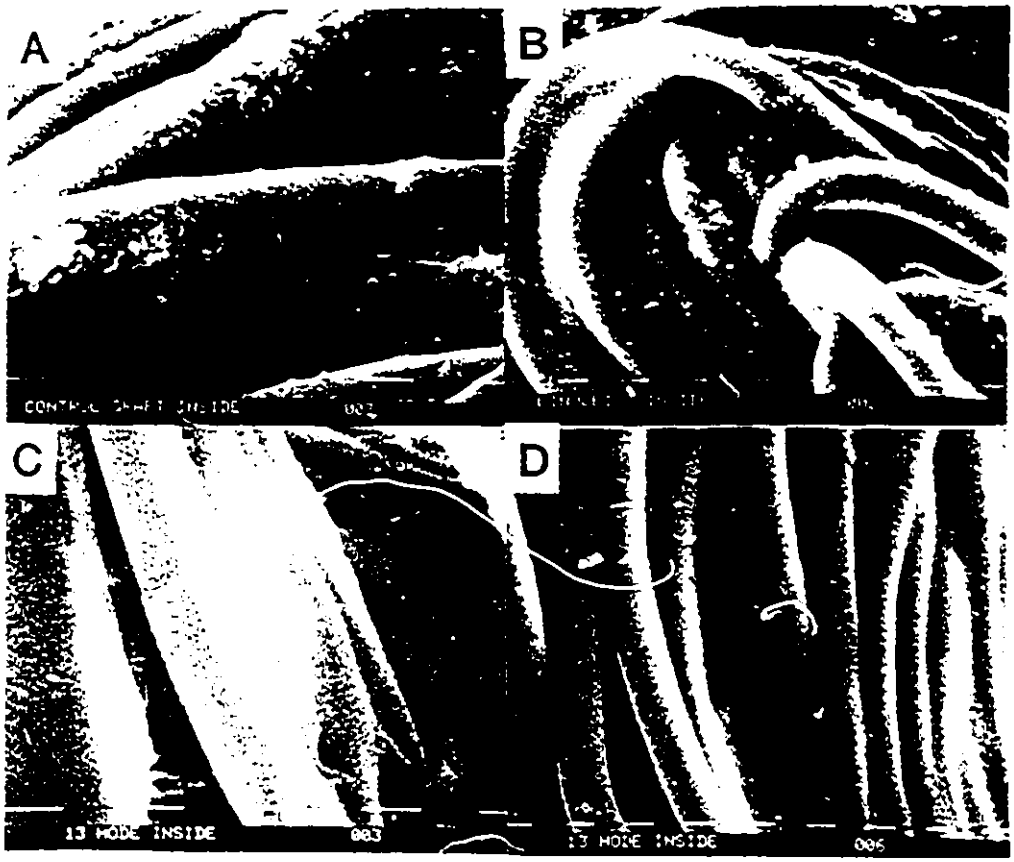


FIGURE 15. Scanning electron micrographs of (A) control, grafts coated with either (B) 2.5 pg/ml linoleic acid (C and D) 2.5 or 304.9 pg/ml 13-HODE, respectively.

BASEMENT MEMBRANE THROMBOGENECITY: IN VIVO STUDIES:

Effect of Drugs: To determine the biological relevance of the *in vitro* basement membrane experiments, experiments were performed *in vivo* in rabbits treated with either salicylate or dipyridamole to either inhibit or enhance vessel wall 13-HODE synthesis respectively. In control rabbits, $3.9 \pm 0.6 \times 10^6$ platelets/cm^2 adhered to the vessel wall surface, 4 hours after air injury, at a time when the vessels produced 14.9 ± 4 ng of 13-HODE/cm^2 (Table 34). When the rabbits were treated with salicylate for 7 days before air-injury, platelet accumulation increased 2-fold, $p < 0.001$. This increase was associated with a 67% decrease in vessel wall 13-HODE levels, $p < 0.001$ (Table 34). When the rabbits were fed a dipyridamole-rich diet for 3 weeks before air-injury, platelet accumulation on the injured carotid arteries decreased to $2.1 \pm 0.3 \times 10^6$ platelets/cm^2, $p < 0.001$. This decrease was associated with a 40% increase in vessel wall 13-HODE (Table 34).

The changes in platelet-vessel wall interactions associated with the different drug treatments were confirmed by SEM (Figure 16, pg 161). Platelet accumulation on the injured arteries of control rabbits 4 hours after air-injury is shown in Figure 16A. Platelet accumulation was markedly decreased on the dipyridamole-treated rabbit vessels (Figure 16B) and markedly increased on the salicylate-treated rabbit vessels (Figure 16C).

TABLE 34. Platelet accumulation on and 13-HODE synthesis by carotid arteries in control, salicylate- and dipyridamole-treated rabbits.

Treatment	Platelet Accumulation (#x10^6/cm^2)	13-HODE (ng/cm^2)
Control	3.9 ± 0.6	14.9 ± 4
Salicylate	8.0 ± 1.6*	4.9 ± 2*
Dipyridamole	2.1 ± 0.3*	20.9 ± 4

Sodium salicylate (50 mg/kg b.i.d.) was fed twice daily. Dipyridamole was incorporated into the standard rabbit chow pellets (10 mg/kg pellets). Data are expressed as mean ± SEM, n = 8; *$p < 0.001$.

Platelet accumulation on the injured vessel wall segments correlated significantly with the circulating dipyridamole plasma level; $r = 0.962$, $p < 0.001$ (Table 35). Plasma dipyridamole levels, in turn, correlated with both vessel wall cAMP and 13-HODE levels. The levels of cAMP and

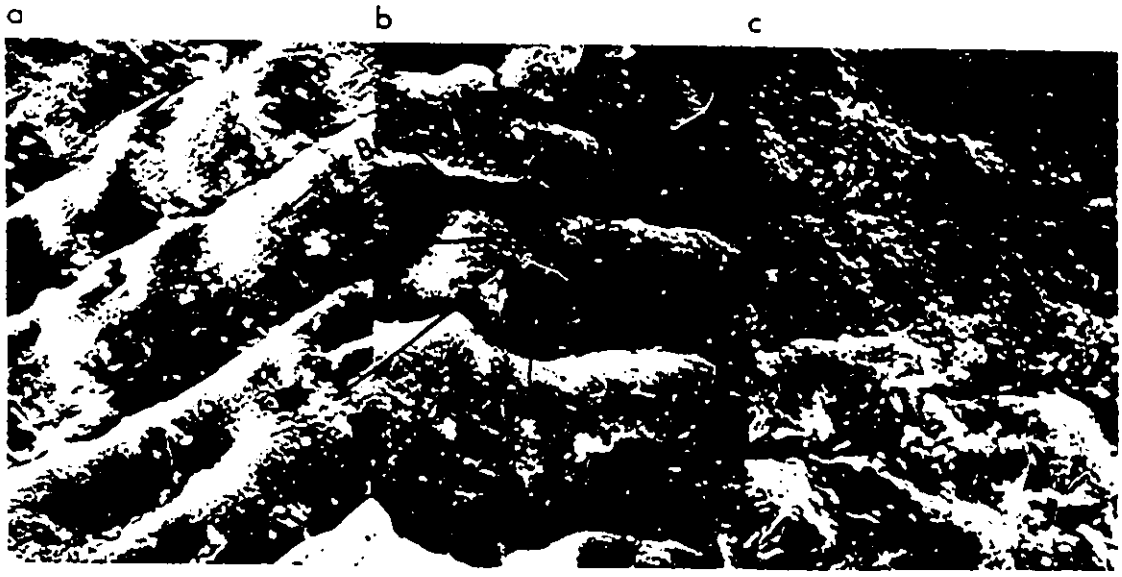


FIGURE 16. Scanning electron micrographs of air-injured rabbit carotid arteries. Air-injured carotid arteries were obtained from (A) control rabbits, (B) dipyridamole treated rabbits, or (C) salicylate treated rabbits.

13-HODE in control vessels were 8.1 ± 2 pmol/cm^2 and 14.9 ± 4 pg/cm^2, respectively. At plasma dipyridamole levels of 197 ± 61 pg/ml, the cAMP level increased by 27% ($r = 0.649$, $p < 0.05$), and the 13-HODE levels increased by 40%, $r = 0.801$, $p < 0.025$ (Table 35). There was no detectable changes in vessel wall cAMP levels in the animals fed salicylate.

TABLE 35. Relationship between dipyridamole plasma levels, vessel wall cAMP levels and 13-HODE synthesis.

		Treatment	
		Dipyridamole	Control
DIP	(pg/mL)	197.0 ± 61	ND
cAMP	(pmol/cm^2)	10.3 ± 3	8.1 ± 2
13-HODE	(ng/cm^2)	20.9 ± 4	14.9 ± 4

Data are expressed as mean ± SEM, n = 8. Correlations: DIP α 13-HODE, $r = 0.7627$, $p < 0.05$; cAMP α 13-HODE, $r = 0.8014$, $p < 0.025$; DIP α platelet accumulation, $r = 0.9619$, $p < 0.005$.

Effect of Diet: The fatty acid composition of diets are summarized under MATERIALS, Table 2. The reader is reminded that the BCO and WO diets were rich in linoleic acid and linolenic acid ($18:3^{n-3}$); the BCO was also rich in γlinolenic acid ($18:3^{n-6}$); and the FO diet was rich in n-3 fatty acids ($18:3^{n-3}$, EPA ($20:^{n-3}$) and $22:6^{n-3}$).

At the onset of the diet studies, all rabbits weighed 2.0 ± 0.2 kg (mean ± SEM). After four weeks diet feeding, all rabbits in all of the groups had a mean weight of 2.8 ± 0.6 kg. There was no significant difference in either weight gain or final weight among the different diet groups.

The fatty acid composition both in the rabbit aortae and the circulating platelets differed according to the diet ingested (Table 36). Thus, when rabbits were fed the FO diet, the vessel wall linoleic and arachidonic acid content were similar as the levels in the vessel wall of lard fed rabbits. Albeit there was some n-3 fatty acids ($18:3^{n-3}$, $18:4^{n-3}$, $20:5^{n-3}$ and $22:6^{n-3}$) detected in their aortae, but disproportionate to the diet content and the amount incorporated into the platelet (see below). When rabbits were fed the linoleic acid rich diets (BCO and WO), vessel wall linoleic and α-linolenic acid levels increased and the arachidonic acid level decreased in comparison to the lard diet (Table 36).

Platelets obtained from rabbits on the FO diet incorporated more EPA as compared to the amount of EPA incorporated into their vessel wall (Table 36). Furthermore,

TABLE 36. Effect of diet on fatty acid composition of rabbit vessel walls and platelets.

VESSEL WALL

	LARD	FO	BCO	WO
16:0	25.1 ± 1.0	23.3 ± 0.8	19.7 ± 0.6	19.2 ± 1.0
18:0	9.3 ± 0.8	9.7 ± 0.6	5.5 ± 0.9	6.3 ± 0.6
$18:1^{n-9}$	31.9 ± 1.2	27.2 ± 0.6	19.9 ± 0.5	22.5 ± 0.7
$18:2^{n-6}$	14.4 ± 0.9	14.1 ± 1.0	28.2 ± 1.8	33.7 ± 1.8
$18:3^{n-6}$	0.1 ± 0.0	0.5 ± 0.3	6.0 ± 0.5	0.4 ± 0.1
$18:3^{n-3}$	1.5 ± 0.1	2.2 ± 0.3	7.0 ± 0.6	5.6 ± 0.7
$18:4^{n-3}$	0.0 ± 0.0	0.2 ± 0.0	0.9 ± 0.1	0.0 ± 0.0
$20:3^{n-6}$	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.1 ± 0.0
$20:4^{n-6}$	6.9 ± 1.3	7.2 ± 0.2	3.3 ± 1.3	3.8 ± 0.9
$20:5^{n-3}$	0.0 ± 0.0	1.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
$22:6^{n-3}$	0.5 ± 0.1	2.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1

PLATELET

	LARD	FO	BCO	WO
16:0	21.5 ± 1.5	23.7 ± 1.6	20.5 ± 1.2	21.2 ± 0.9
18:0	18.4 ± 1.3	19.0 ± 0.6	18.4 ± 1.4	17.0 ± 1.0
$18:1^{n-9}$	21.2 ± 0.7	18.3 ± 4.0	10.8 ± 0.2	12.3 ± 0.5
$18:2^{n-6}$	22.5 ± 0.9	13.7 ± 1.3	22.5 ± 1.1	30.3 ± 2.9
$18:3^{n-6}$	0.0 ± 0.0	0.1 ± 0.1	3.4 ± 0.4	0.0 ± 0.0
$18:3^{n-3}$	0.4 ± 0.1	0.3 ± 0.2	2.3 ± 0.3	2.2 ± 0.3
$18:4^{n-3}$	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0
$20:3^{n-6}$	0.7 ± 0.1	0.5 ± 0.1	5.2 ± 0.6	0.4 ± 0.0
$20:4^{n-6}$	8.7 ± 0.8	6.3 ± 0.7	8.3 ± 0.8	6.0 ± 0.8
$20:5^{n-3}$	0.2 ± 0.0	6.5 ± 0.9	0.1 ± 0.0	0.0 ± 0.0
$22:6^{n-3}$	0.2 ± 0.1	1.7 ± 1.0	0.5 ± 0.5	0.0 ± 0.0

Data expressed as mol % of total lipids, mean ± SEM, n = 6x4.

the ratio of arachidonic acid:EPA in platelets was 1:1, versus 6:1 in the vessel wall. The implications of this ratio difference will be elaborated upon in more detail under Discussion.

Another striking difference between rabbit fatty acid composition in the platelet versus the vessel wall relates to the incorporation of linoleic acid. Whereas linoleic acid was significantly increased in the aortae of BCO and WO fed rabbits (as compared to the lard fed rabbits), there was no increase in linoleic acid content of their platelets (Table 36). Unlike the vessel wall, platelet arachidonic acid levels were not affected by any of the diets, as compared to the lard (control) diet.

Effect of Diet on Vessel Wall and Platelet Cyclo-oxygenase Metabolism and Lipoxygenase: The increases in vessel wall linoleic acid levels correlated with significant increases in vessel wall 13-HODE synthesis, $r = 0.905$, $p < 0.025$ (Table 37). In particular, the 2-fold increase in vessel wall linoleic acid content in the WO and BCO diet fed rabbits was associated with a 2-fold increase in vessel wall 13-HODE synthesis, $p < 0.01$. PGI_2 production was not significantly different among any of the diet groups. The specific changes in platelet fatty acid composition were also associated with specific changes in platelet lipoxygenase-derived 12-HETE synthesis (Table 37). Thus, platelets obtained from the lard

TABLE 37. Effect of diets on vessel wall and platelet fatty acid metabolism.

	DIETS			
	LARD	FO	BCO	WO
Vessel wall				
PGI_2	15 ± 2	21 ± 2	23 ± 3	23 ± 2
13-HODE*	15 ± 4	7 ± 2	30 ± 9	28 ± 7
Platelet				
TXA_2	61 ± 16	50 ± 13	46 ± 10	50 ± 14
12-HETE	3.0 ± 1.0	1.1 ± 0.4	3.0 ± 0.2	3.9 ± 1.1

PGI_2 levels are expressed as pg 6-keto $PGF_{1\alpha}/cm^2$ of vessel wall. 13-HODE levels are expressed as ng 13-HODE/cm^2 of vessel wall. TXA_2 levels are expressed as pg $TXB_2/10^8$ platelets. 12-HETE levels are expressed as ng 12-HETE/10^8 platelets. Data are expressed as mean ± SEM, n = 12.

fed rabbits produced 3.0 ± 1.0 ng 12-HETE/10^8 platelets while platelets obtained from WO or BCO fed rabbits produced 3.9 ± 1.1 and 3.0 ± 0.2 ng 12-HETE/10^8 platelets. In contrast, platelets obtained from FO rabbits (which were rich in EPA), only produced 1.1 ± 0.4 ng 12-HETE/10^8 platelets, $p < 0.05$

(Table 37). As was the case with vessel wall PGI_2 production, platelet TxA_2 production was not significantly different among the diet groups.

Effect of Diet on Rabbit Vessel Wall and Platelet Adhesivity: The dietary effects on platelet and vessel wall lipoxygenase metabolites were associated with differential effects on vessel wall and platelet adhesivity. Thus, 4 hours after air injury, there were 72 ± 12 x 10^5 platelets/cm^2 of injured vessel wall in lard fed rabbits (Table 38). When the rabbits were fed BCO for 4 weeks, vessel wall adhesivity decreased 50%, $p < 0.001$. No significant decrease in vessel wall adhesivity was observed in either the WO or FO fed rabbits.

When platelet adhesivity was measured ex vivo using the in vitro adhesion assay to fibronectin-coated discs, 21 ± 1 x 10^5 platelets/cm^2 adhered, using platelets obtained from lard fed rabbits (Table 38). Adhesion of platelets obtained from either WO or BCO fed rabbits was only marginally decreased while adhesion of platelets from FO fed rabbits (which had marked decreases in 12-HETE synthesis), decreased 57%, $p < 0.001$. Thus, it appears that the FO diet influenced platelet adhesivity, while the BCO diet influenced vessel wall adhesivity.

TABLE 38. Effects of diet on vessel wall and platelet adhesivity.

	DIETS			
	LARD	FO	BCO	WO
VWA[a]	72 ± 12	52 ± 5	36 ± 1	48 ± 9
PLT. ADH.[b]	21 ± 1	9 ± 1	15 ± 2	18 ± 2

Vessel wall adhesivity (VWA) expressed as the number of adherent platelets x $10^5/cm^2$ aorta. Platelet adhesivity (PLT. ADH.) expressed as the number of adherent platelets x $10^5/cm^2$ disc. Data expressed as mean ± SEM, n = [a]24 or [b]6.

13-HODE:HETE RATIOS AND PMN AND TUMOR CELL ADHESION:

PMN Adhesion and 13-HODE Synthesis: Previous studies demonstrated that 5-HETE treatment of endothelial cells did not effect endothelial cell adhesivity for PMNs (Table 26). Therefore, experiments were performed to determine whether treatment of PMNs with 5-HETE had an effect on PMN adhesivity for resting endothelial cells. In addition, experiments were

performed to determine if the levels of 13-HODE in resting PMNs, like that in endothelial cells, inversely correlated with PMN adhesion.

Pre-treatment of PMNs with 20 and 40 μM 5-HETE for 30 minutes, led to 68 ± 12% and 187 ± 36% increases in PMN adhesion to resting endothelial cells, respectively (Table 39). When resting (control) PMNs were treated with ETYA, intracellular PMN adhesion decreased 60 ± 8%, at a time when PMN 13-HODE levels were inhibited by 87 ± 3% (Table 39).

TABLE 39. Effect of pre-treating PMNs with 5-HETE on PMN adhesion to endothelial cells.

	PMN ADHESION		
	5-HETE		
	0 μM	20 μM	40 μM
Untreated PMNs (control)	100 ± 7	168 ± 12	287 ± 36
ETYA-treated PMNs (1 μM)	40 ± 8	77 ± 5	92 ± 12

Adhesion were performed using resting PMNs [i.e. with 13-HODE levels of 505 p/10^6 PMN] and ETYA-treated PMNs [i.e. with inhibited (87 ± 3%) 13-HODE levels]. Data are expressed as % of adhesion of resting PMNs, mean ± SEM, n = 6.

Incubating the ETYA-treated PMNs with 5-HETE reversed this effect and increased PMN adhesion towards control levels (resting PMNs, 0 μM 5-HETE). Exogenous 13-HODE had no effect on PMN adhesion. Furthermore, no 5-HETE was detected in the PMNs under resting conditions. Therefore, these data suggest that endogenous 13-HODE levels in PMNs modulates PMN adhesion under resting conditions while 5-HETE modulates PMN adhesion following stimulation.

Tumor Cell Adhesion and 13-HODE:HETE Ratios: All resting human tumor cells studied, produced basal levels of 13-HODE and 15-HETE. The intracellular levels of 13-HODE and 15-HETE in the human tumor cells were i) 26 ± 3 and < 1 ng/10^7 cells for the U87MG tumor cells; ii) 13 ± 1 and 12 ± 1 ng/10^7 cells for the A549 tumor cells; and iii) 17 ± 2 ng and 51 ± 1 ng/10^7 cells for the HL60 cells, respectively. There was no extracellular 13-HODE nor 15-HETE detected.

The ratio of 13-HODE:15-HETE is plotted against tumor cell adhesion in Figure 17. Under resting conditions, the ratio of 13-HODE:15-HETE in U87MG cells was 21:1, which was associated with < 1 x 10^3 U87MG tumor cells adhering to an endothelial cell monolayer. When the U87MG tumor cells were stimulated with 1 μM fMLP for 10 min, the ratio of 13-HODE: 15-HETE decreased to 1:1, and the number of adherent U87MG tumor cells increased to 2 ± 0.6 x 10^3 cell/monolayer (Figure

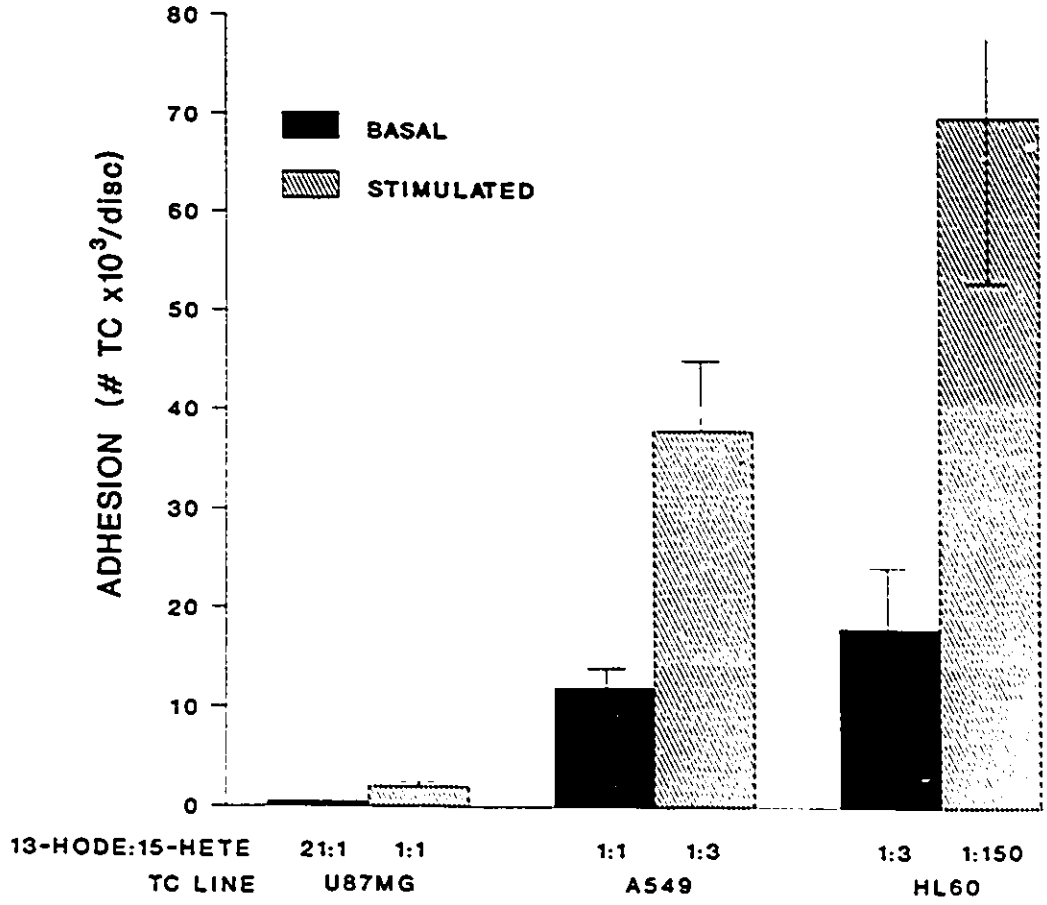


FIGURE 17. Relationship between the 13-HODE:15-HETE ratio of human tumor cell lines and tumor cell adhesion to endothelial cells under both basal and fMLP-stimulated conditions. Data are expressed as mean ± SEM, n = 12.

17). The ratio of 13-HODE:15-HETE in resting A549 tumor cells was 1:1 and 12 ± 2 x 10^3 tumor cell adhered to the endothelial cell monolayers. When A549 tumor cell were stimulated with fMLP, the ratio of 13-HODE:15-HETE decreased to 1:3, and A549 tumor cell adhesion increased to 38 ± 7 x 10^3 tumor cell/ monolayer, $p < 0.01$.

The differences in the 13-HODE:15-HETE ratio and tumor cell adhesion before and after stimulation of transformed HL60 cells were even more marked. The ratio of 13-HODE:15-HETE in resting cells was 1:3 and decreased to 1:150 after stimulation. Under the same conditions, HL60 tumor cell adhesion increased from 18 ± 6 x 10^3 to 70 ± 19 x 10^3 cells/monolayer (Figure 17).

Additional experiments were performed using A549 tumor cells, to determine whether manipulating the intracellular level of cAMP in A549 cells influenced A549 tumor cell 13-HODE synthesis and subsequent adherence. Thus, under resting conditions, A549 cAMP level was 48 ± 4 pmol/10^7 cells (Table 40). When the tumor cells were incubated with 10 or 20 μM dipyridamole for 24 hours, A549 cAMP levels increased to 81 ± 11 and 100 ± 18 pmol/10^7 cells, respectively.

The dose-related increase in A549 cAMP levels were associated with a dose-related increase in A549 13-HODE levels (Table 40). Thus, the intracellular levels of 13-HODE increased from 13 ng/10^7 cells (control), to 17 or 21 ng of 13-HODE/10^7 cells, following a 24 hour incubation with 10 or

20 μM dipyridamole, respectively. These changes were associated with a significant decrease in tumor cell adhesion both to endothelial cells and to endothelial cell-derived basement membrane, $p < 0.01$ (Table 40).

TABLE 40. Effects of dipyridamole treatment of A549 tumor cells on A549 cAMP and 13-HODE levels, and on subsequent A549 adhesion to endothelial cells (ECs) and EC-derived basement membrane (BM).

	DIPYRIDAMOLE TREATMENT		
	0 μM	10 μM	20 μM
cAMP (pmol/10^7 cell)	48 ± 4	81 ± 11	100 ± 18*
13-HODE (ng/10^7 cell)	13 ± 1	17 ± 2	22 ± 2*
A549/EC Adhesion (x10^3 cells/disc)	70 ± 7	55 ± 6	28 ± 6*
A549/BM Adhesion (x10^3 cells/disc)	400 ± 50	250 ± 11	96 ± 16*

Data are expressed as mean ± SEM, n = 4; *$p < 0.01$ versus control (0 μM).

CLINICAL STUDIES:

Clinical Implications of Internal Mammary Artery 13-HODE Levels in Coronary Artery Bypass Surgery: A clinical pilot study was performed to determine whether the inverse relationship between vessel wall 13-HODE synthesis and vessel wall adhesivity that was observed experimentally in both human endothelial cells and in rabbit vessel walls, existed in freshly isolated human vessels. This relationship was examined in internal mammary arteries (IMAs) obtained from 6 patients undergoing elective coronary artery bypass grafting (CABG). 13-HODE levels were determined by HPLC and IMA adhesivity was assessed as the # of ^{3}H-platelets adherent/mm^2 surface area.

The 6 CABG patients ranged in age from 49 to 63 years of age and all patients had discontinued aspirin therapy 7 days prior to surgery, and were given 75 mg dipyridamole orally three times a day starting at 48 hours prior to surgery. The levels of 13-HODE in the IMA segments ranged from 1 to 267 pg/mm^2 and were inversely related to IMA adhesivity, $r = -0.932$, $p < 0.001$ (Figure 18). There was no 15-HETE nor 12-HETE detected in any of the samples. Therefore, it was concluded that the adhesivity of autologous grafts are, in part, influenced by the amount of 13-HODE present.

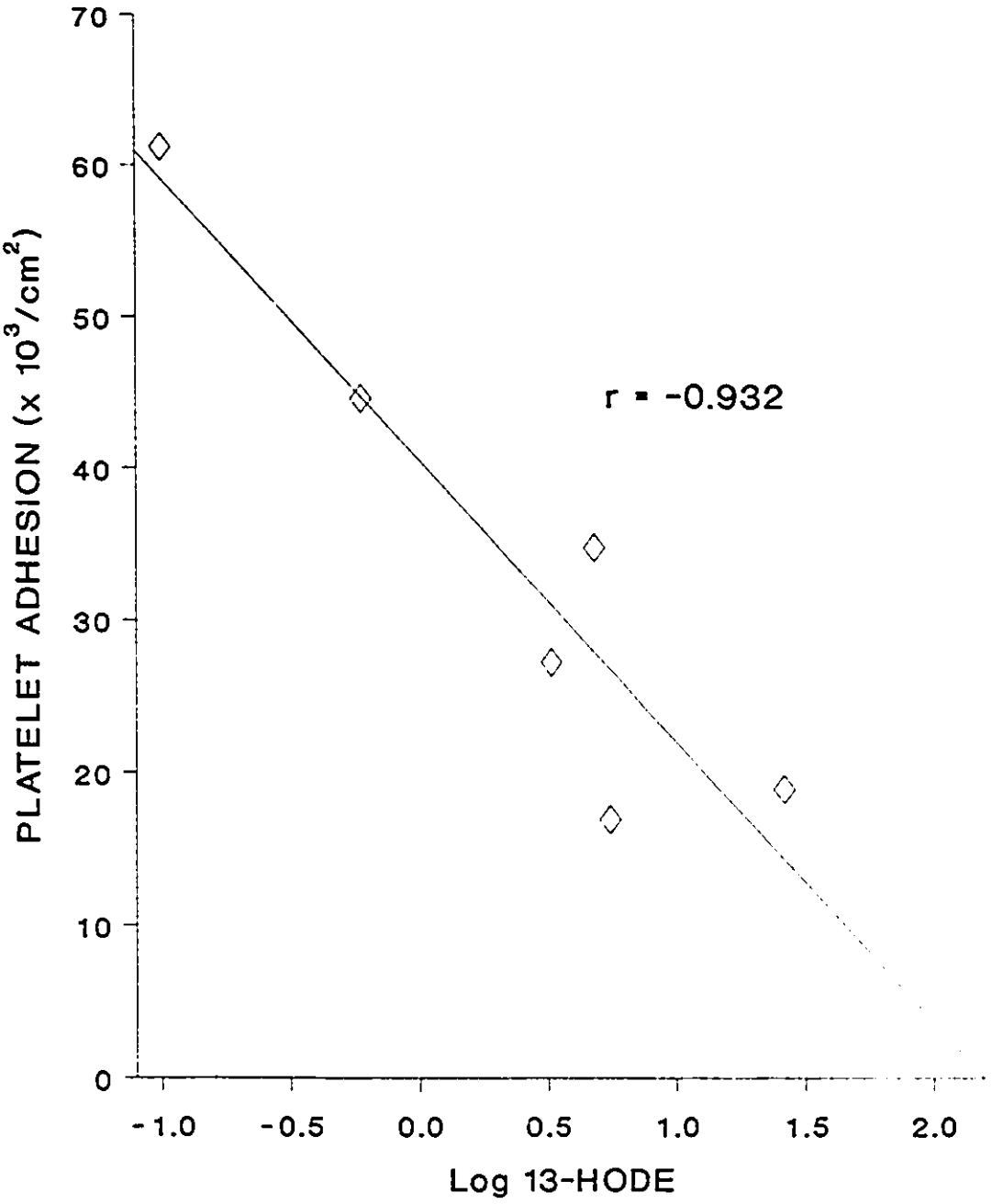


FIGURE 18. Relationship between internal mammary artery (IMA) 13-HODE levels and IMA thrombogenecity.

CHAPTER 5:

DISCUSSION

PREAMBLE:

Platelet interactions with the vessel wall following injury, influence both the acute thrombotic event (platelet-vessel wall adhesion) and the chronic pathogenesis of arteriosclerosis (chronic vessel wall thickening and occlusion). Platelets are thought not to interact with the healthy intact endothelium, but only with vessel wall components exposed following endothelial cell damage and vessel wall injury (Ross, 1981 and 1987; Packham and Mustard, 1986). When platelets adhere to the injured vessel wall, they release a number of constituents which facilitate further platelet activation, activation of the coagulation cascade, and/or are mitogenic, facilitating smooth muscle cell proliferation and hyperplasia (Ross, 1979; Nilsson, 1986; Packham and Mustard, 1986; Ross, 1987). Endothelial cells also synthesize and release a number of constituents which counterbalance the effects of the platelet responses, thereby enhancing fibrinolysis, inhibiting coagulation and preventing platelet aggregation and adhesion (Buchanan, 1988; Collen; 1988; Turpie, 1988). Recently, with the development of endothelial cell culture techniques and a number of molecular biological tools, we have learned that platelets and other blood cells actively interact not only with the injured vessel wall, but also with the intact endothelium itself. Similar cell

interactions and subsequent responses occur in inflammation (leukocyte-vessel wall interactions) and metastasis (tumor cell-vessel wall interactions) (Liotta, 1984; Dustin et al, 1986; Pober et al, 1986; Bevilacqua et al, 1987; Springer et al, 1987; Nicolson, 1988, Rice et al, 1988; Jurgensen et al, 1990; Mortarini et al, 1990).

In this thesis, a number of experiments were performed to consider the possibility that these cell-cell adhesion interactions, which are dependent upon the expression of integrins, are regulated by metabolites derived from the lipoxygenase pathway. Specifically, experiments were performed; i) to identify the cellular source of linoleic acid for 13-HODE synthesis; ii) to confirm our initial observations that there is an inverse relationship between 13-HODE and cell adhesivity; iii) to demonstrate that this relationship is associated with an interaction of 13-HODE and the VnR in endothelial cells; iv) to demonstrate that 13-HODE can alter the adhesivity of other adhesive moieties present in basement membranes and artificial grafts, and; v) to demonstrate that manipulating this pathway by either drugs or diet, alters the adhesivity of the vessel wall for blood cells. Before embarking on these studies it was necessary to develop an appropriate method to measure 13-HODE and other lipoxygenase metabolites. In this chapter I will discuss the results of the experiments in the order of the Specific Objectives as identified on pages 41 to 43.

1. AUTOMATED HPLC EXTRACTION AND QUANTIFICATION PROCEDURE:

The lipoxygenase metabolites have become of interest since their initial discovery in platelets and PMNs (Hamberg and Samuelsson, 1974; Borgeat and Samuelsson, 1979). In an attempt to identify the biological importance of these metabolites, it had become evident that there was a need to develop better assay systems to quantitate these metabolites. Unfortunately, unlike the metabolites of the cyclo-oxygenase pathway which are readily detectable by RIA (Granstrom and Kindahl, 1978), the majority of lipoxygenase metabolites must be quantified either by HPLC or by GC/MS (Bryant and Hwang, 1983).

The quantification of these metabolites by both normal phase-HPLC and by GC/MS requires that the aqueous biological samples first be extracted and then concentrated in organic solvents prior to their measurement. These metabolites also can be analyzed directly from aqueous samples by reverse phase (RP)-HPLC, providing that the metabolite levels are sufficiently high and the protein content sufficiently low (Haas and Buchanan, 1988). However, since most cell suspensions contain low metabolite and high protein levels, sample concentration and/or protein precipitation are still prerequisites for their successful analysis by RP-HPLC.

To overcome these technical problems, lipoxygenase metabolites have been extracted from acidified aqueous samples using organic solvents such as diethyl ether, chloroform-

methanol mixtures, or ethyl acetate (Salmon and Flower, 1982; Powell, 1982). These procedures are both time-consuming and require the evaporation of large volumes of solvent. To overcome these latter problems, a variety of solid phase extraction techniques were introduced using Amberlite XAD resins and octadecyl extraction columns (Powell, 1982; Luderer et al, 1983; Verhagen et al, 1986). These solid phase extraction techniques resulted in good recoveries of lipoxygenase metabolites but were again time-consuming and required additional technical expertise in order to obtain quantitative results.

Recently, Borgeat et al (1984) described a RP-HPLC method that overcame the problem of cell particulates in the sample and reduced the amount of analysis time. This method allowed for a direct analysis of relatively large sample volumes (2 ml) derived from cell suspensions. However, this method had the major limitation that cell suspensions containing protein (eg. 0.35% BSA) could not be assayed, as protein precipitation was not complete. It was therefore, clearly evident that an automated extraction procedure had to be developed which allowed for better recoveries of lipoxygenase metabolites from samples containing protein, while maintaining quantitative and qualitative analysis.

The automated HPLC extraction and quantification procedure that was developed for the present studies, employed a column venting technique, whereby the fatty acids were first

extracted by a pre-column, and proteins and other unwanted material were vented to waste. The pre-column eluent was then directed to the analytical column, and the retained lipoxygenase metabolites eluted off the pre-column and separated on the analytical column. The monohydroxides eluting off the analytical column were then quantitated by UV spectrophotometry.

This automated extraction system was found to be highly efficient for the analysis of metabolites ranging in concentrations from 5 to 200 ng/sample. Their analysis was not affected by either altering the sample volume and/or the presence of protein. In contrast to other extraction techniques, sample acidification was not required and in fact, was detrimental as it led to increased retention of unwanted material and decreased stability of the labile LTs. The quantitative and qualitative results obtained with this automated system are a marked improvement over previous methods for the ranges of sample volume, media, and metabolite levels tested. Finally, the described method is reproducible, reliable and minimizes both the time and cost involved in assaying a sample.

The major limitation of this method however, is that large volumes of plasma can not be analyzed without changing the pre-column. This limitation can be overcome if the pre-column is back-washed and cleaned between each sample run with 1.0 M NaCl. However, the present design of the pre-columns

does not allow for back-flushing. If a new pre-column is designed which allowed for back-flushing, this automated HPLC extraction method may be easily modified to allow for the analysis of these metabolites from plasma.

2. ENDOGENOUS SOURCE OF LINOLEIC ACID IN ENDOTHELIAL CELLS FOR 13-HODE SYNTHESIS:

Experiments were performed to determine which intracellular fatty acid pool in endothelial cells was the endogenous source of linoleic acid for 13-HODE synthesis. Thus, endothelial were incubated with increasing concentrations of exogenous linoleic to determine whether the free fatty acid pool in endothelial cells, which is exchangeable with plasma fatty acids, was the source of linoleic acid. Contrary to what was expected, incubating endothelial cells with low concentrations of exogenous linoleic acid did not result in an increase in intracellular 13-HODE, but rather inhibited 13-HODE synthesis. On the other hand, incubating endothelial cells with high concentrations of exogenous linoleic acid resulted in the synthesis of both extracellular 13-HODE and 15-HETE. It should be noted that these experiments were performed under acute conditions and utilizing high concentrations of exogenous fatty acids. If however, endothelial cells were incubated with low concentrations of linoleic acid for longer incubation times, intracellular 13-HODE synthesis was increased. There are a number of possible explanations for these inconsistencies. First, it is concluded that the high levels of exogenous linoleic acid activated cyclo-oxygenase and stimulated membrane phospholipases to release arachidonic acid. The activation of both these enzymes would explain the synthesis of extracellular

13-HODE and 15-HETE.

In agreement with these data, a number of investigators have reported that endothelial cells produce 15-HETE upon the addition of exogenous arachidonic acid (Hopkins et al, 1984; Kuhn et al, 1985). More recently, Kaduce et al (1989) reported that the predominant endothelial cell-derived monohydroxides of exogenous linoleic acid were 9-HODE and 13-HODE. They suggested that both 9-HODE and 13-HODE were cyclo-oxygenase derived. Funk and Powell (1985) also suggested that vessel wall cells metabolize exogenously added fatty acids predominantly by the cyclo-oxygenase pathway. However, it should be emphasized that these observations were obtained in experiments using exogenous fatty acids. While it is recognized that endothelial cells have the capacity to synthesize 15-HETE from exogenous arachidonic acid and 9-HODE from exogenous linoleic acid, it cannot be concluded that either of these monohydroxides is the predominant metabolite synthesized by the lipoxygenase pathway from intracellular linoleic or arachidonic acid stores, in either resting or stimulated endothelial cells. None of these investigators attempted to measure these metabolites from resting endothelial cells that are utilizing only their endogenous fatty acid stores. While we agree with the above results, when endothelial cells were extracted in the absence of exogenous fatty acids, only 13-HODE was detected, all of which is intracellular.

Furthermore, the lipoxygenase enzyme preferentially metabolized free linoleic acid rather than free arachidonic acid. This was supported by the observation that when endothelial cells were incubated with an equal molar ratio of linoleic and arachidonic acid, 13-HODE was the predominant metabolite. Therefore, it was concluded that 13-HODE is the predominant monohydroxy metabolite produced by resting endothelial cells by the lipoxygenase pathway, and that the plasma-exchangeable free fatty acid pool is not the intracellular source of linoleic acid for 13-HODE synthesis. Experiments were therefore, performed to determine whether the endothelial cell triglyceride pool was the endogenous source of linoleic acid for 13-HODE synthesis.

The triglyceride pool was chosen as the likely endogenous supply of linoleic acid, because; i) Lagarde et al (1984) reported that endothelial cell triglycerides are enriched in linoleic acid; and, ii) Denning et al (1983) and Figard et al (1986) reported that the triglyceride pool in endothelial cells is rapidly turned over in resting cells (i.e. at a time which cAMP levels are high and when phospholipids are inactive). It was also reported that vessel wall smooth muscle cell triglyceride turnover was increased with db-cAMP treatment (Tertov et al, 1986). Therefore, db-cAMP was used to increase endothelial cell triglyceride turnover.

The observation that db-cAMP treatment of endothelial cells resulted in i) marked and dose-related decreases in the

amount of linoleic acid associated with the triglycerides; and, ii) that these observations were observed in both pre- and post-fatty acid labelled endothelial cells, indicated that the increased linoleic acid turnover in the triglycerides was due to an increased hydrolysis and not a decreased incorporation. The observations that dose-related effects of db-cAMP on linoleic acid triglyceride turnover were paralleled with, and were highly correlated with dose-related increases in 13-HODE, provides further evidence that the triglycerides were the endogenous source of linoleic acid for 13-HODE synthesis. The absence of any effect of db-cAMP on PGI_2 synthesis and on arachidonic acid turnover, provided further evidence that 13-HODE was synthesized in endothelial cells when PGI_2 was not, and vice versa.

One anomaly of these experiments which remains unexplained is the observation that following endothelial cell stimulation, 13-HODE disappeared and could not be detected either intracellularly or extracellularly. Furthermore, there was no change in the distribution of linoleic acid among the fatty acid pools, nor were any known metabolites of hydroxy fatty acids detected. It is possible that the lack of detection of free 13-HODE following endothelial cell stimulation is due to either an esterification of 13-HODE into the triglycerides or a degradation of 13-HODE into an unknown metabolite.

3. EFFECT OF ENDOTHELIAL CELL STIMULATION ON 13-HODE LEVELS AND ADHESIVITY:

The results of the previous section confirm that endothelial cells synthesize 13-HODE under basal conditions and PGI_2 following stimulation. These results indicate that the biocompatibility or "non-adhesivity" of the intact endothelium is unlikely to be contributed to by PGI_2, as was initially postulated by Moncada and Vane (1979). In earlier studies, our laboratory provided evidence that the biocompatibility of the endothelial cell was associated in part with the levels of 13-HODE. These preliminary studies were confirmed by the experiments of this thesis. First, it was demonstrated that the decrease in endothelial cell 13-HODE synthesis following stimulation was inversely correlated with endothelial cell adhesivity, not only for platelets, but also for a variety of tumor cells and leukocytes. These results demonstrate that if 13-HODE plays a significant role in modulating endothelial cell "adhesivity", it modulates the adhesivity of the endothelial cell for all blood cells.

The observation that the enhanced endothelial cell adhesivity associated with decreased 13-HODE occurred at a time when PGI_2 synthesis was increased provides further evidence that PGI_2 does not play an important role in regulating endothelial cell biocompatibility in the healthy endothelium. This is consistent with the observations of Schorer and White (1989) who also demonstrated that IL1

stimulated vessel wall adhesivity for platelets was also independent of PGI_2 or TXA_2 production.

Further support for 13-HODE playing a role in modulating endothelial cell adhesivity was provided by the observations that TNF and IL1 stimulation of the endothelium was associated with both decreased 13-HODE synthesis and increased endothelial cell adhesivity for PMNs. In contrast, however, γ-IFN had no effect on either endothelial cell 13-HODE synthesis or adhesivity. These observations are consistent with the differential affects of these 3 cytokines on endothelial cell PMN adhesion receptor expression (Bevilacqua et al; 1985, Gamble et al, 1985; Dustin et al, 1986; Munro et al, 1989). Both IL1 and TNF increased PMN adhesion to endothelial cells, whereas γ-IFN did not. These investigators also demonstrated that the increase in leukocyte adhesion was dependent upon the expression of either ICAM-1 or ELAM-1. However, since γ-IFN also stimulates ICAM-1 expression (Pober et al, 1986a), it seems unlikely that the enhanced PMN adhesion seen in the present studies is dependent upon the expression of ICAM-1.

While the possibility that ELAM-1 expression contributed to the enhanced adhesivity in the present experiments exists, it is unlikely that ELAM-1 was the major adhesion molecule expressed. ELAM-1 has only been shown to facilitate leukocyte adhesion, and has not been shown to facilitate platelet nor tumor cell adhesion (Rice et al, 1989 and 1991).

Furthermore, in our studies, cytokine-induced tumor cell-endothelial cell adhesion was blocked by RGD peptides, peptides which do not alter ELAM-1 mediated adhesion. In addition, Lafrenie et al (1990) recently reported that one of the major adhesion molecules expressed on endothelial cells following cytokine stimulation is the VnR. Thus, as shown in the histoimmunofluorescent studies, this receptor seems to be a more likely possibility.

Regardless of which receptor is involved, these studies provide unequivocal evidence that the enhanced adhesion associated with decreased 13-HODE synthesis is related to the expression of an RGD dependent integrin. From the perspective of tumor cell-endothelial cell adhesion, PGI_2 was thought to act as a natural antimetastatic agent to down regulate tumor cell-vessel wall interactions (Honn and Marnett, 1984). However, earlier data utilizing the infusion of PGI_2 or blocking its synthesis is not consistent with that possibility. In the present studies, it was noted that PGI_2 synthesis following stimulation of endothelial cells with cytokines also occurred at a time when tumor cell-endothelial cell adhesion was increased. It seems more likely that if PGI_2 plays any role in the metastatic process, it is a secondary role.

Finally, the observations that tumor cells adhered to the intact endothelium provide additional support that tumor cells can indeed attach to the intact endothelium and do not

require exposure of the basement membrane. These observations are not contrary to earlier studies in which it was demonstrated that tumor cells avidly adhere to exposed sites of damaged vessel wall and not to the intact endothelium (Liotta et al, 1986; Terranova et al, 1986; Lichtener et al, 1989). Rather, they provide an extension of those observations and raise the possibility that the initial interaction of tumor cells with the vessel wall is in fact with the endothelium.

In conclusion, the data from these experiments has led to the conclusion that; i) endothelial cells have an active lipoxygenase pathway under basal conditions; ii) 13-HODE synthesis is inhibited following stimulation; iii) this decrease in synthesis is associated with increased endothelial cell adhesivity not only for platelets, but also for leukocytes and tumor cells; and finally, iv) PGI_2 probably plays a secondary role in the regulation of these adhesion interactions. The implications of these studies are that 13-HODE acts either as a competitive inhibitor or as an internal regulator of endothelial cell adhesivity.

4. EFFECT OF EXOGENOUS FATTY ACIDS ON ENDOTHELIAL CELL ADHESIVITY:

In the last section, the possibility was raised that 13-HODE either acted as a intracellular regulator of endothelial cell adhesivity, or acted directly in a competitive manner to block cell adhesion. The latter possibility seems unlikely, since in experiments when 13-HODE was added to the outside of the endothelial cell under either basal or stimulated condition, platelet, tumor cell and leukocyte adhesion was unaltered. However, it is recognized that at the high concentration of 13-HODE used, platelet adhesion was decreased. This was in association with a marked stimulation of PGI_2 production. If we conclude from the earlier study that decreased 13-HODE synthesis in endothelial cells results in a non-specific increase in cell adhesion to the endothelial cell, one would expect a similar non-specificity in its ability to block cell adhesion from the outside. Therefore, it is more likely that the apparent decreased platelet adhesion in those experiments was due to the concomitant generation of PGI_2, which in turn, increased platelet cAMP levels and rendered them hypo-responsive. The observations that the enhanced PGI_2 production played little role in leukocyte and tumor cell adhesion to the endothelial cell following pre-exposure to 13-HODE, particularly for the tumor cell, is further evidence that PGI_2 plays, if anything, a secondary role, but not a primary role in adhesion of blood

cells to the endothelium.

It was also curious to note that monohydroxides derived from arachidonic acid, did not influence platelet adhesion with the exception of 5-HETE. Curiously, 5-HETE was the monohydroxide which was incorporated into the endothelial cell, consistent with earlier studies by Richard et al (1986). These studies and those of Richard are in contrast with those of Schafer et al (1986), who reported that endothelial cells specifically incorporate 12-HETE. They presented an argument to support the concept that incorporation of platelet 12-HETE with the vessel wall somehow influences subsequent platelet-endothelial cell interactions. However, an important difference between the observations of Schafer and those by Richard and this laboratory, is that Schafer measured incorporation of the monohydroxy fatty acid to endothelial cells in suspension, whereas experiments in this thesis and those of Richard utilized intact monolayers. Intact monolayers were used in our experiments and should always be used since endothelial cells normally confront circulating blood cells in a monolayer presentation.

The observations that despite 13-HODE, 12-HETE and 15-HETE not binding to the endothelial cell, yet generating PGI_2 production, raises an interesting possibility concerning the requirement of uptake. The lack of uptake of these metabolites, yet their stimulation of these products, raises a possibility that they may act as a pseudo catalysts. Since

they are not, this would suggest that the results of these experiments, albeit real, are *in vitro* artifacts.

These studies, therefore, continue to lead to the conclusion that if monohydroxides in general, and 13-HODE in particular, have any effect on blood cell-endothelial cell interactions, they mediate their effect from inside the cell. Therefore, these studies lead to the hypothesis that intracellular 13-HODE acts either as a secondary messenger to influence endothelial cell adhesivity or alternatively, acts directly with some adhesive moiety influencing its expression. The discussion of the results in Section 5 support the latter possibility.

5. INTRACELLULAR LOCALIZATION OF 13-HODE AND ITS ASSOCIATION WITH THE VITRONECTIN RECEPTOR:

Little is known about the endogenous regulators which influence the expression of adhesion molecules on the external surfaces of endothelial cells and circulating blood cells. The above studies suggest that, at least in endothelial cells, a lipoxygenase metabolite contributes to this process. Other experiments discussed in Section 7, indicate that monohydroxides influence cell adhesion in general. For example, the intracellular levels of HETEs in platelets and in tumor cells up-regulate both platelet and tumor cell adhesivity, respectively.

Similar observations have also been reported when exogenous 13-HODE or 12-HETE is added to stimulated tumor cells (Grossi et al, 1989). The inhibitory effect of 13-HODE on tumor cell adhesion in those studies were associated with decreased $GPII_b/III_a$ receptor expression, whereas the enhancing effect of 12-HETE on tumor cell adhesion was associated with an enhanced $GPII^b/III_a$ receptor expression. Since a receptor similar to $GPII_b/III_a$ receptor, the VNR, has been reported to exist in endothelial cells (Pytela et al, 1985; Van Mourik et al, 1985; Leeksma et al, 1986; Newman et al, 1986; Cheresh, 1987), it was postulated that 13-HODE down-regulated the expression of the VnR in endothelial cells. The histoimmunofluorescent studies provide evidence that 13-HODE and the VnR are in close association in resting endothelial cells.

Furthermore, the depth focusing of these preparations indicated that in the unstimulated cell, both VnR and 13-HODE could only be detected following permeabilization of the membrane. Neither molecule was detectable on the surface of the cell. The fact that the histoimmunofluorescent pattern of staining for 13-HODE (using a fluorescein-tagged second antibody) and the VnR (using à rhodamine red-tagged second antibody) are identical, indicated that both molecules are situated inside the cell in the exact same location. Furthermore, the observation that the LM142 antibody, but not the LM609 antibody detected the VnR in resting endothelial cells indicated that the VnR was present, in association with 13-HODE, in a non-adhesive form. Smith and Cheresh (1988) have demonstrated that the RGD recognizing (adhesive) site of the VnR β chain is masked by its α chain under resting conditions. These data, are compatible with our observation, since the LM142 antibody recognizes the α chain, whereas the LM609 recognizes the RGD recognition site of the β chain. It is also interesting to note that Singer et al (1989) found that other adhesion receptors (laminin and fibronectin receptors) are located in specific granules or "adhesomes" in leukocytes under resting conditions. These observations provide further evidence for the possibility that a mechanism of adhesion molecule regulation by monohydroxides is common to a number of cell types. Therefore, it is difficult to refute the possibility that monohydroxides and the VnR play an

important and interacting role in cell adhesion.

If so, a possible mechanism by which 13-HODE interacts with the VnR to alter endothelial cell adhesion, is shown in Figure 19. The carboxyl terminus of both VnR chains contain long hydrophobic membrane spanning domains, which are required for hydrophobic interactions between the VnR and 13-HODE. In addition, both VnR chains are rich in cysteine, which is necessary for rapid conformational changes (Fitzgerald et al, 1987; Ginsberg et al, 1987; Suzuki et al, 1985 and 1987). Therefore, it is proposed that under resting conditions, i.e. in non-adhesive endothelial cells, the VnR is anchored at the hydrophobic region of the α chain by 13-HODE in vesicles immediately below the plasma membrane (Figure 19a). Under these conditions, 13-HODE maintains the α chain in a conformation such that the α chain masks the RGD recognition site of the β chain. This is consistent with the observations of Smith et al (1988). Upon stimulation, 13-HODE and the VnR α-chain dissassociate, resulting in a conformational change in the α chain which unmasks the adhesive site on the β chain (Figure 19b). At the same time, the VnR relocates to the membrane phospholipids on the apical surface of endothelial cells. The possibility that fatty acid metabolites are capable of rendering conformational changes in the VnR are supported by the recent observations of Conforti et al (1990). These investigators found that the ability of the VnR to recognize various ligands was altered by varying the

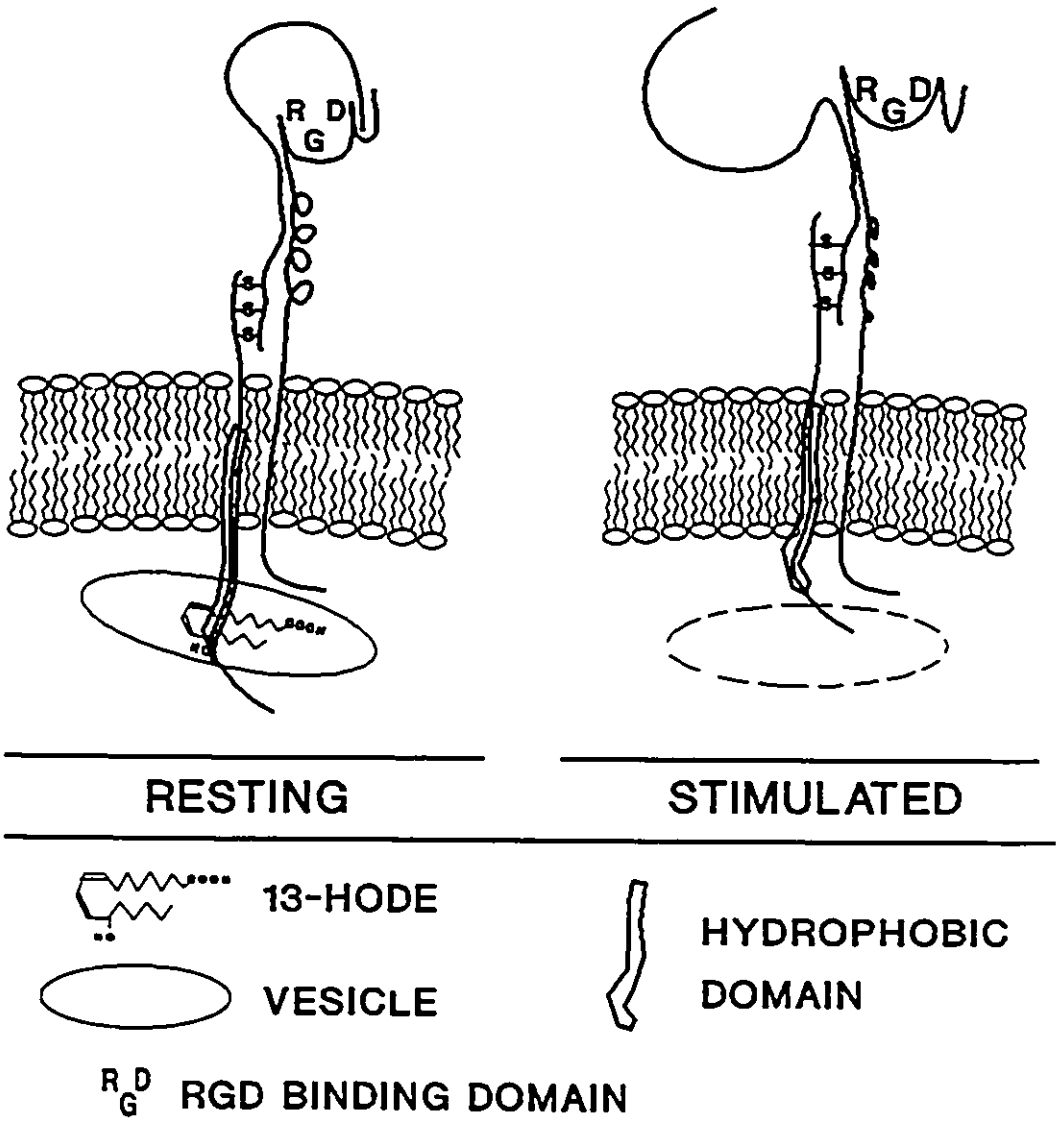


FIGURE 19. Proposed model of how 13-HODE regulates the expression of the VnR in endothelial cells. Under resting conditions, 13-HODE anchors the VnR α chain to cytosolic vesicles, masking the RGD binding domain of the VnR β chain. Following stimulation, the 13-HODE/VnR α chain dissociate and the RGD binding domain is unmasked.

composition of fatty acids present.

In summary, it is concluded that 13-HODE and the VnR are co-localized in specific vesicles in resting endothelial cells in such a manner that the endothelial cells remain non-adhesive. Following endothelial cell stimulation, 13-HODE and the VnR dissassociate and the RGD recognizing adhesive site of the VnR β-chain is expressed on the apical surface of endothelial cells, rendering them adhesive. If our model is correct, it is expected that adding exogenous 13-HODE to extracellular matrixes containing integrins, the hydrophobic sites of which are unmasked, will render those integrins less adhesive. The experiments performed using exogenous 13-HODE and basement membrane and artificial grafts, is consistent with this possibility.

6. 13-HODE AND BASEMENT MEMBRANE THROMBOGENECITY:

A number of studies have demonstrated that a variety of extracellular matrix components modulate platelet-basement membrane interactions. Some of these components include collagen, vWF and fibronectin (Madrid et al, 1980; Houdijk et al, 1985; Sakariassen et al, 1987; Meyer et al, 1987; Sixma et al, 1987a). However, there is some controversy as to whether or not the basement membrane underlying endothelial cells is thrombogenic or thromboresistant. Therefore, experiments were performed to determine whether endothelial cells released 13-HODE into their underlying basement membrane, and if so, whether 13-HODE influenced basement membrane adhesivity.

Adhesivity of the basement membrane was dependent upon the method by which the endothelial cells were removed. Most investigators have prepared basement membranes using NH_4OH, or other toxic chemical to lyze the overlaying endothelial cells. The resulting basement membrane preparations were found to be highly thrombogenic, but their thrombogenecity was thought to be due to the presence of vWF, which facilitates platelet adhesion in flow condition under high shear (Sakariassen et al, 1987; Meyer et al, 1987; Sixma et al, 1987).

It should be noted however, that NH_4OH is a lipid solubilizer. When basement membranes were prepared by non-chemical means, the resulting basement membranes were less adhesive and that adhesivity is inversely related to the amount of 13-HODE associated with it. It is suggested

therefore, that endothelial cells either release 13-HODE continuously to their ablumenal surface or alternatively, release 13-HODE to their ablumenal surface following endothelial cell stimulation. The released 13-HODE interacts with hydrophobic sites, including those on basement membrane associated integrins, rending them non-adhesive. Additional support for this possibility is that when increasing amounts of 13-HODE were added to the surface of artificial grafts containing basement membrane components, there was a concentration-dependent decrease in platelet adhesion to that surface. Furthermore, the decrease in platelet adhesion was achieved at levels of 13-HODE comparable to those found in basement membrane preparation of endothelial cells.

As will be demonstrated later, the observations that the presence of 13-HODE, not only in the basement membranes of endothelial cell cultures, but also in the basement membranes of rabbit vessel walls and human internal mammary arteries, determined the degree of platelet adherence, is compatible with the conclusion that 13-HODE interacts with any or a variety of integrins and/or their ligands in the basement membrane, to render these proteins less adhesive.

These data are in contrast to those of De Graff et al (1989) who concluded "that 13-HODE does not play a major role in platelet subendothelium interaction". Their conclusions were based upon the observation that binding 13-HODE to endothelial cell-derived basement membranes prepared with

NH_4OH, did not render the basement membranes non-adhesive to platelets. However, these investigators presented no data to demonstrate that their basement membrane preparations were either intact or functional. Furthermore, they did not compare the effects of 13-HODE on the adhesivity of basement membranes prepared by other methods. This latter point is of particular important since it is unlikely that NH_4OH would destroy endothelial cell membranes without also destroying components of the basement membranes. Such destructive changes to the basement membranes may prevent 13-HODE from altering the conformational structure of any adhesive component. Furthermore, it should be re-emphasized that the level of 13-HODE associated with endothelial cell basement membranes, prepared by non-enzymatic or non-chemical methods, correlates with decreased platelet adhesivity. Finally, all our observations concerning the relationship between 13-HODE and either endothelial cell or basement membrane adhesivity, have been confirmed by Tloti et al (1991).

If all of the <u>in vitro</u> data have any biological relevance, it would be expected that circulating blood cell-vessel wall interactions would be influenced by either drugs or diets which manipulate 13-HODE synthesis.

7. INFLUENCE OF DRUGS AND DIET ON VESSEL WALL AND PLATELET LIPOXYGENASE METABOLITES AND SUBSEQUENT VESSEL WALL-PLATELET INTERACTIONS:

Drug Effects: Consistent with the above possibility, the adhesivity of injured carotid arteries in rabbits was altered in relationship to changes in vessel wall 13-HODE synthesis.

The observations that salicylate inhibited 13-HODE synthesis and this was paralleled by increased vessel wall adhesivity can explain earlier observations that high dose aspirin, in particular the salicylate moiety, increased thrombosis (Buchanan et al; 1981, Kelton et al, 1978; Czervionke et al, 1978). More importantly, the salicylate data are consistent with the *in vitro* observations that decreasing 13-HODE synthesis increases endothelial cell adhesivity.

The observations that dipyridamole increased vessel wall cAMP and 13-HODE levels, and these effects were associated with decreased vessel wall adhesivity, are consistent with our *in vitro* observations that increased endothelial cell cAMP levels resulted in increased triglyceride turnover, which in turn, increased endothelial cell 13-HODE synthesis and consequently, endothelial cell adhesivity was decreased. These data suggest that manipulation of 13-HODE synthesis *in vivo* has important implications in thrombosis.

The latter observations, i.e. the dipyridamole effect,

raises important issues concerning the use of dipyridamole in the treatment of thrombosis. While numerous experimental studies have demonstrated that dipyridamole impairs platelet function, presumably by blocking phosphodiesterase thereby increasing platelet cAMP levels (FitzGerald, 1987), the beneficial effects of dipyridamole in the clinical setting have been inconsistent. For example, Harker et al (1987) examined the effects of aspirin and dipyridamole in preventing hyperplasia following carotid endartectomies. They concluded that the "antiplatelet" treatment regime was ineffective in preventing chronic hyperplasia and restenosis. In other studies however, the effect of dipyridamole in patients undergoing coronary bypass grafting was examined (Chesebro, 1990). He found that dipyridamole decreased saphenous vein graph hyperplasia. Albeit, the 2 clinical situations are considerably different, their endpoints are similar, i.e. hyperplasia. If one accepts the latter assumption, what is it about the treatment regimes which differ sufficiently to explain for the differences in the drug effect. It was interesting to note therefore, that in the study by Harker, dipyridamole was given 2 hours pre-operatively, while in the study by Chesebro, dipyridamole was given for 48 hours pre-operatively. The combination of aspirin and dipyridamole was given for 12 months post-operatively in both patient populations. Given the results of the <u>in vitro</u> and <u>in vivo</u> data in this thesis, the following possibility may explain the

Harker/Chesebro differences. The 48 hour pre-operative treatment with dipyridamole, increased vessel wall cAMP levels, resulting in increased vessel wall 13-HODE synthesis and decreased vessel wall adhesivity. If so, it is possible that the acute platelet-vessel wall interactions occurring postoperatively were decreased sufficiently to reduce PF4 and PDGF secretion from adherent platelets, thereby reducing subsequent hyperplasia. This is supported by earlier studies in our laboratory, suggesting that dipyridamole treatment requires at least 48 hours before its effects are manifested. This would explain why Harker et al, who only treated their patients 2 hours pre-operatively, did not see a beneficial effect with dipyridamole. The relevance of these observations are a subject of ongoing clinical and experimental studies here at McMaster.

These observations also provide an alternative explanation for the decreased adhesivity of atherosclerotic aorta in Watanabe rabbits (Lawrence et al, 1989). They suggested that thermal injury may have caused a loss of vWF or antithrombotic glycosaminoglycans. However our data, in conjunction with those of Simon et al (1989 and 1989a), provide evidence that the increased vessel wall cell proliferation is associated with increased 15-lipoxygenase activity, and hence 13-HODE synthesis, thereby rendering the vessel wall less thrombogenic. If so, the pharmacological manipulation of vessel wall 13-HODE levels may provide a new approach to the treatment of

arterial thrombosis. Further support for this idea was obtained from the diet studies.

Diet Effects: There is convincing evidence that vascular stenosis, at least in part, is mediated by platelets (Chesebro et al, 1987 and 1990; Harker et al, 1987 and 1990). A number of these platelet constituents that affect smooth muscle cell proliferation and stenosis are: TXA_2, 12-HETE, PDGF, and PF4. Upon exposure of the extracellular matrix, platelets become activated, and adhere to and aggregate on the surface of the exposed matrix. As a consequence, they release a variety of constituents which affect smooth muscle cell proliferation. It has been suggested that if the initial platelet adhesion or aggregation responses are prevented, by using specific anti-platelet agents, smooth muscle cell proliferation and stenosis may be prevented (Sixma et al, 1987; Meyer et al, 1987).

At least 2 platelet glycoproteins, GPIb and the GPIIb/IIIa complex, as well as plasma vWF, are required for optimal platelet adhesion to the exposed extracellular matrix (Sixma et al, 1987; Meyer et al, 1987). The expression of GPIIb/IIIa on the surface of platelets following activation, is also required for platelet spreading on the subendothelium (Weiss et al, 1986). The importance of these GPs for platelet involvement in stenosis is demonstrated by the observations that blockage of these GP receptors with specific antibodies, successfully inhibits acute platelet deposition and subsequent

stenosis (Harker, 1987; Sixma et al, 1987; Meyer et al, 1987). In contrast, other anti-platelet agents such as: aspirin, PGI_2 analogues, and TXA_2 receptor blockers are relatively ineffective in reducing platelet accumulation and stenosis (Harker, 1987; Baumgartner and Hosang, 1988; Metke et al, 1979). It is likely that their ineffectiveness is a result of their action in blocking only the platelet prostanoid pathway, which influences platelet aggregation (Harker, 1987), but not platelet adhesion. Therefore, it would appear that in order to effectively reduce the platelet contribution to stenosis, platelet adhesion must be blocked. This suggests that while both adhered platelets and aggregating platelets are capable of releasing a variety of constituents, only adhered platelets are implicated in stenosis. Adhered platelets may release their constituents directly into the intima and media, where they are closer to the smooth muscle cells and less susceptible to plasma inhibitors, such is not the case for aggregating platelets.

In contrast to TXA_2, 12-HETE is necessary for both platelet adhesion and macrophage adhesion (Spector et al, 1988). 12-HETE is also a potent chemoattractant for, and influences chemokinesis of smooth muscle cells, neutrophils and macrophages. Besides platelets, macrophages, neutrophils and vascular smooth muscle cells are other cellular sources of 12-HETE (Spector et al, 1988). Thus inhibition of platelet or vessel wall (i.e. smooth muscle cell or macrophage) 12-HETE

production may lead to a reduced incidence of stenosis.

In regards to the latter statement, a dietary fish oil, EPA, has recently been shown to have some promise clinically in reducing platelet adhesion to the vessel wall (Casali et al, 1986; Landymore et al, 1985; Dehmer et al, 1988; Slack et al, 1988). EPA is thought to inhibit both TXA_2 and 12-HETE production by displacing arachidonic acid from platelet phospholipids (i.e. the source of arachidonic acid for TXA_2 and 12-HETE synthesis), and by competing with arachidonic acid for both the cyclo-oxygenase and lipoxygenase enzymes (Casali et al, 1986; Dehmer et al, 1988; Nordoy et al, 1985; Knapp et al, 1986). In addition to inhibiting 12-HETE synthesis, EPA also inhibits several endogenous agents thought to be involved in smooth muscle cell-mediated stenosis, namely: leukocyte-derived lipoxygenase metabolites (Lee et al, 1985; Dyerberg, 1986) and PDGF (Fox and DiCorleto, 1988). In agreement with EPA reducing the rate of stenosis, there is a reduced incidence of cardiovascular disease in Eskimos and Japanese fishermen whose diets are rich in EPA (Dyerberg and Bang, 1978; Fitzgerald, 1987; Fitzgerald et al, 1987; Ballard-Barbash and Callaway, 1987).

There is also a reduction in cardiovascular complications in atherosclerotic patients fed diets enriched in linoleic acid (Dehmer et al, 1988), the substrate for 13-HODE.

These various studies formed the basis for performing the diet studies to compare the potential antithrombotic

effects, not only of linoleic acid, but also of EPA. The results of these experiments provide evidence that addresses 3 specific issues. First, these studies were the first to demonstrate ir the same animal that dietary linoleic acid is selectively incorporated into the vessel wall, whereas EPA is selectively incorporated into the platelet. The ramifications of these observations will be discussed momentarily. Second, the observation that dietary intervention can selectively alter vessel wall 13-HODE synthesis and as a consequences alter vessel wall adhesivity, provides further evidence that vascular wall 13-HODE synthesis is biologically important. Third, the observation that EPA is selectively incorporated into the platelet and that this is associated with decreased platelet 12-HETE synthesis and platelet adhesivity, but does not result in a significant "antithrombotic" effect (as measured by vessel wall adhesivity), raises some interesting issues concerning the present rationale for using "anti-platelet" agents, and in particular, for using EPA as an antithrombotic agent.

The concept of repairing the vessel wall as a strategy to decrease thrombogenesis has been limited primarily to cardiologic and surgical intervention, such as angioplasty and coronary artery bypass grafting. These treatments also require adjunct antiplatelet and anticoagulant therapy (Dehmer et al, 1988; Brister et al, 1990; Chesebro, 1990; Harker et al, 1990). However, the experimental data reviewed above,

suggest an alternative strategy, namely altering vessel wall thrombogenecity. A number of earlier studies have proposed rendering the vessel wall less thrombogenic after injury by targeting specific thrombin inhibitors at thrombin bound to fibrin or the injured vessel wall (Jand et al, 1990; Marzec et al, 1990; Okwusidi et el, 1990; Eisenberg et al, 1991). Others have suggested seeding genetically altered endothelial cells on artificial grafts or damaged vessel walls (Dichek et al, 1989; Johnson et al, 1991). We propose an alternative strategy that may change the inherent thrombogenic properties of the vessel wall, rather than attempting to counter the thrombogenic components already expressed on the vessel wall surface. Part of this strategy includes manipulating 13-HODE in an attempt to regulate adhesion molecule expression, not only on the apical surface of the endothelium, but also on the subendothelial surface which is exposed following injury. Interestingly, a number of studies suggest a possible vehicle with which to deliver such agents to potential thrombogenic sites in the vessel wall. Mizushima et al (1990 and 1991) reported that certain lipid microspheres containing PGE_1 (lipo-PGE_1) are selectively incorporated in areas of vascular damage in the spontaneous hypertensive rat and the atherosclerotic rabbit, suggesting that a specific effect could be targeted in a cardiovascular system. It is interesting to note that in these latter studies, the lipid microspheres carrying PGE_1, were 55% linoleic acid. Data from the present

study demonstrates that linoleic acid is selectively incorporated by the vessel wall, which suggests that delivering of the Lipo-PGE_1 to the target area is achieved by this selective uptake process. These latter experimental data provide evidence therefore, to suggest not only that manipulating vessel wall thrombogenecity is an alternative approach to achieve an antithrombotic effect, but also the technology to deliver specific agents to thrombogenic sites. Further studies exploring this alternative approach of antithrombotic therapy merit further investigation.

It can be concluded from these studies that there is selective incorporation of linoleic acid by the vessel wall resulting in a selective increase in 13-HODE synthesis therein. The observation also provides the basis for altering vessel wall adhesivity to achieve an antithrombotic effect.

The selective incorporation of EPA into the platelet, resulting in decreased platelet 12-HETE synthesis and decreased platelet adhesivity, is consistent with previous studies that 12-HETE is necessary for platelet adhesion (Buchanan et al, 1986). The observation that EPA had little or no effect, not only on vessel wall PGI_2 production, but also on platelet TXA_2 production, suggests that the predominant effect of EPA on platelet function is associated with interfering with the lipoxygenase pathway. In vitro studies demonstrate that EPA competes with, has a higher affinity for, and is metabolized slower by cyclo-oxygenase than arachidonic acid (Crawford,

1983; Powell and Funk, 1987). The results from our diet study suggest a similar competition of EPA for 12-lipoxygenase. Less 12-HETE was produced in platelets with a 1:1 EPA:arachidonate ratio. This notwithstanding, would suggest that decreasing platelet adhesivity is not as an effective approach to achieve an antithrombotic effect as altering vessel wall adhesivity. This may explain the lack of a significant and a consistent effect of EPA in clinical studies.

In summary, the in vitro, ex vivo and in vivo experimental studies provide collective evidence that vessel wall 13-HODE regulates vessel wall adhesivity. While the utilization of specific 15-lipoxygenase inhibitors (i.e. analogues of linoleic acid, 15-lipoxygenase antibodies, or 15-lipoxygenase anti-sensed endothelial cells), may be required to prove unequivocally that 13-HODE regulates adhesion molecule expression, the observation that vessel wall 13-HODE synthesis is inversely related to vessel wall adhesivity in a variety of settings and under an assorted number of conditions, provide rather conclusive proof that this relationship is causal. Furthermore, the observations that the opposite relationship occurs in the platelet (12-HETE synthesis versus platelet adhesion), provide support to the hypothesis that the relative ratio of linoleate:arachidonate monohydroxides regulate integrin expression and/or their adhesivity in cells

in general. The anomaly to this hypothesis is that endothelial cells and platelets only synthesize one of these metabolites. Nonetheless, the results of the leukocyte and tumor cell adhesion studies, support the general hypothesis.

8. ROLE OF 13-HODE/HETE RATIOS IN PMN AND TUMOR CELL ADHESION:

It has been reported by Singer et al (1989), that the laminin and fibronectin receptors were located in specific granules or "adhesomes" in PMNs under similar resting conditions, similar to the localization of the VnR in endothelial cells. Soberman et al (1985), also demonstrated that PMNs synthesize 13-HODE under resting conditions and 5-HETE following stimulation. These 2 observations are consistent with our earlier and present studies, that adhesion receptors are internalized in "non-adhesive" cells, at a time when 13-HODE synthesis is ongoing, and that 13-HODE synthesis decreases and arachidonic acid metabolism increases following stimulation, at a time when the cells are adhesive. For example, when the ratio of 13-HODE:HETEs was decreased in either PMNs or tumor cells, their adhesivity increased. In particular, 13-HODE predominated in the resting non-adhesive cells, and HETEs predominated in the stimulated adhesive cells.

Other investigators have demonstrated that cytokines, such as IL1 and TNF, enhance PMN and tumor cell-endothelial cell adhesion by facilitating the expression of adhesion receptors on endothelial cells (Rice et al, 1988; Dustin et al, 1986; Pober et al, 1986a; Jurgensen et al, 1990; Mortarini et al, 1990). Other studies demonstrate that exogenous 12-HETE (the platelet-derived lipoxygenase metabolite) facilitates GPIIb/IIIa receptor expression on tumor cells,

resulting in increased tumor cell-endothelial cell adhesion (Grossi et al, 1989). These investigators also demonstrated that 13-HODE blocked the 12-HETE induced GPIIb/IIIa expression and subsequent tumor cell-endothelial cell interactions. These observations provide evidence that endothelial cell derived 13-HODE not only blocks platelet and PMN-endothelial cell adhesion, but also platelet-induced tumor cell-endothelial cell adhesion. Thus, platelet and endothelial cell lipoxygenase metabolites appear to have opposing roles in regulating tumor cell-endothelial cell interactions through integrin receptor expression.

If these in vitro observations, can be extrapolated to the in vivo experimental or clinical situation, they suggest that altering the intracellular ratio of 13-HODE:15-HETE in leukocytes and tumor cells and/or 13-HODE levels in endothelial cells may be a mechanism whereby pharmacological intervention may prevent tumor cell-endothelial cell interactions, and subsequent metastasis. In agreement with this conclusion, Burns et al (1987) found that tumor cells were more sensitive to chemotherapy drugs when the tumor cells were enriched with linoleic acid (the substrate for 13-HODE), raising the possibility that fatty acid modifications of tumor cells is a useful adjunct to the currently used chemotherapy agents.

9. CLINICAL IMPLICATIONS OF MONOHYDROXY FATTY ACID:

Clinical Implications of Internal Mammary Artery 13-HODE Levels in Coronary Artery Bypass Surgery: A characteristic feature of coronary arteries with age and/or injury, is that there is diffuse thickening of the intima and subsequent formation of atherosclerotic lesions (Clarkson et al, 1987; Stary, 1989). These lesions obstruct the lumen of the coronary arteries and consequently, reduce blood flow, which in turn, results in starvation and hypoxia of myocardial tissues. In an attempt to restore blood flow to the myocardial tissues, in order to prevent future tissue damage and a potentially fatal myocardial infarctions, the occluded or stenosed vessels can be replaced by autograph patient graphs, e.g. with saphenous veins or internal mammary arteries (IMAs). Of these 2 arteries, a number of studies indicate that the IMA may be the conduit of choice in patients undergoing coronary bypass surgery (Loop et al, 1986; Barner et al, 1985; Pkies et al, 1984). Other studies demonstrate that autologous graft occlusion is largely due to thrombus formation and subsequent graft atherosclerosis (Grondin et al, 1985; Merrilees et al, 1988).

While testing the clinical relevance of the experimental data is beyond the scope of this thesis, it was of interest to perform preliminary studies to determine whether 13-HODE/vessel wall adhesivity existed in tissues

obtained from patients undergoing elective coronary artery bypass grafting (CABG). Again, consistent with all the experimental data, the adhesivity of the IMAs was inversely related to IMA 13-HODE synthesis. Furthermore, no 9-HODE, 12-HETE nor 15-HETE was detected in any of the samples. This latter observation is important since Rosolowsky et al (1990) reported that stenosed and normal canine coronary arteries produce primarily 12-HETE and 15-HETE. Finally, subsequent studies in another 51 patients demonstrated the consistency of this relationship and provide the additional information that 13-HODE synthesis decreases with age in this patient population (Brister et al, 1991). These observations, in combination with our in vivo studies manipulating vessel wall 13-HODE synthesis by either drug or diet, provide the basis for future clinical studies in these and other patient populations with vessel wall disease.

10. SUMMARY

The data presented in this thesis demonstrates that monohydroxy lipoxygenase metabolites influence blood cell-vessel wall interactions. In general, the ratio of endogenous linoleate:arachidonate monohydroxides modulate integrin expression and subsequent cell-cell adhesion. Furthermore, the presence of monohydroxides in the basement membrane underlying endothelial cells may alter the ability of these integrins to interact with their ligands. The results of these studies a better understanding of the regulation of cell-cell adhesion and suggest new approach to treat and prevent inflammation, metastasis and thrombosis.

11. FUTURE DIRECTIONS

While it has been consistently demonstrated that there is an inverse correlation between vessel wall 13-HODE levels and vessel wall adhesivity, a true causal relationship has not been demonstrated due to i) the lack of specific 15-lipoxygenase inhibitors, and ii) the inappropriateness of using add-back experiments (exogenous 13-HODE does not bind to endothelial cells nor influence their reactivity). Recently, the cDNA for 15-lipoxygenase has been cloned and sequenced. Thus, it is now possible to use molecular cloning techniques to both up- and down-regulate endothelial cell 15-lipoxygenase activity as a means of specifically influencing endogenous 13-HODE levels.

Future studies will make use of these tools to up- and down-regulate 15-lipoxygenase gene expression, to further confirm that there is a causal relationship between 13-HODE and vessel wall adhesivity. Other studies will be performed to determine whether 13-HODE directly alters the ability of the vitronectin receptor to recognize its ligands.

These above 2 experimental approaches should confirm a causal and a direct effect of 13-HODE in regulating the expression of the vitronectin receptor on the surface of cells. Once a causal and direct effect have been established, it will be recessing to ideally the mechanism by which 13-HODE influences vitronectin receptor expression. These above experiments should answer a great majority of the questions

which are raised by but are beyond the scope of this thesis.

These future experiments and the ongoing clinical studies are part of my and my colleagues endeavors.

APPENDIX

APPENDIX 1: ABBREVIATIONS

ABREV.	NAME
18:2	Linoleic acid
20:4.	Arachidonic acid
ACD	Acid citrate dextrose
BCO	Black currant seed oil
BSA	Bovine serum albumin (fatty acid free)
db-cAMP	N^6,2'-O-dibutyryl cAMP
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycolbis-β-aminoethylether-tetraacetate
ELAM-1	Endothelial cell leukocyte adhesion molecule-1
EPA	Eicosapentaenoic acid
ETYA	Eicotetraynoic acid
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FN	Fibronectin
FO	Fish oil
GC	Gas Chromatography
GP	Glycoprotein; e.g. GPIIb/IIIa
HBSS	Hank's balanced salt solution
HETE	Hydroxyeicosatetraenoic acid; e.g. 15-HETE
HODE	Hydroxyoctadecadienoic acid; e.g. 13-HODE

HPLC	High performance liquid chromatography
IFN	γ-Interferon
ICAM-1	Intercellular adhesion molecule-1
IL1	Interleukin 1α
IMA	Internal mammary artery
LT	Leukotriene; e.g. LTC_4
LFA-1	Lymphocyte function-associated antigen-1, CD11a/CD18
Mac-1	Macrophage antigen 1, CD11b/CD18
MW	Molecular weight
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PG	Prostaglandin; e.g. PGI_2
PF4	Platelet factor 4
PLT	Platelet
PMN	Polymorphonuclear leukocyte (or neutrophils)
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SEM	Scanning electron microscopy, or Standard error of mean
TNF	Tumor necrosis factor
TXA_2	Thromboxane A_2
VN	Vitronectin
VnR	Vitronectin receptor
vWF	von Willebrand factor
WO	Walnut oil

APPENDIX 2: BUFFERS, REAGENTS AND STOCK SOLUTIONS

Alkaline Reagent: 2 g anhydrous Na_2CO_3, 400 mg NaOH, 160 mg NaK tartrate and 100 µl sodium lauryl sulphate dissolved in 100 ml purified water.

Alkaline-Copper Reagent: 100 parts Alkaline Reagent, 1 part Copper Reagent.

Borate Buffer: 0.1 M Na Borate, pH 9.0, saturated with O_2 at 20°C for ten minutes.

BSA Stock Solution (5 mg/ml): 50 mg fatty acid free bovine serum albumin was dissolved in 10 ml water.

Copper Reagent: 4 g $CuSO_4.5H_2O$ dissolved in 100 ml water.

Dilute S-Stock: 1 part E-Stock mixed with 9 parts Borate Buffer.

Folin Reagent: Dilute 2 N phenol reagent solution 1:1 with purified water.

HBSS+: HBSS containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM Hepes, pH 7.35.

10% Magnesium nitrate solution: 10 g of $Mg(NO_3)_2.6H_2O$ in 100 ml HPLC grade methanol.

Reagent A: 4.2% ammonium molybdate in HCl, was prepared by dissolving 4.2 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 100 ml 4.5 M HCl.

Reagent B: 0.3% (w/v) malachite green solution, was prepared by dissolving 900 mg malachite green in 300 ml purified water just prior to use.

Reagent C: Dye-molybdate reagent, was prepared by mixing 100 ml reagent A with 300 ml reagent B, stirring the reagent for 3 hours and then filtering the reagent through a Whatman No. 1 filter paper. Reagent C was stored in the dark at 22°C for up to 3 weeks.

Reagent D: 1% (v/v) Triton X-100, was prepared by diluting 1 ml Triton X-100 in 99 ml purified water. Reagent D was stored at 4°C for up to 1 month.

S-Stock: 10 mg soyabean lipoxygenase type 1 dissolved in 10 ml of 1 mM Na Borate, pH 9.0.

Solvent X & Y: 20 ml chloroform, 20 ml methanol and 18 ml 0.9% NaCl in purified water were mixed in a separatory funnel and after phase separation, the upper aqueous phase (Solvent X) was separated from the lower organic phase (Solvent Y).

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