# Vascular transcellular signaling

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Receptors on the cell surface are targets for ligands that direct cell–cell interactions. Receptors can detect a variety of signal molecules such as catecholamines, cytokines, neurotransmitters, and eicosanoids. Ligand binding initiates, intracellular signaling processes and a cascade of biochemical events that induce changes in the cell. A current fundamental problem is to define the mechanisms involved in the regulation of transmembrane signaling processes, how they achieve specificity, and how dysregulation may influence disease. Two such diseases that appear linked with one another and share many common alterations in metabolic events are atherosclerosis and thrombosis.

Atherosclerosis may be defined as a progressive disease process involving large to medium-sized muscular and large elastic arteries. The advanced lesion consists of elevated focal intimal plaques with a necrotic central core containing lysed cells, cholesteryl ester crystals, lipid-laden foam cells, and plasma proteins such as fibrinogen/fibrin. Overlying the central core is a cellular zone containing smooth muscle cells, macrophages, and T-lymphocytes. The lesion is capped superficially by fibrous tissue. Vascularization of peripheral areas of the plaque is often observed. These fibro-fatty plaques are found in the abdominal aorta, coronary and internal carotid arteries, and other critical areas of the vasculature. The presence of atheroma renders the patient thrombosis-prone, presumably because the plaques gradually enter a complicated phase when they become calcified, fissured, or ulcerated. The latter surfaces act as powerful agonists for platelet activation, and recruitment and thrombus formation. Fifty percent of deaths in the United States are attributable to complications of atherosclerosis; of these 50% are directly due to coronary thrombosis with consequent myocardial infarction.

Platelet adherence, activation, and release of agonists, including growth factors, are the inciting events for the predisposition to thrombosis—an event that accompanies and defines the terminal stage of untreated atherosclerosis. Further evidence in support of the thrombosis component concept has recently been provided. In a study of expression of the thrombin receptor in arterial tissue by in situ hybridization and immunohistochemistry, this recep-

tor was widely expressed in human atheroma. It was identified in macrophage-rich areas and in locations rich in vascular smooth muscle cells. Thus, activation of the thrombin receptor may contribute to fibrosis and the inflammatory reaction occurring in the vasculature during development of atherosclerosis and also in the restenosis complicating surgical bypass procedures (1). It is now evident that atherosclerosis and thrombosis are parallel events that must be studied together in order to comprehend basic aspects of pathology and develop therapeutic modalities (2–6).

In discussing the novel, "Madame Bovary," Vladimir Nabokov stated the following: "Three forces make and mold a human being: heredity, environment and the unknown agent X. Of these the second, environment, is by far the least important, while the last, agent X, is by far the most influential. In the case of characters living in books, it is of course the author who controls, directs and applies the three forces." (7). These statements are very applicable to the problem at hand. Consider myocardial infarction: heredity does come into play if we acknowledge a positive family history, hypertension, diabetes, and a cholesterol over 200 mg/dl. We could also add elevated LDL and reduced HDL levels. Elevations of Lp[a] comprise another possibly inherited risk factor for coronary artery disease. Environment would include cigarette smoking and probably obesity. However, in the

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setting of the aforementioned known factors, "agent X" remains strongly influential. Therapeutics for both atherosclerosis and thrombosis are not as satisfactory as they should be, proving that the pathogenesis is unclear and emphasizing the importance of "agent X" (7).

One approach toward learning more about "agent X" is through appreciation of the multicellular nature of the developing atherosclerotic plaque. The concept is valid whether one subscribes to the "response to injury" hypothesis or to the idea that monocyte recruitment followed by smooth muscle proliferation are earlier events in the arteriopathy. In any case, there is a multicellular inflammatory response consisting of platelets, neutrophils, macrophages, and fluid phase reactants secreted from these cells such as lysosomal enzymes, cytokines, coagulation proteins, and growth factors.

Relatively recently, it has become apparent that cells participating in the inflammatory response to evolving atherosclerotic plaques and the thrombi that form as a complication are not only reacting with components of the lesion but with each other. This is in the form of metabolic and functional cell–cell interactions as well as a subdivision of this process, known as transcellular metabolism. Transcellular metabolism can be defined as a biological process wherein one or many metabolite(s) from one cell can be utilized by another cell as substrate for a reaction or as an agonist for a reaction sequence. In this review, metabolic products with biologic activity originating from a variety of interacting cells will be discussed with regard to their potential to promote thrombo-atherosclerosis and induce alterations in lipid metabolism. These products include eicosanoids, growth factors, and cytokines (6).

## THE EICOSANOID SIGNALING PATHWAY

An eicosanoid is an enzymatically oxygenated, biologically active derivative of arachidonic acid or, rarely, from other 20-carbon polyunsaturated fatty acids (the term eicosa is the Greek locution for 20). Mammalian tissues cannot desaturate oleic acid toward the methyl end of the chain due to absence of $\Delta 9$-desaturase. Therefore, linoleic acid must be supplied in the diet from plant sources. Mammals can convert dietary linoleic acid (18:2) to linolenic acid (18:3) and thence to arachidonate. Arachidonate ($\Delta 5,8,11,14:20:4\omega 6$) can be obtained from the diet or from linoleate indirectly through linolenic acid. Arachidonic and linoleic acid are therefore "essential" components of the diet, the chronic absence of which will lead to a well-defined disease known as Essential Fatty Acid Deficiency (8). Arachidonate and linoleate contain a double bond, 6 carbons from the methyl group. Therefore, they are classified as omega-6 (n-6) fatty acids. Arachidonic acid contains 20 carbon atoms and 4 double bonds. On the other hand linoleic acid, which is the metabolic precursor

of arachidonate, contains 18 carbon atoms and two double bonds. The "short-hand" designation for these fatty acids is 20:4 and 18:2 for arachidonate and linoleate, respectively. In contrast to the omega-6 fatty acids mentioned above, eicosapentaenoate (20:5) and docosahexaenoate (22:6) are omega-3 (n–3) fatty acids. These polyunsaturated fatty acids are found in fish oils and are considered by many to be clinically important because they are metabolically incorporated into mammalian tissues and give rise to eicosanoids that have mild antiatherosclerotic, antithrombotic, and antiinflammatory properties. Populations such as Eskimos in Greenland, who ingest large quantities of fish oils in their diet are thought to demonstrate a relative paucity of atherosclerotic and thrombotic diseases. Recent clinical metabolic studies have provided evidence for a protective effect of omega–3 fatty acids (9).

Formation of eicosanoids in stimulated cells and tissues is a ubiquitous process, which may indicate that these mediators are of major importance in regulation of host defense mechanisms, hemodynamics, vascular permeability and smooth muscle contraction. Other functions attributed to eicosanoids include control of ion transport across membranes, modulation of synaptic transmission, and neutrophil chemotaxis. Eicosanoids are only produced in nanomolar quantities and are low molecular weight substances, but their potent biological properties are quite disproportionate to their quantitative and qualitative characteristics.

Eicosanoids fall into the pharmacologic category of autacoids. These are highly evanescent biologic moieties, formed locally in the microenvironment of perturbed organs or tissues. Their effects take place at the site of origin. This differentiates them from hormones, which are synthesized and secreted by endocrine glands and subsequently transported in the vasculature to a distant target organ or tissue. Hormones are usually stimulators and "set tissues into motion." In contrast, an autacoid can be either stimulatory or inhibitory (10).

There are four major classes of eicosanoids: prostaglandins, thromboxanes, leukotrienes, and lipoxins (**Fig. 1**). A series of experiments by Samuelsson and associates (11) led to the discovery that prostaglandins were derivatives of arachidonic acid. This research documented the presence of unstable intermediate endoperoxides produced by the cyclooxygenase in the cell, forming immediately after oxygenation of free arachidonate, and their subsequent conversion within seconds to prostaglandins ($PGE_2$, $PGF_{2\alpha}$, and $PGD_2$). Discovery of the transient endoperoxides ($PGG_2$ and $PGH_2$) also led to detection and characterization of thromboxane $A_2$ and prostacyclin. These novel metabolic pathways were highly significant for understanding the role of eicosanoids in the pathogenesis of thrombosis and atherosclerosis (4).

Subsequent research led to elucidation of a comparable
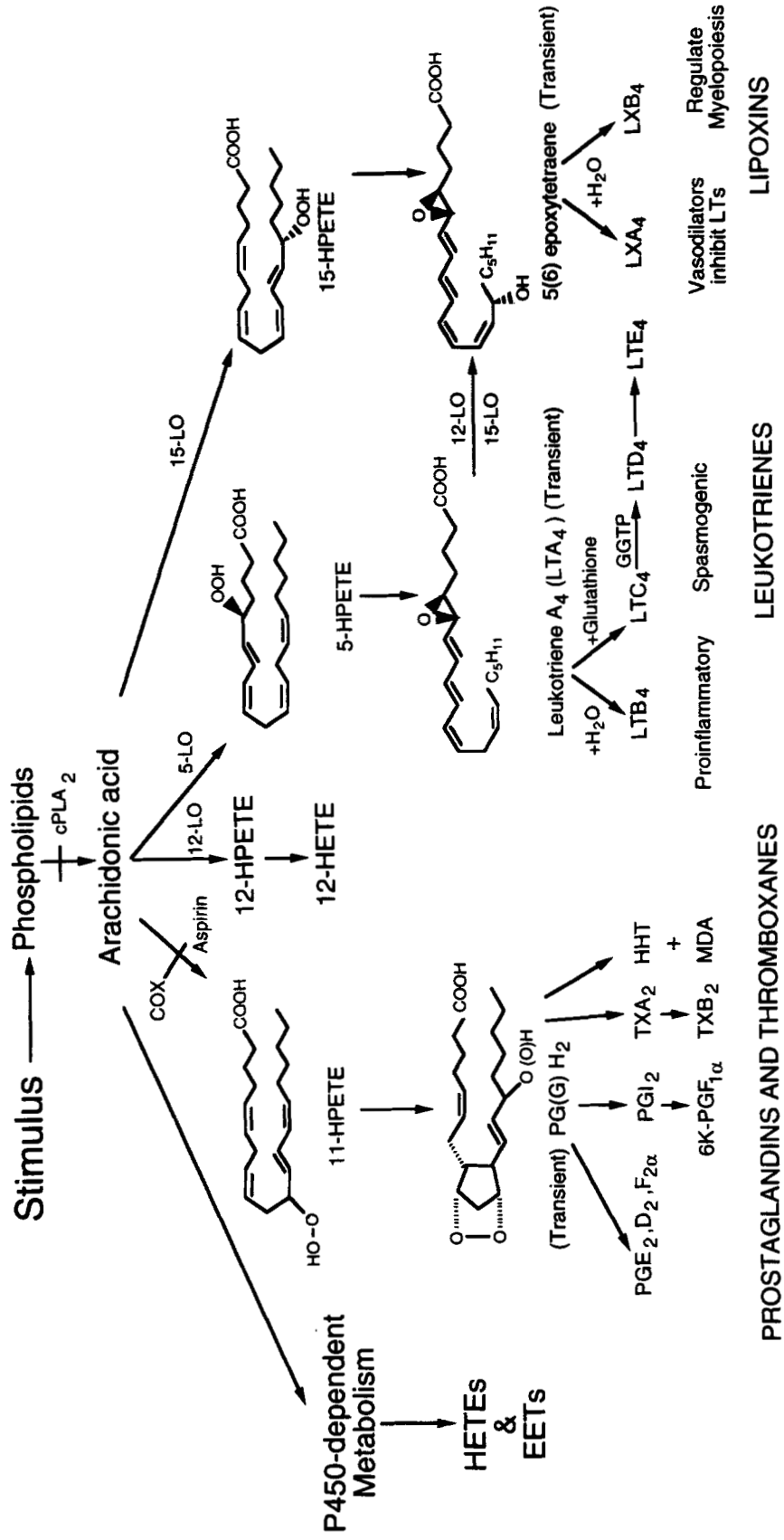
**Fig. 1.** Eicosanoid metabolism—1993. Depicted herein are the general classes and individual groups of eicosanoids as well as the intermediate compounds involved in their biosynthesis. With regard to the prostaglandin and thromboxane pathway, two PGH synthase isozymes have recently been described in murine cells: PGH synthase-1 and -2. This new observation may have functional implications (14, 17–19). PGH synthase is a *bis*-oxygenase which catalyzes insertion of oxygen at carbon 11 and 15 of arachidonate. PGH synthase is inhibited by aspirin, which acetylates the SER[530] residue. The bulk of the acetyl group blocks binding of arachidonate to the active site of PGH synthase. Further transformation of the transient endoperoxide intermediate depends upon enzymes present in the cell of origin. Depicted on the right is oxygenation of arachidonate as catalyzed by three lipoxygenase enzymes in mammalian tissues: 5-, 12-, and 15-lipoxygenases. Each lipoxygenase inserts molecular oxygen into a stereoselective position within arachidonate. Thus, 5-lipoxygenase inserts molecular oxygen into the carbon 5 position. Leukocytes convert arachidonate predominantly to 5-lipoxygenase-derived products. Importantly, the 5-lipoxygenase is also responsible for generation of leukotriene $A_4$. $LTA_4$ is a 5,6-epoxide-containing intermediate which is rapidly transformed to leukotriene $B_4$, the most potent proinflammatory eicosanoid. Alternatively, $LTA_4$ can be conjugated with glutathione to generate leukotrienes $C_4$, $D_4$, and $E_4$ in mast cells, basophils, or eosinophils. The latter constitute the slow-reacting substances of anaphylaxis (SRS-A) (25). The far right panel depicts formation of lipoxins. These eicosanoids can be generated by initial oxygenation at C-15 to form 15-HPETE, which can be subsequently converted to 5,6-epoxytetraene via the action of a 5-lipoxygenase. This 5,6-epoxytetraene or its equivalent can be generated from leukotriene $A_4$ when it is converted by either the 12- or 15-lipoxygenases. Lipoxins can also be generated during cell-cell interactions or by individual cell types. The panel at the extreme left indicates that arachidonic acid can be transformed by cytochrome P450-dependent mechanisms. In this pathway, arachidonate is oxygenated at each of the major *cis*-pentadiene sites. $A_4$ and $B_4$ possess a number of biologic properties that include vasodilation, inhibition of leukotriene activity, and regulation of myelopoiesis in bone marrow cells (25, 102). The panel at the extreme left indicates that arachidonic acid can be transformed by cytochrome P450-dependent mechanisms. In this pathway, arachidonate is nonallylic and more stable than leukotriene $A_4$ or 5,6-epoxytetraene. Biologic The P450 enzyme system can also oxygenate arachidonate and insert alcohol groups or form epoxides which are nonallylic and more stable than leukotriene $A_4$ or 5,6-epoxytetraene. Biologic activities of the P450 metabolites of arachidonate have been studied in great detail in the kidney, blood vessels, platelets, and bone marrow (15, 16). (From Dr. Charles Serhan, with permission.)

series of reactions in neutrophils occurring via an unstable epoxide intermediate, leukotriene $A_4$, produced by lipoxygenase. The enzymatic hydration product of the latter is leukotriene $B_4$, the most powerful proinflammatory substance yet described. Furthermore, addition of glutathione to leukotriene $A_4$ leads to formation of leukotriene $C_4$, a powerful vasoconstrictor. Leukotriene $C_4$ is then metabolized to leukotrienes $D_4$ and $E_4$ by elimination of a gamma-glutamyl residue and glycine. These cysteine-containing leukotrienes actually represent the slow-reacting substance of anaphylaxis (SRS-A) (12, 13). Another pathway in the arachidonic acid cascade leads to production of epoxy and dihydroxy fatty acids. This cytochrome P450-modulated system culminates in production of lipoxygenase-like metabolites such as 12(R)-HETE and 20-HETE (14–16).

## ENZYMATIC OXYGENATION AND PROCESSING OF FREE ARACHIDONATE

Arachidonate is never present in free form in the cell. It is esterified in the *sn*-2 position of cellular phospholipid. Cell activation results in cleavage and release of arachidonate mainly via action of a phospholipase $A_2$ in the cytosol. After receptor-mediated activation has taken place, the phospholipase is regulated by a specific G-protein. Unesterified arachidonate is immediately oxygenated by the cyclooxygenase (PGG/H synthase), a group of lipoxygenases or a cytochrome P450 mono-oxygenase. Thus, the initial rate-limiting step in eicosanoid biosynthesis is availability of unesterified arachidonate (14).

The quantity of cleaved arachidonate far exceeds that which is oxygenated to form eicosanoids. Unprocessed arachidonate leaves the cell, probably by facilitated diffusion (14). Because of its lipophilic nature, arachidonate will penetrate other cells in proximity. Depending upon their state of activation and qualitative enzyme content, proximal cells will metabolize arachidonate to other eicosanoids. This phenomenon is known as "transcellular metabolism" (13).

## CYCLOOXYGENASE (PGG/H SYNTHASE) AND LIPOXYGENASE

The cyclooxygenase is particulate, requires heme for its activity, and it is inactivated following acetylation by aspirin. Lipoxygenases are cytosolic, insensitive to aspirin, and retain activity so long as free arachidonate is available. Nevertheless, these two enzymes have features in common, and their catalytic activity results in metabolites that play a critical role in the thrombotic and atherogenic processes. Both enzymes recognize the 1,4-*cis*-pentadiene structure in polyunsaturated fatty acids. They also abstract a methylenic hydrogen from arachidonate in order to insert molecular oxygen. There are two PGG/H synthase isozymes, i.e., PGH synthase-1 and -2 (14). PGH synthase-1 is completely inhibited by aspirin. When PGH synthase-2 is aspirin-treated, it metabolizes arachidonate to 15-hydroxyeicosatetraenoic acid instead of $PGH_2$. PGH synthase-2 is only expressed after cell activation (17–19). An unstable intermediate results from the initial arachidonate transformation, and this is further metabolized by tissue-specific enzymes into biologically active eicosanoids. In the case of platelets, isomerization of $PGH_2$ to thromboxane $A_2$ is catalyzed by thromboxane A synthase. $PGH_2$ is also fragmented by the thromboxane synthase to hydroxyheptatrienoic acid (HHT) and malondialdehyde (MDA). The significance of the latter two compounds for thrombosis and atherosclerosis has never been defined. The primary structure of human thromboxane synthase has been determined, and it has properties characteristic of a cytochrome P450 (20). The platelet thromboxane synthase has recently been cloned (21). New information derived from cloning will help clarify mechanisms of action of eicosanoids at the biochemical/pharmacologic interface. As will be discussed, the unstable endoperoxide intermediates play a critical role in transcellular metabolism.

Thus far, three main lipoxygenases have been identified in human tissues, and they have all been cloned and characterized (12, 22). These lipoxygenases catalyze hydrogen abstraction and insert molecular oxygen in a similar manner. Each lipoxygenase generates a hydroperoxyeicosatetraenoic acid that carries a hydroperoxy group (mainly in the S configuration) at the lipoxygenase-specified carbon [5(S)HPETE, 12(S)HPETE, 15(S)HPETE]. Importantly, the 5-lipoxygenase possesses another activity that abstracts the 10-pro R hydrogen, with formation of the highly critical intermediate $LTA_4$ [a 5(6) epoxide]. In addition, the 5-lipoxygenase requires calcium, ATP and the 5-lipoxygenase activating protein (FLAP) (23, 24) (**Fig. 2**). When neutrophils are activated, 5-lipoxygenase in the cytosol translocates and becomes associated with FLAP on the cell membrane. It is not known whether this reaction occurs with the other lipoxygenases. However, the translocation presents an opportunity to intervene pharmacologically with agents that can block association of soluble 5-lipoxygenase with membrane-associated FLAP, upon which it becomes activated (Fig. 2) (25).

## TRANSCELLULAR METABOLISM AS A MECHANISM OF EICOSANOID BIOSYNTHESIS

Although eicosanoids are relatively simple molecules, their biologic properties are profound. They promote the inflammatory response, participate in hypersensitivity reactions and can be agonists for prothrombotic and
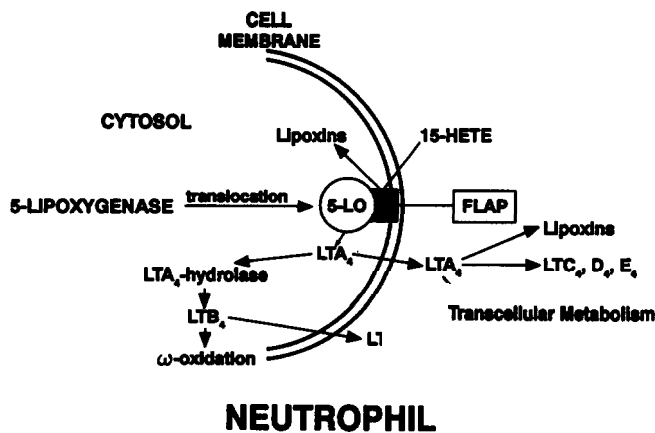
**NEUTROPHIL**

**Fig. 2.** Lipoxgenase reactions in neutrophils. These leukocytes contain a 5-lipoxygenase, but not a 12-lipoxygenase as found in platelets. In addition to calcium and ATP, the 5-lipoxygenase requires a membrane-associated protein for full activity. This protein is known as the 5-lipoxygenase activating protein (FLAP) (23, 24). The 5-lipoxygenase is a cytosolic enzyme, but upon neutrophil activation, it translocates to the membrane where it associates with membrane-bound FLAP. The 5-lipoxygenase catalyzes formation of the 5(6)-epoxide, $LTA_4$ (12, 25). The $LTA_4$-hydrolase will catalyze formation of $LTB_4$ which then functions as a powerful proinflammatory autacoid. In addition, $LTA_4$ can leave the neutrophil and initiate formation of lipoxins and the components of SRS-A in these cells. Thus, $LTA_4$ can penetrate other cells in proximity such as erythrocytes, platelets, endothelial cells, and mast cells for production of metabolites which neither cell can produce alone (transcellular metabolism) (13, 27, 103, 104).

proatherogenic processes. On the other hand, eicosanoids such as $PGI_2$ and $PGD_2$ act as "thromboregulators" by elevating cyclic AMP in platelets and vascular cells (6, 26). This probably keeps platelets from aggregating excessively in vivo. Early studies had established metabolic patterns of eicosanoid generation in individual cells (10). Maclouf and colleagues (27, 28) suggested that biosynthesis of arachidonic acid metabolites be viewed as a two-step reaction. The initial step involves generation of labile, biochemically reactive species that are then secondarily transformed by specific enzymes to yield a biologically active eicosanoid. Later, it became obvious that the two steps could take place in two separate cell types. Thus, two cells in proximity could enzymatically process a biochemically reactive intermediate (such as endoperoxide or leukotriene $A_4$) as it traversed the fluid-phase between the cells. It would penetrate the second cell and be processed to a product that neither cell could produce alone. Alternatively, the intermediate could increase the quantity of a metabolite that both cells produce together. As already mentioned, this form of cell–cell communication represents transcellular biosynthesis (**Fig. 3**). The initial example was biochemically demonstrated as transcellular biosynthesis of prostacyclin by endothelial cells that had obtained endoperoxides from activated platelets (29). This process had been suggested by Vane, Anggard, and Botting (30). In this instance, more prostacyclin was

produced than the quantity biosynthesized by endothelial cells alone.

Maclouf, Fitzpatrick, and Murphy studied the metabolism of leukotriene $A_4$ in cells that had no 5-lipoxygenase activity. Thus, erythrocytes could process $LTA_4$ into $LTB_4$ and were also able to produce $LTB_4$ from stimulated neutrophils. This was also the case for human endothelial cells, which transformed exogenously provided $LTA_4$ into leukotriene $C_4$. Because this reaction is aspirin-insensitive, it would mean that aspirin-treated platelets could still induce vasoconstriction in the vicinity of an inflamed atherosclerotic plaque. Comparable reactions have been demonstrated with smooth muscle and endothelial cells (27, 31). Stimulated platelet-neutrophil suspensions can also generate lipoxins, a unique class of biologically active eicosanoids derived from the 15-lipoxygenase pathway (25).

## SIGNIFICANCE OF CELL–CELL INTERACTIONS AND TRANSCELLULAR METABOLISM IN THROMBOSIS AND ATHEROSCLEROSIS

There is essentially no baseline production of eicosanoids. Detection of an eicosanoid in vivo or in vitro is thought to reflect activation of a single cell known to be capable of producing a given eicosanoid. If we consider recent data from studies of transcellular metabolism, another concept can be appreciated. As shown in **Fig. 4,** a donor cell capable of producing an unstable intermediate does require activation. However, the recipient cell need not be activated in order to produce a new eicosanoid with different biological properties or more of a metabolite common to the two cells. Thus, formation of

**EICOSANOID PATHWAY INTERACTIONS:
PLATELETS, NEUTROPHILS, ENDOTHELIUM**
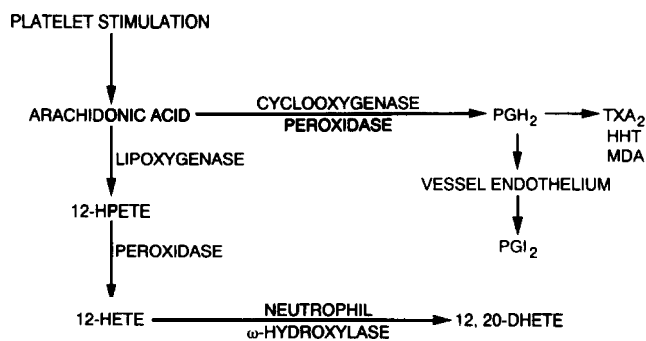


**Fig. 3.** Cell–cell interactions in the eicosanoid pathway. Precursors, intermediates, and end-products derived from one cell type can be utilized by another cell in proximity to it for increased synthesis of a metabolite. Alternatively, a new eicosanoid can be produced with a novel function that neither cell can synthesize alone. Examples of these possibilities are shown herein and discussed in the text.
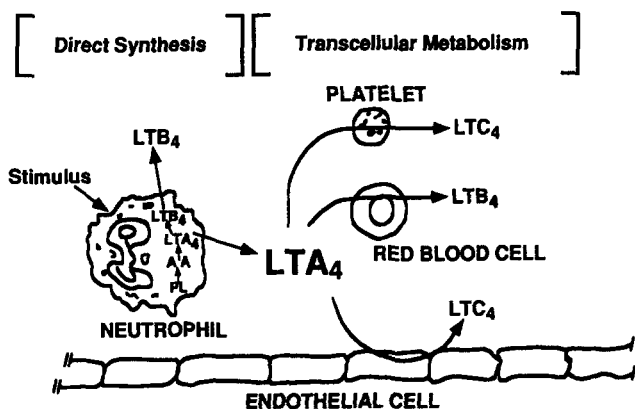
**Fig. 4.** Transcellular metabolism of leukotriene $A_4$ generated by direct synthesis in the activated neutrophil (donor cell). Recipient cells in this instance are the platelet, erythrocyte, and endothelial cell, respectively. The latter cells do not require activation and the biologically active product is a reflection of biosynthetic processes in the acceptor cell. The acceptor cells *do not have the capacity to generate the end products* shown from arachidonate. However, they can do so from the epoxide intermediate, leukotriene $A_4$. Leukotriene $C_4$ which has formed in the platelet and endothelial cell is a coronary vasoconstrictor and is aspirin-insensitive. Transcellular metabolism endows erythrocytes with the capacity to participate indirectly in the inflammatory response via $LTB_4$ production (from refs. 27 and 28).

a new eicosanoid by transcellular metabolism is actually the end result of agonist stimulation of the donor cell. This is particularly pertinent for production of leukotriene $B_4$ and $C_4$ from neutrophil-derived leukotriene $A_4$ by erythrocytes, platelets, and endothelial cells, respectively (Fig. 4) (28).

This new information concerning transcellular metabolism also has therapeutic implications. Inhibition of leukotriene $A_4$ production or its uptake by an acceptor cell could prevent platelets and endothelial cells from producing leukotriene $C_4$. This would be pertinent for patients with unstable angina who are unresponsive to aspirin because of continued $LTC_4$ production and coronary vasoconstriction (31). Inhibition of the $LTC_4$ receptor

might represent another approach toward blockage of vascular damage at sites of fissured atherosclerotic plaques.

Cell–cell interactions and transcellular metabolism in the eicosanoid pathway are now amenable to an orderly classification that allows one to conceptualize the phenomena and also derive possibilities for therapeutic intervention in those interactions that represent excessive prothrombotic or proinflammatory activities (**Table 1**). An example of the Type IA category would be utilization of platelet-derived endoperoxides by aspirin-treated endothelial cells for the formation of prostacyclin (29, 32). Prostacyclin production in these experiments is accompanied by unresponsiveness of platelets to all agonists. We now know that such platelet inhibition is also due to production of endothelium-dependent relaxing factor (EDRF/NO) (33) and to an ecto-ADPase (apyrase) on the endothelial cell surface that metabolizes released platelet ADP (4, 34). A variant of Type IA is processing of released arachidonate from activated neutrophils by stimulated platelets to produce more thromboxane $A_2$ (35).

For Type IB, processing of neutrophil leukotriene $A_4$ by erythrocytes, platelets, and endothelial cells as shown in Fig. 4 represents a prime example. This also applies to lipoxin formation during neutrophil-platelet interactions wherein platelet 12-lipoxygenase can transform leukotriene $A_4$ into lipoxins (36). In each case, the recipient cell could not produce the end product alone (13).

The Type II cell–cell interactions are stimulus-specific (Table 1). Thus, platelet 12-HETE is used by activated neutrophils with production of 5S,12S-DiHETE when both cells have been exposed to a common stimulus, for example, the model agonist ionophore A23187 (Type IIA). 5S,12S-DiHETE production diverts neutrophils from synthesizing leukotriene $B_4$, and in this way the process functions as an antiinflammatory mechanism. Importantly, neither platelets or neutrophils can produce 5S,12S-DiHETE alone. The versatility of these Type IIA reactions is demonstrable by an experiment in which addition of radiolabeled 5-HETE to activated platelets will result

TABLE 1. Classification of cell–cell interactions and transcellular metabolism in the eicosanoid cascade[a]

Type I: Different cells in proximity metabolize a transient eicosanoid intermediate
   IA: A cell produces an intermediate endogenously, but can also obtain this intermediate from a stimulated vicinal cell, thereby enabling synthesis of more final metabolite

   IB: A cell that is incapable of synthesizing an intermediate can obtain it from a neighboring cell and process it to an eicosanoid

Type II: A cell can convert an eicosanoid end product synthesized from one in proximity to a new compound which neither cell can synthesize alone
   IIA: The cells involved have been activated by a common stimulus
   IIB: An activated cell synthesizes an eicosanoid that penetrates an unstimulated vicinal cell which then synthesizes a new metabolite

Type III: Reactive transient intermediates or eicosanoids synthesized by one cell are capable of acting as agonists or inhibitors for synthesis of a new type of eicosanoid from another cell

[a]This is mainly a working classification. Occasionally a particular reaction can fit into one or more categories.

in formation of 5S,12S-DiHETE. In other words, the neutrophil itself is not necessary, but an eicosanoid derived therefrom will suffice (13, 37).

The Type IIB cell–cell interaction differs from Type IIA in that only one of the two cells under study is activated. If thrombin or collagen is added to a combined suspension of unstimulated neutrophils and platelets, the neutrophils will metabolize 12-HETE to 12,20-DiHETE, two lipoxygenase products that have lipolytic activity in vascular cells (see next section). In appropriate control experiments, addition of thrombin or collagen to neutrophils in the absence of platelets does not result in eicosanoid formation (37).

In Type III, cell–cell interactions, an eicosanoid or intermediate synthesized by one cell type can act as an agonist or inhibitor for production of eicosanoids in other cells. For example, leukotrienes $C_4$, $D_4$, and $E_4$ can induce thromboxane release from perfused lung preparations (38). Also, leukotriene $A_4$ can enhance calcium mobilization from neutrophils (25).

It is now clear that the physical and biological behavior of one cell can be markedly altered by the presence of another. This is due to direct cell contact or secretory products of one or more cell types, especially after activation by specific agonist(s). To date, pharmacologic therapy directed against single cell types has not been completely satisfactory. Perhaps control of newly recognized cell–cell interactions via development of inhibitors of precursors or through receptor blockade will result in a more definitive therapeutic approach to thrombosis (26). This concept is developed in subsequent sections of this review.

## CELLULAR INTERACTIONS INVOLVING TRANSMEMBRANE SIGNALING: ROLE OF EICOSANOIDS AND CYTOKINES

The interaction of inflammatory cells such as neutrophils and macrophages with cytokine-activated endothelial cells constitutes a mechanism by which soluble mediators alter smooth muscle cell and macrophage cholesterol metabolism. From numerous in vivo studies, we know that platelets and/or platelet products are abundant in fatty streaks of hypercholesterolemic, nonhuman primate vessel walls (39). It has been proposed that platelets serve as cholesterol donors for both smooth muscle cells and macrophages (40). Platelet components such as ADP, serotonin, fibrinogen, and fibronectin enhance LDL receptor activity and inhibit scavenger receptor activity in human macrophages (40). Importantly, these receptors regulate cholesterol delivery to both smooth muscle cells and macrophages (41).

Transcellular metabolism of eicosanoids can occur when these cells are in close proximity. Recent data have been summarized which describes the co-culture of platelets or neutrophils with either endothelial or smooth muscle cells (6), where transcellular metabolism of $LTA_4$ to $LTC_4$ or 12-HETE to 12,20-diHETE occurs during platelet neutrophil interactions (6). 12,20-diHETE stimulates cholesteryl ester (CE) hydrolytic activity (42) as does $PGI_2$ and 12-HETE (42, 43). This effect is regulated by the protein kinase A cascade (44) (**Fig. 5**). Such data support the concept that interactions of blood cells are pivotal in generation of mediators that are capable of altering cholesterol metabolism in arterial cells during atherogenesis. Lipoxygenase and/or cyclooxygenase-derived eicosanoids participate in regulation of processes leading to lipid accumulation in the vessel wall after endothelial cell activation or injury. These results have enhanced our understanding of the pathogenesis of foam cell development. Under normal circumstances, eicosanoids derived from the vessel wall, such as $PGI_2$ and related compounds, have been documented to play a role in the "barrier" function of intact endothelium (40). They also inhibit permeability of the vessel wall to large macromolecules such as LDL (40). Several types of eicosanoids also promote monocyte adhesion, diapedesis, and modulation of smooth muscle cell phenotype and proliferation (40). Subacute or chronic injury may exacerbate these events, culminating in smooth muscle cell proliferation, and a potential increase in CE deposition. These processes clearly occur following release of, and response to, potent inflammatory mediators including cytokines, growth factors, and those eicosanoids which are elaborated by activated endothelium (6).

The role of each cytokine and growth factor in the atherogenic response to injury is quite complex. Some of these biological response modifiers appear to be pro-atherosclerotic. These include PDGF, FGF, IL-1, TGFβ, TNF, IFN-γ, and GM-CSF. This subject area has been extensively reviewed elsewhere (6).

Eicosanoids synthesized by the normal vessel wall in response to local humoral stimulation act to antagonize the influence of pro-atherogenic factors, and to some degree they mediate the influence of anti-atherogenic factors by maintaining the endothelium in a "quiescent state." This is manifested as a non-thrombotic, non-adhesive surface that maintains a low degree of permeability to circulating macromolecules. Examples of these types of eicosanoids are $PGI_2$, $PGD_2$, and $PGE_2$, three major cyclooxygenase metabolites produced in the vascular bed (40). This is in contrast to the HETEs and leukotrienes, including $LTB_4$ and $LTC_4$ which have pro-atherogenic properties (6, 40). Paradoxically, aspirin inhibits eicosanoid but not leukotriene production.

It is clear that intact $PGI_2$ biosynthetic capacity is important for modulation of pro-atherogenic stimuli. Factors that inhibit vascular production of $PGI_2$, such as aspirin or dietary alterations, result in reduced vascular eicosanoid production. Because the biological activities of $PGI_2$ and related eicosanoids are mediated through
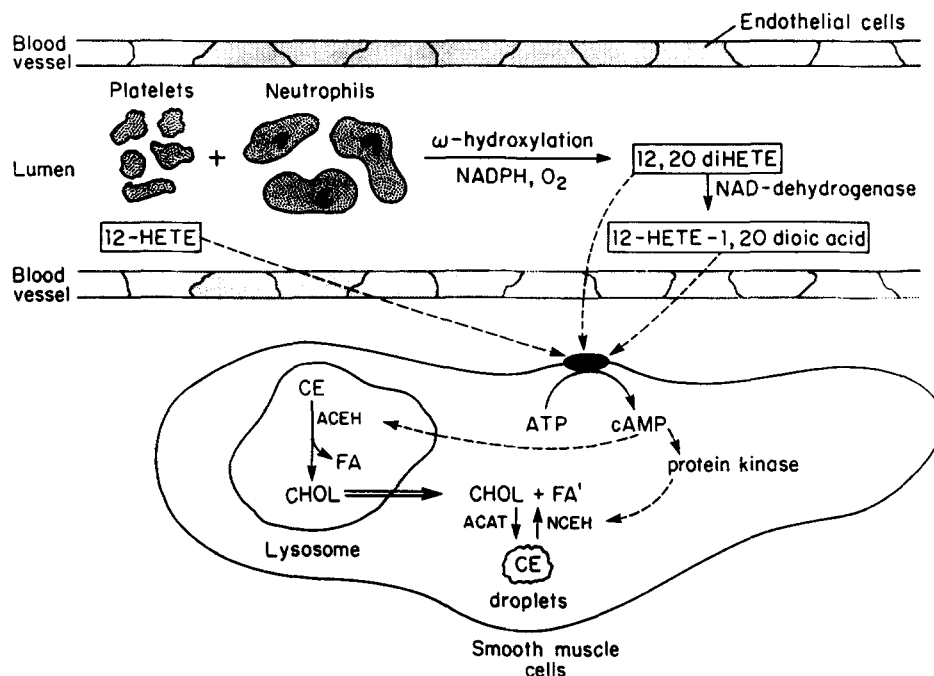
**Fig. 5.** Platelet–neutrophil–arterial smooth muscle cell interactions. The interaction of platelets with neutrophils produces two dihydroxy acids by omega hydroxylation from platelet-derived 12-HETE. All three P450-dependent metabolic products have the capacity to stimulate cyclic AMP in arterial smooth muscle cells which, in turn, can activate lysosomal (acid) CE hydrolytic activity (ACEH). Alternatively, cyclic AMP can activate protein kinase A and therefore stimulate cytoplasmic (neutral) CE hydrolytic activity (NCEH). These activities can influence CE retention in the cell.

generation of cyclic AMP, the importance of the eicosanoid-cyclic AMP linkage is paramount to control of lipolysis in atherogenesis (45). We have shown that accretion of CE in smooth muscle cells can be modulated by exogenously provided eicosanoids such as $PGI_2$ and 12-HETE (42, 43). These autacoids elevate CE hydrolase in the cell by elevating intracellular levels of cyclic AMP, whereas $PGE_2$ inhibits CE synthetic (ACAT) activity (42, 43, 46) (**Fig. 6**).

Eicosanoids are one of the most prolific second messenger systems synthesized in response to humoral stimulation. The impact of eicosanoids on the net response to growth factor/cytokine stimulation is dependent upon regulation of eicosanoid biosynthesis, the spectrum and quantity of eicosanoids elaborated in response to agonists, and the influence of eicosanoids on other second messenger systems. This concept is highlighted below.

One example is the interaction of a peptide hormone with its receptor. This results in activation of numerous intracellular proteins. The principal mechanism by which this occurs is phosphorylation by the calcium and phospholipid-dependent protein kinase known as protein kinase C. The latter is activated by tyrosine kinase intrinsic to the peptide hormone receptor. This mechanism has been elucidated in detail for signal transduction of PDGF upon interaction with its receptor (47) (**Fig. 7**). In addi-

tion, protein kinase C is activated in endothelial cells by endotoxin, TNF, and IL-1 (48). Activated protein kinase C then may phosphorylate numerous proteins including phospholipase C (PLC). Phosphatidylinositol is a preferred substrate for PLC which is hydrolyzed to 1,2-diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$). Diacylglycerol has several biological activities including stimulation of protein kinase C as part of a positive feedback to stimulate eicosanoid biosynthesis (49). Diacylglycerol is also a cofactor for adenylate cyclase, which when phosphorylated can stimulate ATP conversion to cyclic AMP. Normally, inositol phosphates activate calcium channels. Calcium permits the association of soluble phospholipase $A_2$ with the cell membrane, suggesting that membrane association is a requisite for phospholipase activation and arachidonic acid release (50). Eicosanoid biosynthesis occurs subsequent to this metabolic effect. Indeed, protein kinase C activation can occur in parallel with phospholipase $A_2$ activation (51).

As mentioned earlier, eicosanoid biosynthesis is under stringent metabolic control. Only a small percentage of released arachidonic acid is actually oxygenated and converted to eicosanoid metabolites. Eicosanoid generation is limited by the activity of acyl-CoA lysophosphatide acyltransferase and cyclic AMP (49). Cyclic AMP itself inhibits further autacoid release, and negative feedback
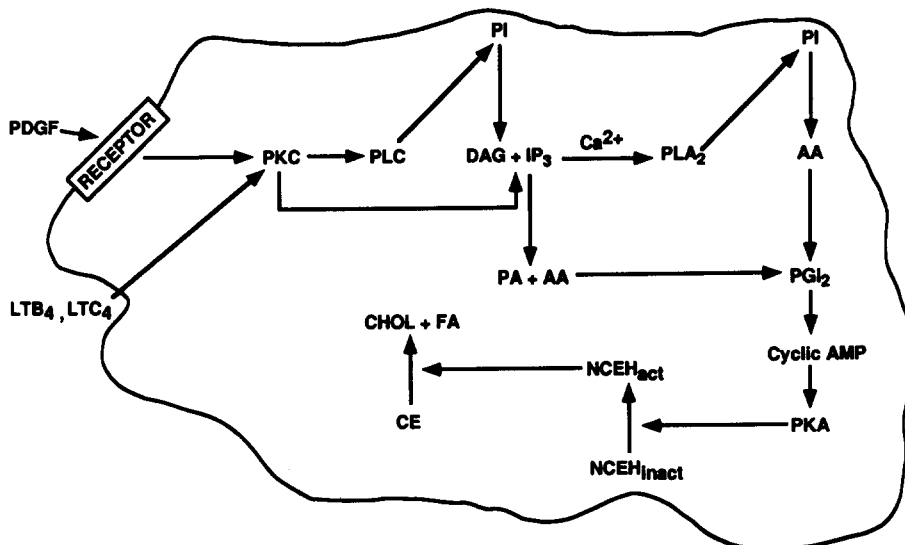
## ARTERIAL SMOOTH MUSCLE CELL
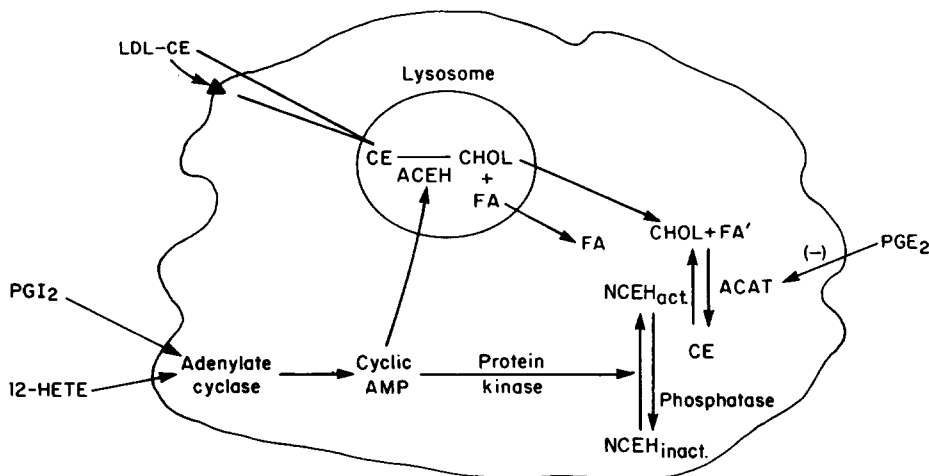
**Fig. 6.** Regulation of cholesteryl ester hydrolysis by eicosanoids. The cell is challenged with CE in the form of LDL-CE where the CE, once internalized, is degraded by ACEH in the lysosomes to free cholesterol. This free cholesterol is esterified by another fatty acid (FA) to form cytoplasmic CE droplets by the enzyme, ACAT. $PGE_2$, another major cyclooxygenase metabolite produced by the vessel wall, inhibits ACAT activity in the cell. Prostacyclin ($PGI_2$) and 12-HETE elevate ACEH and NCEH activities by elevating intracellular cyclic AMP. The cyclic AMP-dependent protein kinase activates the NCEH by covalent phosphorylation. NCEH is inactivated by protein phosphatases in the cell.

can occur via inhibition of calcium movement and by activation of protein kinase A (52). In fact, the biological activity of the major cyclooxygenase product, viz. $PGI_2$, appears to reflect its ability to regulate other second messenger systems, notably cyclic AMP. Almost all the biological activity of $PGI_2$ can be attributed to stimulation

of cyclic AMP production. The significance of such observations relates to the fact that cyclic AMP can inhibit a variety of cellular processes including smooth muscle cell proliferation (53). Cyclic AMP can also enhance apoptosis, indicating that it is a very important secondary messenger in the cell. Agents that elevate cyclic AMP, such as

**Fig. 7.** Signal transduction processes affecting lipolysis in smooth muscle cells. Growth factors (e.g., PDGF) and eicosanoids (e.g., $LTB_4$, $LTC_4$) have the capacity to stimulate the DAG-$IP_3$ (diacylglycerol-inositol trisphosphate) system in the cell by activating PKC (protein kinase C). Phosphatidylinositol (PI) is also used to synthesize $IP_3$. $IP_3$ can be converted to phosphatidic acid (PA) and arachidonic acid (AA). Arachidonic acid is required for production of prostacyclin ($PGI_2$). $PGI_2$ can stimulate cyclic AMP production which, in turn, activates protein kinase A. As depicted in Fig. 6, this protein kinase can activate cytoplasmic lipolysis.

interferon (54) and PGE₁ (55), can also inhibit smooth muscle cell proliferation. In fibroblasts, TNF and IL-1 increase cyclic AMP, which is followed by a reduction in expression of *c-myc* messenger RNA and inhibition of thymidine incorporation into DNA (56). Furthermore, while thrombin stimulates endothelial cell proliferation and PGI₂ synthesis, agents that stimulate cyclic AMP also inhibit induction of *c-sis* by thrombin and TGF-β (57) and inhibit thrombin-induced PDGF-B chain synthesis (58). Thus, agents that stimulate cyclic AMP may be doing so via PGI₂ generation. Therefore, loss of PGI₂ synthetic capacity could conceivably result in unregulated smooth muscle cell proliferation. This is another paradox when we consider that aspirin blocks PGI₂ production.
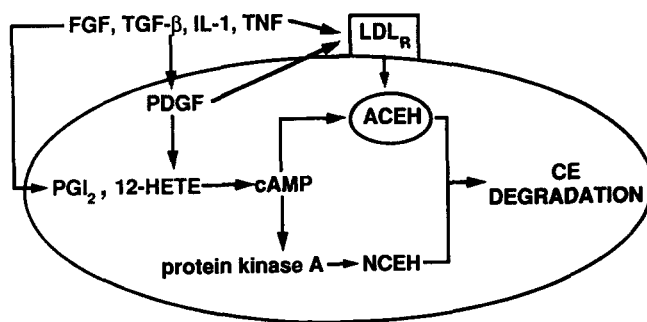
The regulation of cellular cholesterol metabolism via lysosomal CE is also under cyclic nucleotide control (43). We and others have demonstrated that lysosomal CE hydrolase activity in arterial smooth muscle cells is activated by cyclic AMP (43, 59). In addition, we and others have characterized a neutral CE hydrolase activity in smooth muscle cells and macrophages (60, 61). Activity of this enzyme is stimulated by eicosanoids through activation of protein kinase A. Up-regulation of the LDL receptor gene, LDL receptor expression (62), and HMG-CoA reductase activity (63) is also dependent upon activation of protein kinase C (44, 60, 61). Thus, the complex nature of the protein kinase A and C systems can impact very specifically on CE metabolism in the cell.

As discussed elsewhere (6, 40), growth factors and cytokines derived from endothelial cells may modulate smooth muscle cell and macrophage function when these cells interact with each other. Growth factors, including PDGF, acidic and basic FGF, and transforming growth factor-beta (TGFβ) are synthesized by vascular cells and platelets. PDGF is secreted in a biologically active form from stimulated endothelial cells, smooth muscle cells, and macrophages. Agonists include endotoxin, thrombin, and TGFβ. However, basic FGF is released intracellularly or is bound to extracellular matrix. Expression of basic FGF activity may require cell injury or exposure of extracellular matrix to proteases. TGFβ is secreted in latent form, and must be activated by proteases (64). Interestingly, endothelial and smooth muscle cells, when co-cultured together, produce activated TGFβ. In contrast, endothelial and smooth muscle cells cultured alone only produce the latent form (65). PDGF and basic FGF are potent smooth muscle cell mitogens (66-68). TGFβ can either increase or decrease smooth muscle cell proliferation in vitro, depending on the culture conditions (69, 70). Cytokines including IL-1, TNF, and granulocyte macrophage colony-stimulating factor (GM-CSF) or M-CSF are synthesized by inflammatory cells and by activated arterial endothelial and smooth muscle cells. Each of these cytokines has been documented to affect cholesterol trafficking in the cell; most often with regard to LDL-CE

entry into the cell (**Fig. 8**). Recent data from our laboratory have shown that TGFβ and TNF have the capacity to enhance the binding of LDL to the LDL receptor on smooth muscle cells and HepG₂ cells; and, increase the RNA transcript levels for the LDL receptor gene (71).

The intracellular signals arising from interaction of growth factors with cell receptors are rapidly induced. We know of three distinct mammalian cell systems, including: *1)* the cyclic AMP pathway, *2)* the growth factor receptor tyrosine kinase pathway, and *3)* the phosphatidylinositol-diacylglycerol cascade (6). The most studied growth factor in terms of its effect on smooth muscle cell proliferation and migration is PDGF. It modulates smooth muscle cell proliferation, migration, chemotaxis, and chemokinesis (72). The importance of this growth factor in smooth muscle cell hyperplasia is evidenced by increased production of PDGF by proliferating smooth muscle cells, and also by the observation that antibodies to PDGF inhibit neointimal smooth muscle cell accumulation after angioplasty (73). PDGF has a significant impact on smooth muscle migration as well (73). Consistent with this concept is the increase in PDGF messenger RNA transcripts in cholesterol-laden aortic atheromatous tissue, but not in normal, uninvolved aortic tissue (74). We also know that macrophages recruited into atherosclerotic lesions possess significant quantities of PDGF B chain messenger RNA (72). The latter is reduced after the macrophages have subsequently accumulated lipid (72). More information is needed to ascertain whether foam cell development from smooth muscle cells found in vivo occurs prior to, simultaneously with, or subsequent to smooth muscle cell proliferation.

In addition to PDGF, other growth factors synthesized by vascular cells mediate smooth muscle cell proliferation. These include TGFβ and FGF as mentioned. TGFβ can either inhibit or stimulate smooth muscle cell proliferation (75) (**Fig. 9**). TGFβ and PDGF have been implicated in regulation of cholesterol metabolism (76) (Fig. 8). Both



**Fig. 8.** Cytokine regulation of cholesterol trafficking. This figure shows that growth factors (FGF, TGFβ) and cytokines (IL-1, TNF) can elevate intracellular levels of PDGF. This growth factor can activate the LDL receptors (LDL_R) on the surface of the cell to endocytose CE. PDGF can also increase PGI₂ and 12-HETE in the cell which, as shown in Fig. 6 can increase cyclic AMP. This secondary messenger is an activator of lipolysis in the cell.
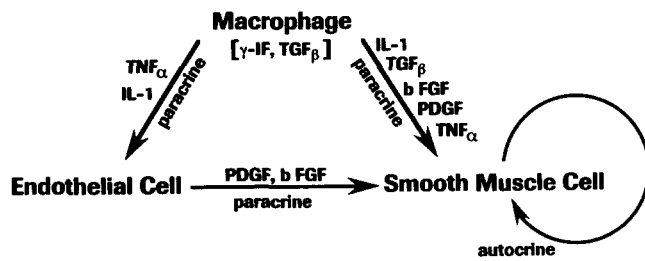
**Fig. 9.** Major autocrine and paracrine effects of cytokines and growth factors on smooth muscle cell proliferation. Cell–cell interactions occur between macrophages and vascular cells such as endothelial and smooth muscle cells. Macrophages can secrete a variety of cytokines (TNFα, IL-1, INFγ) and growth factors (TGFβ, bFGF, and PDGF) which can affect neighboring endothelial cells or smooth muscle cells. PDGF and bFGF can enhance smooth muscle cell proliferation. Smooth muscle cells can also produce these biological response modifiers in an autocrine manner. Unlike TNFα and IL-1, macrophage-derived INFγ and TGFβ have not been shown to induce PDGF or bFGF production in endothelial cells. These observations indicate that there is cellular specificity for the secretion and effects of these cell-derived mediators.

PDGF and FGF promote binding and uptake of LDL into smooth muscle cells (76, 77) (Fig. 8). In fibroblasts, PDGF increases LDL receptor gene transcription (78). It can also increase fluid-phase endocytosis in the absence of a storage pool of cellular cholesterol (79). Our laboratory has demonstrated that PDGF stimulates cholesteryl ester (CE) hydrolase activity (80), whereas TGFβ activates both LDL receptor activity and the CE cycle in arterial smooth muscle cells (76) (Fig. 8). We have proposed that, teleologically, growth factors stimulate the LDL receptor due to the cell's requirement for additional cholesterol in order to synthesize new cell membrane for progeny cells. There is a paucity of information on effects of these growth factors on activity or expression of the scavenger receptor in the macrophage. The role of the latter in cellular proliferation is unclear but its role in promoting accumulation of intracellular CE is of major importance.

It is noteworthy that there is a whole body of information on the effects of basic FGF on smooth muscle cell proliferation. It is beyond the scope of this review to highlight all of these reports as this has been recently reviewed (81). We have recently shown that smooth muscle cell-derived foam cells have increased RNA transcript levels and protein for basic FGF compared to normal cells (82).

The concept that cytokines may modulate the atherogenic response to injury is based on a number of in vivo studies. First, TNF, GM-CSF, interferon, and IL-2 have all been shown to lower serum cholesterol in humans (83, 84). Second, interferon inhibits atherogenesis in rabbits (85). The cellular mechanism(s) involved in effects of cytokines still remain undefined. One hypothesis is that they promote cholesterol deposition and clearance by the spleen and reticulo-endothelial cell system, thereby depleting plasma of LDL-CE and reducing the net cholesterol content of the arterial vasculature (6). Accord-

ingly, many in vitro studies have focused on the role of known cytokines in cholesterol clearance with special reference to endothelial cells, smooth muscle, and macrophages. Cytokines can also down-regulate receptor-mediated uptake of LDL. In one study, it was reported that products of activated lymphocytes can decrease activity of the LDL and scavenger receptors on monocytes (86). The significance of these findings is undefined, as decreased cellular clearance of modified LDL may provoke increased interstitial LDL levels with concomitant increases in plasma LDL. Interestingly, both TNF and IL-1β increased LDL binding to HepG$_2$ cells and increased message levels for the LDL receptor. M-CSF and GM-CSF had no effect in these cells; however, M-CSF did increase scavenger receptor activity in monocyte/macrophages (87). Further research is needed in this area in order to define the role of the LDL and scavenger receptors, perhaps working in parallel in cholesterol trafficking, under the influence of cytokine challenge.

## CROSS–TALK BETWEEN CYTOKINES AND THE EICOSANOID NETWORK: ROLE OF CELL–CELL INTERACTIONS

Data generated from in vivo studies suggest that diet-induced atherosclerosis results in decreased PGI$_2$ production by the vessel wall (40). This is important because PGI$_2$ is a vasodilator and inhibitor of platelet reactivity (40). Reduced PGI$_2$ production could mean less protection against a thrombotic event on a fissured plaque in the vessel wall (6). Activated endothelium itself produces PGI$_2$, PGF$_{2\alpha}$, and PGE$_2$ and to a lesser extent, hydroxyeicosapentaenoic acids (HETEs) (40). The endothelium can convert neutrophil-derived LTA$_4$ to LTC$_4$ during transcellular metabolism, which, in turn can stimulate endothelial cell PGI$_2$ synthesis from free arachidonic acid. In contrast, endothelial cells can incorporate 12-HETE into cellular phospholipids that can actually inhibit PGI$_2$ synthesis. Endothelial cell-derived eicosanoids have been implicated in processes related to cell proliferation, cell adhesion, permeability, migration, chemotaxis, phenotypic changes, and the regulation of sterol metabolism (6, 40). These eicosanoid-modulated events will require further correlation with endothelial cell production of endothelium-dependent relaxing factor/nitric oxide (EDRF/NO).

In macrophages, eicosanoids derived from the cyclooxygenase and lipoxygenase pathways can influence cholesterol metabolism in a manner similar to smooth muscle cells (6) (Fig. 6). PGE$_2$ inhibits CE accumulation presumably by inhibiting ACAT activity. It is important to note that PGI$_2$ and PGE$_1$ (in vitro) will also reduce LDL receptor activity and cholesterol synthesis in macrophages (88, 89). However, PGE$_1$ does not contribute

significantly to total vascular eicosanoid production as its precursor, dihomo-gamma-linoleic acid, is a very minor fatty acid in vascular tissue.

Recent evidence indicates that the major cytokines produced in vascular cells such as interferon, IL-1, colony-stimulating factor (CSF), and TGF$\beta$ have a significant effect on metabolic regulation of eicosanoid metabolism. IL-1, an important cytokine derived from activated endothelial cells and macrophages, can stimulate PDGF production (90) or eicosanoid synthesis (91). Eicosanoid synthesis can also be initiated via direct phospholipase activation (92) or by increased transcription and translation of cyclooxygenase messenger RNA (91). Other phospholipase activators such as bradykinin and thrombin are synergistic with cytokines in this activity (93). Cytokines such as TNF and IL-1 will also stimulate endothelial cell eicosanoid biosynthesis by induction of cyclooxygenase. In fact, TNF and IL-1 can either be additive (94) or synergistic (95) in terms of their effects on cyclooxygenase activity in vascular cells. If the endothelium can be activated to produce specific cytokines, and these cytokines can, in turn, have an effect of eicosanoid biosynthesis, the interaction of cytokines with the eicosanoid pathways should have a significant impact on cholesterol trafficking in the vessel wall (Fig. 8).

Eicosanoids also regulate several aspects of macrophage function, particularly with regard to the cytokine network. Endotoxin challenge in these cells can stimulate IL-1 production. Phorbol ester treatment can up-regulate TNF gene expression through synthesis of LTB$_4$ whereas up-regulation of TNF gene expression by phorbol ester is blunted by PGE$_2$ (96). This mechanism may represent a positive feedback loop, as LTB$_4$ stimulates IL-1 and TNF release (97). In addition to stimulating PGE$_2$, TNF can also stimulate colony-stimulating factor-1 (CSF-1) synthesis (98). It appears that one response modifier can affect another, with the ultimate expression of a significant signal in the cell. From the literature, we know that cyclooxygenase-generated eicosanoids antagonize the effects of cytokine activation whereas lipoxygenase-derived eicosanoids mediate or amplify the effects of cytokine activation (6). Thus, an important concept has now been identified and developed. The involvement of growth factors follows a metabolic pathway similar to cytokines. We now know that PDGF can stimulate eicosanoid production by activating phospholipase A$_2$ (99) (Fig. 7); PDGF can also promote eicosanoid generation by stimulation of transcription and translation of cyclooxygenase and PGI$_2$ synthase genes (100). PDGF is synergistic with serotonin in stimulating smooth muscle cell PGI$_2$ production (101). However, PDGF does not stimulate endothelial cell eicosanoid production presumably because large vessel endothelium does not possess receptors for PDGF. The effect of FGF on endothelial cell ei-cosanoid biosynthesis apparently differs from that of other growth factors in that FGF will inhibit endothelial cell eicosanoid production by reducing the cellular content of cyclooxygenase and production of PGI$_2$ (6). Thus, the FGF effect is antagonistic to the action of PDGF on eicosanoid biosynthetic enzymes.

## SUMMARY

Thus, it is apparent that humoral factors released during inflammation can affect cholesterol metabolism in arterial cells during atherogenesis. These humoral factors released from the macrophage, endothelium, or smooth muscle can modify the cytokine/growth factor/eicosanoid network in the vessel wall in either a paracrine or autocrine manner (6, 40). We also postulate that this could result in alterations in native LDL induced by endothelium (6, 40). Therefore, regulation of the cytokine/growth factor network by eicosanoids may represent an important aspect of arterial responsiveness to injury, as well as progression of intimal hyperplasia and CE deposition in a setting of inflammatory cell activation. Recent understanding of the biochemistry of eicosanoids and the metabolic consequences of these biological response modifiers has helped us to further develop this concept as it relates to mechanisms involving cholesterol delivery and trafficking within the vessel wall during thrombo-atherosclerosis.

In this review, we have attempted to highlight recent data which support our classification system for cell–cell interactions, and document that eicosanoids and cytokines released from one cell can activate corresponding receptors on neighboring cells. They can interact with each other in this "cross talk" phenomenon during transmembrane signaling. Recent evidence demonstrating that phosphorylation reactions involving protein kinases A and C and tyrosine protein kinase, coupled with the highly regulated eicosanoid pathway and the DAG-phosphatidylinositol system, appears to have a major impact in our understanding of at least three processes related to atherogenesis: *1)* cholesterol delivery, *2)* intracellular cholesterol processing, and *3)* cholesterol efflux. Identification of these diverse pathways associated with transmembrane signaling have helped us to define processes related to thrombosis since they share common pathways in a complex arteriopathy during atherogenesis.

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