INTRODUCTION

The oxygenated, biologically active derivatives of the 20-carbon essential fatty acid arachidonate are now defined as eicosanoids. These include prostaglandins, which were the first to be described, followed by thromboxane, prostacyclin (PGI2), leukotrienes, and other hydroxy acids (1–4). Although eicosanoid formation is seemingly ubiquitous in mammalian tissues, their precise physiological role cannot as yet be defined. They can be classified as autacoids, i.e., substances which are evanescent and exert their effects locally in the microenvironment of the tissues where they were generated. Such effects might include regulation of vascular tone and permeability of capillaries and venules, contraction or relaxation of muscle, stimulation or inhibition of platelet function, activation of leukocytes, regulation of renal blood flow and mineral metabolism, and possible control of growth and/or spread of malignant cells.

Eicosanoids are not stored in cells and must always be newly synthesized in response to perturbation. The precursor, arachidonic acid, is present only in esterified form and must be hydrolyzed prior to utilization for eicosanoid synthesis. The exact mechanisms governing release of arachidonic acid upon cell stimulation are incompletely understood at present. Released arachidonate can be processed in several ways (5). It may leave the cell and become available for metabolism by another cell or it may be bound by plasma albumin. Most importantly, arachidonate can be enzymatically oxygenated by a particulate cyclooxygenase and/or a cytoplasmic lipoxygenase with the formation of unstable intermediate compounds (2). These include endoperoxides (PGG2/PGH2) for prostaglandin formation and hydroperoxides and epoxides (LTA4) for hydroxy acid and leukotriene production (2, 3). Ultimately, the derivatives formed will depend on the type of converting enzymes present in a tissue. Pathways of formation of prostaglandins, thromboxanes, and leukotrienes from arachidonic acid are summarized in Fig. 1. Also shown in the figure are reactions which may be pharmacologically inhibitable.

METHODOLOGY IN EICOSANOID RESEARCH

In studying eicosanoid metabolism in a given cell type, it is important to ascertain the full pattern of products produced by that cell under various conditions of stimulation. Such base line information may then set the stage for identification of a new, previously unrecognized eicosanoid. In some tissues (e.g., platelets) more so than others, low concentrations of free arachidonate initiate oxygenation reactions. In other cells, model stimuli such as ionophore A23187 are required to give rise to eicosanoid generation. Obviously, profile identification is compounded in multicellular tissues such as lung and kidney where different cell types may synthesize different metabolites. In addition, the cells may biochemically modify each others' precursors, intermediates, and end products (6–8). It should also be stressed that under pathological conditions, at sites of tissue injury or inflammation, migration of stimulated or unstimulated hematologic cells may further complicate the profile of eicosanoids formed.

If the eicosanoid pattern of a tissue is known, the investigator is in a better position to understand the biological effects observed when that tissue is stimulated. For example, cultured human endothelial cells produce prostacyclin and PGE2, both of which are vasodilators and inhibitors of platelet function (6). However, these cells also synthesize PGE2, and PGF2a, which may have physiological effects in the vasculature which have not as yet been appreciated.

The methodologic approach to characterization of eicosanoids produced by cells under investigation still follows the general pattern established in the early 1960's (for historical review, see ref. 1). A tissue or specific cell type is incubated with radiolabeled arachidonate with or without a stimulus and the metabolites formed are identified and isolated by thin-layer (TLC)
or high-performance liquid chromatography (HPLC). Structure of new products is determined by ultraviolet spectrophotometry and a combination of gas–liquid chromatography and mass spectrometry (GLC–MS) (2). If formation of transient intermediate compounds that will undergo attack by water or alcohol is suspected, short-term incubations can be performed and the O-alkyl derivatives can be trapped with methanol or ethanol. If the presence of an unstable intermediate is validated, an attempt can be made to isolate or synthesize it. It can then be tested for inherent biological activity and, in addition, utilized for detection of new physiologically active eicosanoids. Thus, endoperoxides were shown to be transformed into thromboxane A2 (TXA2) in platelets and to prostacyclin in vascular tissues. The epoxide LTA4 is the precursor of the dihydroxy acid leukotriene B4 (LTB4) and the cysteinyl-containing leukotrienes C4, D4, and E4 (2).

Elucidation of the structure of an eicosanoid, along with the ability to isolate and/or synthesize it in pure form and in sufficient quantities (1–5 mg), allows for development of highly sensitive radioimmunoassays (RIA) (9). This is particularly important because eicosanoids are present in only minute quantities in stimulated tissues and plasma. In some instances RIA can be carried out on a sample without previous extraction and purification, which is a distinct advantage. However, specificity may be improved by prior extraction with organic solvents and chromatographic purification. RIA’s are less useful for studying the eicosanoid profile of a cell or tissue because they measure single compounds and do not provide information concerning unknown metabolites. Impurities, unknown eicosanoids, or high levels of cross-reacting substances may on occasion bind to the antibody and produce a falsely elevated value. Therefore, direct assays should be monitored by performing control RIA’s on extracted, purified material for purposes of comparison (9).

Historically, dating back to the original discovery of prostaglandins by Kurzrok (1), almost every eicosanoid was initially detected in a bioassay system. The bioassay response is immediate [or slightly delayed in the case of slow-reacting substance (SRS-A)] and therefore the action of eicosanoids with short half-lives can be observed. In
addition, a new compound can be identified prior to full characterization, and model eicosanoids can be tested. The utility of bioassay was maximized by the superfusion cascade system developed by Vane (9) in which several different tissues, each with a characteristic response to specific compounds, is studied. Combinations of tissues can be devised in which almost all eicosanoids can be qualitatively identified. Specificity can be improved by addition of appropriate antagonists for interfering compounds and by extraction and purification of the metabolite under study (9).

Whenever possible, eicosanoids should be measured by multiple complementary techniques which will mutually compensate for the disadvantages of each. Ideally this would include bioassay, RIA, HPLC, GLC-MS, and, in special cases such as platelets and leukocytes, cell aggregation studies.

THE CYCLOOXYGENASE REACTION AND ITS BIOLOGICAL CONSEQUENCES

The cyclooxygenase is a substrate-activated, heme-containing enzyme which requires molecular oxygen, free arachidonic acid, and possibly a hydroperoxide activator (10-12). It should be noted that arachidonate is by far the preferred substrate for cyclooxygenase. For example, eicosapentaenoic acid (20:5) is 92% less efficiently converted to its metabolites than is arachidonate. This has implications for dietary manipulations of the eicosanoid pathway in vascular diseases. The initial step in endoperoxide formation involves insertion of molecular oxygen at the C-11 position of arachidonate (Fig. 1). This 11-peroxy derivative (11-HPETE) becomes an endoperoxide via addition of another oxygen at C-15, formation of a new carbon-carbon bond between C-8 and C-12 (cyclopentane ring), and finally establishment of an oxygen bridge between C-11 and C-9. This 15-hydroperoxy endoperoxide PGG_2 is rapidly reduced to the 15-hydroxy derivative PGH_2 because peroxidase is present in cyclooxygenase (11, 12). As summarized in Fig. 1, endoperoxide formation then gives rise to many different types of eicosanoids with specific biological activities (11, 12). The biochemical reactions, as elucidated in the landmark experiments of Samuelsson and associates (11), formed the basis of subsequent research in prostanooids and later in hydroxy acids and leukotrienes as well (2). Of particular pertinence is the action of acetyl salicylic acid (aspirin) on cyclooxygenase. The acetyl group is covalently bound to the active site of the enzyme thereby inducing total inhibition. The acetylation defect can be corrected by enzyme replacement in all tissues except platelets which cannot synthesize new protein. Therefore, platelets cease to produce their major cyclooxygenase product thromboxane A_2, which induces aggregation and vasoconstriction, for the remainder of their lifespan. However, 12-HETE, the platelet lipoxygenase product, is not inhibited by aspirin. The aspirin-treated platelet is totally unresponsive functionally to arachidonic acid stimulation in vitro and can no longer respond fully to usual concentrations of other standard platelet agonists. In vivo, this is manifested as a mild prolongation of the bleeding time. It should be emphasized that this situation is not synonymous with an "anti-thrombotic" state (13). However, aspirin treatment does seem to offer some degree of partial protection for patients at risk for occlusive vascular disease, based on an accumulation of positive but generally not statistically significant experience in clinical trials (4, 13). That aspirin exerts a beneficial effect in thrombotic diseases that is unrelated to cyclooxygenase inhibition cannot be ruled out.

Prostacyclin (PGI_2) was discovered during a series of experiments wherein blood vessel segments or microsomes generated a new eicosanoid when incubated with arachidonate or endoperoxide (1, 12). This product induced blood vessel relaxation and inhibited platelet aggregation by elevating cyclic AMP and blocking calcium mobilization (12). Subsequently the material was structurally identified, synthesized, and shown to have the same biological properties as the natural compound (1, 12). Prostacyclin is labile in aqueous media below pH 7, which has made it difficult to utilize for clinical purposes (12). It was originally thought that vascular homeostasis might be modulated by a "balance" between prostacyclin and thromboxane production since they have opposite biological effects on both platelets and blood vessels. This led to additional concern that aspirin treatment would serve to inhibit endoperoxide production by blood vessels, thus eliminating prostacyclin synthesis, an effect which might be potentially harmful. The concept may require reevaluation as a working hypothesis for several reasons: production of other eicosanoids by platelets and vascular tissues may not have been duly considered, the concept of cell-cell interactions in the eicosanoid pathway involving platelets, leukocytes, and endothelium had not as yet emerged, and some clinical trials wherein high-dose aspirin seemed successful were carried out in the setting of low to absent prostacyclin production (4, 5, 13).

THE LIPOXYGENASE PATHWAY
HYDROXY ACIDS AND LEUKOTRIENES

In general, lipoxygenases are heterogeneous cytoplasmic enzymes. As in the cyclooxygenase system, the initial catalytic step involves formation of a hydroperoxy derivative of arachidonate. Plant lipoxygenases had been
well studied prior to their description in mammalian tissues, and provided a methodologic background for the ensuing eicosanoid research. The 12-lipoxygenase in platelets was the first to be described in mammalian tissues. Since then lipoxygenase activities specific for other positions on the arachidonate molecule such as C-5, C-11, and C-15 have been described in other cells, especially those involved in inflammatory responses (2, 3).

Studies of eicosanoid metabolism in inflammatory cells were initiated by Borgeat and Samuelsson in 1976, when they incubated radiolabeled arachidonate with rabbit polymorphonuclear leukocytes (2, 11). Following extraction, purification by silicic acid chromatography, and HPLC, new metabolites were identified by GLC-MS and UV spectrophotometry. The eicosanoids identified were all products of the lipoxygenase pathway and included two enzymatically produced and biologically important compounds, 5-HETE and LTB₄. The UV spectrum of LTB₄ and its accompanying dihydroxyeicosatetraenoic acid isomers demonstrated the now well-known conjugated triene pattern of absorption bands (11). The structure and stereochemistry of LTB₄ and its isomers, and experiments with isotopic oxygen, suggested the possibility of their formation from a transient hydrolyzable intermediate. This gave rise to short-term (30 sec) incubation experiments with arachidonate and leukocytes in which the products were trapped with methanol or ethanol. Results of these experiments demonstrated the existence of the proposed intermediate, LTA₄, and indicated that it was converted enzymatically to LTB₄ and nonenzymatically to the isomers of LTB₄. The epoxide structure of LTA₄ (Fig. 1) was confirmed by chemical synthesis and it has since been isolated from polymorphonuclear leukocytes (2). As shown in Fig. 1, the suggested pathway from arachidonic acid to LTA₄ is via the intermediate compound 5-HPETE. An interesting analogous situation exists between the cyclooxygenase and lipoxygenase pathways. Thus, the unstable endoperoxides are specifically metabolized to prostanoids and the unstable epoxide LTA₄ can be processed to different types of leukotrienes.

Slow-reacting substance of anaphylaxis (SRS-A) is a biological material which induces a slow but long-lasting contractile response in smooth muscle. It has been implicated in the pathogenesis of immediate hypersensitivity reactions, asthma, and other proinflammatory responses (3). The activity was originally recovered from perfused lung following injection of cobra venom (probably containing phospholipase A₂) or antigenic challenge (2, 14). Several lines of experimental evidence indicated that SRS-A might have been an eicosanoid of the leukotriene type. 1) Radiolabeled arachidonate was incorporated into SRS-A in basophilic leukemia cells after stimulation with ionophore A23187. 2) This response was inhibited by ETYA (eicosatetraynoic acid) but not by indomethacin. 3) The UV spectrum of SRS-A was similar to that of the leukotrienes (2–4). The above similarities led to the hypothesis that SRS-A and the previously characterized leukotrienes might originate from a common intermediate eicosanoid, specifically the unstable epoxide LTA₄ (2). Fortunately it was then possible to prepare sufficiently pure quantities of SRS-A for structural and functional analyses through utilization of a murine mastocytoma cell system. Since SRS-A was known to be a sulfur-containing lipid, the cells were incubated with labeled arachidonic acid or labeled cysteine and stimulated with ionophore A23187. Purification was achieved by protein precipitation, alkaline hydrolysis, and column and HPLC. The pure product induced a typical contractile response in guinea pig ileum which was reversed by FPL55712, an SRS-A inhibitor. Although the UV spectrum of this material resembled LTB₄, there was a 10-nm shift in the maximum wavelength, consistent with a sulfur component alpha to a conjugated triene. Presence of the sulfur substituent at C-6 was verified by additional experimental data. The pure compound (LTC₄) was shown to contain glycine and glutamic acid in addition to cysteine. Corresponding cysteinylglycine (LTD₄) and cysteiny1 (LTE₄) derivatives were also described (2). SRS-A is now thought to consist of the parent compound LTC₄ and its metabolites LTD₄ and LTE₄. The biosynthetic pathway begins with LTA₄ formation from arachidonate by way of 5-HPETE and then glutathione conjugation of LTA₄ as catalyzed by glutathione-S-transferase. Formation of LTD₄ and LTE₄ is catalyzed, respectively, by gamma glutamyl transpeptidase and dipeptidase (2, 3).

The leukotrienes represent a new class of autacoids with remarkable biological properties. The dihydroxy acid LTD₄ is the most powerful chemotactic and chemokinetic eicosanoid thus far described. It also promotes adhesion of neutrophils to vascular endothelium. In addition, LTD₄ is a complete secretagogue for neutrophils, induces their aggregation, and increases microvascular permeability. These activities represent important components of the inflammatory response (14, 15).

The important biological actions of SRS-A (LTC₄, LTD₄, LTE₄), apparently conferred by the presence of a cysteine-containing residue at C-6, are largely related to its smooth muscle-stimulating activity. These include bronchoconstriction, wheezing, and reduced expiratory flow in the pulmonary system. SRS-A also induces a vasoconstrictor response in the coronary circulation and in terminal arterioles in other tissues. In addition to its spasmodogenic functions, an increase in local vascular permeability has been observed following intradermal injection of SRS-A. Thus, in human skin, a local wheal
followed by an erythematous flare occurred at the injection site (14). Availability of pure SRS-A for in vitro and in vivo studies will furnish additional information concerning its functional parameters.

Future endeavors in leukotriene research will include development of specific antagonists of their action, inhibitors of their production, additional methods of assay, and characterization of cell receptors and physiologic stimuli.

CELL-CELL INTERACTIONS IN EICOSANOID PATHWAYS

Due to the labile nature of eicosanoids, these autacoids exert their most important biological effects in the microenvironment of cells participating in their production (1). Usually a given physiological or pathological event involves multiple cell types in close proximity. As previously discussed, mechanisms for processing arachidonic acid are ubiquitous, but also reflect individual cell and tissue specificities. Heretofore eicosanoid research has been focusing mainly on metabolites produced by individual cells. It may now be important to recognize the potential for conversion of eicosanoid intermediates and end products to new metabolites which cannot be synthesized by a single cell type and which may have important biological properties. An illustration of this concept is the hemostatic platelet plug or arterial thrombus. Platelets, leukocytes, and endothelial cells, all of which have active eicosanoid pathways, are brought into close apposition during the process (5). In addition, circulating leukocytes and blood vessels are early participants in the inflammatory process (3).

In 1980 we directly demonstrated, with the use of radioisotope labeling techniques, that platelet-derived endoperoxides could be utilized by endothelial cells in the formation of prostaclin (6). This line of investigation was extended to studies of platelet-neutrophil interactions, wherein combined suspensions of [3H]arachidonate-labeled platelets and unlabeled neutrophils were stimulated with ionophore A23187. Several radioactive eicosanoids, including 3H-labeled LTB4 and 5-HETE, that could not have been produced by platelets alone were identified (7). Also detected was a dihydroxy-eicosatetraenoic acid (5S,12S-DiHETE) synthesized from platelet 12-HETE by stimulated neutrophils.

The platelet-neutrophil experiments were then modified in order to examine the effects of thrombin and collagen—platelet stimuli known to occur in vivo. In contrast to ionophore, thrombin and collagen do not initiate eicosanoid metabolism in neutrophils. Under these experimental conditions (i.e., stimulated platelets and unstimulated neutrophils) a new metabolite of arachidonic acid, 12S,20-dihydroxyeicosatetraenoic acid (12,20-DiHETE), was formed from released platelet 12-HETE as demonstrated by both thin-layer radio-chromatography and HPLC. Since 12,20-DiHETE could be formed by a platelet-neutrophil system from aspirin...
treated donors, it was next demonstrated that unstimulated neutrophils could hydroxylate purified platelet 12-
HETE to 12,20-DiHETE. Structural elucidation of the product was accomplished by Drs. Thomas D. Oglesby and Robert R. Gorman. The isolated material was hydrogenated and oxidized and analyzed by GLC-MS (8). The reactions described above are summarized in Fig. 2.

Thus cell-cell interactions involving both the cyclooxygenase and lipoxygenase pathways have been docu-
mented experimentally. They may provide explanations for in vivo phenomena which thus far have not been elucidated by studies of eicosanoid metabolism in single cell types in vitro.

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tion in relation to prostacyclin and thromboxane phys-