REVIEW ARTICLE

THE PROSTANOIDS IN HEMOSTASIS AND THROMBOSIS

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The Prostanoids in Hemostasis and Thrombosis

A Review

J. Bryan Smith, PhD

THE RECENT DISCOVERY and study of novel compounds derived from prostaglandin endoperoxides, referred to in this review as the prostanoids, has provided new insights into the mechanisms regulating the functions of blood platelets. Thromboxane A_2, discovered in 1975 by Hamberg, Svensson, and Samuelsson,[119] is capable of inducing platelet aggregation and constricting blood vessel walls. Counterbalancing these effects, prostacyclin (PGI_2), discovered just one year later,[155,220] acts to inhibit platelet aggregation and dilate the vessel wall. These properties, and the great facility with which platelets make thromboxane A_2 and endothelial cells make prostacyclin, implicate these novel prostanoids in both hemostasis and thrombosis. The purpose of this review is to bring together the many different aspects of this new area of research, which range from the consumption of essential fatty acids to the elevation of adenosine 3′:5′-cyclic phosphate (cyclic AMP). A major aim will be to impress the reader with the great potential that management of the production or effects of these prostanoids offers for the treatment of thrombosis.

Research on prostaglandins has gone forward at an ever-increasing pace, and the number of publications has become so enormous that a reviewer with good intentions faces a tremendous task in doing justice to all those concerned. Nevertheless, I have attempted to do just that and apologize to those whom I may have missed. I begin by reviewing the effects of the most active prostanoids on vascular smooth muscle and platelets and then turn to a discussion of the possible involvement of the prostanoids in hemostasis. Since hemostasis is a very complicated event (see Figure 3) it seemed only correct to summarize the factors that are presently known to contribute to hemostasis. In this way the contribution made by the prostanoids can be put in perspective. Arterial thrombosis is even less well understood than hemostasis. I have attempted to review briefly the events that are presently believed to be involved in arterial thrombosis and lead to acute myocardial ischemia. Evidence is growing that formation of

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thromboxane A_2 (TxA_2) may present a serious complication in thrombosis. If this is true, mechanistic approaches aimed at reducing thromboxane A_2 formation or effects or supplementing the formation of PGI_2 may prove to be of great value in reducing the mortality associated with this prevalent occurrence. Therefore, I have given special emphasis to the potential therapeutic approaches that might be taken based on these new developments. A biochemical coverage of the prostanoids may not seem appropriate for a review in a journal of pathology. However, most of the major developments to date have been biochemical, and there seems to be little question that future progress will depend on a thorough knowledge of prostanoid biochemistry. I have therefore included as an appendix a section on the biosynthesis of the prostanoids to allow individuals who need more detailed information to obtain it more easily.

Effects of the Prostanoids

The prostanoids have a great number of biologic actions. Those actions directly relevant to hemostasis and thrombosis involve effects on the vasculature and platelets and are summarized in Table 1.

Effects on Vascular Smooth Muscle

The prostaglandin endoperoxides PGG_2 and PGH_2 are formed from arachidonic acid and are 100–200 times more potent than PGE_2 in causing contraction of the rabbit aorta.[120] Although endoperoxides produced from dihomo-γ-linolenic or 5,8,11,14,17-eicosapentaenoic acids also contract the aorta, they are less active than PGG_2 or PGH_2.[234,235,288] The potent "rabbit aorta-contracting substance" (RCS) that is released from guinea pig lungs during anaphylaxis[277] or during arachidonic acid infusion[363] cannot be PGG_2 or PGH_2, because it is significantly more unstable.[119] It is presently believed that the activity of RCS is largely attributable to TxA_2.[119,235,342,343] When human platelets are incubated with arachidonic acid[119,365] or thrombin,[74] they also produce a potent constrictor of vascular smooth muscle, which is probably TxA_2. Studies in which TxA_2 was generated by incubating PGH_2 with platelet microsomes

Table 1—Effects on Prostanoids

Vascular smooth muscle	
Constrictors	$TxA_2 > PGG_2 \simeq PGH_2 > PGE_2$
Dilators	$PGI_2 \simeq PGE_2 \simeq PGE_1$
Platelet aggregation	
Inducers	$TxA_2 > PGG_2 > PGH_2 > PGE_2$
Inhibitors	$PGI_2 > PGD_2 > PGE_1$

showed that as little as 10 ng (30 pmole) of TxA_2 causes marked constriction of rabbit aorta.[235] It was concluded that TxA_2 is approximately 50 times more potent than PGH_2[235] (ie, 5000 times more potent than PGE_2). TxA_2 has been found to contract vascular smooth muscle isolated from all species so far examined.[74,234,260,342-344]

PGG_2 and PGH_2 constrict porcine, cat, and canine coronary arteries but relax bovine coronary vessels.[234,260] The cause of the paradoxical vasodilation of bovine coronary arteries by arachidonate, PGH_2, and PGG_2 was elucidated by Kulkarni et al [176] and Raz and associates,[286] who demonstrated that they are converted into a potent but unstable vasodilator by the bovine vessels. This vasodilator was subsequently identified as PGI_2.[234] It is now known that PGI_2 dilates isolated mesenteric,[70] celiac,[41] and coronary arteries.[260] It lowers blood pressure when infused into mammals[11] and in this respect is similar to PGE_1 or PGE_2.

Effects on Platelets

To study platelet function, blood is usually collected into an anti-coagulant, such as sodium citrate or heparin, and centrifuged at low g forces to prepare platelet-rich plasma (PRP). Suspensions of platelets prepared in this manner aggregate after addition of agents such as adenosine diphosphate (ADP), epinephrine, collagen, and thrombin.[34]

Three prostanoids, PGE_1, PGD_2, and PGI_2, have been shown to be potent inhibitors of platelet aggregation.[19,41,99,100,106,125,155,167,171,172,194,204,212,213,247,331,348,366,386] PGE_2 is less active and in low concentrations stimulates ADP-induced aggregation of rat and pig platelets [171] and enhances the second wave of ADP-induced aggregation of human platelets.[316] In heparinized PRP, PGE_2 actually causes the aggregation of pig platelets.[194] The inhibitory effect of PGE_1 on platelet aggregation was first demonstrated by Kloeze,[171] who showed that concentrations as low as 3×10^{-8} M are effective. PGD_2 is about twice as active as PGE_1 as an inhibitor of the aggregation of normal human platelets [247] but is relatively inactive in inhibiting the aggregation of platelets from patients with myeloproliferative disorders [56] or from most animals.[247,331,386]

The discovery of PGI_2 resulted from observations by Moncada et al [220] that an unstable factor that inhibits platelet aggregation is formed when PGH_2 or PGG_2 is incubated with microsomes obtained from blood vessels. They noted that the conversion of PGG_2 or PGH_2 into PGI_2 catalyzed by aortic microsomes is high (80–90%), while little or no PGI_2 (> 1%) is produced from added arachidonic acid. However, PGI_2 is formed spontaneously by specimens of human arterial or venous tissues.[223] The potency of PGI_2 as an inhibitor of aggregation is 10–20 times that of PGE_1 or PGD_2,

and it has been suggested that the formation of PGI_2 explains the lack of platelet adhesion to the intact endothelium of blood vessels.[106]

The inhibition of platelet aggregation of PGI_2, PGE_1, and PGD_2 is mediated by elevation of cyclic AMP in platelets.[19,99,204,212,366] PGI_2, the most potent inhibitor of platelet aggregation, is also the most powerful activator of adenylate cyclase in intact platelets and isolated membranes.[19,99,348] The inhibitory effects of all three prostaglandins are potentiated by drugs which cause the elevation of intracellular cyclic AMP by inhibiting cyclic AMP phosphodiesterase.[213,367] High affinity binding sites for PGI_2 and PGE_1 have been identified on human platelets.[309,317] Pharmacologic studies,[195,382] biochemical measurements of increases in cyclic AMP,[212,214] and binding studies [309,317] all indicate that PGI_2 and PGE_1 have a common receptor site on platelets. PGD_2 appears to activate adenylate cyclase by acting at another receptor site.

How increases in intracellular levels of cyclic AMP suppress platelet function is the subject of intensive investigation. A cyclic-AMP–dependent calcium pump has been recognized in platelet membranes, and it has been suggested that cyclic AMP acts by reducing the intracellular level of free calcium.[125,160] Further, it has been shown that increases in intracellular cyclic AMP are associated with the phosphorylation of a platelet protein (mol wt 24,000 daltons) which is present in the membrane fraction that can take up calcium ions.[31,125,160,161,383]

Hamberg and associates [120] discovered that the prostaglandin endoperoxides PGG_2 and PGH_2 induce platelet aggregation. This discovery provided explanations for the earlier observations that platelet aggregation results when arachidonic acid is incubated with PRP,[321] and for the labile aggregation-stimulating substance (LASS) produced when arachi donic acid is incubated with an endoperoxide synthetase preparation from seminal vesicles.[388,390] PGH_1, PGH_3, and their precursors dihomo-γ-linolenic acid and 5,8,11,14,17-eicosapentaenoic acid apparently do not induce platelet aggregation.[288,321,390] PGG_2 is about 3 times more potent than PGH_2 as an aggregating agent, and its threshold concentration for inducing aggregation in human citrated PRP is about 0.3 μM.[120,199] In studies of the biochemical transformation of arachidonic acid or PGG_2 into TxA_2 by platelets, it was noted that the aggregating activity disappeared rapidly with a half-life similar to that of TxA_2 (about 30 seconds at 37 C) and was greater than could be accounted for by remaining PGG_2 or PGH_2. It was therefore proposed that TxA_2, as well as being a potent constrictor of arterial smooth muscle, induces platelet aggregation.[119] It has been shown subsequently that the conversion of PGH_2 into TxA_2 by platelet microsomes or solubilized thromboxane synthetase from platelet mi-

crosomes is associated with an increase in platelet aggregating activity.[235,397]

The mechanism by which PGH_2, PGG_2, and TxA_2 induce aggregation is unknown. These prostanoids, as well as ADP and epinephrine, are inhibitors of PGE_1-stimulated cyclic AMP accumulation in human platelets but have no effect on the basal levels of either cyclic AMP or cyclic GMP.[100,211] It is therefore unlikely that TxA_2 induces platelet aggregation by an effect on cyclic nucleotide metabolism.

Platelet aggregation by arachidonic acid or prostaglandin endoperoxides is associated with the secretion of ADP, adenosine triphosphate (ATP), and serotonin from platelet storage granules.[49,321,328,390] It has been suggested that platelet aggregation by these compounds is mediated solely by released ADP. However, studies have shown that the addition of arachidonic acid, prostaglandin endoperoxides, or TxA_2 to platelet suspensions can induce aggregation without nucleotide secretion, provided extracellular plasma or fibrinogen is present.[49,148,169] Fibrinogen is known to be required for the aggregation of platelets by ADP.[35,333] It seems probable that PGH_2, PGG_2, and TxA_2 have their own receptor site(s) on platelets, since the antagonists 15-deoxy-9,11-epoxyimino-PGH_2[82] and pinane thromboxane A_2,[242] which abolish platelet aggregation by these prostanoids, do not block aggregation by ADP. Furthermore, there is evidence that ADP and the prostanoids act synergistically in inducing platelet aggregation.[168,325,373] The positive feedback loops operative during aggregation by ADP and TxA_2 are illustrated in Figure 1.

Dissociations Between Effects on Coronary Vasculature and Platelets

A number of studies indicate that the receptors for prostanoids on platelets and coronary vessels are not identical. PGE_1 inhibits the aggregation of platelets from all species examined[386] but dilates porcine coronary arteries and constricts coronary arteries in cows and cats.[234,260] Al-

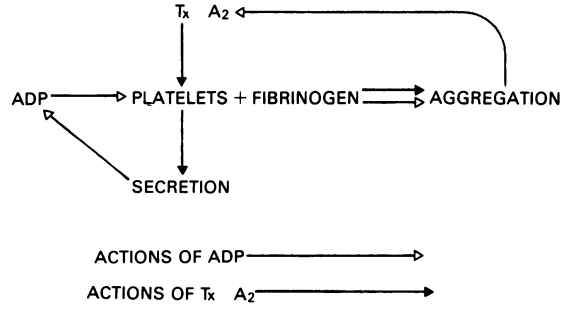


Figure 1—Positive feedback loops for aggregation of platelets by TxA_2 and ADP. The formation of thromboxane A_2 during ADP-induced primary platelet aggregation in the presence of fibrinogen induces the secretion of ADP and a change in the platelets such that they will undergo secondary aggregation. Although the secretion of ADP is not essential for secondary aggregation, its presence may activate a positive feedback loop that enables more thromboxane A_2 formation.

though PGH_3 is converted by platelet thromboxane synthetase into TxA_3, which is a potent rabbit aorta-contracting substance, neither PGH_3 nor TxA_3 appear to cause platelet aggregation.[235,288] By contrast, the prostaglandin endoperoxide formed from an unnatural 19-carbon fatty acid (19:4, n-6) induces platelet aggregation but does not contract the aorta after incubation with platelets.[288] The effects of different unsaturated fatty acids and their endoperoxides on platelet aggregation and on the rabbit aorta before and after incubation with thromboxane synthetase are summarized in Figure 2.

Dissociations between platelet and vessel wall receptors have also been noticed with stable analogs of the prostanoids. A sulfur-containing analog of PGI_2, 6,9-thia-PGI_2, is a potent inhibitor of platelet aggregation but constricts isolated cat coronary arteries.[241] Similarly, the stable analog of PGH_2, 15-deoxy-9,11-epoxyimino-PGH_2, antagonizes the effects of TxA_2 and PGH_2 on platelet aggregation but constricts the rabbit aorta.[82]

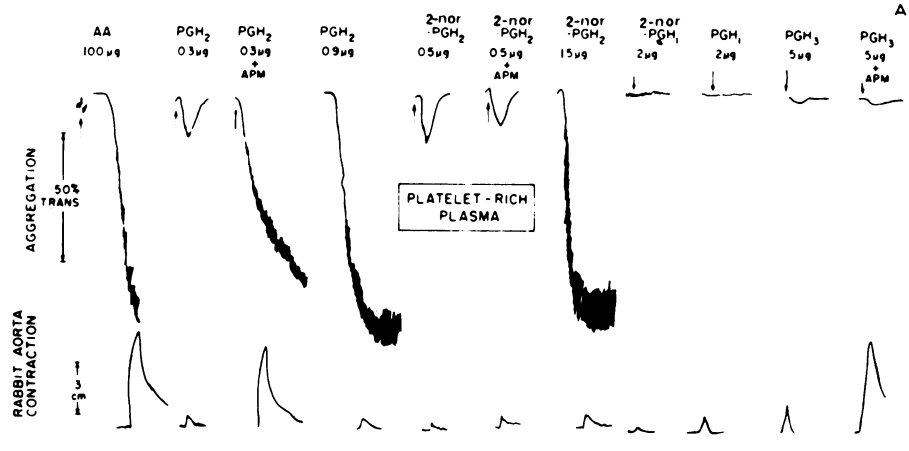


Figure 2—Effects of prostaglandin endoperoxides and thromboxanes on aggregation of platelet-rich plasma (top panel) and the simultaneous generation of rabbit aorta contractile activity (bottom panel). Aggregation (37 C, stirring) was carried out with 0.4 ml of platelet-rich plasma with the use of a Payton aggregometer. The arrow indicates the addition of agonist to plasma. Aggregation induced by arachidonic acid was performed by adding the required amount of sodium arachidonate solution (5 mg/ml, pH 8.5) to 0.4 ml of plasma in the aggregometer cuvette. Aggregation induced by the endoperoxides was measured by the evaporation of an aliquot of the endoperoxide solution (25–50 µg/ml acetone) in the cuvette, followed immediately by addition of 0.4 ml of plasma. Thromboxanes were generated by preincubation (in the cuvette) of the endoperoxide (in 40 µl of 0.05 M phosphate buffer, pH 7.8) with 10 µl of aspirin-treated platelet microsomes at 0 C for 2 minutes followed by the addition of 0.4 ml plasma. When testing for rabbit aorta contracting activity the contents of the aggregometer cuvette were removed 2 minutes after the initiation of the reaction and injected over a rabbit thoracic aorta strip. The small rabbit aorta contracting activity produced by the addition of the endoperoxides to plasma is due to the direct constrictor activity of the endoperoxides. The following abbreviations were employed: 2-nor-PGH_2, prostaglandin H_2 obtained from $^{C}19{:}4$ acid; 2-nor-PGH_1, prostaglandin H_2 obtained from $^{C}19{:}3$ acid; APM, aspirin-treated platelet microsomes prepared as described previously; AA, sodium arachidonate. Used with permission.[288]

Synthetic Mimetics of the Prostanoids

The methyl ester of PGH_2 has been chemically synthesized.[156] Several stable analogs of the prostaglandin endoperoxides also have been synthesized [39,57] and shown to mimic the effects of the naturally occurring PGG_2, PGH_2, and TxA_2 (Table 2).[198,330] The 9,11-azo-analog of PGH_2 is several times more potent than PGH_2, both in inducing platelet aggregation and in constricting the rabbit aorta,[57] and has activity comparable to TxA_2.[235-236] The epoxy-methano analogs of PGH_2[39,330] also induce platelet aggregation and rabbit aorta contraction. The effects of these stable compounds suggest that the chemically unstable prostaglandin endoperoxides and TxA_2 exert their effects without chemical interaction with their cellular receptors.

Although a large number of stable analogs of PGI_2 have been synthesized, all of these have proved to be less potent than the parent compound as inhibitors of platelet aggregation.[40,58,241] The most active compounds are 6,9-thia-PGI_2[241] and 6,9-imino-PGI_2.[40] Compounds lacking the 5,6 double bond of PGI_2 have extremely low biological activity in most cases.[58] A derivative of PGE_1 has been shown to be more potent and more stable *in vivo* than the parent compound and may be of value therapeutically.[238]

Hemostasis

The major pieces of evidence that prostanoids are involved in hemostasis are that patients with platelet cyclooxygenase deficiency have slightly prolonged bleeding times,[179,199] as do normal subjects who have ingested aspirin.[159,210,338,376] In this section the mechanisms of hemostasis are summarized, the actions of agents active in hemostasis are discussed, and a rationale for the effect of aspirin is developed.

Mechanisms of Hemostasis

Hemostasis by definition includes all of the processes that arrest blood loss from the lumen of a vessel once all the cellular layers of its wall have

Table 2—Effects of Stable Analogs of PGH_2 and PGI_2

Compound	Aorta constriction	Platelet aggregation
9, 11-azo-PGH_2	+	+
9, 11-methanoepoxy-PGH_2	+	+
9, 11-epoxymethano-PGH_2	+	+
6, 9-thia-PGI_2	(+)	−
6, 9-imino-PGI_2	?	−

+, induces; −, inhibits.

been ruptured (see Figure 3). It is well recognized that effective hemostasis in mammals requires the simultaneous presence of circulating viable platelets and an intact coagulation mechanism.[231,374] One of the earliest events observed following injury to a vessel is the formation of a small aggregate of platelets at the breach in the vessel wall. The platelets at this stage are loosely packed and contain many granules. Within seconds, a large clump of platelets forms and the platelets lose their granules. Subsequently, the formation of fibrin and the entrapment of red cells occurs. Usually, within 3–4 minutes the plug is solid enough to withstand the pressure present in the vessel lumen, and the loss of blood is prevented.[174,381,400]

While hemostatic plugs are formed from constituents of the blood, the initial cause of platelet aggregation must come from a change in the vessel wall. This change appears to be the exposure of collagen fibers. Platelets are seen adhering to collagen fibers in the mesentery about injured vessels,[174] and collagen induces platelet aggregation *in vitro*.[334,401] The aggregation of platelets by collagen is associated with the loss of platelet granules and the appearance in the supernatant fluid of the aggregating agent ADP.[138,144,334] Platelets will adhere to collagen in the absence of divalent cations, but the aggregation of platelets by released ADP [91] requires extracellular calcium or magnesium ions.[35] Collagen–platelet interactions are discussed further in a later section.

The generation of thrombin is also important for normal hemostasis.

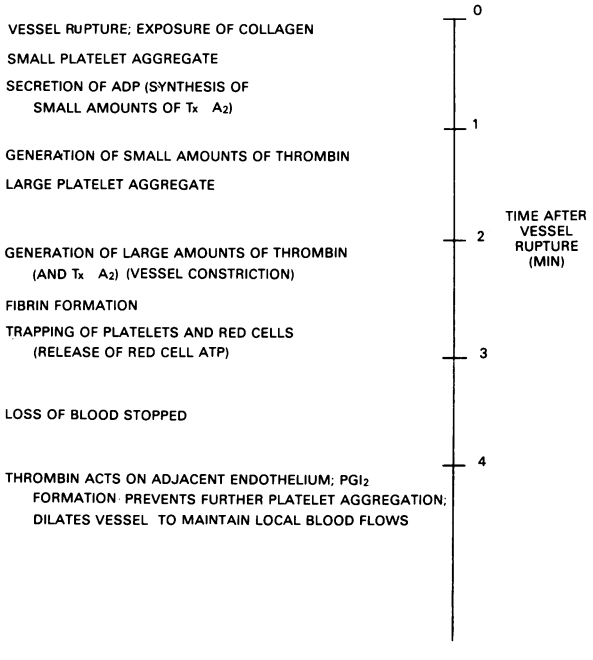


Figure 3—Possible sequence of events involved in hemostasis.

When there is a defect in the coagulation mechanism, the platelet plug that forms at the breach in the vessel wall is unstable.[32] One action of the thrombin is probably to generate fibrin, which can act to anchor the platelet aggregates. It has been shown that platelets adhere to polymerizing fibrin.[245] Besides this, however, thrombin is also known to aggregate platelets at concentrations too low to cause fibrin formation [351] and to cause the degranulation of platelets.[139] Moreover, platelet aggregation by thrombin is synergistic with that induced by ADP.[270] Most investigators accept the concept that tissue thromboplastin becomes available immediately following vessel injury and can activate the extrinsic coagulation system leading to thrombin generation.[239] Additional mechanisms of thrombin generation also become available during hemostasis because collagen can activate Factor XII, and platelets that have been activated by collagen are capable of activating Factor XI independently of Factor XII.[372] The effects of thrombin on platelets and endothelial cells are discussed in the next section.

Bleeding disorders are frequently linked to a lack of circulating platelets (thrombocytopenia) or to abnormal circulating platelets.[45,80,148,200,231,374] When the platelet count falls below one tenth of normal (250,000/cu mm of blood) hemorrhage is usually observed. In the hemorrhagic disorder known as thrombasthenia, the platelet count is normal and the platelets adhere to the damaged vessel. However, the platelets fail to aggregate when exposed to agents such as ADP, collagen, or thrombin.[45,355,404] This disorder has been linked to the absence of certain platelet membrane glycoproteins.[254,255,273] In a second platelet disorder, known as storage pool disease, the platelets aggregate in response to ADP but fail to aggregate normally with collagen. This defect is due to an absence in the platelets of the granules that normally secrete ADP.[148] In another hemorrhagic disorder, von Willebrand's disease, platelets fail to form a mass adequate to bridge the gap in the transected vessel but aggregate normally *in vitro* in response to ADP, collagen, and thrombin.[80] This bleeding disorder can be corrected by the transfusion of normal plasma and appears to be due to the absence of a portion of the Factor VIII molecule necessary for the adhesion of the platelets to the damaged vessel wall.[375] The prolonged bleeding times noted in patients with platelet cyclooxygenase deficiency [179,199] implicates the prostanoids in hemostasis.

Actions of Thrombin on Platelets and Endothelial Cells

Platelets and Thrombin

Thrombin is a proteolytic enzyme with a molecular weight of 37,500 daltons that catalyzes the hydrolysis of a single arginyl-glycine peptide

bond in the A_α and B_β chains of fibrinogen. The polymerization of the fibrin monomers so formed constitutes clotting. Pure thrombin is considered to have a specific activity of 2600 U/mg in a fibrinogen clotting assay,[77] and so it can be calculated that 1 U/ml thrombin is equivalent to a concentration of 11 nM.

Thrombin, in concentrations of 0.1–0.3 U/ml, induces a change in the shape of platelets; and in stirred citrated plasma this change is followed by platelet aggregation.[52,351] The aggregation can be prevented by heparin in the presence of heparin cofactor [52] or by the thrombin antagonist hirudin but not by the ADP antagonist ATP.[193] Platelets that are treated with thrombin and then washed and recovered in an elaborate procedure respond normally to ADP but no longer respond to thrombin.[166,290]

In 1954, Bigelow [22] demonstrated that thrombin releases serotonin from platelets. Subsequently, it has been shown that serotonin is stored in dense granules in platelets [279] together with ATP, ADP, and calcium [140,141] and that all of these components are secreted in response to thrombin.[48,68,101,149,228] Also released are components of alpha granules, including hydrolytic enzymes [215] (glycosidases and cathepsin), fibrinogen,[101] a heparin-neutralizing protein [232] (platelet factor 4), and a protein that stimulates the proliferation of smooth muscle cells.[298] It has been demonstrated that secretion from platelets is a selective process differing markedly from cell lysis.[101,139]

Aggregation by thrombin is not dependent on the secretion of ADP,[193,269,270] and secretion by thrombin is not dependent on aggregation. When platelet suspensions are not stirred, or contain ethylenediaminotetraacetate (EDTA), thrombin causes secretion without aggregation.[48]

The mechanism by which thrombin induces platelet aggregation and secretion is still the subject of intensive investigation. Trypsin and papain also induce secretion from platelets, while thrombin treated with diisopropyl fluorophosphate (DFP) neither clots fibrinogen nor induces secretion.[65,205] These findings suggest that the proteolytic activity of thrombin is the basis for its action on platelets. On the other hand, the substrate for thrombin on the platelet surface does not appear to be fibrinogen, since enzymes isolated from several snake venoms clot fibrinogen but fail to aggregate platelets.[65] Recently a glycoprotein on the surface of platelets (mol wt 89,000) has been implicated as the initial site of proteolytic attack by thrombin.[272]

Several workers have demonstrated the presence of binding sites for thrombin on platelets.[92,197,352,394] One class of binding site has a dissociation constant of 0.02 U/ml and a capacity of about 500 molecules per platelet,

while a lower affinity class of site has a dissociation constant of 2.9 U/ml and binds about 40,000 molecules per platelet.[197] Thrombin inactivated with DFP binds in an identical way to platelets and competes with active thrombin for binding.[197] From a kinetic analysis of the secretion of Ca^{2+} and ATP from platelets, it has been suggested that thrombin does not turn over when it triggers platelet reactions.[68] A model has been proposed that involves reversible binding of thrombin to a receptor and reversible catalytic modification of this receptor complex leading to platelet activation.[206]

During secretion induced by thrombin, ATP in the platelet cytoplasm is converted into hypoxanthine.[90,149] It appears that this conversion is a reflection of the inability of platelets to adequately compensate for energy-consuming reactions by rapid ATP resynthesis. It has been shown that phosphorylation of myosin light chain occurs concomitantly with secretion induced by thrombin.[2,62,124,191] Since thrombin-induced secretion occurs in the presence of EDTA, it must be independent of external calcium ions. However, the release of calcium ions from intracellular binding sites on the inner platelet membrane may play an important role in aggregation and secretion. Whether these binding sites are protein [31] or lipid (see below) in nature is presently not known.

Formation of Prostanoids During Thrombin–Platelet Interaction

Smith and Willis [323] showed that the treatment of platelets with thrombin results in the formation of nanogram amounts of PGE_2 and $PGF_{2\alpha}$. Since aspirin abolished prostaglandin formation but did not inhibit the release of serotonin, adenine nucleotides, or hydrolytic enzymes, it was concluded that the formation of prostaglandins is not essential for secretion. Serum has a higher content of PGE_2 and $PGF_{2\alpha}$ than plasma because thrombin acts on platelets when whole blood clots.[320]

Hamberg and associates [118] showed that microgram amounts of 12-L-hydroxy-eicosatetraenoic acid (HETE), 12-hydroxy-5-*cis*, 8,10-*trans*-heptadecatrienoic acid (HHT), and TxB_2 are formed when suspensions of washed platelets are treated with 5 U/ml thrombin. The amounts of PGE_2 and $PGF_{2\alpha}$ recovered from these incubations were much lower. The addition of aspirin or indomethacin to the suspensions markedly inhibited HHT and thromboxane B_2 formation; and when aspirin was ingested by the platelet donor, the production of these metabolites was reduced by 95%. By contrast, aspirin and indomethacin increased the formation of HETE by as much as 300%.[117,118]

Material that reacts with thiobarbituric acid to form a pink pigment was shown to be formed when platelets were treated with thrombin.[261]

This material is probably malondialdehyde, because it is formed in amounts to approximately equal those of HHT (see Figure 5) and its synthesis is inhibited by aspirin.[118,326]

While the amount of TxB_2 in normal plasma is less than 0.5 nM, the content of TxB_2 in normal serum obtained from blood clotted at 37 C is 0.6 to 1.2 μM.[87]

Thrombin and Platelet Phospholipase Activities

The above observations are consistent with the now established concept that thrombin can cause the hydrolysis of endogenous arachidonate from phospholipids in the platelet membrane. A part of the arachidonic acid is converted by prostaglandin synthetase into PGH_2, and the majority of this PGH_2 is converted by thromboxane synthetase into TxA_2, HHT, and malondialdehyde. Much of the remaining free arachidonic acid is converted by platelet lipoxygenase into 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE), which is then reduced by enzymes in the platelets to HETE.

To identify the source of the arachidonic acid used for thromboxane synthesis, several workers have studied the effects of relatively high concentrations of thrombin (5–10 U/ml) on platelets prelabeled with radioactive arachidonic acid. In studies with human [23-25,185,292-294,302,311] and horse platelets [182,183] the majority of radiolabel was incorporated into phospholipids, and after thrombin stimulation the major changes were seen in phosphatidylcholine (PC) and monophosphatidylinositol (MPI). One study concluded that PC was the major source of arachidonic acid for TxB_2 synthesis.[23] Free arachidonic acid was shown to accumulate when platelets were treated with thrombin in the presence of oxygenase inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA),[24] while, in its absence, radioactive TxB_2, hydroxy fatty acids and other products were produced.[24,182,183] It has also been reported that some arachidonic acid is incorporated into the plasmalogen form of phosphatidylethanolamine after thrombin stimulation [293] and that trypsin can induce prostanoid formation.[275]

Studies by Bills et al [25] indicated that a phospholipase A_2 in platelets acts only on 2-arachidonyl-PC. Platelets containing labeled arachidonate showed a decrease in PC after thrombin stimulation, while platelets labeled with oleate or linoleate showed no such decrease in PC. The specific activity of arachidonate in PC decreased in accordance with a specific deacylation. On the other hand, Bills et al [25] noted that the decrease in MPI was not fatty-acid–selective. More complete studies of the changes in phosphoinositides in response to thrombin have now been made by

other workers [182,185,291] and indicate that the decrease in MPI after thrombin stimulation is largely a consequence of the activation of a phospholipase C activity, MPI phosphodiesterase. Little arachidonic acid is released from MPI by phospholipase A_2 activity.

Within 10 seconds of the addition of low concentrations of thrombin to platelets there is a conversion of triphosphoinositide (TPI) into diphosphoinositide (DPI).[162,185,189] This change is also noted after the addition of ADP to platelets,[190] and it has been speculated [185] that this loss of phosphate from TPI may be the chemical trigger that releases the intracellular calcium required for intracellular contraction (eg, phosphorylation of myosin [62,191]) and stimulation of MPI-phosphodiesterase and phospholipase A_2 activities. Also within a few seconds of the addition of thrombin there is a decrease in MPI and an approximately fivefold increase in diglyceride.[291] The diglyceride is rapidly rephosphorylated to phosphatidic acid and then reconverted to MPI, DPI, and TPI.[185] At these early times with these low concentrations of thrombin there is little hydrolysis of PC, which only occurs later or after the addition of higher concentrations of thrombin.

A schematic representation of these changes and their hypothetical consequences is presented in Figure 4. Changes 1 and 2 are considered to

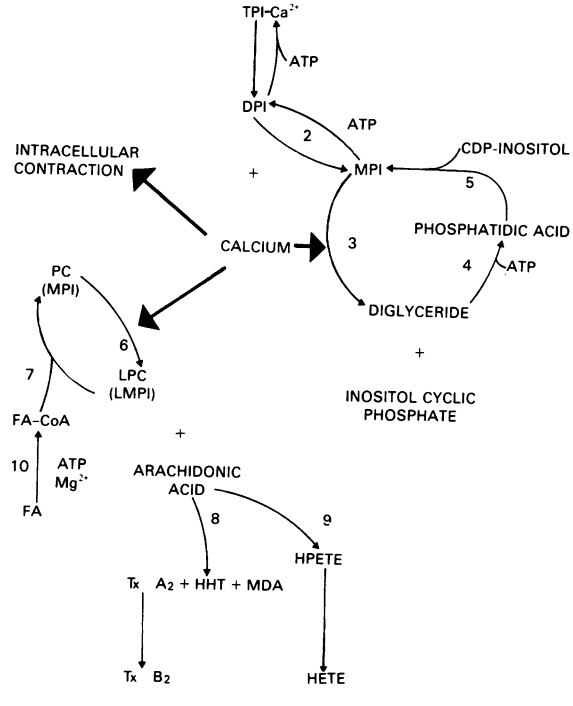


Figure 4—Hypothetical changes in platelet phospholipids elicited by thrombin. TPI, DPI, and MPI, tri-, di-, and monophosphoinositides, respectively; FA, fatty acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

be reversible and involve the liberation of calcium ions from acidic phospholipids [10] into the cytoplasm, followed by their resequestration with the resynthesis of TPI. The formation of inositol cyclic phosphate in Step 3 could be the membrane-destructive step associated with platelet secretion. Changes 8 and 9 are irreversible, occur at higher intracellular calcium concentrations, and are associated with the formation of the prostanoids. The formation of the prostanoids is not essential for secretion by thrombin since normal release of serotonin is observed in the presence of ETYA.[89] On the other hand, ETYA does not block the changes in the phosphoinositides and phosphatidic acid.

Phospholipase A_2 has been detected in [152] and isolated from human platelet membranes.[153] The enzyme has an absolute requirement for calcium ions and will hydrolyze PC or PE containing oleate or linoleate at the 2 position. Since studies with intact platelets indicate that 2-arachidonyl PC is the major lipid cleaved after thrombin stimulation, it seems possible that this specificity is conferred not by enzyme itself but by a specific localization of arachidonyl-PC in the vicinity of the phospholipase A_2. It is known that phospholipids are asymmetrically distributed in the platelet membrane.[47,310] Gerrard and associates [94] have localized prostaglandin endoperoxide synthetase in platelets immunochemically using 3'3-diaminobenzidine to detect peroxidase activity. Their results indicate that prostanoid synthesis in platelets occurs in the dense tubular system that lies beneath the platelet membrane. This tubular system also appears to be the major sequestration site for calcium ions.[160,161,383] The suggestion that thromboxane A_2 may act as a physiological ionophore to transport calcium out of these membranes [94] seems inconsistent with the concept that phospholipase A_2 needs to be activated by calcium prior to thromboxane formation.

Thrombin and Platelet Oxygen Consumption

Shortly after the addition of thrombin to platelet suspensions there is a burst in oxygen consumption.[89,147,226,227,274] This burst is insensitive to inhibitors of oxidative phosphorylation, such as antimycin or cyanide, but is reduced by inhibitors of prostanoid biosynthesis, such as aspirin or ETYA.[89,227] The burst of oxygen consumption appears to occur only after the secretion of platelet constituents is almost completed.[227]

In conclusion, thrombin causes both morphologic and biochemical changes in platelets. The biochemical changes noted so far include the secretion of the components of certain granules, the proteolysis of a membrane glycoprotein, the phosphorylation of myosin and diglyceride, and the hydrolysis of TPI, MPI, and PC. It appears that the release of arachidonic acid from PC induced by thrombin and its subsequent oxygenation

occurs only at a late stage and that the formation of TxA_2 in response to thrombin does little to modify *in vitro* platelet responses.

Endothelial Cells and Thrombin

Endothelial cells synthesize and release that part of the Factor VIII molecule necessary for adhesion of platelets to the damaged vessel wall, which is absent in the bleeding disorder von Willebrand's disease. Thus, endothelial cells possess a synthetic capacity that appears to be essential for normal hemostasis. On the other hand, endothelial cells possess ADPase activity,[201] and Saba and Mason [303,304] showed that treatment of umbilical venous endothelium with several platelet-active agents, including thrombin, was associated with the release of an inhibitor of platelet function. Subsequent studies with suspensions of these endothelial cells have established that the release of this inhibitory activity is attributable to the synthesis and release of prostacyclin.[59,60,61,203,378,379]

Thrombin is a potent stimulator of PGI_2 formation by endothelial cells obtained from the human umbilical vein.[60,61,379] Remarkably, the mechanism of the action of thrombin on these cells has many characteristics in common with its action on platelets. Treatment of thrombin with DFP markedly reduces its capacity to stimulate PGI_2 formation,[61,379] while the effect of thrombin can be mimicked by trypsin.[379] Moreover, thrombocytin, a proteolytic enzyme from the venom of *Bothrops marajoensis* that aggregates platelets but is inactive on fibrinogen,[244] also causes PGI_2 formation.[61] Reptilase-R, which clots fibrinogen, is inactive.[61] Just as with platelets, endothelium from the human umbilical cord vein has binding sites for thrombin.[12,13] Whether thrombin also stimulates phosphorylation of myosin or activation of MPI phosphodiesterase in these venous endothelial cells as it does in platelets is presently not known. It will be of interest to learn more about how thrombin causes the stimulation of phospholipase A_2 in these cells.

Thrombin has not been found to stimulate PGI_2 formation by other cell types as yet. These include endothelial cells from porcine aorta [196] and smooth muscle cells and fibroblasts from human umbilical arteries and veins.[87] On the other hand, endothelial cells from the aorta and the umbilical artery can synthesize PGI_2 from arachidonic acid.[87,196,203] In one report it was noted that a plasma factor stimulates PGI_2 formation by the endothelial cells from porcine aorta.[196]

Effect of Ionophore A23187 on Platelets and Endothelial Cells

Perhaps the major piece of evidence for the hypothesis that intracellular calcium is involved in platelet function has been the finding that the divalent cation ionophore A23187 [289] induces platelet aggregation and

secretion.[78,166,209,384] Further, it has been shown that in the absence of extracellular calcium ions this ionophore mobilizes more arachidonic acid than does thrombin [182,276,292] and that this release is accompanied by a marked increase in oxygen consumption.[276] While elevation of intracellular cyclic AMP inhibits the activation of phospholipase A_2 in platelets induced by thrombin,[93,183,216] it does not inhibit the activation caused by ionophore.[292]

Furthermore, while both the secretory reaction and the activation of phospholipase A_2 in platelets by thrombin depends upon the availability of metabolic ATP, the ionophore A23187 can induce activation of phospholipase A_2 in ATP-depleted platelets even though there is no secretion.[292] Thus, activation of phospholipase A_2 in platelets appears to depend solely on an increase in free intracellular calcium ions, while secretion appears to depend on both this increase and the availability of ATP. A23187 is less active than thrombin in causing the conversion of MPI into phosphatidic acid.[182,185]

The ionophore A23187 is also a potent stimulator of PGI_2 formation by endothelial cells from human umbilical veins [379] and of prostaglandin formation by polymorphonuclear leukocytes.[380] Thus, a generalization appears to exist that phospholipase A_2 in cells is activated by an increase in free intracellular calcium ions.

Collagen–Platelet Interactions

The protein collagen has a molecular weight of about 215,000 daltons and is composed of 3 polypeptide chains, each containing about 1000 amino acid residues.[151,312] Although five genetically different chains have been recognized so far, they are all very similar and have molecular weights of about 100,000 daltons. Collagen exists as a rod-like triple helix 1.5 nM in diameter and 300 nM long. It has a polymorphic amino acid content but contains glycine in every third position and the unusual amino acids, hydroxylated proline and hydroxylated lysine. While collagen may exist as individual molecules at 4 C and acid pH, it tends to self-polymerize into fibrils at higher temperatures and neutral pH. Platelets adhere rapidly to microgram (nanomolar) amounts of these collagen polymers, and after a lag phase they secrete the contents of dense granules, synthesize TxA_2 and aggregate.[138,144,215]

Platelet aggregation and secretion induced by collagen can be inhibited by many steroidal anti-inflammatory drugs, including aspirin, phenylbutazone, fenoprofen, mefenamic acid and indomethacin.[75,256,338,376,402,403] It is now accepted that these drugs produce these effects by inhibiting prostanoid biosynthesis. Thus, although thromboxane formation seems to be

of little importance for thrombin-induced platelet aggregation and secretion, it apparently plays a major role in aggregation and secretion induced by collagen. Nevertheless, higher concentrations of collagen can overcome the inhibitory effects of aspirin, indicating that collagen can induce aggregation and secretion by a pathway independent of thromboxane formation.[48,402]

The reason thromboxane formation is important for aggregation by collagen as yet is only partially understood. Platelets from patients with storage pool disease synthesize prostanoids but aggregate poorly in response to collagen, a fact that suggests that the secretion of ADP is important for collagen-induced aggregation.[148] On the other hand, these platelets aggregate normally in response to arachidonic acid [148] in keeping with the findings that thromboxane A_2 can induce platelet aggregation without nucleotide secretion.[49,169] The greater dependence of collagen on the secretion of ADP may be due to a "positive feedback loop" in which released ADP and the prostanoids act synergistically in inducing aggregation (see Figure 1).[137] It is important to note that this peculiar dependence on both thromboxane formation and ADP secretion for aggregation is observed only when platelets are in contact with a surface,[eg,208] for example, when they are adhering to collagen or to each other (aggregation), in which case it leads to a second wave of aggregation.[215] The synergism between ADP and prostanoids [168,325] may explain why a mixture of aspirin-treated and storage-pool–deficient platelets aggregates almost normally in response to collagen [385] and why PGE_2 potentiates the aggregation of aspirin-treated platelets (which can release ADP) but not storage-pool–deficient platelets.[377]

There has been only one report of the changes in phospholipids that occur after incubation of platelets with collagen.[29] These changes seem worthy of further study, since these changes may not be identical to those caused by thrombin.

Prostanoids and Bleeding Time

Probably the most commonly used clinical test of hemostasis involves measuring the time required to stop bleeding after a small incision has been made through the dermis and subcutaneous tissues.[210] This skin bleeding time has been shown to be almost entirely dependent on the number and viability of platelets and to be relatively independent of coagulation disorders usch as hemophilia. It is well established that aspirin produces a statistically significant prolongation of the bleeding time in normal subjects [210,338,376] and can cause prolonged bleeding in hemophiliacs or in patients with von Willebrand's disease (VWD).[159,282] These

effects of aspirin suggest that thromboxane formation is of some importance in maintaining hemostasis in normal subjects, and becomes extremely important when platelet adhesion (eg, in VWD) or thrombin formation (eg, in hemophilia) is defective. Ultrastructural studies indicate that the major effect of aspirin on the formation of hemostatic plugs is to reduce the extent of platelet degranulation.[381] Therefore, it seems possible that the major function of TxA_2 in hemostasis is to facilitate the adhesion-induced secretory reaction of platelets.

Since aspirin inhibits endoperoxide synthetase, it may not only prevent thromboxane formation by platelets but potentially could reduce prostacyclin formation by endothelial cells. The removal of this inhibitor of platelet aggregation might shorten the bleeding time. Several studies indicate that the prostaglandin synthetase of vessel walls is less susceptible to inhibition by aspirin than that of platelets.[14,42,59,60,97] Therefore, it has been suggested, but not yet proven *in vivo*, that aspirin in clinical doses does not compromise PGI_2 formation.[14]

The significance of the thrombin stimulation of thromboxane formation by platelets or of prostacyclin formation by endothelial cells from the human umbilical vein is a matter of speculation at this time. It is possible that the generation of TxA_2 acts to constrict the vessel wall and aids in the early events of hemostasis and that TxB_2 is involved in the later events of hemostasis, or perhaps in inflammation, since it is chemotactic for leukocytes.[170] On the other hand, the generation of PGI_2 may act to maintain blood circulation once blood loss has been prevented. The author's observations with endothelial cells from various sources indicate that venous umbilical endothelium is an unusually active producer of PGI_2 in response to thrombin and may indicate that this is a response which exists in a vessel with a low rate of blood flow acting to inhibit venous thrombosis.

Thrombosis

Thrombosis can be caused either by alterations in the vessel wall or by intravascular stimuli.[231] It is important to note that while all of the cellular layers of the vessel wall are ruptured in hemostasis, only minimal damage to the vessel wall may be involved in thrombosis.[158] Since occlusive arterial thrombi in man are almost always associated with breaks in the lining of atherosclerotic plaques,[55,86] the adhesion of platelets to exposed collagen or other plaque constituents may be the initial stimulus for thrombus formation. There have been many reports showing that platelets rapidly adhere to exposed subendothelium once there is a separation or loss of endothelial cells.[231] While the role of platelets is most prominent in the arterial system where flow rates are high and vascular lesions are

common, it appears that they are also involved in the incipient steps leading to venous thrombosis.[315]

Myocardial Infarction and Thrombosis

The severity of acute myocardial ischemia is determined by the local balance between oxygen supply and demand.[130] When myocardial oxygen is increased, it is usually compensated for by increased coronary blood flow due to relaxation of smooth muscle in precapillary arterioles (resistance vessels). Angina pectoris (chest pain) is thought to be due to an imbalance created by a temporarily enhanced oxygen demand in the presence of a fixed, restricted supply of oxygen because of atherosclerotic vessels. Acute myocardial infarction will result if this imbalance is prolonged. Typical agina pectoris with electrocardiographic ST segment depression is commonly observed in patients during physical exertion or emotional stimulation, and ischemia is most intense in the subendocardial region of the left ventricle. On the other hand, Prinzmetal's variant angina is typically characterized by ST-segment elevation on the electrocardiogram recorded during chest pain and is associated with coronary-artery spasm occurring at rest (often in the early morning). The formation of platelet aggregates during angina could facilitate infarction of the myocardium either by direct mechanical obstruction of capillaries, or infarction could result from the release of vasoactive substances.[283]

In a recent study of patients with angina at rest it was observed that the electrocardiographic changes that develop prior to anginal attacks are identical to those that develop prior to myocardial infarction.[207] Furthermore, it was observed, at post-mortem examination of patients, that those branches of the coronary artery that underwent vasospasm were those that finally underwent complete thrombotic occlusion. Therefore, it was suggested that heart attacks do not result from circulating platelet emboli,[eg,109,395] but rather vasospasm, by reducing blood flow through a narrowed atherosclerotic vessel, causes arterial thrombosis and leads to myocardial infarction.[207]

While this suggestion obviously has some foundation, it is important to bear in mind that in patients with variant angina coronary-artery spasm appears to occur at the site of a fixed atherosclerotic lesion.[281] It therefore seems possible that the sequence of events leading to myocardial infarction in variant angina might involve limited platelet adhesion at the break in the lining of an atherosclerotic plaque with the release of platelet constituents and the synthesis of the vasoconstrictor TxA_2. The subsequent aggregation and vasoconstriction could cause ischemia and the damage of more endothelium, leading to more aggregation [eg,368] and eventually com-

plete thrombotic occlusion. On the other hand, during angina pectoris developing on exertion, the initial damage might result from the ischemia due to limited oxygen supply but could develop into an infarction as described above.

Thromboxane A_2 and Thrombosis

Ellis and associates [74] demonstrated that the release of TxA_2 from platelets induced by thrombin caused marked constriction of isolated coronary arteries and suggested that TxA_2 may be involved in unstable angina. We recently employed a radioimmunoassay for TxB_2 to examine the levels of this stable derivative of TxA_2 in the circulation. We found that the plasma levels of this compound were below our detection levels (<0.5 nM) in normal subjects but were frequently elevated in 59 plasma samples obtained from 6 patients with variant angina (average 15 nM).[187] In a study of 14 patients with angina pectoris, little or no TxB_2 was detected during rest (average 0.53 nM), but plasma levels increased during cardiac pacing and peaked 5 minutes after pacing at the time of maximal cardiac lactate production (ischemia).[188] These preliminary observations suggest that TxA_2 may play a causative role in arterial thrombosis.

Fatty Acids and Thrombosis

Massive thrombosis can be produced by the infusion of saturated fatty acids into the circulation,[54] although the thrombogenicity of the fatty acids is decreased when they are bound to albumin.[134] The subcutaneous injection of adrenocorticotropin into rabbits caused thrombosis which was associated with high plasma-free fatty acid levels.[135] Although it is possible that fatty acids are thrombogenic, because they can directly lead to platelet aggregation,[123] it now seems more likely that they cause thrombosis because they damage the endothelium, leading to the exposure of platelet-active constituents.[96,192,313]

Of several fatty acids injected into the ear vein of rabbits by Silver and associates,[319] only arachidonic acid led to sudden death with platelet aggregates in the heart and lungs. This effect appeared to be a direct consequence of prostanoid formation by platelets, since it was abolished by aspirin.

Aspirin and Thrombosis

There have been several reports that aspirin can reduce the extent of ischemic injury in animals subjected to coronary artery occlusion [368] and in patients with myeloproliferative disorders or malignant disease.[21,280,371] On the other hand, aspirin does not seem to affect the increased number

of circulating platelet aggregates present during myocardial ischemia.[136] Two retrospective case control studies by the Boston Collaborative Drug Surveillance Group [36] reported that myocardial infarction occurs less frequently in subjects who take aspirin. However, firm evidence for a therapeutic value of aspirin in myocardial infarction must await the results of the large number of prospective studies now under way.

Potential Approaches to Thrombosis

Although our understanding of the role of prostanoid synthesis in controlling platelet function is still meager, it suggests several potential mechanistic rationales for reducing thrombotic conditions such as pulmonary embolism, stroke, and myocardial infarction. Potential approaches include the following.

Substitution of Other Fatty Acids for Arachidonic Acid

Normally, platelets have large amounts of arachidonic acid in their phospholipids and barely detectable amounts of dihomo-γ-linolenic or 5,8,11,14,17-eicosapentaenoic acid.[53,202] Increasing the level of one of these latter fatty acids in platelets [64,72,164,389] might diminish the tendency toward thrombosis for several reasons: 1) the pool of platelet arachidonic acid might be reduced, and so less TxA_2 would be formed; 2) if released from platelet phospholipids, these fatty acids could compete with arachidonic acid for the platelet prostaglandin endoperoxide synthetase; 3) there is only limited conversion of dihomo-γ-linolenic acid into the vasoconstrictor TxA_1, while the formation of the inhibitor of aggregation PGE_1 is favored [76]; 4) while 5,8,11,14,17-eicosapentaenoic acid may be converted into TxA_3, this thromboxane is less effective in causing platelet aggregation and vasoconstriction.

It has been suggested that ingestion of di-homo-γ-linolenic acid might be harmful because it cannot be converted biosynthetically into PGI_1 and might diminish the formation of PGI_2 by endothelial cells.[72] On the other hand, 5,8,11,14,17-eicosapentaenoic acid can be converted into PGI_3, which is an inhibitor of platelet aggregation.[72,243] This fatty acid is present in the lipids from Eskimos,[71] and Eskimos have a diminished thrombotic tendency.[72]

The major fatty acid in linseed oil is α-linolenic acid (18:3, n-3), which can be chain-elongated in mammals into 5,8,11,14,17-eicosapentaenoic acid.[5,108] When rats are fed a mixture of saturated fat and linseed oil, there is a reduction in the arachidonate content of the phospholipids in their plasma and platelets and an accumulation of an unidentified 20-carbon unsaturated fatty acid.[249] Feeding with linseed oil causes a significant re-

duction in the number of pulmonary platelet thrombi detected in the lungs 1 minute after the injection of a large dose of ADP.[248,249] It seems possible that this antithrombotic effect of linseed oil is due to reduced production of TxA_2 and increased production of TxA_3 and PGI_3.

Inhibition of Phospholipase Activity

Prevention of the release of arachidonic acid from platelet phospholipids would abolish thromboxane formation. However, such an inhibitor of phospholipase A_2 might also act on endothelial cells to reduce PGI_2 formation. There is evidence that steroids can inhibit phospholipase activity in some cell types and not in others by a mechanism that depends on RNA and protein synthesis.[63] Therefore, it may be possible to selectively inhibit platelet phospholipase A_2 by using a drug that selectively acts on megakaryocytes.

Inhibition of Prostaglandin Endoperoxide Synthetase

Of course, aspirin inhibits cyclooxygenase and holds promise as an antithrombotic agent because of its persistent effect on platelets and apparently reduced effects on other cells. However, it can compromise PGI_2 formation, and it has been shown that high doses of aspirin do promote venous thrombosis in rabbits.[163] A cyclooxygenase inhibitor (perhaps sulfinpyrazone [46,97,350]) with even greater selectivity for platelets would therefore be desirable.

Inhibition of Thromboxane Synthetase

Certain synthetic analogs of the endoperoxide PGH_2, which inhibit thromboxane synthetase, have been found to inhibit platelet aggregation induced by arachidonic acid or PGH_2 in a competetive fashion.[81,98] Such compounds have the additional advantage that they will allow the PGH_2 formed by platelet endoperoxide synthetase to be converted into inhibitors of platelet aggregation such as PGD_2 in plasma [324] or PGI_2 in endothelial cells.[220] Unfortunately, the synthetic analogs of PGH_2 investigated to date cause constriction of the rabbit aorta, which may preclude their use as antithrombotic agents.

Imidazole [219] and its acidic derivatives,[396] which inhibit thromboxane synthetase in a noncompetitive fashion, are poor inhibitors of platelet aggregation.[81,233,396] This poor inhibition has been attributed to the relatively weak activity of the parent compound and to the impermeability of platelets to its acidic derivatives.[396] Nevertheless, the development of a selective thromboxane synthetase inhibitor is one of the most promising new rationales for antithrombotic therapy.

Thromboxane Antagonists

The synthesis of 9,11-iminoepoxy-PGH_2 [82] and pinane thromboxane A_2 (PTA_2) [242] and the demonstration that they antagonize the platelet aggregating effects of prostaglandin endoperoxides, opens yet another avenue for the potential treatment of thrombosis. It can be anticipated that the development of similar compounds with a fuller evaluation of their therapeutic potential will be made in the near future.

Another approach may be to immunize high-risk subjects with a protein conjugate of a stable derivative of thromboxane A_2. The body's own defense mechanism would then develop antibodies that bind thromboxane A_2 and so neutralize its effects. Antibodies developed in rabbits against a stable analog of PGH_2 have been shown to inhibit competitively platelet aggregation induced by arachidonic acid or PGH_2 *in vitro*.[83] Our own experience [329] indicates that such antibodies would also be effective *in vivo*.

Use of Inhibitory Prostaglandins

The rapid metabolism of PGE_1 and PGI_2 in the circulation dictates that their antiplatelet effects can only be maintained by continuous infusion. The infusion of PGE_1 has been shown to be of value for short-term antithrombotic treatment during cardiopulmonary bypass or renal dialysis.[1,336] It seems probable that more stable derivatives of these compounds such as the inter-m-phenylene-PGE_1,[238] 6,9-thia-PGI_2,[241] or 6,9-imino-PGI_2 [40] will be marketed as antithrombotic drugs in the future.

Conclusions

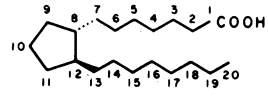
The rapid progress in prostanoid biochemistry has left behind a plethora of biological questions still to be answered. The exact roles that TxA_2 and PGI_2 play in hemostasis and thrombosis still cannot be defined with any certainty. Why is so much TxA_2 produced by platelets in response to thrombin when it plays no part in thrombin-induced aggregation or secretion? Is it being produced to cause vessel constriction? What is the nature of the remarkable synergism that occurs between ADP and prostanoids (the positive feedback loop) during adhesion-induced platelet aggregation? Are PGD_2 and PGE_1 physiologically or pathologically important inhibitors of aggregation? How much PGI_2 is being continually released from the endothelium, and what are the physiologic regulators of its output? If PGI_2 is not a circulating hormone, what is its primary function? Is PGI_2 formation more important in veins, where blood flow is slow, or in arteries, where blood flow is fast and vascular lesions are common? Above all, will the great potential that the management of these prostanoids offers lead to an effective treatment for thrombosis? Regardless of the many

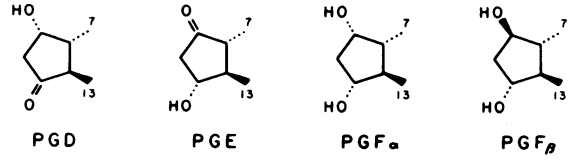
questions that have been raised, a new dimension of research has been uncovered. It seems likely that continued effort in this area can do little but good.

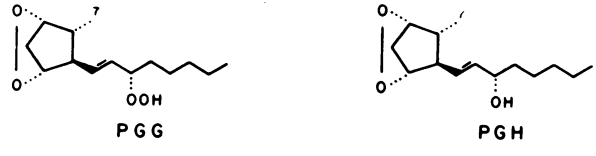
APPENDIX: BIOSYNTHESIS OF THE PROSTANDOIDS

Nomenclature

The term "prostanoids" has been used in the preceding review to include all compounds that can be derived from prostaglandin endoperoxides, including prostaglandins, thromboxanes, and hydroxy fatty acids. The name "prostaglandin" (PG) was used first by Von Euler [369] and has become generic for a number of related fatty acids that all possess an identical carbon skeleton. This basic structure (prostanoic acid) was elucidated by Bergstrom et al [17] and consists of a five-membered carbon ring with two (seven- and eight-membered) carbon side chains, comprising 20 carbon atoms in all (Figure 5). As is conventional, carbon numbering begins at the carboxyl group. Different prostaglandins are classified according to the functional groups attached at carbon-9 and carbon-11 in the five-membered ring (see Figure 5). The designations PGF_α and PGF_β in-







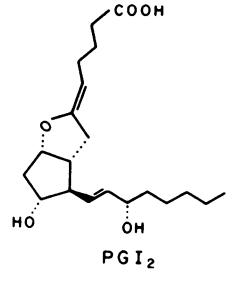


Figure 5—Prostanoic acid and different types of prostaglandins.

dicate whether the hydroxyl group at carbon-9 points down below the plane of the paper (α) or out (β). The hydroxyl group at carbon-15 (as shown for PGG_2, PGH_2 and PGI_2) also points below the plan of the paper and this is known as an S-configuration. Numerical subscripts (eg, PGE_1, PGE_2, and PGE_3) indicate the number of double bonds in the two side chains.

The name thromboxane was introduced by Hamberg et al [119] to describe an unstable vasoconstrictor formed from prostaglandin endoperoxides which did not have the basic prostanoic acid structure. This compound was named thromboxane A_2 (TxA_2) because it is formed by thrombocytes (platelets), possesses an oxane:oxetane ring structure, and contains two double bonds in its side chains (Figure 6). In buffer solutions it rapidly incorporates one molecule of water, and the stable compound so produced, originally named PHD,[117] by analogy was renamed thromboxane B_2 (TxB_2).[119] The hemiacetal hydroxyl group of TxB_2 can be in the α or β configuration because of the equlibrium with its acyclic derivative. This equilibrium is indicated in Figure 6 by an irregular line.

Biosynthesis of the Prostanoids

The prostanoids are not stored in cells but are biosynthesized rapidly once substrate fatty acids are made available to the appropriate enzymes. The various steps involved in the formation of the prostanoids are summarized below.

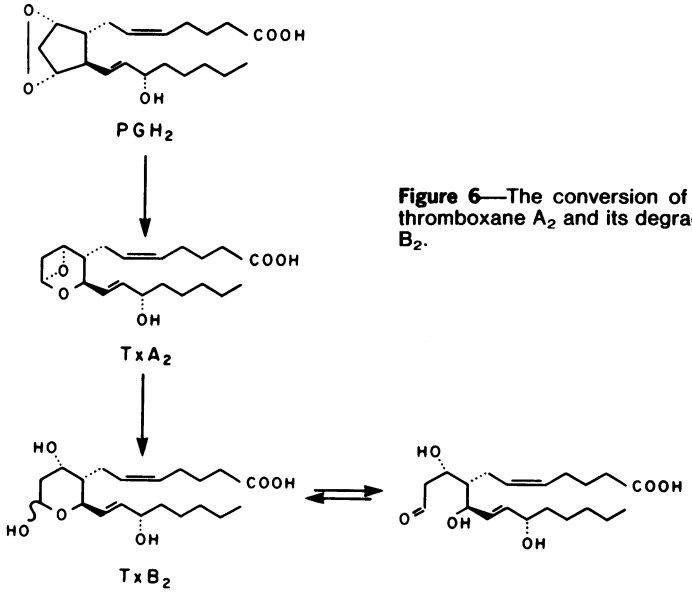


Figure 6—The conversion of prostaglandin H_2 into thromboxane A_2 and its degradation to thromboxane B_2.

Biosynthesis of Fatty Acid Precursors

It was realized in 1929 that certain fatty acids are essential in the diet.[44] These essential fatty acids (linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid) contain two or more *cis* double bonds commencing 6 carbon atoms from their methyl end and are designated the n-6 family (Figure 7). The first member linoleic acid (18:2, n-6) may be formed from oleic acid (18:1, n-9) in plants but cannot be formed in man or animals [5,108] because additional bonds are introduced between the existing double bond and the terminal methyl group in plants, whereas further desaturation only occurs between the existing double bond and the carboxyl group in mammals. The biosynthesis of arachidonic acid from linoleic acid proceeds via desaturation to γ-linolenic acid, chain elongation to dihomo-γ-linolenic acid, and then by further desaturation to arachidonic acid.[18] Arachidonic acid is present in high concentrations in ester form in most animal fats and so can be assimilated by man directly. Other related fatty acids, eicosapentaenoic acid (20:5, n-3) and culpanodonic acid (22:5, n-3) cannot be biosynthesized from linoleic acid in animals [5,18,108] but are present in high concentrations in fish.[15,108] These also can be assimilated by man, and eicospaentaenoic acid is found in high concentrations in lipids from Eskimos.[71] Although the content of dihomo-γ-linoleic acid is high in seminal vesicles,[181] arachidonic acid is the dominant fatty acid of the n-6 family in most other mammalian tissues,[5] including platelets.[53,202]

The naturally occurring unsaturated fatty acids in mammals contain

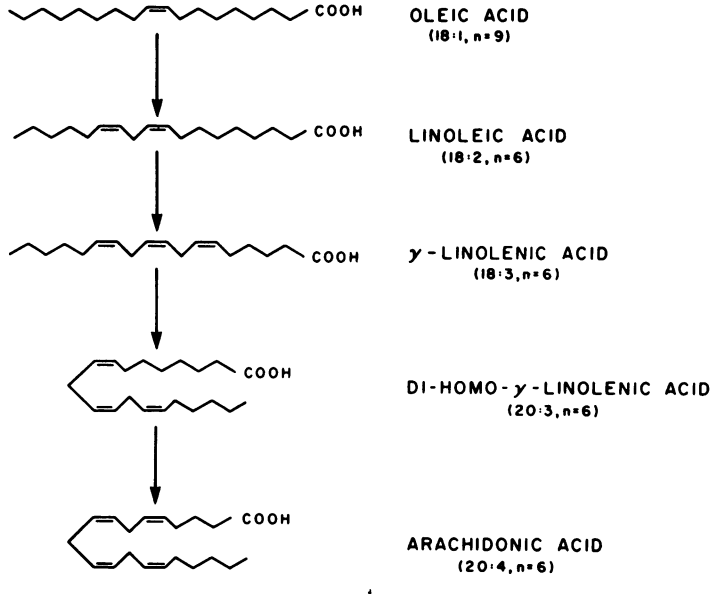


Figure 7—Biosynthesis of arachidonic acid from linoleic acid in mammals.

mainly double bonds of the *cis* configuration, which has the effect of introducing unique kinks in their structural conformation.

Phospholipase A_2

The fatty acid precursors of prostanoids are not free as carboxylic acid in cells, but they are abundant in ester linkage at the 2-position of phospholipids. Since esters are not substrates for prostaglandin synthetase,[181,370] it was postulated that the triggering event in prostaglandin formation by cells is the activation of phospholipase A activity to liberate the fatty acid precursors.[177] The importance of endogenous phospholipase A_2 (EC 3.1.1.4) in controlling prostaglandin synthesis has recently been established in many cells and tissues including fibroblasts,[37,142,230] macrophages,[146] spleen slices,[85] perfused rabbit hearts,[145] and perfused guinea pig lungs,[30] and has been studied most intensively in platelets, as discussed earlier. In most of these studies, the phospholipids in the membranes of cells were prelabeled with radioactive arachidonic acid; and release of radioactivity from phospholipids, with concomitant prostaglandin formation, was shown to occur in response to stimuli such as mechanical disturbance, ischemia, histamine, bradykinin, thrombin, or the calcium ionophore A23187.

Prostaglandin Endoperoxide Synthetase

The carbon chain length of arachidonic acid (C20:4, n-6), together with its unique "U" form of physical conformation, caused by the *cis* double bonds, suggested that it might act as a precursor for prostaglandins.[358] The biosynthetic conversion of arachidonic acid into PGE_2 was simultaneously demonstrated by two groups in 1964.[16,360] Subsequently, the precursors of PGE_1 and PGE_3 were shown to be dihomo-γ-linolenic acid (20:3, n-6) and eicosapentaenoic acid (C20:5, n-3), respectively.[252,337]

Seminal vesicles (bovine or ram) have been found to be a rich source of prostaglandin endoperoxide synthetase.[16,50,73,116,127,217,218,253,259,357,360,361] The enzyme (EC 1.14.99.1) is present in the microsomes and has been solubilized and purified to a high extent.[127,217,218,259,357] Its molecular weight determined from the Stokes radius and sedimentation coefficient is 124,000 daltons. On sodium dodecyl sulfate polyacrylamide gel electrophoresis a single polypeptide of 72,000 daltons is observed, indicating that the enzyme has two subunits.[357] The mechanism of prostaglandin endoperoxide formation from arachidonic acid [252] initially involves the stereospecific abstraction of a proton from carbon-13, followed by a lipoxygenase-like reaction [307] with the introduction of a molecule of oxygen at carbon-11 (Figure 8). This peroxy fatty acid is subsequently transformed by intramolecular rearrangement (with the introduction of a second molecule of oxygen [306]) into PGG_2. The cyclic endoperoxide PGG_2 has a 15S-hydro-

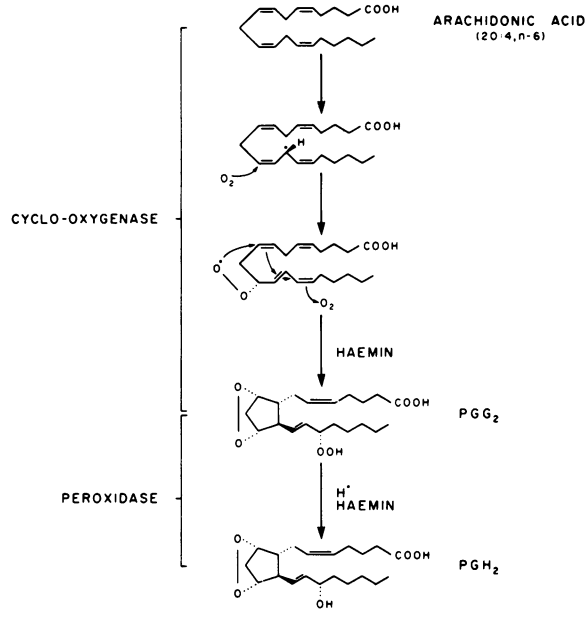


Figure 8—Formation of prostaglandin H_2 from arachidonic acid by prostaglandin endoperoxide synthetase.

peroxy group and is converted enzymatically into PGH_2 with a 15S-hydroxy group. These unstable cyclic endoperoxides, PGG_2 and PGH_2, were first isolated in 1973 by Nugteren and Hazelhof [253] and Hamberg and Samuelsson.[116,120]

Highly purified prostaglandin endoperoxide synthetase contains both cyclooxygenase activity (which produces PGG_2) and peroxidase activity (which converts PGG_2 into PGH_2).[218,357] The cyclooxygenation and peroxidation reactions require added hemin or a similar metallo-protein which is probably lost during the purification of the enzyme.[259,357] The peroxidase reaction also requires a suitable electron donor, and many phenolic compounds such as hydroquinone, propylgallate, tryptophane, serotonin, and epinephrine will suffice. The natural hydrogen donor has not been determined, but in the absence of a hydrogen donor the accumulation of PGG_2 is associated with rapid inactivation of the enzyme.[73]

While the majority of studies on endoperoxide synthetase have been carried out with the enzyme isolated from seminal vesicles, it appears that the same enzyme with similar cofactor requirements is present in most tissues,[50] with relatively high concentrations occurring in kidney medulla,[20] kidney papilla,[104] spleen,[105] lung,[112,346] and platelets.[117,118,122]

Transformation of Prostaglandin Endoperoxides

The prostaglandin endoperoxides can be stored in organic solvents at reduced temperatures, but they are unstable under aqueous condi-

tions [116,120,253] and decompose with a half-life (t ½) of 5 minutes in buffer at pH 7.4 and 37 C. Prostaglandin endoperoxides are also the substrates for a number of enzymes.[341] The presently known pathways for the transformation of PGH_2 into different prostanoids are illustrated in Figure 9 and are discussed below.

Prostaglandin E_2

PGE_2 is the major stable product formed when PGH_2 decomposes nonenzymatically in buffer solutions at neutral pH.[120,253] Moreover, an enzyme (PGH-PGE isomerase, EC53.99.3) that catalyzes the isomerism of PGH_2 into PGE_2 has been solubilized and isolated from the microsomes of bovine seminal vesicles.[217,258] This enzyme requires glutathione as a cofactor, explaining the earlier observation that glutathione, above all other sulfur-containing compounds, promotes the formation of E-type prostaglandins.[252,287] PGH_1 is an equally good substrate for this enzyme (producing PGE_1), while PGG_2 and PGG_1 are converted less efficiently into 15-hydroperoxy-PGE_1 and 15-hydroperoxy-PGE_2, respectively.[258] This suggests that the major pathway for the formation of E-type prostaglandins is PGG → PGH → PGE, although the pathway PGG → 15-hydroperoxy-PGE → PGE has been suggested.[308] High concentrations (~ 40 μg/ml) of PGE_1 and PGE_2 are present in human semen [349] and PGE_2 of renal origin (200 ng/day) is present in human urine.[95] The formation of small amounts (3 ng/ml) of PGE_2 has been detected during platelet aggregation.[327]

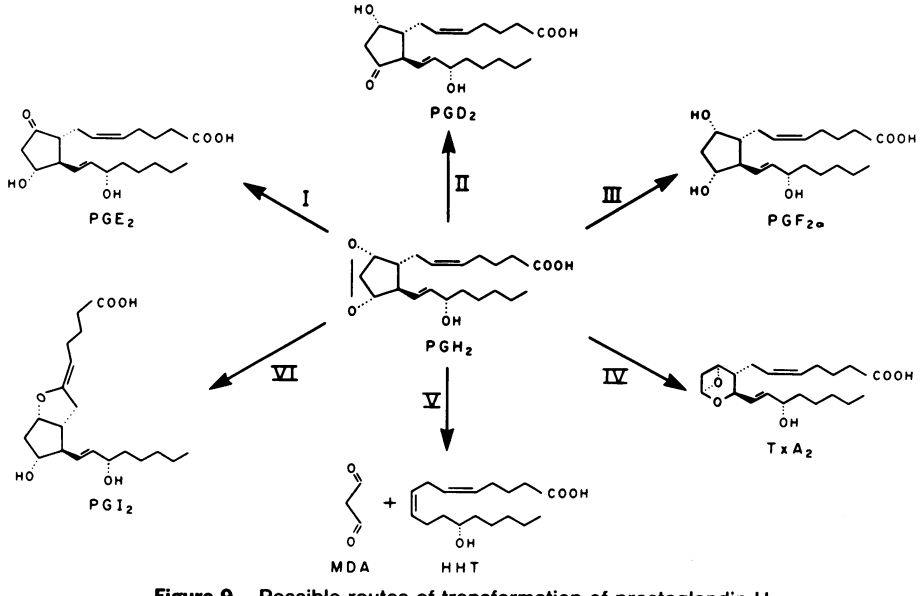


Figure 9—Possible routes of transformation of prostaglandin H_2.

Prostaglandin D_2

PGD_2 is the second major product formed when PGH_2 decomposes nonenzymatically in buffer solutions at neutral pH. It was initially discovered as one of the products of incubations of arachidonic acid with seminal vesicles in the absence of glutathione.[103,252] The isomerism of PGH_2 into PGD_2 in buffer solutions is accelerated by albumin,[51,113,324] probably due to a fatty acid binding site, and certain albumins, particularly bovine, favor the formation of PGD_2.[51,113] Homogenates of several rat tissues and an enzyme in sheep lung have been reported to catalyze the conversion of PGH_2 into PGD_2 in the presence of added glutatione.[253] Platelets are capable of synthesizing PGD_2.[6,257]

Prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$ is formed in small amounts when PGH_2 decomposes in buffer at neutral pH.[253] Its formation is markedly enhanced when mild reducing agents such as stannous chloride are added [116,253] or when the combination of glutathione and glutathione-S-transferases from rat liver are present.[51] Perhaps of greater significance is the fact that PGE_2 or PGE_1 can be reduced to $PGF_{2\alpha}$ or $PGF_{1\alpha}$ by enzymes identified in many tissues, including kidney, brain, liver, spleen, heart, and lung.[114,128,184,186] These enzymes require NADH (cytoplasmic) or NADPH (microsomal) as cofactors and may regulate the balance between E- and F-type prostaglandins. Relatively small amounts of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ are present in human semen ($\sim$ 5 μg/ml), and $PGF_{2\alpha}$ has been identified in human urine.[95]

Thromboxane A_2 (TxA_2) and Thromboxane B_2 (TxB_2)

Little or no formation of TxA_2 from PGH_2 occurs nonenzymatically, but an enzyme that catalyzes the formation of TxA_2 has been observed in platelets,[7,69,117,118,119,132,236,339,343,397] in lung,[112,343,345,346,391] in lung fibroblasts,[37,143] in spleen,[105] and in brain.[392]

TxA_2 is very labile ($t_{1/2} \simeq 30$ seconds at pH 7.4) and as yet has not been isolated or chemically synthesized. The incorporation of one molecule of water into TxA_2 to produce TxB_2 is shown in Figure 6.

Thromboxane synthetase is present in the membrane fraction of platelets [69,132,236,339] and lungs [345,391] and has been solubilized and separated from prostaglandin synthetase.[122,345,391,397] Eicosapentaenoic acid (C20:5, n-3) is converted in good yield by platelet membranes via PGH_3 into TxA_3.[234,235,288] However, PGH_1 is a poor substrate for thromboxane synthetase, and incubation of dihomo-γ-linoleic acid (C20:3, n-6) with platelets or platelet membranes results in only a low yield of TxA_1 or TxB_1.[76,180,234,288]

Hydroxy Fatty Acids and Malondialdehyde

The hydroxy fatty acid 12-hydroxy-5-*cis*,8-*trans*,10-*trans*-heptadecatrienoic acid (HHT), and the 3-carbon fragment malondialdehyde are formed together in equimolar amounts when PGH_2 decomposes in aqueous medium, particularly under acidic or basic conditions [253] or when boiled proteins are present.[263] A similar hydroxy fatty acid, 12-hydroxy-8-*trans*,10-*trans*-heptadecadienoic acid (HHD) and malondialdehyde are formed from PGH_1.[253] The formation of these products occurs by a fragmentation reaction of the reverse Diels-Alder type.[307]

The formation of HHT and malondialdehyde from PGH_2 is also catalyzed by the solubilized thromboxane synthetase from platelet membranes.[397] Recent studies [7,69,285] indicate that two molecules of PGH_2 interact with thromboxane synthetase to produce approximately equimolar amounts of TxA_2, HHT, and malondialdehyde by a dismutase reaction. These studies also indicate that HHT and malondialdehyde are not breakdown products of TxA_2. Platelets also convert dihomo-γ-linolenic acid (C20:3, n-6) into HHD and malondialdehyde, but this conversion is less efficient than that from arachidonic acid.[76,180]

Prostacyclin (PGI_2) and 6-Keto-$PGF_{1\alpha}$

PGI_2 is not formed in significant amounts during the decomposition of PGH_2. The conversion of PGH_2 into PGI_2 is catalyzed by an enzyme originally shown to be present in the microsomal fraction of porcine aorta.[220,305] The discovery of PGI_2 is credited to Bunting, Moncada, Vane, and associates,[41] and the elucidation of the structure of PGI_2 was accomplished in elegant experiments by Johnson and associates.[154,155] Prior to these studies, several other investigators unknowingly may have been studying PGI_2.[176,267,303,304] PGI_2 is stable in aqueous solutions at pH 8.4 and above, but it is unstable at pH 7.4 ($t_{1/2}$ 10.4 minutes at 22 C) and is hydrolyzed to 6-keto-$PGF_{1\alpha}$. Like TxB_2, 6-keto-$PGF_{1\alpha}$ exists in equilibrium between an open form and a lactone form (Figure 10). It has recently been demonstrated that at acid pH, under strictly anhydrous conditions, PGI_2 methyl ester can be converted into a tricyclic derivative.[240]

PGH_1 cannot be converted into PGI_1 by prostacyclin synthetase because cyclization between carbon-6 and carbon-9 requires the presence of the 5,6-*cis* double bond in PGH_2. PGH_3 is converted efficiently into PGI_3.[72,234,243] PGI_2 is the main prostanoid formed from arachidonic acid in the isolated perfused rabbit and rat hearts,[67,150] and significant formation of PGI_2 has been detected in renal cortex [387] and papillae,[104] stomach,[262,267] lung,[66,107,224] and fetal arteries.[265] The highest prostacyclin synthetase activity in blood vessels is present in the intima.[129,222] Cultured endothelial

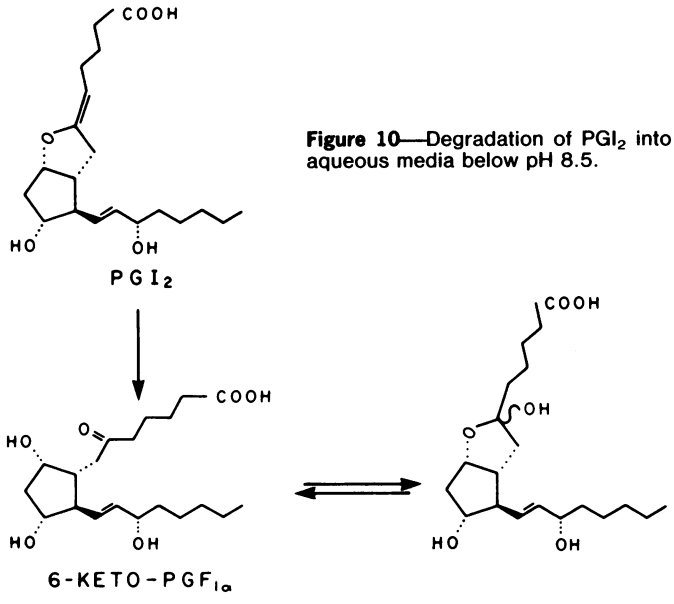


Figure 10—Degradation of PGI_2 into 6-keto-$PGF_{1\alpha}$ occurs in aqueous media below pH 8.5.

cells from human umbilical veins and arteries,[87,60,61,203,378,379] bovine [378] and porcine aorta,[196] and rat liver [353] readily convert arachidonic acid into PGI_2. Reports concerning cultured macrophages,[146,229] smooth muscle cells, and fibroblasts [14,196,347,379] are not consistent, but in general they seem to be much less active in producing PGI_2. There is evidence that prostacyclin synthetase activity is greater in rat arteries than in rat veins.[322] On the other hand, we have observed that cultured endothelial cells from human umbilical veins contain significantly more of both endoperoxide synthetase and prostacyclin synthetase activities than cells from human umbilical arteries.[87]

Lipoxygenase Activities

Platelets contain an ω-8 lipoxygenase which transforms arachidonic acid into 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE).[117,118,251] This hydroperoxy fatty acid is reduced to 12L-hydroxy-eicosatetraenoic acid (HETE) before it leaves the cells (Figure 11). The lipoxygenase is present mainly in the soluble fraction of platelets [251] but also may be associated with platelet membranes.[133] The enzyme prefers arachidonic acid as substrate but will act on other 20-carbon fatty acids which possess at least two *cis* double bonds at carbons n-9 and n-12.[251] As with plant lipoxygenase [278,301] the peroxidation reaction appears to depend upon ferric ion.[3] HETE has been reported to be chemotactic for human

polymorphonuclear leukocytes *in vitro*.[356] Recently it was reported that platelets could convert HPETE into trihydroxy fatty acids rather than into HETE [157] (Figure 11).

Rabbit polymorphonuclear leukocytes have been found to contain a lipoxygenase that transforms arachidonic acid into 5L-hydroxy-6,8,11,14-eicosatetraenoic acid, and 8,11,14-eicosatrienoic acid into 8L-hydroxy-9,11,14-eicosatrienoic acid.[33]

Transport and Metabolism of the Prostanoids

The prostanoids are rapidly inactivated once they enter the circulation. This section summarizes the evidence for the theory that prostanoids act as local hormones.

Transport Mechanisms

Prostanoids do not diffuse freely across cell membranes,[27] so that once biosynthesis occurs, and they are released from a cell or tissue into the blood, a transport system is required to remove them from the circulation. Transport mechanisms for PGE- and PGF-type prostaglandins have been identified and studied in lung and kidney cortex. The transport is rapid, saturable, and dependent on energy and temperature.[26]

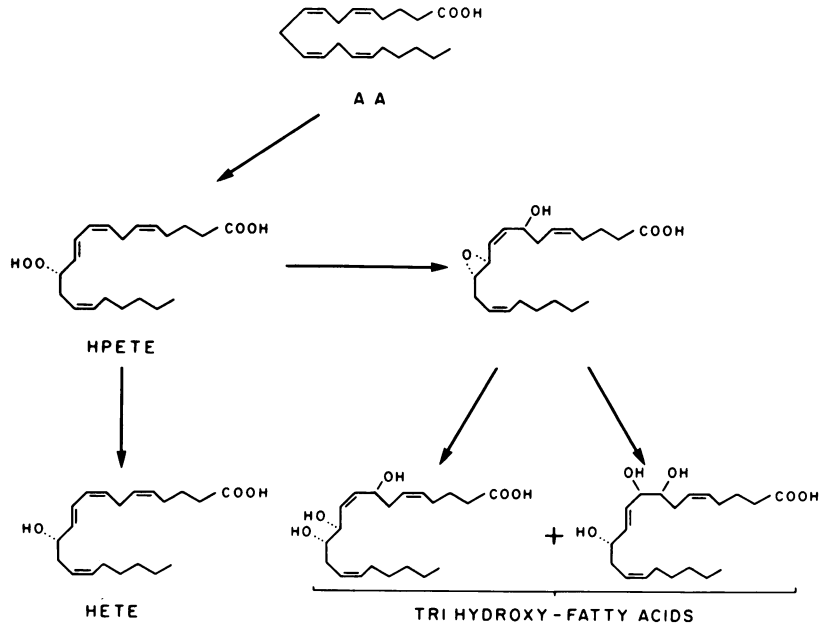


Figure 11—Lipoxygenase pathways in platelets.

Metabolism of E and F Types of Prostaglandins

The lungs take up and metabolize prostaglandins of the E and F types by the action of 15-hydroxy-prostaglandin dehydrogenase.[8] The biologically inactive 15-keto-prostaglandins so formed are subsequently reduced at carbon-13 by the action of prostaglandin reductase (see Figure 12). The rapid metabolism of PGE_2 and $PGF_{2\alpha}$ in man has been demonstrated.[102,111,115] Tritium-labeled PGE_2 was injected intravenously into an arm vein, and 90 seconds later venous blood from the opposite arm was collected. This sample contained very little of the injected prostaglandin but did contain large amounts 15-keto-13,14-dihydro-PGE_2[115] (see Figure 12). Both 15-hydroxy-prostaglandin dehydrogenase and prostaglandin reductase are cytoplasmic enzymes, and they have been found in several tissues, especially spleen and kidney cortex, as well as lung.[9] Siggins [318] demonstrated significant prostaglandin dehydrogenase activity in arterioles, and recently it has been detected in arteries and veins.[393] In one report dehydrogenase activity was demonstrated to be greater in atherosclerotic regions of the aorta than elsewhere.[284]

The metabolism of E- and F-type prostaglandins proceeds further in the liver with β-oxidation (loss of two carbon atoms) of the carboxyl carbon chain and ω-oxidation of the methyl end carbon chain. The major urinary metabolites excreted when E- or F-type prostaglandins are injected intravenously in man have been determined.[102,110,111,115] Reactions involved in the formation of the major urinary metabolite of PGE_2 are summarized in

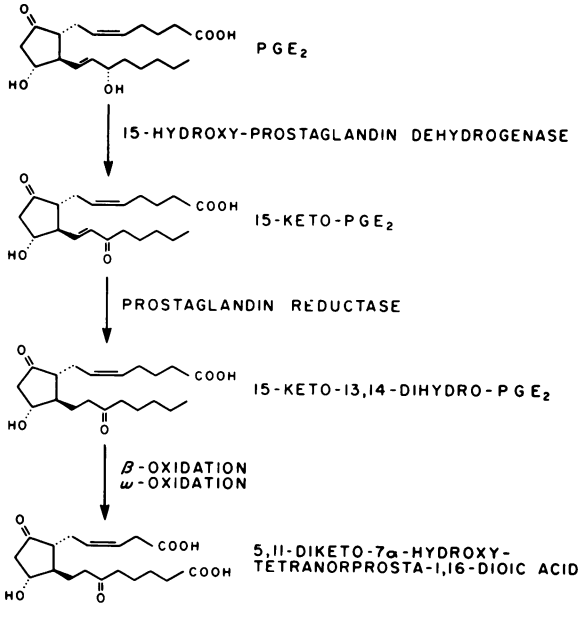


Figure 12—Reactions involved in the formation of the major urinary metabolite of PGE_2 in man.

Figure 12. The qualitative determination of the basal rate of urinary excretion of the major metabolites of the E-type or F-type prostaglandins has provided an estimate of the total body turnover of these compounds in man. The daily production of PGE_1 plus PGE_2 is 50–330 μg in men and 20–40 μg in women.[110] The daily production of PGF_α is 40–230 μg and 40–60 μg in men and women, respectively.[111]

There is presently no information on the metabolism of D-type prostaglandins.

Metabolism of Thromboxane B_2

The metabolism of intravenously injected, tritium-labeled TxB_2 has been studied in monkeys.[165,295,296] TxB_2 is eliminated from the circulation with a half-life of about 10 minutes after an initial rapid clearance. The dominant compound in all blood samples, even when recovered 20 minutes after the initial injection, is TxB_2, and the initial rapid clearance is due to uptake into tissues.[165]

Analysis of urinary metabolites of radioactive TxB_2 in monkeys [295,296] indicates that TxB_2 is not a substrate for 15-hydroxy-prostaglandin dehydrogenase. Unconverted TxB_2 has been detected in urine, and the major metabolite present is dinor-TxB_2, the result of one step of β-oxidation. Changes in the thromboxane ring structure apparently can occur *in vivo*, and these new metabolites then become substrates for 15-hydroxy-prostaglandin dehydrogenase, prostaglandin reductase, and β-oxidation. Some of the presently known pathways for the metabolism of TxB_2 are illustrated in Figure 13.

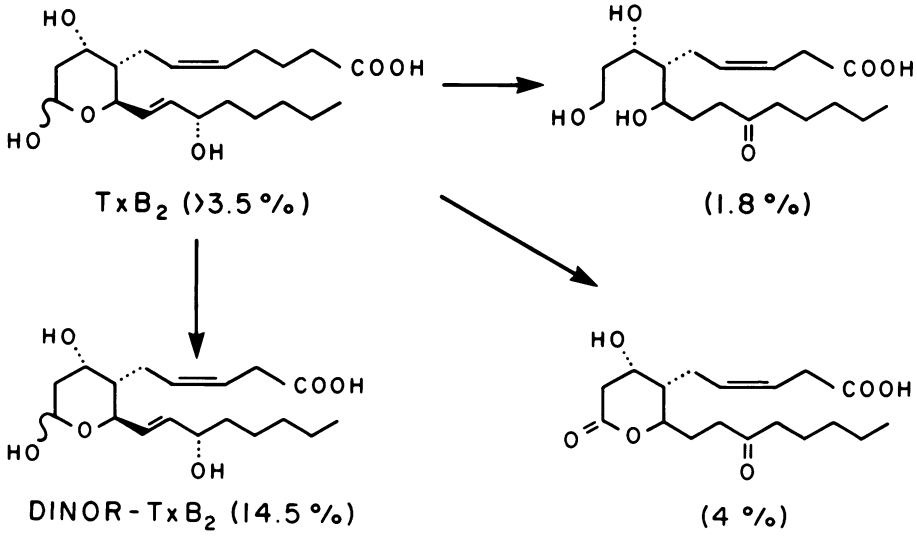


Figure 13—Relative abundance of radioactive thromboxane B_2 and some of its radioactive metabolites of urine after intravenous injection into monkeys.[296]

The chemical instability of TxA_2 has precluded studies of its metabolism. A report on the release of prostanoids from guinea pig lungs in response to anaphylaxis [66] indicated that 15-keto-TxB_2 (PG numbering) is produced, suggesting that perhaps TxA_2 is a substrate for 15-hydroxy-prostaglandin dehydrogenase. On the other hand, endogenously formed TxB_2 has been detected in the circulation of certain patients [187] and in rabbit plasma after the injection of arachidonic acid.[46] These findings show that at least part of endogenously formed TxA_2 must be hydrolyzed to TxB_2 *in vivo*, and it seems probable that spontaneously hydrolysis is the major route of inactivation of TxA_2.

Metabolism of Prostacyclin and 6-Keto-$PGF_{1\alpha}$

The two principal urinary metabolites of 6-keto-$PGF_{1\alpha}$ excreted by rats both still possess a hydroxyl group at carbon-15, indicating that 6-keto-$PGF_{1\alpha}$ is not rapidly metabolized by 15-hydroxy-prostaglandin dehydrogenase.[268] Several groups have confirmed that, in contrast to the E- and F-type prostaglandins, 6-keto-$PGF_{1\alpha}$ is a poor substrate for 15-hydroxy-prostaglandin dehydrogenase in homogenates of the lung, kidney, or blood vessels.[126,340,393]

On the other hand, PGI_2 is an excellent substrate for the 15-hydroxy-prostaglandin dehydrogenase present in homogenates of lung, blood vessels, and other tissues.[393] The major metabolite is 6,15-diketo-$PGF_{1\alpha}$. Inactivation of PGI_2 appears to occur rapidly in the liver and in the hindquarters, and there is little or no disappearance or metabolism of PGI_2 on passage through intact lungs,[11] because even though PGI_2 is a substrate for the dehydrogenase in the lungs, it is not a substrate for the pulmonary transport system and so never leaves the circulation to come in contact with the enzyme.[126]

Since at physiologic pH, PGI_2 is fairly rapidly hydrolyzed to 6-keto-$PGF_{1\alpha}$, it might be expected that urinary metabolites of PGI_2 would reflect the action of 15-hydroxy-prostaglandin dehydrogenase on PGI_2 in tissues other than the lung (ie, metabolites with a 15-keto-group) and the lack of action of this enzyme on 6-keto-$PGF_{1\alpha}$ (ie, metabolites still possessing a 15-hydroxyl group). This has been confirmed in rats subjected to long-term continuous intravenous infusion of PGI_2.[340] Five compounds possessing a 15-keto group were isolated from urine. These were all 13,14-dihydro-15-keto derivatives of PGI_2 that had undergone one step of β-oxidation (ie, loss of two carbon atoms) and various degrees of ω-oxidation. Two compounds, accounting for about 30% of the excreted metabolites, were derivatives of 6-keto-$PGF_{1\alpha}$ and had retained both the 15-hydroxyl group and the 13, 14 double bond. Both had undergone one step of β-oxidation, and one had also undergone ω-hydroxylation. The structure and

relative abundance of these metabolites in rat urine are shown in Figure 14.

The duration of action of intravenously injected PGI_2 is very short,[11] indicating that PGI_2 is rapidly inactivated in the body. It has been suggested that the major reason for this rapid disappearance of PGI_2 is due to the metabolic capacity of the blood vessels themselves.[393] Although evidence has been presented that PGI_2 is a circulating hormone that is released from the lungs,[107,224] we were unable to detect any changes in blood pressure when antibodies which bind PGI_2 were infused into cats.[329]

Inhibitors of Prostanoid Biosynthesis

A number of naturally occurring molecules and drugs have been shown to influence the biosynthesis of prostanoids. The use of these compounds is of value in elucidating the role of the prostanoids. Perhaps more impor-

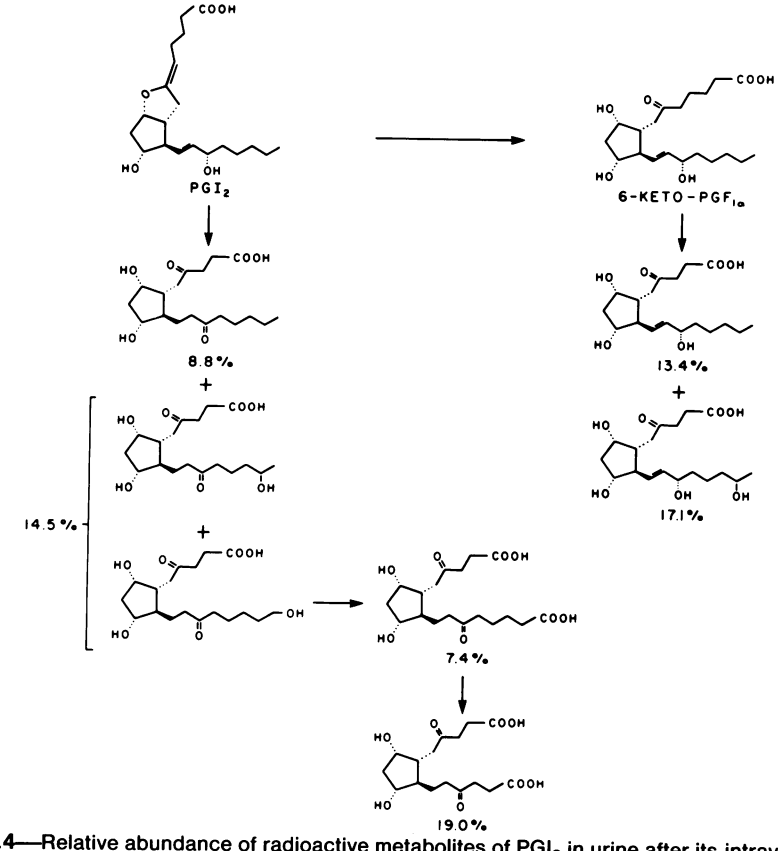


Figure 14—Relative abundance of radioactive metabolites of PGI_2 in urine after its intravenous infusion in rats.[340]

tant the rational use of such compounds might be of therapeutic value in the treatment of thrombosis.

Naturally Occurring Fatty Acids

Linoleic acid (18:2, n-6) [266] and certain unsaturated fatty acids that accumulate during essential fatty acid deficiency are competitive inhibitors of endoperoxide synthetase.[398,399] These latter fatty acids belong to the n-9 pathway, in which oleic acid (18:1, n-9) is desaturated to α-linoleic acid (18:2, n-9); this desaturation is followed by chain elongation and further desaturation, producing eicosatrienoic acid (20:3, n-9).[18] When essential fatty acids are lacking from the diet, 5,8,11-eicosatrienoic acid accumulates, and it can replace arachidonic acid in several tissues.[5,88] Rat platelets have been shown to have reduced capacity for the synthesis of endogenous TxA_2 during essential fatty acid deficiency.[38]

Lipid hydroperoxides produced by plant lipoxygenase, such as 15-hydroperoxy-arachidonic acid, have been found to be selective inhibitors of prostacyclin synthetase.[221,305] On the other hand, HPETE produced by the platelet lipoxygenase selectively inhibits thromboxane synthetase.[122]

Synthetic Fatty Acids

An acetylenic analog of arachidonic acid, 5,8,11,14-eicosatetraynoic acid (ETYA), which has triple bonds instead of double bonds, is a potent inhibitor of endoperoxide synthetase.[4] It is believed to act by forming a highly reactive intermediate with the enzyme in the presence of oxygen.[361] ETYA also inhibits plant lipoxygenase [4] and platelet lipoxygenase.[117] On the other hand, another acetylenic compound, 5,8,11-eicosatriynoic acid, has little effect on the cyclooxygenase and selectively inhibits platelet lipoxygenase.[121]

The 8-*cis*,12-*trans*, 14-*cis* analogs of dihomo-γ-linolenic and arachidonic acids are competitive inhibitors of endoperoxide synthetase. The fact that they can be recovered unchanged at the end of incubations with the enzyme indicates that they are not enzyme substrates.[250,359]

Nonsteroidal Anti-inflammatory Drugs

In 1971, three papers were published simultaneously that reported that the nonsteroidal anti-inflammatory drugs aspirin and indomethacin, but not sodium salicylate, inhibited prostaglandin biosynthesis in guinea pig lung homogenates,[362] isolated spleen preparations,[79] and in human platelets.[323] Aspirin and indomethacin were found to be especially active on human platelets, and platelets isolated after volunteers had ingested therapeutic amounts of either of these drugs were shown to have a reduced

capacity for prostaglandin biosynthesis.[323] These findings have been widely confirmed and extended.

Both aspirin and indomethacin inhibit highly purified endoperoxide synthetase from bovine and ram vesicular glands.[84,332,354] Aspirin selectively acetylates this enzyme,[297,300] while the effect of indomethacin is noncovalent in nature and may be reversible.[335] Acetylation by aspirin is associated with loss of cyclooxygenase activity and can be prevented by substrate (arachidonic acid) or by prostaglandin synthetase inhibitors (linoleic acid or indomethacin).[297,300] Aspirin does not inhibit the peroxidase activity in endoperoxide synthetase. Incubation of platelets with aspirin containing radiolabel in its acetyl group leads to time-dependent incorporation of radioactivity into a protein present in platelet membranes that on gel electrophoresis has approximately the same molecular weight as the endoperoxide synthetase (85,000 daltons).[43,299] That this protein is endoperoxide synthetase is strongly suggested by the findings that acetylation is saturable by 30 μM aspirin in 15 minutes at 37 C and can be prevented by indomethacin, or by the oral ingestion of aspirin.[43]

The effect of aspirin on prostaglandin synthesis by platelets persists for several days *in vivo*, whereas that of indomethacin is relatively short-lived.[173] Since platelets are released into the circulation from megakaryocytes in bone marrow as anucleate cells with essentially no capacity for protein synthesis, this persistent effect of aspirin is probably caused by permanent acetylation of the endoperoxide synthetase in platelets. It has been observed that after volunteers ingested a single tablet of aspirin only 11% of the membrane protein in their platelets could be acetylated by radioactive aspirin. Since no increase in the capacity of their platelets to be acetylated was noted when blood was drawn two days later, it was suggested that the ingested aspirin also acetylated their megakaryocytes.[43] These findings should be compared with other observations showing that after aspirin is incubated with endothelial cells, the ability of the cells to produce prostaglandins returns within a few hours.[14,59,60]

Several groups have noted the high sensitivity of the endoperoxide synthetase in platelets to inhibition by aspirin.[43,271,323] The daily ingestion of 20 mg of aspirin (1/16 of a tablet) by volunteers inhibits acetylation of their platelets by radioactive aspirin by more than 50%. The endoperoxide synthetase in other tissues or in cells in culture is inhibited by aspirin but only in higher concentrations.[14,42,97] It has been shown that the ingestion of aspirin, indomethacin, and even sodium salicylate in therapeutic amounts reduces the excretion of urinary metabolites of PGE_1 and PGE_2 in man.[110]

A number of other nonsteroidal anti-inflammatory drugs, including meclofenamic acid, flufenamic acid, and naproxen, have been shown to in-

hibit prostaglandin synthetase.[84,354] These compounds all possess a free carboxyl group [84] and probably act at the substrate binding site.

Synthetic Prostanoids

Stable analogs of prostaglandin endoperoxides have been found to be potent inhibitors of thromboxane and prostacyclin synthetase. These include 15-deoxy-9, 11-azo-PGH_2[81,98,233] and 15-deoxy-9,11-methanoepoxy-PGH_2.[339] Recently two prostanoid analogs, 15-deoxy-9, 11-epoxyimino-PGH_2 [82] and pinane-thromboxane A_2 (PTA_2) [242] have been synthesized and shown to inhibit selectively prostacyclin synthetase or thromboxane synthetase, respectively.

Miscellaneous Inhibitors

Other compounds, as well as the stable prostanoid analog mentioned above, have been found to inhibit thromboxane synthesis. Sodium-p-benzyl-4 (1-oxo-2-[4-chlorobenzyl]-3-phenylpropyl) phenylphosphonate (N-0164),[175] nordihydroquaiarcetic acid, 2-isopropyl-3-nicotinylindole, and imidazole [81,219,233,237] selectively inhibit thromboxane synthesis by platelets. Derivatives of imidazole containing 1-carboxylalkyl substituents are potent inhibitors and act in a noncompetitive fashion.[396]

An inhibitor of monoamine oxidase, tranylcypromine, has been reported to selectively inhibit prostacyclin synthesis.[41] 1-Phenyl-3-pyrazolidone inhibits both the cyclooxygenase and lipoxygenase pathways in lung and platelets.[28]

Corticosteroids

Recent reports indicate that corticosteroids inhibit prostaglandin biosynthesis by certain tissues and cells and that they do so by inhibiting the activation of phospholipase A_2. Corticosteroids inhibit the release of arachidonic acid from phospholipids of transformed 3T3 mouse fibroblasts normally elicited by serum, bradykinin, or thrombin.[142] They also inhibit the release of arachidonic acid from guinea pig lungs, which occurs spontaneously or can be induced by histamine.[30,246] On the other hand, they do not suppress the release of arachidonic acid induced by bradykinin in the lungs [30] and fail to suppress arachidonic acid or prostanoid release from disrupted cells or rat carrageenin granuloma fibroblasts.[230] Evidence has been presented that the inhibitory activity of corticosteroids depends on RNA and protein synthesis,[63] which may explain the lack of effect of hydrocortisone on prostaglandin synthesis by platelets.[323] The mechanism of action of these compounds is complex and has not yet been solved.

Inhibitors of Phospholipase A_2

The antimalarial drug mepacrine [364] and several local anaesthetics, including tetracaine and procaine,[178] have been reported to inhibit phospholipase A_2. The concentrations of these compounds required to inhibit the enzyme are rather high (1 mM) and suggest that inhibition of phospholipase A_2 may be secondary to other effects. The compounds probably act by reducing the fluidity of the phospholipid bilayer [314] and so making the substrate less available for enzyme attack. However, many may also act by preventing the availability of the calcium ions that are necessary for the activity of phospholipase A_2.

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