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An Eicosanoid-Centric View of Atherothrombotic Risk Factors

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Abstract

Cardiovascular disease is the foremost cause of morbidity and mortality in the western world. Atherosclerosis followed by thrombosis (atherothrombosis) is the pathological process underlying most myocardial, cerebral, and peripheral vascular events. Atherothrombosis is a complex and heterogeneous inflammatory process that involves interactions between many cell types (including vascular smooth muscle cells, endothelial cells, macrophages and platelets) and processes (including migration, proliferation, and activation). Despite a wealth of knowledge from many recent studies using knockout (KO) mouse and human genetic studies (GWAS and candidate approach) identifying genes and proteins directly involved in these processes, traditional cardiovascular risk factors (hyperlipidemia, hypertension, smoking, diabetes mellitus, sex and age) remain the most useful predictor of disease. Eicosanoids (20 carbon polyunsaturated fatty acid derivatives of arachidonic acid and other essential fatty acids) are emerging as important regulators of cardiovascular disease processes. Drugs indirectly modulating these signals, including COX-1/COX-2 inhibitors, have proven to play major roles in the atherothrombotic process. However, the complexity of their roles and regulation by opposing eicosanoid signaling, have contributed to the lack of therapies directed at the eicosanoid receptors themselves. This is likely to change, as our understanding of the structure, signaling and function of the eicosanoid receptors improves. Indeed, a major advance is emerging from the characterization of dysfunctional naturally occurring mutations of the eicosanoid receptors. In light of the proven and continuing importance of risk factors we have elected to focus on the relationship between eicosanoids and cardiovascular risk factors.

Keywords

eicosanoids; atherothrombosis; prostaglandins; prostanoids; platelets; hypertension; hyperlipidemia; oxidative stress; diabetes mellitus

Atherothrombosis

Atherothrombosis, the leading cause of morbidity and mortality globally [1], is a complex inflammatory disease of the arterial wall [2] in which a sclerotic plaque of lipid and fibrous tissue is deposited over time, often leading to rupture and thrombus formation. Such vascular lesions create a depot for circulating lipids, prompting an immune response, and developing feedback amplification of inflammatory mediators further enhancing material deposition [3]. As the sclerotic plaque remains relatively innocuous while stable, the onset of a thrombotic event is highly unpredictable in both occurrence and severity [4]. Initiated by fatty streak deposition with oxidized low-density lipoprotein [5, 6], the atherosclerotic lesion development is driven by inflammation [7] and is pathologically enhanced by

dyslipidemia [8, 9]. Thrombosis results from platelet interaction with the plaque [10]. In dyslipidemic mice, lesion-prone vasculature shows enhanced expression of endothelial cell adhesion molecules, VCAM-1 and P-selectin, prior to atherosclerotic plaque deposition [11]. Cell adhesion markers provide attachment points for circulating platelets and leukocytes [12, 13]. Platelets are ubiquitous throughout lesion initiation, plaque growth, and thrombosis [14–19]. The resulting thrombosis can manifest as unstable angina, myocardial infarction, or sudden death [20–22]. Platelet activation is the primary target for anti-thrombotic therapy [10], with clopidogrel inhibition of adenosine receptors and aspirin inhibition of thromboxane generation being most effective [23]. Relationships between eicosanoids and cardiovascular disease risk factors have been long recognized [24]. The following review focuses on the biology of eicosanoid signaling, and their roles in modifying and regulating critical processes involved with the major risk factors associated with heart disease.

Eicosanoids

Eicosanoids are oxidative metabolites of arachidonic acid involved in highly concerted and largely self-regulated cellular signaling. Liberation from arachidonic acid (AA) from lipid membrane, by phospholipase A₂ (PLA₂) initiates a signaling cascade with diverse downstream second messenger amplification steps promoting multiple potentially contradictory cellular behaviors. Culminating effects are largely dependent upon the availability of specific enzymes and the receptors with which the various members of this ligand family can interact (Table 1). The formation and activity of these ligand families has been extensively reviewed elsewhere [25–27]. Briefly, AA is immediately oxidized into one of three primary pathways via cyclooxygenase, lipoxygenase, or cytochrome P450, generating upstream substrates for the prostaglandins, leukotrienes, or epoxyeicosanoids, respectively (Figure 1).

In addition to the direct agonism of these ligands at their cognate receptors, the resulting signals also involve promiscuous mechanisms including agonism and antagonism at receptors for other eicosanoids, competition for synthesis at processing enzymes, and transactivation of non-canonical pathways. Further complexity arises when precursor substrates can be delivered to nearby cells for transcellular processing [28] and the convergence of signaling pathways on shared second messengers with competing cellular responses determined by the composite effect of multiple ligands. It is important to consider these intricacies when facing the myriad of seemingly contradictory effects and incomplete phenotypes (when pathway components are knocked out). Unraveling such an orchestration is beyond the scope of this review and is currently being intensely studied. Rather, we will focus on how specific portions of these intricate pathways influence known cardiovascular risk factors, where large gaps of knowledge remain in tying together the cardiovascular influence of eicosanoid signals (Figure 2), and where considerable opportunities in deciphering eicosanoid biology remain ripe for active research (Table 2).

Prostaglandins (PG) are products of arachidonic acid where cyclooxygenase 1 (COX-1) or cyclooxygenase 2 (COX-2) adds two oxygen to form the 5-member ring structure of prostaglandin G₂ (PGG₂), and spontaneously sheds an oxygen to give prostaglandin H₂ (PGH₂), which can then be converted to any of the prostaglandin signaling molecules by one of the specific synthases. Isoprostanes are prostaglandin-like eicosanoids formed by peroxidation by free radicals and reactive oxygen species [29], primarily of arachidonic acid, but also form from other lipid and fatty acid precursors [30]. Predominant effects of prostaglandins and isoprostanes in the context of cardiovascular hemostasis include effects on vasculature, platelets, and leukocytes. Prostanoid receptors mediate homeostatic responses including vasoconstriction, predominantly through thromboxane A₂ (TxA₂), with similar effects from TxB₂ and PGF₂α, effects which are countered by vasodilation via PGE₂, PGI₂,

LTC₄, LTD₄, and LTE₄ [31]. Many of the same components regulate inflammatory responses including platelet activation (TxA₂) and inhibition (PGD₂, PGI₂), as well as leukocyte induction (TxA₂, LTB₄) and inhibition (PGD₂, PGE₂).

Leukotrienes (LT) are formed in a more linear fashion through a lipoxygenase-generated intermediate, hydroperoxy-eicosatetraenoic acid (HPETE), which gives rise to leukotriene A₄, via 5-HETE. Addition of water to LTA₄ gives LTB₄, whereas addition of cysteine-based glutathione, by glutathione-S-transferase, produces leukotriene LTC₄, metabolized to cysteine-containing LTD₄ and LTE₄. These cysteinyl leukotrienes signal through two specific receptors CysLT₁ and CysLT₂ which, though more well-studied in the context of asthma (contractile and proinflammatory) in the cardiovascular system, LTC₄ and LTD₄ are hypotensive [32]. Leukotriene receptors are expressed on platelets [33], but cysteinyl leukotrienes are also importantly known to directly interact with P2Y₁₂ receptors [34], which are the target receptors of clopidogrel.

Epoxyeicosanoids (EETs) are products of cytochrome P450 epoxidation of arachidonic acid, and provide a major anti-inflammatory signal [35, 36]. The formation and activity of EETs on endothelial dysfunction has been recently reviewed [37]. Whereas cytochrome epoxidases (predominantly CYP family 2C) [38] synthesize epoxyeicosatrienoic acids in endothelia and promote vasodilation, ω -hydroxylase gives rise to hydroxyeicosatrienoic acids (HETEs) promoting vasoconstriction [38]. Interestingly, when acting on linoleic acid rather than arachidonic acid, Cyp2C generates reactive oxygen species [39, 40].

Risk Factors

Significant reductions in coronary heart disease over recent years stem from improved risk factor profiles [41]. The Framingham Heart Study is among the most influential of cardiovascular clinical studies, providing comprehensive health evaluations for over 10,000 individuals across three generations [42]. The study was used to develop a multivariate prediction model of coronary heart disease risk, identifying the major risk factors of hypertension, cholesterol, smoking, diabetes mellitus, age, sex, and race. Identification of these risk factors has guided clinical mitigation of coronary disease progression with considerable success. Although collectively addressing patient risk factors has decreased the overall prevalence of cardiovascular events [43], individually addressing hypertension or glucose imbalances in diabetic patients has been less effective than anticipated at improving outcomes [44–48]. Together these observations indicate that although each risk factor can individually contribute to pathology, the complexity and severity of cardiovascular disease stems from the multifaceted interactions between these complicated factors.

Recognition of the complexity of risk factor contributions leads to two specific approaches for managing cardiovascular events. The current approach is to combine therapies directed at distinct risk factors. Multiple points of intervention have the advantages of providing tight control of each specific response. However, there remain inherent limitations and potential dangers with a combined therapy approach including drug/drug interactions and side effects from multiple drugs [49, 50]. An alternative strategy would be to identify a common denominator in the mechanism of multiple risk factors for focused intervention. To employ either approach effectively requires improved understanding of the underlying pathophysiology and detailed investigation into the underlying pathways through which these risk factors intersect. Interestingly, despite the eicosanoids playing key roles in the regulation of many of these cardiovascular risk factors, no eicosanoid-based drug is currently being used both in the treatment and prevention of atherothrombosis. This is in part due to the complex and ubiquitous nature of their actions, leading to many potential side effects. Understanding tissue specific regulation of eicosanoids, the corresponding signaling

pathway-specific actions, and the conformational changes required for such signaling may ultimately lead to development of tissue/receptor/pathway-selective drugs required to precisely activate or inactivate a tissue specific function.

Gender

There is a clear difference in the influence of risk factor categories on coronary heart disease in males and females [51]. The risk contribution of HDL cholesterol, for instance, is stronger in females than in males [51], whereas cigarette use shows much higher risk contribution for males [52]. Postmenopausal increases in androgens [53] are associated with increased risk for cardiovascular events in females [54] and support the protective role of estrogens relative to androgens. Such a protective effect has been demonstrated by estrogen through stimulation of prostacyclin synthesis [55]. This may arise, at least in part, from a well-defined estrogen response element in the promoter of the human prostacyclin receptor [56]. Therapeutic treatment with aspirin (a low-dose COX-1 inhibitor reducing platelet thromboxane production) shows a similar divergence, providing protective effects against strokes in women and against myocardial events in men, but not vice versa [57]. Increased thromboxane synthesis with decreased prostacyclin synthesis is associated with atherosclerotic lesion burden in female ApoE^{-/-} mice, relative to both female wild-type and male ApoE^{-/-} mice, despite reduced cholesterol levels [58]. Similarly, PGE₂, prostacyclin, and thromboxane production are higher in female mouse macrophages than in male [59].

Production of the cytochrome P450 eicosanoid 20-HETE correlated with increased blood pressure and sodium retention in male rats maintained on a high fat diet relative to those fed a control diet [60]. Female rats, however, did not induce 20-HETE on a high fat diet unless they were treated with exogenous androgens. Increased 20-HETE production is associated with oxidative stress [61] and endothelial dysfunction [62]. Specific inhibition of 20-HETE reduces postmenopausal hypertension [63]. Interestingly, however, 20-HETE inhibits COX-2 up-regulation [64], and 20-HETE and other vasoconstrictors are preferentially formed with COX-2 inhibition [65].

Increasing blood pressure increases 20-HETE production by both neutrophils and platelets [66]. 20-HETE contributes to vascular smooth muscle depolarization by inhibiting potassium-calcium channels [67] and promoting calcium influx via L-type calcium channels [68]. Vasoconstriction is mediated by calcium flux induced depolarization of vascular smooth muscle [69, 70]. Interestingly, the androgen driven elevation in blood pressure related to 20-HETE production is directly tied to sodium retention [60]. In general, the evidence suggests that hormonal regulation of eicosanoids contributes to both hypertensive response in males and premenopausal cardioprotection in females. There remains much to be explored on differential regulation of eicosanoids in cardiovascular disease and risk factors.

Hypertension

The most consistent cardiovascular risk factor across genders is hypertension [71, 72], contributing to age-related coronary heart disease risk more than any other single risk factor [73]. Lipidomic profiling of hypertensive versus normotensive individuals indicates reduced membrane arachidonic acid content [74], which might suggest increased lipid mobilization [75], though numerous other mechanisms may be responsible for the disparity. In coordination with many other vasoactive agents, systemic blood pressure is largely maintained by balancing the vasodilatory effects of vascular wall-generated PGI₂ and nitric oxide with the vasoconstrictive effects of platelet-derived TxA₂ [76].

Though blood pressure is maintained through the combined efforts of numerous mechanisms beyond the scope of this review [77–81], baseline hypertension is largely influenced by vascular wall biology, where the common mechanistic pathway responsible for vasoconstriction of the vasculature (increased peripheral resistance) is vascular smooth muscle cell depolarization causing contraction. The depolarization is caused by intracellular calcium flux, which is primarily opposed by cyclic AMP formation, blocking calcium influx [82]. PGI₂, PGD₂, and PGE₂ can also promote vasodilation via Gs stimulation of adenylyl cyclase (AC), generating cyclic adenosine monophosphate (cAMP), hyperpolarizing VSMC. In the opposing direction, TxA₂ stimulates intracellular calcium release, promoting VSMC constriction via membrane depolarization.

Prostacyclin and thromboxane

Prostacyclin is a potent vasodilator with critical involvement in vascular disease (anti-atherothrombosis, cardioprotective) which has recently been reviewed [83]. Despite having a robust pro-inflammatory influence in the context of rheumatoid arthritis, prostacyclin (PGI₂) serves anti-inflammatory roles in vasculature, inhibiting atherosclerotic formation [84]. The predominant effects of prostacyclin are a result of limiting the effect of thromboxane [85]. In accordance with the overwhelming vasodilatory role of prostacyclin, mice with predominant COX-1 prostanoid formation (analogous to selective COX-2 inhibition) develop systolic hypertension from high salt (reversible with prostacyclin receptor agonist) despite increased formation of all other major prostanoids [86]. Indeed, a dysfunctional prostacyclin receptor arising from a human genetic variant (R²¹²C) was associated with an increased prevalence of hypertension [87]. This variant also accelerates progression of atherothrombotic disease in humans [87].

Prostaglandin E₂

In addition to the roles of PGI₂ and TxA₂, prostaglandin E₂ (PGE₂, also known as dinoprostone) also demonstrates clear hemodynamic influence. Reducing PGE₂ synthesis by genetic deletion of microsomal PGE synthase-1 (mPGES-1) causes severe hypertension and impaired sodium excretion [88] through PGE₂ effects on aquaporin [89]. This response is attributed to the EP₂ and EP₄ receptor subtypes which, like the prostacyclin receptor (IP), are coupled to Gs [90].

However, the role of PGE₂ in cardiovascular disease is multifaceted [91], largely due to the presence of four cell surface receptors (EP_{1–4}), with different secondary messengers often leading to opposing downstream effects. EP₁ receptor deficiency, for instance, decreases resting systolic blood pressure [92] with compensatory increases in pulse rate and renin activity in males, through reduced angiotensin II hypertension [93]. The primary effector for EP₁ is Gq, promoting intracellular calcium release [90]. The primary EP₃ secondary messenger is Gi, decreasing cellular cAMP formation. The opposing stimulus, cAMP formation via adenylyl cyclase activation, is carried out by EP₂ and EP₄ coupling to Gs (the same effector as IP). The ability of PGE₂ to stimulate opposing pathways complicates defining a clear role of PGE₂ in hypertension.

EETs

The cytochrome P450 (Cyp)-derived epoxyeicosatrienoic acids (EETs) utilize alternative mechanisms to influence vascular tone. EETs induce vasodilatation through hyperpolarization [94] of vascular smooth muscle cells [95] via activation of large conductance calcium-activated potassium channels [96], further contributing to the salt-sensitivity of angiotensin-dependent hypertension [97]. Vascular benefits of EETs extend from reducing platelet aggregation [98] and adhesion [99], to reducing the endothelial inflammatory response [35]. Some EET isomers inhibit cyclooxygenase, reducing

thromboxane formation; however all EETs reduce platelet aggregation [100]. EETs also interfere with platelet adhesion to endothelia by hyperpolarizing platelets [101]. Angiotensin II, a prothrombotic vasoconstrictor, impairs Cyp2C synthesis of EETs [102, 103] while increasing EET catabolism to lower activity dihydroxyeicosatrienoic acids (DHETs) [104] by soluble epoxide hydrolase [94, 105] causing a loss in systemic EET availability.

The EETs directly stimulate PPAR γ [106] and competitively antagonize the thromboxane A₂ receptor [107]. Eicosapentaenoic acid (EPA), a putatively cardioprotective ω 3-series fatty acid [108, 109], induces endothelial EET formation by Cyp2J2 via PPAR γ , while inhibiting the exaggerated VSMC growth observed in spontaneously hypertensive rats [110]. EPA also reduces platelet activation in both CAD patients [111] and healthy subjects [112].

Cytochrome P450 epoxygenases 2C9 and 2J2 are directly inhibited by H₂O₂, reducing EET production under oxidative conditions [113] such as ROS. Interestingly, nitric oxide produces a similar inhibition of EET formation [114]. As oxidative stress can cause hypertension [115] and hypertension promotes oxidative stress [116], it is not surprising that lifestyle recommendations for reducing the risk of high blood pressure and cardiovascular events, each reduce oxidative stress, including smoking cessation, weight loss, exercise, reducing alcohol consumption, consuming a healthy diet, and reducing stress.

Smoking and Oxidative Stress

The very nature of eicosanoids as oxidative metabolites of ubiquitous membrane components implies a direct relationship to oxidative stress. The relationship between smoking, oxidative stress, and inflammation is well established, but complex. Smoking increases risk of coronary heart disease alone [117], but also greatly increases risk in combination with other risk factors [43, 118]. Cigarette smoke is a complex mixture of molecules, each with a distinct pharmacological response. Nevertheless, many direct influences of habitual smoking promoting heart disease are modulated by eicosanoids: hypertension [89, 119], tachycardia [120–123], reduced cardiac output [124], cholesterol accumulation [125], vessel damage [126], and promotion of clot formation [127].

Cigarette smoke contains multiple agents contributing directly to inflammation. Lipid-soluble smoke particles consisting of DMSO-soluble particles (DSP), nicotine, and lipopolysaccharides, upregulate G protein-coupled receptors for potent contractile agents, endothelin and thromboxane, [128] which contribute to endothelial dysfunction [129]. In addition to upregulating contractile receptors, LPS exposure simultaneously increases activation of inflammatory and contractile thromboxane and angiotensin I receptors while inhibiting anti-inflammatory and dilatory prostacyclin and acetylcholine receptors [130].

Isoprostane production

Smoking increases oxidative stress markers malondialdehyde (MDA) [131] and isoprostanes [132] through free-radical catalyzed peroxidation of arachidonic acid and AA metabolites [133, 134], as well as inflammatory markers C-reactive protein, tumor necrosis factor- α , interleukin-6 [131]. Elevated circulating levels of homocysteine are also associated with smoking [135] and constitute a strong risk for endothelial dysfunction [136] and associated cardiovascular events [135]. Homocysteine is generated through methionine metabolism, with elevated levels most commonly associated with vitamin B deficiencies [137]. Elevated homocysteine increases thromboxane synthesis [138] and isoprostane formation [139], enhancing platelet activity [140]. Nevertheless, attempts to lower homocysteine levels have failed to reduce cardiovascular events [141, 142], despite significant reductions in homocysteine levels.

With numerous potential avenues leading from smoke to reactive oxygen species [131, 143], the resulting effect is an increased systemic inflammatory state. Direct effects of increased oxidation on eicosanoids include vascular and platelet alterations [144]. In addition to stimulating inflammatory response through the thromboxane A₂ receptor [145], isoprostanes contribute to angiotensin II induced vasoconstriction [146]. F₂-isoprostanes are elevated in both chronic and acute smoking, accompanied by decreased plasma arachidonate. A role for oxidative stress-derived isoprostanes [147] in activation of the thromboxane receptor may explain some cases of observed aspirin resistance [148]. The thromboxane receptor is critical to these events and is targeted by isoprostanes. This suggests that TP receptor antagonists may be protective, but these are not yet widely used. Specific antagonists of the thromboxane A₂ receptor are in development. The PERFORM study of stroke patients demonstrated no benefit over aspirin treatment [149], but lacked a sufficiently severe population (e.g., aspirin-resistant or diabetic patients) to observe the anticipated benefits of such an approach [150]. An additional potential drawback to thromboxane receptor inhibition is that combined inhibition of COX-2 and TP can destabilize plaques [151]. This could interfere with the therapeutic utility of such an approach, since smaller and less stable plaques are more likely to precipitate acute clinical events as they lack protective collateral formation [152].

HETEs in Oxidative Stress

Generally, the detrimental effects of smoking are best summarized as oxidative damage promoting chronic inflammation. In addition, hypercholesterolemia, diabetes mellitus, visceral obesity, and hyperhomocysteinemia all contribute to inflammatory signals promoting formation of reactive oxygen species. In addition to the direct increase in formation of 8-iso-PGF₂α from oxidative stress, HETE levels are also elevated with chronic smoking, which show no further increase with acute smoking [153]. Additional eicosanoid influence from oxidative damage includes reduction of beneficial EETs [113], and formation of 12-HETE by 12-LOX oxidation of AA [154]. Smoking cessation among heavy smokers (at least 20 cig/day) improves platelet function by increasing sensitivity to PGE₁, and resulting in decreased mean platelet volume (less basal activation) without altering the total number of platelets [155]. The influence of smoking modifies production of each eicosanoid class and occurs predominantly through an increased inflammatory state caused by the combined effects of inhaled mediators and oxidative stress.

Metabolic Syndrome

Individuals frequently exhibit multiple metabolic risk factors for cardiovascular disease. This suggests that these risk factors may arise from a common underlying etiology, but the molecular basis for the clustering of risk factors is not yet understood. “Metabolic syndrome” is defined as having three or more of these risk factors, which include central obesity, insulin resistance, hyperglycemia, hypertension, excessive blood clotting, and dyslipidemia. While patients diagnosed with metabolic syndrome are not treated differently—the individual risk factors are still addressed accordingly—this classification is important because it identifies a subset of patients at the greatest risk. The presence of metabolic syndrome corresponds to a doubling of cardiovascular disease risk, along with a five-fold increased risk for type 2 diabetes mellitus [156]. As measures and mechanisms can be distinguished between these risk factors, each is discussed separately; however, there is considerable overlap between their roles, as each is considered to support a proinflammatory state.

Diabetes mellitus

Whereas oxidative stress and hypertension have direct and obvious influences on the cardiovascular system, the linkage between glucose regulation and cardiovascular disease has been conceptually more challenging, though no less important. The insulin-resistance and associated glycemic dysregulation from type 2 diabetes mellitus doubles the risk of coronary heart disease [157] and related vascular complications, as well as increasing the risk of normotensive patients [158]. However, efforts to control glucose levels have proven much less effective than anticipated in decreasing cardiovascular events in diabetes [158–160], and aggressive glucose control therapy has at times been associated with worsening outcomes from severe hypoglycemic vascular events, likely due in some cases to off-target effects of specific medications [161, 162].

Platelet hyperactivity has been a recognized response to hyperglycemia for decades [163], and is responsible for numerous diabetic complications [164]. It is difficult to tease out differences from diabetic and oxidative contributions to atherothrombosis, as they are intimately related through the observation that hyperglycemia produces ROS [165]. Nevertheless, in a rat model of type 2 diabetes mellitus, the formation of an eicosanoid oxidative stress marker, 8-iso-PGF₂α, was decreased by anti-oxidant vitamin E or anti-diabetic PPAR agonist troglitazone [166]. Hyperglycemia from diabetes mellitus increases oxidative stress, enhancing lipid peroxidation [167]. Increased thromboxane synthesis and isoprostane production have been noted in type-2 diabetic patients [168, 169], and circulating levels of these eicosanoids can be reduced with intense glucose lowering [170]. These positive feedback signals result in persistent platelet activation in diabetic patients, marked by increased urinary 11-dehydro TxB₂ in patients with DM, hypercholesterolemia, and hypertension, and correlated with increased vascular events [170] [171]. A recent study revealed that high glucose increases both thromboxane synthesis and TP expression through an aldose reductase-dependent mechanism [169].

One particularly important and commonly measured isoprostane, 8-iso-PGF₂α, is a peroxidation product of AA, and is increased in diabetic patients with levels correlating to both blood glucose and thromboxane metabolite levels [170]. Both thromboxane and isoprostane are effective activators of the thromboxane A₂ receptor (TP), leading to recent interest in using TP antagonism as a therapeutic approach for diabetes [172]. However, there remain significant and unidentified differences in thromboxane and isoprostane signaling, as sCD40L release appears to be selective for thromboxane activation of platelets [173]. Further, antioxidant therapy with vitamin E reduced both isoprostane and thromboxane formation in diabetic patients [170], whereas aspirin has no influence on isoprostane formation. As both thromboxane and isoprostanes activate one of the critical positive feedback signals in platelet activation the TP receptor is becoming recognized as a critical link between insulin resistance and cardiovascular disease [27, 169, 172].

Obesity

As with diabetes mellitus, obesity (often measured by body mass index, BMI) has been a long-recognized risk factor in cardiovascular disease [174], associated with dyslipidemia, oxidative stress, increased thromboxane production [175], and hyperactive platelets [176]. The inverse association of exercise habits with cardiovascular events [177] is often attributed to the resulting obesity. Interestingly, while moderate obesity is associated with atherosclerotic burden and increased thromboxane production, morbid obesity is often associated with reduced atherosclerotic burden and decreased thromboxane production. In cases of extreme obesity, higher BMI measures seem to confer a slight protective effect relative to moderate obesity, though still elevated relative to non-obese [178]. However,

studies found that while hs-CRP and leptin levels increased with BMI, TxB₂ was reduced in both lean subjects and insulin-sensitive morbidly obese patients [175]. Perhaps this sheds some light on the obesity paradox in which morbid obesity reduced risk relative to moderate obesity [179]. Insulin-resistant morbidly obese patients had higher elevated circulating inflammatory markers than those who were insulin sensitive [180]. Furthermore, they found increased ERK phosphorylation and NFκB activation in biopsied visceral adipose tissues of the insulin resistant group, consistent with the increased systemic inflammation, and responsible for downstream alterations in IRS-1 and Akt. These findings support the opinions of others who have long viewed insulin-resistance as a condition of chronic inflammation [181]. More recently an 11 year longitudinal study acknowledged that changes in patient fitness provide substantial long-term benefits in cardiovascular prognosis irrespective of BMI levels [182]. Acute exercise increases F₂-isoprostane formation which is short lived; however, chronic exercise leads to sustained decreases in isoprostane levels [183]. A similar pattern of inflammatory cytokine production with acute exercise and reduction with extended training has been reported [184]. Isoprostanes provide one convergence point between dyslipidemia, oxidative stress, and platelet hyperactivity.

Dyslipidemia

Hypercholesterolemia is known to enhance thromboxane A₂ synthesis [185, 186]. Notably, atherosclerotic plaques in LDL receptor knockout mice on a high fat diet have increased TxAS and TxA₂ levels [187]. The LDL receptor is responsible for cellular uptake of cholesterol rich low-density lipoprotein [188]. Low density lipoprotein (LDL) levels are a risk factor closely related to obesity. Oxidation of low-density lipoprotein generates platelet stimulating lipid mediators including F₂-isoprostane and lysophosphatidic acid (LPA) [189]. While reduction of LDL cholesterol levels by statins has beneficial effects, LDL itself remains harmless until oxidized [190]. Oxidized LDL is a component a plaques, but the oxidation of LDL also releases isoprostanes [191]. Conversely, high density lipoprotein (HDL) has protective effects relative to LDL, largely by promoting cholesterol efflux [192]. Interestingly, whereas isoprostanes are produced from oxidized LDL, HDL is the major lipoprotein carrier of plasma isoprostanes, reducing free isoprostane effects by providing means of both sequestering and inactivating isoprostanes [193]. Infusion of HDL has been shown to reduce atheroma volume in cardiac catheterization patients [194].

The omega-3 fatty acids EPA and DHA have been cautiously reported as having generally beneficial effects in cardiovascular disease [195], by shifting lipid metabolism to less inflammatory pathways, reducing blood pressure and platelet activity. They similarly associate with decreases in the plasma oxidative stress marker, F₂-isoprostanes [196]. Short-term treatment with DHA modifies the prostanoid synthesis profile of megakaryocytes [197], reducing pro-inflammatory arachidonic acid derivative formation. However, simply changing the substrate lipid does not entirely avoid oxidative processing, and platelets treated with DHA possess both pro- and anti-oxidant activities [198]. Although generally favoring less inflammatory eicosanoids over their AA-derived counterparts, extended omega-3 fatty acid consumption can also increase vascular endothelial permeability induced by platelet activating factor [199]. A detailed comparison of the specificities and potencies of 2-series AA-derived and 3-series EPA-derived prostaglandins indicates that EPA is an effective inhibitor of AA oxygenation by PGHS-1 and that 3-series derivatives had reduced efficacy and partial agonism at EP receptors, suggesting a number of possible signaling benefits of omega-3 supplementation. However, the same study also highlights important limitations: namely, that TP activation remains nearly identical, and that PGHS-2 preferentially metabolizes AA series compounds [200].

An important consideration in obesity research is the different characteristics across types of adipose tissue. Adipocytes are generally categorized as white adipose or brown adipose tissue, driven largely by mitochondrial content [201]. Large numbers of active mitochondria (with high iron content) cause some adipocytes to have a brown tint [202] and result in a highly metabolically active cell with many small thermogenic lipid processing droplets, as opposed to the white adipocytes consisting primarily of a single large lipid droplet for long term energy storage. The effect of lipid composition on adipocyte phenotype is not limited to pharmacological manipulation, but occurs endogenously as specific fatty acids are selectively mobilized from adipocyte tissue during lipolysis as a function of the chain length and degree of saturation [203], resulting in preferential mobilization of eicosanoid precursor forms for PGE₂ and PGI which potently influence adipocyte development [204] and lipolysis. Such findings suggest additional opportunities in seeking influence from omega-3/omega-6 lipid balance on adipocyte fate through altered eicosanoid production. This works both ways of course, with white adipocytes promoting PGE₂ biased lipolysis [205], maintaining low cAMP levels relative to brown adipose tissue. Investigating the decision making process behind fate determination in preadipocyte mesenchymal progenitors, Vegiopoulos et al. identified COX-2 as a critical component in the induction of brown adipose tissue formation [206].

Over-expressing COX-2 shifts progenitor cells toward a brown adipose phenotype (with a high metabolic capacity measured by mitochondrial thermogenic genes (UCP1, CIDEA, CPT1B, DIO2). Use of the prostacyclin analog carbaprostacyclin has a similar effect, whereas COX-2 inhibition results in a nearly complete population of white adipose cell types. The same phenotypic shift, when driven by β -adrenergic activity, was also interrupted with COX-2 inhibition, suggesting that this effect was also mediated by eicosanoid production. Additionally, the β -adrenergic receptors, like the prostacyclin IP receptor, couple to Gs, stimulating adenylyl cyclase to produce cyclic AMP. Cyclic AMP stimulates lipolysis [207], whereas cyclic nucleotide phosphodiesterases, stimulated by intracellular calcium flux, hydrolyze cyclic AMP and terminate the signal. Compared to normal weight controls, obese individuals have higher PGE₂ levels, formed predominantly by adipocyte phospholipase A₂ (AdPLA) in white adipose tissue [208]. Removing AdPLA or selectively blocking EP₃ receptors blocked increased PGE₂ expression typically induced by a high fat diet and prevented both weight gain and cAMP suppression.

Platelet Hyperactivity and Interactions with Endothelial and Vascular Smooth Muscle Cells

Many of the risk factors and associated eicosanoids modulate the activity of circulating platelets [209]. There are clear pathophysiological links between platelet alterations and gender [210] [211], hypertension [212–214], chronic smoking [215–217], diabetes [169, 218, 219], and obesity [220–222]. Platelet hyperactivity, in particular, is an effective predictor of a poor prognosis for patients with a recent myocardial infarction [223].

Thromboxane and Prostacyclin

In the context of platelet activity, the most profound eicosanoid effects are observed with thromboxane and prostacyclin. Thromboxane stimulates platelet activation and aggregation through a self-perpetuating positive feedback loop via Gq stimulation of intracellular calcium release. Prostacyclin overrides this process, providing an anti-activation cAMP signal via Gs stimulation of adenylyl cyclase. The platelet activation signal converges on intracellular calcium mobilization, largely through TP and related pathways exacerbate atherosclerosis [224]. Of many downstream effects, TP activation promotes an additional convergence point between dyslipidemia and platelet hyperactivity through CD40/CD40L

stimulation [225]. This response is independent of aspirin [226], highlighting the overlapping integration of multiple activating pathways.

The role of thromboxane as a primary feedback pathway of platelet activation [227] is central to cardiovascular diseases and has been recently reviewed [228]. The TP receptor is activated and stabilized by ROS [229], and TxA₂ ligand is similarly induced by hyperglycemia via ROS [169]. Furthermore, TP stimulation also increases production of ROS [230], revealing yet another level of thromboxane positive feedback amplification. Acute glucose exposure reduces the ability of COX inhibitors to inhibit platelet aggregation regardless of the stimuli [231], reducing the effectiveness of aspirin and exaggerating platelet responses under hyperglycemic circumstances.

TP knockout mice on an apoE null background have reduced atherogenesis, whereas the IP receptor knockout mice develop accelerated atherogenesis [232]. These atherogenic effects include effects on smooth muscle cells, but also on platelet responses. Proliferative and migratory effects of thromboxane on smooth muscle cells are enhanced by platelet-released serotonin (5-HT) at sub-threshold concentrations [233], suggesting an important role for platelet thromboxane release in restenosis. Thromboxane may affect migration in other cell types as well. Mesenchymal stem cells derived from human adipose tissue become migratory and proliferative in response to thromboxane receptor agonism (with U46619) and show markers suggesting differentiation into smooth-muscle like cells [234] via ERK and p38 MAPK. A similar pro-migratory effect of thromboxane receptor activity has been recognized in endothelia [235] where either COX-2 inhibition or TxA₂ antagonism attenuated FGF-induced corneal angiogenesis, and TxA₂ agonism reconstitutes the angiogenic response.

Prostaglandin E₂

The effects of PGE₂ on platelets are less profound and less specific, but no less important. As with hemostasis, the influence of PGE₂ platelet signaling involves counteracting signaling. EP₂ and EP₄ provide a prostacyclin-like Gs/cAMP response, whereas EP₁ elicits a pro-aggregatory thromboxane-like Gq/calcium response, with the additional complication of EP₃-mediated Gi inhibition of adenylyl cyclase. Owing to the presence of multiple receptor targets, PGE₂ provides a modulating effect, shifting the balance between the pro-aggregatory Gq and calcium signal (via EP₁) [236] and the anti-aggregatory Gs-cAMP signal (via EP₂ and EP₄) [237], tempered by an inhibitory influence on cAMP through Gi via EP₃ [238]. The predominant effects on platelets are via the EP₃ and EP₄ receptors [236], though these alternative signaling pathways likely play integral roles in other tissues.

Despite a general observation of PGE₂ inhibiting platelet aggregation [239], specific EP₃ antagonism is often required to observe platelet aggregation. Interestingly, decreasing PGE₂ formation via PGE₂ synthase-1 decreases plaque burden [240], supporting an important, if unclear, role for PGE₂. The platelet inhibitory effect occurs primarily through EP₄, attenuating calcium release [241], though selective stimulation of EP₂ receptors has the same effect [242] on ADP and collagen aggregation [243]. Similar self-regulation with opposing receptor functions is observed in platelets where prostanoid EP₂ and EP₃ receptors differentially modulate activation [244]. An EP₃-selective PGE₂ analog, 11-deoxy-16,16-dimethyl PGE₂, exaggerates intracellular calcium release by ADP, whereas EP₂ selective agonist butaprost inhibits the secondary wave of ADP-induced aggregation.

Similar studies, in attempt to identify the differential influence of EP₂/EP₄ platelet stimulation and EP₃ platelet inhibition, have also indirectly provided insight into unraveling the complexity of the downstream signaling pathways of thromboxane. Stimulating platelets with the thromboxane analogue U46619 in combination with selective EP₃ or EP₄

antagonists showed that EP₃ antagonism eliminated the synergistic aggregation effect without affecting aggregation directly [245]. The logic behind this study was to evaluate the therapeutic potential of PGE₂ inhibition owing to the overlapping ligand selectivity of these two receptors. They noted relatively low levels of plaque PGE₂, limiting the therapeutic implications, but highlighting the role of EP₃ stimulation in the process and supporting the notion that Gi stimulation does not produce aggregation on its own, but makes the cell more sensitive to calcium stimulation by reducing cAMP.

PGE₂, however, is found at lower levels in atherosclerotic lesions than PGE₁ [246]. The relative abundance of these ligands deserves consideration in light of their overlapping specificities and their complex effects. PGE₁ and PGE₂, for instance, both stimulate EP₃ receptors, and both balance the resulting Gi effect by stimulating Gs via either IP, in the case of PGE₁, or EP₂/EP₄ in the case of PGE₂ [237]. These redundant checks and balances keep the system in a dynamic equilibrium maintained through altering both ligand and receptor repertoires in response to each other. Despite the potent influence of prostaglandins on vasoconstriction and vasodilation, the influence of eicosanoids on hypertensive tone is neither limited to prostaglandins nor to the calcium/cAMP balance they maintain.

C-reactive protein

An emerging risk factor in cardiovascular disease is the biomarker of inflammation: C-reactive protein (CRP) [247, 248]. Though less predictive than the combination of traditional risk factors such as age, family history, and smoking [249], CRP may be a superior biomarker to the more commonly used LDL [250] and has been recently recognized as an independent risk factor for coronary heart disease [247, 251] as well as diabetes [252]. C-reactive protein increases along with inflammation, exists in circulating pentameric or bound monomeric forms [253], and instigates complement activation in response to exposed membrane phosphocholine [254].

As with many of the pathways involved in inflammation, positive feedback functions in both directions. Platelet activation converts native circulating pentameric CRP to the inflammatory monomeric CRP [255]. CRP similarly activates platelet aggregation and ATP release in a dose dependent manner [256]. From the perspective of eicosanoid signaling, C-reactive protein modulates the COX-prostanoid pathway, suppressing prostacyclin synthesis and promoting thromboxane receptor transcription [257]. Additionally, CRP blood levels can be reduced by directly altering eicosanoid production with either aspirin treatment [258] or with omega-3 (EPA and DHA) supplementation [259]. Thus, CRP can repress cardioprotective eicosanoids, and eicosanoids can modulate CRP levels.

Leukotrienes

Leukotrienes are highly abundant in atherosclerotic plaques [260] and the 5-lipoxygenase activating protein (FLAP) gene (ALOX5AP) variants (13q12-13) increase leukotriene B₄ (LTB₄) production and confer risk for MI and stroke. [261]. Though CysLT receptors are predominantly found on mast cells, eosinophils, and endothelia, CysLT₁ receptors exhibit a potent smooth muscle contractile response [262]. CysLT₂ antagonist decreased neutrophil infiltration and leukocyte adhesion molecule expression reducing ischemia-reperfusion (I/R) damage from myocardial infarction, a process resulting from reactive oxygen species formation as part of the innate inflammatory response [263]. CysLT₂R acts exclusively through Gq calcium mobilization in endothelia [264], whereas CysLT₁R promiscuously couples with Gq and Gi [265]. Overexpression of CysLT₂R increases endothelial permeability and aggravates I/R injury [266] [267], where selective CysLT₂R antagonism reduces post ischemia-reperfusion. Similarly, CysLT₁ antagonism with Monetlukast reduces ischemic injury [268] and associated histopathological alterations [269].

The BLT₁R receptor, a Gq coupled, LTB₄ sensitive receptor in VSMC [270], when knocked out, decreases atherosclerosis in a hyperlipid rat model [271], via role in smooth muscle recruitment [272] [273]. Similarly, BLT antagonism improves atherosclerotic burden in mice [274] via reduced monocyte recruitment and foam cell formation. A CysLT₁R antagonist, montelukast, inhibits atherosclerotic lesion size and intimal hyperplasia through effects on VSMC proliferation [275, 276]. Montelukast treatment also improves endothelial cell function, reduces vascular ROS production, and ameliorates plaque generation in mice [277]. Just as hyperglycemia promotes oxidative stress, dyslipidemia leads to hyperglycemia via insulin resistance. As with hyperglycemia, hypercholesterolemia is also associated with hyperactive platelets [278], likely through increased isoprostane and thromboxane production [191].

Prostaglandin D₂

Like leukotrienes, Prostaglandin D₂ is more commonly associated with asthma and allergy [279] than with cardiovascular disease, though PGD₂ does play an increasingly recognized roll in hemostasis [280] [281]. Serum levels of Localin-type prostaglandin D synthase (L-PGDS) are strongly associated with hypertension [282] and are predictive of coronary artery disease severity in patients undergoing diagnostic angiography [283]. Another clinical study showed that increased serum L-PGDS correlates with increased coronary artery intima-media complex thickness and brachial-ankle pulse wave velocity [284]. In summary, increased serum L-PGDS associates with overall risk factor profile and measures of atherosclerotic progression.

Receptors DP₁ and newly discovered CRTh₂ (DP₂) have distinct structural characteristics and ligand selectivity [285], and are both stimulated by PGD₂. CRTh₂ is also activated by TxB₂, the metabolite of TxA₂ [286]. PGD₂ is formed by immune cells [287] which accumulate in atherosclerotic lesions [288]. PGD₂ inhibits iNOS expression [289] and L-PGDS knockout mice display aggravated obesity and atherosclerosis [290].

Challenges and Opportunities in Eicosanoid Receptor Pharmacology

Only recently have the importance of eicosanoid roles in the development of clinical human atherothrombosis become recognized. These became particularly apparent after identifying the cardioprotective role of selective COX-1 inhibition (e.g. low dose aspirin) [291] and the clear cardiovascular adverse effects of selective COX-2 inhibition (e.g. rofecoxib) [292]. These have all been further supported by many elegant mouse studies [85, 293, 294]. It now remains to be seen whether direct targeting of the downstream receptors will be of any further benefit (and cost effective). For such advances, understanding the structure/function of each receptor in a defined tissue is critical. Naturally occurring mutations can be used clinically as “human genetic functional knockouts” to study effects on human tissues and disease; however, it is becoming apparent that each individual mutation may provide specific structural perturbations that may lead to quite different signaling defects (TABLE 2). Moreover, deciphering such processes as dimerization and alternative splicing, and understanding defects in such processes, will provide insights into the modulation of human disease. Indeed, targeting eicosanoid-related disease processes at the level of their respective GPCRs may require conformation-specific ligands. Owing to the complexity of receptor structure and function [295], in combination with the complexity of the inflammatory basis of atherothrombosis [2], the intimate involvement of platelets [10] and many other cardiovascular cells, and the overarching influence of risk factors such as dyslipidemia [296], investigations into eicosanoid signaling will continue to play a central role in seeking a unifying picture of atherothrombotic development.

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Figure 1.

Arachidonic acid is oxidized by various means to produce downstream signaling mediators. The complexity of these pathways result from differential processing of each of the major signaling classes (prostaglandins, epoxyeicosanoids, and leukotrienes) producing ligands with overlapping and counteracting receptor interactions. These interactions predominantly converge on two opposing intracellular signals resulting in cellular hyperpolarization (via cAMP) or cellular depolarization (via intracellular calcium flux).

Figure 2.

A subset of eicosanoids provides convergent stimuli from dominant risk factors. Sex-specific hormones influence hypertension through differential effects on the production of cardioprotective prostacyclin and related mediators. Smoking and metabolic syndrome both promote reactive oxygen species. A common downstream effect of each mechanism includes contributing to platelet hyperactivity. Inflammatory cytokines released from platelets exaggerates these effects through positive feedback signaling.

Table 1

Eicosanoid Receptors involved in Atherothrombosis

Receptor	Eicosanoid Ligand	Primary Effectors	Vascular Expression	Influence
IP (PTGIR)	Prostacyclin (PGI ₂)	Gs	Endothelia, VSMC, Platelets	Vasodilation, Anti-aggregation
TP (TBXA2R)	Thromboxane (TxA ₂), isoprostanes, PGH ₂	Gq/G ₁₁	Platelets, VSMC, Macrophage	Vasoconstriction, Aggregation
EP ₁ (PTGER1)	PGE ₂	Gq	VSMC, Endothelia	Vasoconstriction, Aggregation
EP ₂ (PTGER2)	PGE ₂ , OxPAPC	Gs	Platelets, VSMC	Vasodilation, Anti-aggregation
EP ₃ (PTGER3)	PGE ₂	Gi	Platelets, VSMC, Endothelia	Vasoconstriction, Aggregation
EP ₄ (PTGER4)	PGE ₂	Gs	Platelets, VSMC, Endothelia	Vasodilation, Anti-aggregation
DP ₁ (PTGDR)	PGD ₂ , PGJ ₂	Gs	Endothelia, VSMC, Platelets,	Vasodilation, Anti-aggregation
CRTh2 (DP2, GPR44)	PGD ₂ , 11-dehydro-TxB ₂ , OxPAPC	Gi/Go	Endothelia	Vasoconstriction
FPa (PTGFR)	PGF _{2a}	Gq/G ₁₁	Endothelia, VSMC	Vasoconstriction
FPb	PGF _{2a}	Gs	Endothelia, VSMC	Vasodilation
CysLT ₁ R (Cyslt1)	LTC ₄ , LTD ₄ , LTE ₄	Gq	Endothelia, Leukocytes, Platelets	Vasoconstriction, Aggregation
CysLT ₂ R	LTC ₄ , LTD ₄ , LTE ₄	Gq/G ₁₁	Endothelia, Platelets	Vasoconstriction, Aggregation
GPR17 (UDP/CysLT)	LTC ₄ , LTD ₄ , UDP	Gi, Gq/G ₁₁	Endothelia	Vasoconstriction
BLT ₁ R (Ltb4r)	LTB ₄	Gq/G ₁₁	Leukocytes, Endothelia, Macrophage	Vasodilation
BLT ₂ R (Ltb4r2)	LTB ₄	Gq/G ₁₁	Macrophage, Endothelia	
OXE (GPR170)	5-oxo-EET	Gi	Leukocytes, Macrophage	

Table 2**Challenges and Opportunities in Eicosanoid Receptor Pharmacology**

Challenge	Effected Receptor	Phenomenon and Implications	References
Alternative Splicing	TP		
	TP	Opposite AC coupling, different R ⁶⁰ L sensitivity, desensitization, regulation by NO and PGI ₂ , timing of ERK activation	[297–301]
	EP ₃		[302]
	FP	Altered drug response	[303]
Heterodimerization	IP/TP	Form new iPE ₂ III binding site	[304]
	IP/TP	Co-regulation via endocytosis	[305]
	IP/TP	Promote TxA ₂ cAMP response	[306]
	TP α /TP β	Enhance isoprostane signal	[307]
	CysLT ₁ /CysLT ₂	Altered internalization	[308]
	CysLT ₁ /GPR17	Negative regulator	[309, 310]
Genetic Variants	IP: R ²¹² C	Accelerated atherothrombosis through Dominant-negative influence	[87, 311, 312]
	IP: R ²¹² H	pH sensitivity	[313]
	IP: R ⁷⁷ C, R ²¹⁵ C	Impaired cAMP production	[314]
	IP: M ¹¹³ T, L ¹⁰⁴ R, R ²⁷⁹ C	Misfolding, impaired binding	[315]
	TP: R ⁶⁰ L, D ³⁰⁴ N	Severe bleeding, abolished ligand binding	[316]
	CysLT ₁ : G ³⁰⁰ S	Asthma, atopy	[317]
	CysLT ₂ : A ⁶⁰¹ G	Reduced ligand affinity, Atopy	[318, 319]