I. Biochemistry and Cell Biology

A. Cyclooxygenase (COX) localization – COX I and COX II are located on the luminal faces of endoplasmic reticulum (ER) membranes, and on the intermembrane faces of the nuclear envelope (NE). (The bilaminar (double) NE is continuous with the ER.) In contrast to most integral membrane proteins, such as glucagon and epinephrine receptors and the LDL receptor, COX I and COX II do not pass through membranes. They are said to be monotopically inserted into membranes, and are exposed on only one side, in this case the luminal side, of the membrane. Eicosaenoic acids (cyclooxygenase substrates such as arachidonic acid) reach the COX active site by diffusing through a short patch of membrane into a “protein tunnel” that is part of the enzyme-membrane contact region. Differences in the structure of this tunnel in COX I and COX II have contributed to the design of specific inhibitors.

B. 5-Lipoxygenase (5-LO) and PLA2 localization – Leukotriene A4 (LTA4) synthesis occurs predominantly, if not exclusively, on the nuclear envelope (NE). However, in unstimulated cells, 5-lipoxygenase (5-LO), the enzyme catalyzing the conversion of arachidonic acid to LTA4 is localized in the cytosol and nucleoplasm. Stimulation of leukotriene producing cells such as neutrophils, macrophage, and mast cells, results not only in activation of phospholipase, as in prostaglandin producing cells, but also in 5-LO translocation to the NE. FLAP (5-Lipoxygenase Activating Protein), a protein that spans the NE membrane, appears to recruit 5-LO to the NE. Interestingly, a phospholipase A2 (PLA2) isoform is also recruited from the cytosol to FLAP, and is presumed to generate arachidonic acid (or other eicosaenoic acids) from phospholipids in the NE membrane.

Accumulation of the PLA2 isoform at the NE (and ER) also occurs following stimulation of prostaglandin producing cells. Although the mechanics of 5-LO and PLA2 translocation are not well characterized, the process is of considerable interest as both an additional natural control point in eicosanoid biosynthesis, and as a potential target of new NSAIDs.

C. Regulation of eicosanoid biosynthesis – Until recently it was assumed that the major, and perhaps only, controls of eicosanoid biosynthesis were at the levels of phospholipase activation (including translocation), and where appropriate, COX II transcriptional induction or repression. The remaining enzymes of the biosynthetic pathways were assumed to be present at fixed, constitutive levels, specific to certain cell types (for example; PGI2 synthase in endothelial cells, TXA2 synthase in platelets, and PGE2 synthase in a wide variety of cell types). It now appears that in at least some eicosanoid mediated biological processes, such as inflammation (a complex process that includes vasodilation, increased vascular permeability, increased leukocyte-endothelial cell adhesion, increased leukocyte synthesis, and chemotaxis), the levels of both COX II and some synthases are coordinately regulated.

In regard to COXII transcriptional induction, recent studies suggest that both insulin and estrogen induce COXII gene transcription and subsequent PGI2 production in endothelial cells. These results support previous findings indicating that insulin and estrogen are antithrombotic.

D. Eicosanoid transport – Prostaglandins (including PGIs and TXAs) probably exit producing
cells by facilitated transport through a single transporter, while LTB$_4$ and LTC$_4$ exit through two different specific transporters. The prostaglandins, LTB$_4$ and LTC$_4$, are all produced intracellularly, while the conversion of LTC$_4$ to LTD$_4$ and LTE$_4$ occurs extracellularly. Since LTD$_4$ may be the most potent of the leukotrienes in stimulating airway smooth muscle cell contraction (bronchoconstriction), the potential ability to inhibit its synthesis with drugs that can remain extracellular may be particularly valuable for the treatment of asthma.

II. Signaling Pathways

A. G protein-coupled receptors – The eicosanoids that have been studied (including essentially all the major prostaglandins and leukotrienes) signal predominantly, and perhaps exclusively, through specific heterotrimeric G Protein-Coupled Receptors (GPCRs). Specifically, binding of the eicosanoid to an extracellular domain of the receptor (which contains seven membrane spanning hydrophobic $\alpha$-helices) allows the receptor to stimulate the exchange of GTP for GDP on an associated intracellular heterotrimeric G-protein ($G_\alpha$-GDP)($G_{\beta\gamma}$). The heterotrimer then dissociates to $G_\alpha$-GTP and $G_{\beta\gamma}$, both of which are capable of interacting with particular effectors. There are many isoforms of $G_\alpha$ (more than twenty) with different receptor and/or effector specificities, as well as different tissue distributions. For example, $G_s$-GTP stimulates adenylyl cyclase (also referred to as adenylate cyclase), $G_i$-GTP inhibits this cyclase, and $G_q$ stimulates a particular isoform of phospholipase C ($PLC_\beta$). (Glucagon receptors and $\beta$-adrenergic receptors like the one for epinephrine, each coupled to $G_s$, are two more familiar examples of GPCRs).

Prostaglandin (including prostacyclin and thromboxane) receptors are designated with the letter P preceded by a standard PG abbreviation, such as E, D, F, I (prostacyclin), T (thromboxane), etc. A subscript after the P indicates that there is more than one receptor specific for that PG. Leukotriene receptors are designated with the letters LT, preceded by a standard LT abbreviation such as B or Cys (for any of the cysteine containing LTs, specifically, LTC$_4$, LTD$_4$, and LTE$_4$).

There are at least ten PG and four LT receptors. Their specificities and $G$ partners where known are listed below:

- EP$_1$ ($G_q$); EP$_2$ ($G_s$); EP$_3$ ($G_i$); EP$_4$ ($G_s$)
- DP$_1$ ($G_s$); DP$_2$
- FP($G_q$)
- IP ($G_s$)
- TP$_\alpha$ ($G_q$); TP$_\beta$ ($G_q$)
- B-LT$_1$; B-LT$_2$
- CysLT$_1$; CysLT$_2$

B. Steroid hormone family-like receptors – A subclass of the steroid family of nuclear transcriptional regulating receptors, known as the PPAR subfamily, binds some eicosanoids.
(Other members of the steroid receptor family bind steroids, retinoids, thyroxine, or vitamin D.) However, efficient binding requires micromolar eicosanoid concentrations rather than the nanomolar levels typical for GPCRs, and thus it is not clear whether eicosanoids are bona fide endogenous PPAR ligands.

C. Some examples of specific eicosanoid signaling pathways – The following outlines present partial pathways (some more fleshed out here than others), that illustrate the biological functions of some prostaglandins and leukotrienes.

1. Smooth muscle contraction:

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\text{PGF}_2 \to \text{FP (vascular smooth muscle)} \to \text{G}_{q}\text{-GTP} \to \text{PLC} \to \text{second messengers (diacyl glycerol; inositol trisphosphate)} \to \text{increased intracellular (Ca}^{++}\text{)} \to \text{activation of actin-myosin complexes} \to \text{contraction}
\]

\[
\text{TXA}_2 \to \text{TP}_\beta (\text{vascular smooth muscle}) \to \text{G}_{q}\text{-GTP} \to \text{PLC} \to \text{second messengers (diacyl glycerol; inositol trisphosphate)} \to \text{increased intracellular (Ca}^{++}\text{)} \to \text{activation of actin-myosin complexes} \to \text{contraction}
\]

\[
\text{LTD}_4 \to \text{CysLT}_1 (\text{airway smooth muscle}) \to [\text{G}_q\text{-GTP}] \to \text{contraction}
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2. Smooth muscle relaxation:

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\text{PGE}_2 \to \text{EP}_2 (\text{smooth muscle}) \to \text{G}_s\text{-GTP} \to \text{adenylate cyclase} \to \text{second messenger (cAMP)} \to \text{PKA} \to \text{inhibition of actin-myosin complexes} \to \text{relaxation}
\]

\[
\text{PGI}_2 \to \text{IP (vascular smooth muscle)} \to \text{G}_s\text{-GTP} \to \text{adenylate cyclase} \to \text{second messenger (cAMP)} \to \text{PKA} \to \text{inhibition of actin-myosin complexes} \to \text{relaxation}
\]

3. Chemoattraction; increased cell-cell adhesion:

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\text{LTB}_4 \to \text{B-LT}_1 (\text{neutrophils}) \to [\text{G}_q\text{-GTP}] \to \text{chemoattraction}
\]

\[
\text{LTB}_4 \to \text{B-LT}_1 (\text{neutrophils}) \to \text{increased expression of cell surface molecules that stimulate neutrophil-endothelial cell adhesion}
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\[
\text{LTD}_4 \to \text{CysLT}_1 (\text{endothelial cells}) \to \text{increased expression of cell surface molecules that}
\]
stimulate leukocyte-endothelial cell adhesion.

D. The clinical and basic science relevance of receptor studies – The identification and cloning of receptors represents a major advance in eicosanoid research. “Knockouts” of all of the receptors noted here have been generated in mice, and their examination is yielding major insights into eicosanoid function. (For example, IP-/- mice are viable, but exhibit extremely robust blood clotting responses.) In addition, knowledge of receptor structure is allowing the design of specific agonists and antagonists, such as the CysLT1 antagonist montelukast (Singulair) which is effective in treating some forms of asthma and allergic inflammation. In analogy with studies of a wide variety of other cell signaling receptors, it is also assumed that genetic variants of eicosanoid receptors will be identified and correlated with a variety of known and yet to be characterized medical conditions. Finally, it appears that the expression of at least some eicosanoid receptors may be regulated. For example, insulin increases IP levels in platelets while retinoic acid decreases TP levels. These results suggest that insulin and retinoic acid are both antithrombotic.

III. Some Clinical Correlations Related to Aspirin and COXIBs

The roles of prostaglandins in mediating fever (through EP3 receptors on neurons), pain (through EP1 and IP receptors on neurons), thrombosis (through IP and TP receptors on platelets and vascular smooth muscle), and inflammation (in conjunction with leukotrienes and through a variety of receptors on leukocytes and endothelial cells) are relatively well established. Not so well established are reports of increased COX II expression and prostaglandin production (notably PGE2) in certain tumors, particularly those of the colon and breast, the observation that this increased expression stimulates cell survival and angiogenesis, as well as estrogen production in the breast, and a multitude of clinical trials that suggest that a variety of NSAIDs (including aspirin and specific COX II inhibitors, the so-called COXIBs) may prove beneficial in treating and/or preventing colon cancer. Also not so well established are reports that PGE2 can increase the expression of amyloid precursor protein in glial cells and that some NSAIDs may be beneficial in treating Alzheimer patients. (Alzheimer patients produce increased levels of a proteolytic peptide derived from amyloid precursor protein.) Whether the NSAID data are related to decreased precursor protein levels, or to an indirect anti-inflammatory effect, is not known. Rather than trying to reconcile all of the often conflicting data on the effectiveness of NSAIDs in cancer therapy and dementia treatment, the following discussion will outline some of the more accepted clinical correlations related to eicosanoid metabolism.

A. Aspirin and COXIB usage

1. Aspirin usage – Aspirin treatment is generally divided into three categories based on dosage:

   **Aspirin I.** Low dose. In the range of 50-100 mg/day. Reduces platelet TXA2 production; anti-thrombotic; primarily inhibits COX I.

   **Aspirin II.** Intermediate dose. 2-4 g/day. Analgesic, antipyretic and mildly anti-inflammatory; globally inhibits COX I and COX II.
Aspirin III. High dose. 6-8 g/day. Analgesic, antipyretic, anti-inflammatory (as effective as cortisone); serum concentration in millimolar range; globally inhibits COX I and COX II.

Since Cox I is more sensitive to aspirin than COX II (the isoform that mediates fever, pain and inflammation), analgesic, antipyretic and anti-inflammatory aspirin levels hit COX I hard. The so-called “housekeeping” cyclooxygenase, COX I, is present in many tissues and regulates such diverse processes as stomach HCl secretion (inhibition), thrombosis (stimulation) and kidney function. Therefore, high, intermediate, and in some cases, even low dose aspirin treatment, can result in unwanted side effects such as ulcers (primarily gastrointestinal) and/or bleeding.

2. COXIB usage – Selective COX II inhibitors such as celecoxib (Celebrex) and rofecoxib (Vioxx), known collectively as COXIBs, generally bypass the unwanted side effects attributed to aspirin but may exhibit side effects of their own (see below). Nevertheless, they have proven to be particularly effective in managing the chronic pain and inflammation associated with a variety of conditions, including rheumatoid arthritis and osteoarthritis.

B. Risk-benefit considerations of aspirin and COXIB usage – How can one analyze whether the benefit ascribed to a particular NSAID outweighs the potential risk of its usage? At the least one needs to know what percentage of a random population clearly benefits from drug treatment, and what percentage clearly exhibits an increase in unwanted side effects. However, since each patient is unique, one also needs to consider genetic predisposition, personal history, and the fact that some people are more than willing to reduce a low chance of developing what they consider a serious condition in exchange for a higher chance of inducing one they hope will be less serious. The analysis is not easy, since not all of the statistics are accepted, and so the following three considerations are a bit subjective.

1. Cardiovascular disease and aspirin – There appears to be no doubt that low dose aspirin reduces myocardial infarction frequency in men, but even at low doses, gastrointestinal side effects are more frequent than had been expected. It has been suggested that low dose aspirin treatment is statistically most beneficial for patients who are either genetically predisposed, or have a personal history of cardiovascular disease.

   For example, high risk patients (men and women) with previous myocardial infarctions, ischemic strokes, or transient ischemic attacks, generally have a five to ten percent annual risk of having another serious “vascular event”, while low risk patients (without a previous history or genetic predisposition to cardiovascular disease) have less than a one percent annual risk. Low dose aspirin treatment reduces the occurrence of myocardial infarctions in both high and low risk groups by about thirty percent, which corresponds to the prevention of about 10-20 attacks per 1000 patients/year in the high risk group and 1-2 attacks per 1000 patients/year in the low risk group. However, long term low dose aspirin therapy approximately doubles the risk of a serious extracranial bleeding episode in both groups, which corresponds to about 1-2 excess events per 1000 patients/year for each group. (For the high risk group the above data applies to both men and women using daily aspirin dosages of 75-100 mg. For the low risk group, the data also appears to apply to both men and women, if one selects to de-emphasize the “Women’s Health Study” which used an aspirin dose of 100 mg every other day.)

   Finally, an immediate dose of 150-200 mg aspirin is recommended in clinical settings for
individuals who are having, or who have just had, a heart attack or ischemic stroke. Outside of a clinical setting one tablet (300 mg) is the usual dose.

2. Colon cancer and aspirin or COXIBs – A variety of studies evaluating the effects of many NSAIDs in the prevention and treatment of human colon cancer have been reported or are in progress. As noted previously, the results are controversial, but suggest that aspirin (80 mg to 300 mg/day) may be beneficial, especially for patients with a genetic predisposition or personal history of the disease, where unwanted side effects are worth the risk. Surprisingly, COXIBs and other NSAIDs are not as effective, except for COXIBs in patients with a severe genetically inherited form of colon cancer (Adenomatous Polyposis Coli).

3. Fever, pain, inflammation, and aspirin or COXIBs – Intermediate and high dose aspirin is quite effective in controlling fever, pain and inflammation. However, treatment if prolonged, is often associated with side effects that result in an unacceptable risk/benefit ratio. Some of the risks (for example, bleeding) can be reduced or relieved by the use of reversible COX inhibitors such as ibuprofen, indomethacin, or acetaminophen, or as noted previously, by the use of COXIBs.

An unusual aspirin usage risk is known as Aspirin Sensitive (Intolerant) Asthma. About five percent of asthma sufferers (specifically those who tend to have high leukotriene levels) exhibit even higher leukotriene levels and severe broncho-constriction following aspirin usage. The ability of aspirin to increase leukotriene levels is due to increased biosynthesis through a so-called “shunting” mechanism. Leukocytes stimulated by asthma inducing agonists produce arachidonic acid that is channeled through either the COX or 5-LO pathways. Irreversible COX inhibition essentially blocks one pathway, and allows the shunting of the excess arachidonic acid through the other.

COXIB treatment for pain and inflammation should bypass aspirin related risks, but high dose, long term usage of most drugs is rarely problem free. For example, COX II is the major cyclooxygenase in PG\(_{\text{I}2}\) producing endothelial cells, and there are suggestions that long term COXIB usage can promote thrombosis. For safety, long term COXIB therapy should probably not be prescribed for individuals with either a genetic predisposition or personal history of cardiovascular disease.

IV. Some Clinical Correlations Related to Leukotriene Synthesis and Action Inhibitors

The new leukotriene synthesis and action inhibitors, referred to collectively as “leukotriene modifiers” or “antileukotrienes” are either five lipoxygenase inhibitors (such as Zileuton) or CysLT\(_1\) receptor antagonists (such as Singulair). At least in the case of severe asthma, they have not proven to be as effective as expected, but are of value in treating mild to moderate asthma and aspirin intolerant asthma. The problem here may relate to the fact that leukotrienes are not the only bronchoconstricting agents in asthmatics, and to the possible presence of lipoxygenase and receptor genetic variants. Unexpectedly, however, leukotriene modifiers have proven to be quite effective in treating some inflammatory conditions such as allergic rhinitis. Also, since leukotrienes have recently been proposed to be major contributors to the inflammatory component of atherogenesis, leukotriene modifiers may be of value in the treatment of vascular
V. Actions of Aspirin and COXIBs That Are Independent of COX Inhibition

When the mechanism of aspirin function as an irreversible COX I inhibitor was discovered in the 1970's (COX II was not identified until 1991), and the COXIBs were designed as COX II inhibitors in the 1990's, it was assumed that these drugs would be specific for cyclooxygenases. However, they may not be as specific as hoped. For example, high dose levels of aspirin and other salicylates inhibit a kinase (IKK) that normally activates a transcription factor (NFkB) that stimulates the transcription of a variety of pro-inflammatory genes, including COX II. NFkB also stimulates the transcription of other genes that promote cell survival. Taken together, the inhibition of NFkB by salicylates results in cyclooxygenase independent anti-inflammatory and anti-cell survival effects. It was certainly unexpected that two structurally and mechanistically unrelated enzymes such as COXs and IKK would be salicylate targets, or that the inhibition of either of them could result in similar phenotypes (anti-inflammatory/anti-cell survival). None of the COXIBs or other reversible COX inhibitors studied inhibits IKK, but Celebrex has been reported to inhibit another cell survival promoting kinase (PDK). It is not clear whether any of these non-COX specific inhibitions are physiologically relevant, but their discovery may lead to new areas of pharmacological research.