INTRODUCTION

INFLAMMATION

Inflammation usually occurs as a normal defensive mechanism of the body to injury and comprises a complex series of vascular, humoral, and cellular events at or near the site of injury. Agents and means of provoking the response include mechanical trauma (especially crushing), radiation (thermal, UV, radioactive emanations), direct chemical damage (caustic and corrosive chemicals), secondary chemical or biochemical damage (metabolic inhibitors, anoxia), invading organisms (viruses, bacteria, parasites) and last but by no means least antibody-antigen reactions (Bowman and Rand, 1980; Guyton and Hall, 1996; Gallin and Snyderman, 1999). Inflammatory reactions are generally divided into two types: acute and chronic inflammation (Bowman and Rand, 1980).

Acute inflammation is the initial response to tissue injury; it is mediated by the release of autacoids. Some of the autacoids involved are histamine, serotonin, bradykinin, prostaglandins and leukotrienes (Katzung and Furst, 1998). Histamine and serotonin are believed to mediate the initial phase of inflammation (1 - 1½ h), and kinins the second phase (1½ - 2 h), PGs probably exert their proinflammatory effects in the late phase of inflammation (2½ - 6 h) (Lee and Katayama, 1992). Acute inflammation is characterized by the classic signs of pain, heat, redness, swelling and impairment of function. Microscopically, it involves a complex series of events including dilation of arterioles, capillaries and venules with increased permeability and blood flow; exudation of fluids, including plasma proteins; and leukocytic migration (polymorphonuclear leukocyte, PMNs) into the inflammatory focus (Gallin and Snyderman, 1999). If the initiating stimuli for an inflammatory reaction are not eliminated by the reaction or controlled adequately, a continuing state of inflammation persists (Bowman and Rand, 1980). Duration of the process is usually measured in hours or days (Furst and Munster, 2001).

Chronic inflammation is a long lived reaction, the reaction persisting for weeks or months after the initial exposure to the damaging agent (Hurley, 1983). Characteristically, there is an abundance of exudate, granulomatous tissue, monocytosis with many multi-nuclear giant cells formed by their fusion, lymphocytosis and accumulation of plasma cells. The connective tissue invasion results in the formation of much fibrous tissues (Bowman and Rand, 1980). Chronic inflammation involves the release of a number of mediators that are not prominent in the acute response. Some of these are interleukins 1, 2, 3 and 6, tumor necrosis factor alpha (TNF-α) and interferons. One of the most important conditions involving these mediators is rheumatoid arthritis, in which chronic inflammation results in pain and destruction of bone and cartilage that can lead to severe disability and in which systemic changes occur that can result in shortening of life (Katzung and Furst, 1998).

There is morphological variation in acute and chronic inflammation: (1) serous inflammation, this reaction marked by the outpouring of a thin fluid that, depending on the size of injury, is derived from either the blood serum or the secretions of mesothelial cells lining the peritoneal, pleural, and pericardial cavities; (2) fibrinous inflammation, this occurs as a consequence of more severe injuries, with the resultant greater vascular permeability allowing larger molecules (fibrinogen) to pass the endothelial barrier. Fibrinous exudates may be degarded by fibrinolysis and the accumulated debris may be removed by macrophages, resulting in restoration of the normal tissue structure. However, failure to completely remove the fibrin results in the ingrowth of fibroblasts and blood vessels, leading ultimately to scaring; (3) ulceration, this refers to a site of inflammation where an epithelial surface (skin, gastric epithelium, oolonic mucosa. bladder epithelium) has become necrotic and eroded, often with associated subepithelial acute and chronic inflammation. This can occur as a consequence of toxic or traumatic injury to the epithelial surface (e.g., peptic ulcer). The peptic ulcer of the stomach or duodenum illustrates the typical finding. There is usually an early intense neutrophillic infiltration with associated vascular dilation. In contrast, with chronic lesions, the area surrounding the ulcer develops fibroblastic proliferation, scaring, and

the accumulation of chronic inflammatory cells (lymphocytes, macrophages, plasma cell) (Mitchell and Cotran, 1997: Collins, 1999)

MEDIATORS OF INFLAMMATION

The criteria used to determine whether an endogenous substance can be positively considered as an inflammatory mediator, were first considered by Dale (1911) and restated by Vane (1972). These criteria are as follows:

1. The mediator should be detectable, at the site of inflammation, at the right time, in amounts adequate to account for the effect under consideration.
2. The mediator, when administered in concentrations of the order of those found in the lesion, should produce the observed effects, and no other.
3. Specific blocking agents or antagonists of the effects of the proposed mediator should prevent or attenuate the effect.
4. Prevention of release of the mediator should abolish or prevent the effect.
5. Agent or procedures preventing the breakdown or removal of the mediator should prolong or potentiate the effect.

Mediators which suit the above criteria and are specified as inflammatory mediators are as follows:

I. Vasoactive Amines

Histamine and serotonin (5-Hydroxytryptamine)

Histamine, which is present throughout body tissues, is formed by decarboxylation of the natural amino acid, histidine. Histamine is stored primarily in mast cells, which are usually found alongside blood vessels, and also in circulating basophils especially at site of potential tissue injury (Owen, 1987; Burkhallter *et al.*, 1998). Histamine release is based on acute injury caused by each of a wide variety of noxious stimuli ranging from anaphylaxis through to both chemical and physical insults. In recent years, the capacity of histamine to modify lymphocyte function which may be relevant to inflammation has also become appreciated. Studies conducted *in vitro* have

suggested potentially important anti-inflammatory activity of histamine on leukocytes, although demonstration of important *in vivo* consequences of this action has proved elusive. Histamine is clearly important in acute inflammation associated with mast cell degranulation in non-rodent species including man (Owen, 1987), whereas in certain rodents, serotonin may be of equal or greater importance because these substances have in common the structural features of a amine group and share common functional effects on blood vessels (Abbas *et al.*, 1997). Serotonin causes dilatation of arterioles, together with constriction of venules, with the result that capillary pressure rises and fluid escapes from the capillaries. In large vessels, both arteries and veins, are usually constricted by serotonin, though the sensitivity varies greatly. This is a direct action on vascular smooth muscle cells, mediated through 5-HT_{2A} receptors. Serotonin can also cause vasodilatation by several mechanisms, all operating through 5-HT_1 receptors (Rang *et al.*, 1995).

The acute inflammatory response to histamine comprises vasodilatation, an increase in microvascular permeability and edema formation. Pharmacological analysis of the receptor involvement in these component parts of the inflammatory response has shown that the vasodilatation involves both H_1- and H_2- receptors. Histamine can also cause pain and itching. An alternative role for histamine might be as a co-mediator of inflammation. In acute inflammation, histamine could both act as the vasodilator and increase vascular permeability but in chronic inflammation would only fulfil the vasodilator role, perhaps serving to potentiate the increase in microvascular permeability caused by second mediators such as prostaglandins. Histamine is relatively unimportant in the later stages of the response. Thus, inhibition of histamine responses delays but does not prevent the inflammatory response (Owen, 1987).

II. Plasma protease

Kinins

Kinins are a family of small peptides formed in blood and biological fluids. They are formed enzymatically by the action of enzymes known as kallikreins or kininogenases acting on protein substrates called kininogens. Three kinins have been

identified in mammals: bradykinins, kallidin (lysylbradykinin) and methionyllysylbradykinin. Note that each kinin contains bradykinin in its structure (Reid, 1998). A variety of factors including tissue damage, allergic reactions, viral infections and other inflammatory events activate a series of proteolytic reactions that generate bradykinin and kallidin in the tissue (Babe and Serafin, 1996). The kinins generated locally contribute to the acute and possibly the chronic phase of the inflammatory reaction by producing vasodilation, local edema, pain and synthesis of prostaglandins (Regoli, 1987; Babe and Serafin, 1996). Kinins may also modulate migration of white blood and tissue cells that take part to the inflammatory process. Kinin are among the most potent activators of prostaglandin, histamine and/or 5-hydroxytryptamine release and indeed some of their major actions - for instance the endothelium mediated vasodilation, the production of pain, the smooth muscle contraction or relaxation in various organs - are associated with release of prostaglandin. It has also been shown that kinins promote the release of prostacyclins from vessels and from cell cultures (rat adipocyte, human endothelium cell) possibly by interacting with membrane phospholipases. Recent findings indicate also that kinins and other peptides activate PGE_2 production by deriving their arachidonic acid from phospholipid.(Regoli, 1987). There are at least two distinct receptors for kinins, which have been designated B_1 and B_2 (Babe and Serafin, 1996). B_2 receptors mediate a large number of rapidly occurring biological effects, particularly the symptoms and signs of inflammation, while activation of B_1 receptors produces a range of proinflammatory effects including edema, pain and promotion of blood-borne leukocyte trafficking (Regoli, 1987; Babe and Serafin, 1999; Calixto *et al.*, 2000). Considerable effort has been directed toward developing kinin receptor antagonists, since such drugs have considerable therapeutic potential as anti-inflammatory and antinociceptive agents. Actions of kinins mediated by prostaglandin generation can be blocked nonspecifically by inhibitors of prostagladin synthesis such as aspirin (Reid, 1998; Reid, 2001).

Complement

The complement system of blood plasma and extravascular tissue fluid plays an important role in many immune defense reactions and absence of a functional complement system reduces many inflammatory reactions. Activation of complement is mediated by either of two distinct pathways, known as the classical and alternative pathway. The classical pathway is initiated by antigen-antibody complexes while the alternative pathway is typically activated by bacterial cell wall carbohydrates. Complement activation promotes acute inflammation, recruitment of leukocytes and killing of pathogens by phagocytosis, and lysis or release of toxic products. The large fragment of C3 cleavage, C3b, binds covalentry to the activator and promotes the important defence reactions of immune adherence and phagocytosis. Addition of C3b to C3 convertases results in the formation of a C5 convertase which cleaves C5 into C5a and C5b. This is the last recognised enzymatic step in the complement cascade because the larger fragment, C5b, and the later components, C6, C7, C8 and C9, from the lytic complex by a series of hydrophobic interactions (Collins, 1999).

Among the complement components, C3 and C5 are the most important inflammatory mediators. C3 and C5 can be activated by several proteolytic enzymes present within the inflammatory exudate. These include plasmin and lysosomal enzymes released from neutrophils. Thus, the chemotactic effect of complement and the complement-activating effects of neutrophils can set up a self-perpetuating cycle of neutrophil emigration (Collins, 1999). C3a and C5a are anaphylatoxins because they release histamine and stimulate smooth muscle contraction. The actions of C3a include histamine release and increase vascular permeability in human skin. In addition, C5a may also contribute to inflammatory reactions by stimulating the release of other mediators. Apart from the release of histamine, C5a may also release protein mediators such as degenerative enzymes, cationic proteins and IL-1. Similarly, C5a can release lipid mediators such as LTs and PAF. PG synthesis has been reported to be stimulated by C5a and vasodilator PGs are well known to potentiate inflammatory reactions. Thus, inflammatory responses begun by complement activation may be prolonged and

potentiated by the actions of mediators released by C5a. The C5- derived peptides have potent effects on leukocytes and promote neutrophil endothelial interactions which lead to neutrophil accumulation and associated edema formation (Jose, 1987).

III. Arachidonic Acid Metabolites

Arachidonic Acid (AA) is a 20 carbon polyunsaturated fatty acid derived primarily from dietary linoleic acid and present in the body only in esterified form as a component of cell membrane phospholipids. It is released from these phospholipids via celluiar phospholipases that have been activated by mechanical, chemical, physical stimuli, or by inflammatory mediator such as C5a (Mitchell and Cotran, 1997). AA metabolites, also called eicosanoids, are synthesized by two principal enzyme pathways, the cyclooxygenase, and the lipoxygenase pathway (Salmon and Higgs, 1987; Campbell and Haluska, 1999; Collins, 1999). The scheme of the major metabolic transformations of AA is shown in Figure 1.

The cyclooxygenase pathway produces prostanoids, including prostaglandins (PGs) and thromboxane (TXs) (Collins, 1999; Fantone and Ward, 1999). During inflammation, PGE_2 and PGI_2 are the most important. These products are both potent vasodilator and hyperalgesic agents and since they have been detected at sites of inflammation, it is believed that they contribute to the erythema, edema and pain which are characteristics of the inflammatory response (Salmon and Higgs, 1987). PGE_2 is also a powerful pyrogenic substance and its production is thought to account for the fever induced by interleukin-1 (IL-1), an endogenous pyrogen (Bernheim *et al.*, 1980). Although PGs do not appear to have direct effects on vascular permeability, both PGE_2 and PGI_2 markedly enhance edema formation and leukocyte infiltration by promoting blood flow in the inflamed region in combination with mediators such as bradykinin and histamine. Moreover, they potentiate the pain-producing activity of bradykinin and other autacoids. Similarly, the combination of PGE_2 or PGI_2 with chemotactic factors results in plasma leakage from the microcirculation by a mechanism dependent on circulating polymorphonuclear leukocytes (PMNs) (Salmon and Higgs, 1987; Campbell and

Halushka, 1996). TXA_2 is a major product of arachidonic acid metabolism in platelets which promotes platelet aggregation and vasoconstriction (Campbell and Halushka, 1996).

It is now clear that there are two isozymes of cyclooxygenase (COX) called COX-1 and COX-2. Both isozymes catalyze the conversion of arachidonic acid to PGH_2, the most important step in the formation of both PGI_2 and TXA_2. COX-1, also called constitutive COX, is present in the platelets, endothelium, kidney and stomach mucosa whereas COX-2, also called inducible COX, may be induced by an inflammatory stimulus in macrophages or other cells (Antonio and Souza Brito, 1998). COX-1 has long been thought to be the site of action of NSAIDs. Ideal anti-inflammatory drugs should have an inhibitory action on PG synthesis mediated by COX-2 but not by COX-1. Thus, an inhibitor of COX-2 may be anti-inflammatory drug without the side effects of reducing renal function or producing gastric ulcerations (Antonio and Souza Brito, 1998).

The lipoxygenase pathway mediated by three different lipoxygenases (Collins, 1999). 5-lipoxygenase is the most important of these enzymes. It leads to the synthesis of the leukotrienes (LTs) (Campbell and Halushka, 1999). The LTs can be divided, on the basis of their chemical structures and pharmacological actions into LTB_4, LTC_4, LTD_4, LTE_4 and LTF_4 (Piper and Samhoun, 1987). LTB_4 is the strongest candidate as an inflammatory mediator. It has powerful effects on PMN function; it is a potent chemokinetic, chemotactic and degranulating agent for PMNs. The actions of LTB_4 on PMNs are stereospecific, are not shared by other LTs. In human skin, LTB_4, LTC_4 and LTD_4 cause transient wheal and flare responses either by a direct action or through the release of other endogenous mediators (Salmon and Higgs, 1987). LTC_4 and LTD_4 appear to act on the endothelial lining of postcapillary venules to cause exudation of plasma. They also are bronchoconstrictors in man (Campbell and Halushka, 1996).

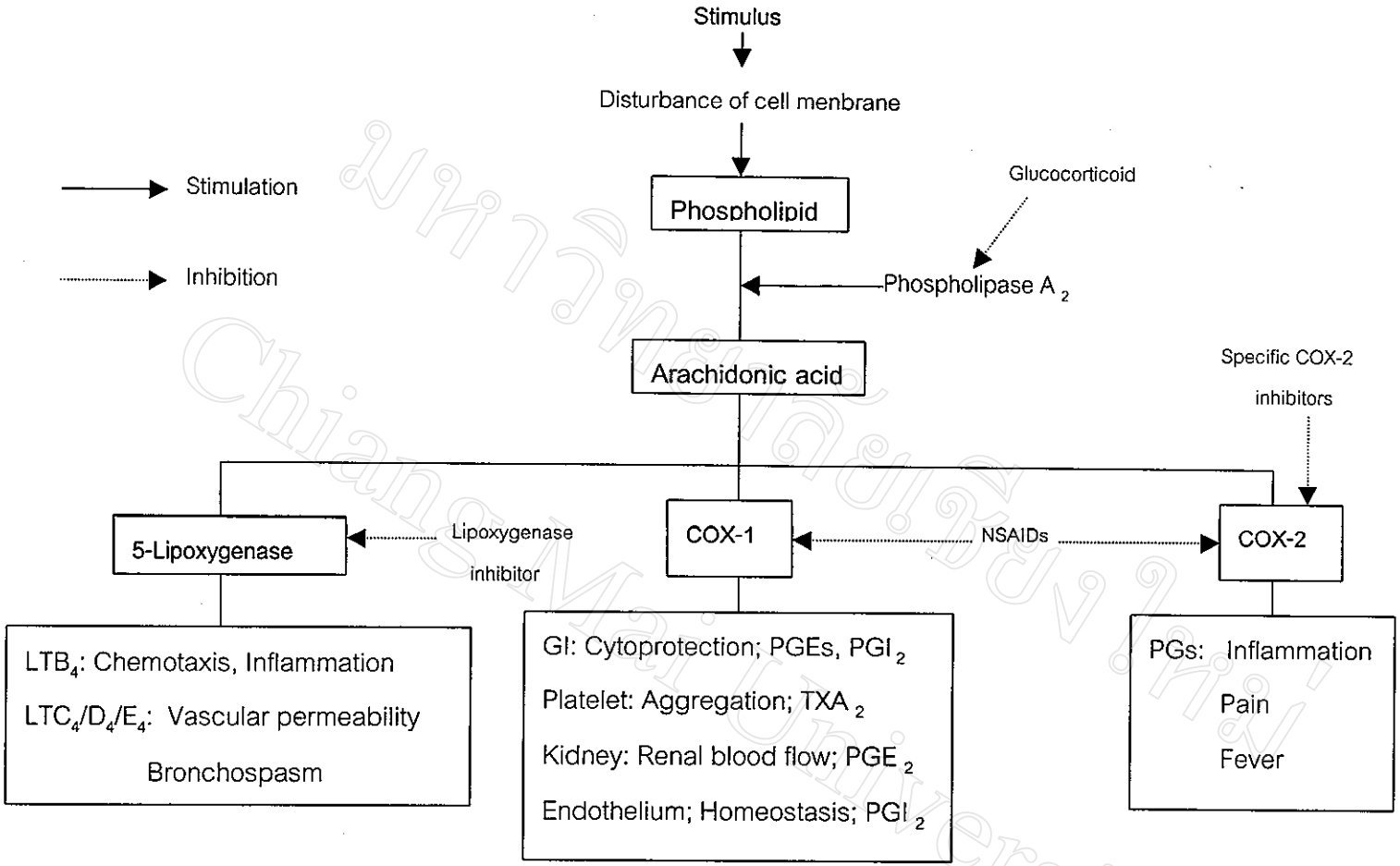


Figure 1. Scheme of the major metabolic transformations of arachidonic acid

COX = cyclooxygenase, LT = leukotriene, NSAIDs = non-steroidal anti-inflammatory drug, GI = gastrointestinal tract

IV. Platelet-activating factor (PAF)

PAF is a potent mediator formed by eosinophils, macrophages, neutrophils, vascular endothelium and tissue (Prescott *et al.*, 1999). Its biosynthesis involves the acetylation of a precursor released from membrane phospholipids by activated phospholipase A_2 (Vargaftig and Braquet, 1987). Two pathways have been described for PAF synthesis: *remodeling* and *de novo*. The remodeling was described first and probably is the more important in inflammation. PAF is not produced by this pathway in resting cells; they must be activated to initiate the synthesis. The synthesis is initiated by phospholipase A_2 and arachidonate release and PAF synthesis are closely coupled. The arachidonic acid is converted to eicosanoids, which also have diverse, potent actions. The lyso-PAF is converted to PAF by specific acetyltransferase, which is activated by phosphorylation on cell stimulation (Prescott *et al.*, 1999). PAF activates most inflammatory cells and induces a variety of *in vivo* effects related to inflammation, particularly to immediate hypersensitivity and accordingly to bronchial asthma (Vargaftig and Braquet, 1987). Like the eicosanoids, PAF is not stored in cell but is synthesized in response to stimulation. It is elaborated by leukocytes and mast cells and exerts proinflammatory effects. Signs and symptoms of inflammation include increased vascular permeability, hyperalgesia, edema and infiltration of neutrophils (Campbell and Halushka, 1996). PAF may be of particular importance in late phase reactions, in which it can activate inflammatory leukocytes. In this situation, the major source of PAF may be basophils or the surface of vascular endothelial cells (stimulated by histamine or leukotrienes) rather than mast cell (Abbas *et al.*, 1997). It is still impossible to determine clearly the role of PAF as a potential mediator in inflammation. The possibility that it plays an important role is nevertheless as likely, if not more so than in case of the eicosanoids (Vargaftig and Braquet, 1987).

V. Cytokines

Cytokines are a potent polypeptide/proteins (glycoproteins) with both local and (frequentry) systemic effect that produces specific receptor-mediated effects on target cells or on the producer cell. It is important to note that most cytokines are not

constituitively produced, but require cell activation for their synthesis. This definition therefore includes the interleukins (ILs), growth factors, colony-stimulating factors, tumor necrosis factor (TNF) etc. Cytokines are responsible for important homeostatic functions such as the maintenance of normal levels of circulating blood leukocytes (lymphocytes, myelomonocytic cells and erythrocytes) and the acute-phase response to infections; it is now clear that they can also be responsible for tissue pathology in a variety of diseases ranging from acute infectious lesions to chronic infectious diseases and to chronic idiopathic diseases, and chronic destructive arthritis (Sunday *et al.*, 1999)

IL-1, IL-2 and TNF are the inflammatory cytokines which play key roles in the acute-phase respone to infection and tissue injury. As well as their important local effects, the cytokines produced by macrophages and neutrophils have long range effects that contribute the host defense. One of these is the elevation of body temperature which is caused by TNF-α, IL-1, IL-6 and other cytokines. These are termed "endogenous pyrogens" (Janeway *et al.*, 1999). IL-1 and TNF produce many of same proinflammatory responses which include mobilization and activation of PMNs; induction of cyclooxygenase and lipoxygenase enzymes; increase in adhesion molecule expression; activation of B-cells, T-cells, and natural killer cells; and stimulation of production of other cytokines (Insel, 1999). TNF is capable of inducing IL-1 release. Clearly, many of the events associated with acute inflammatory reaction can be mediated by IL-1 and TNF (Billingham, 1987). Other actions of these agents likely contribute to the fibrosis and tissue degeneration of chronic proliferation phase of inflammation; stimulation of fibroblast proliferation, induction of collagenase and activation of osteoblasts and osteoclasts (Insel, 1999). On occasions, IL-1 or TNF may be the sole mediators; it seems more likely that the cytokines act in concert with other classes of inflammatory mediators in defense of the host. Where the cytokines differ from so many other classes of inflammatory mediators, however, is in their potential to mediate the tissue destruction in chronic diseases such as rheumatoid arthritis (Billingham, 1987).

ANTIINFLAMMATOY DRUGS

The treatment of patients with inflammatory disease involves two primary goals; first, the relief of pain which is often the presenting symptom and the major continuing complaint of the patient; and second, the slowing or - in theory - arrest of tissue-damaging processes (Katzung and Furst, 1998).

Non-steroid anti-inflammatory drugs (NSAIDs)

Aspirin and NSAIDs such as indomethacin, ibuprofen, naproxen, etc. are used to suppress signs and symptoms of inflammation. These drugs also exert antipyretic and analgesic effects, but it is their anti-inflammatory properties that make them most useful in the management of disorders in pain, which is related to the intensity of inflammatory process (Katzung and Furst, 1998). Inhibition of cyclooxygenase (COX), the enzyme responsible for the biosynthesis of the prostaglandins (PGs) and certain related autacoids, generally is thought to be a major facet of the mechanism of action of NSAIDs (Insel, 1999). During therapy with these drugs, inflammation is reduced by decreasing of release of mediators from granulocytes, basophils and mast cells. The NSAIDs decrease the sensitivity of vessels to bradykinin and histamine, affect lymphokine production from T lymphocytes and reverse vasodilation (Katzung and Furst, 1998). In addition to sharing many therapeutic activities, NSAIDs share several unwanted side effects. The most common is a propensity to induce gastric or intestinal ulceration.

There are two isozymes of COX (COX-1 and COX-2). Both isozymes catalyze the conversion of arachidonic acid to PGH_2, the most important step in the formation of both PGs and thromboxane A_2 (TXA_2). COX-1, also called constitutive COX, is a wide-ranging essential enzyme which produces PGs involved in cytoprotective and regulatory functions in gastrointestinal mucosa, platelet and renal cells whereas COX-2, also called inducible COX, produces PGs that mediate pain and inflammation (Antonio and Souza Brito, 1998).

All NSAIDs inhibit COX-1 and COX-2, each to varying degrees. This observation has lead to the hypothesis that the therapeutic effects of NSAIDs are achieved by COX-

2 inhibition, but many of the toxic effects, most commonly gastroduodenal ulcer, result from the inhibition of COX-1. Animal data suggest that inhibition of COX-2 is the basis for analgesic and anti-inflammatory effects of NSAIDs. As a result, new strategies for treating inflammation and pain have focused on the development of therapeutic agents with selective or specific inhibition of COX-2 (Katzung and Furst, 1998).

Anti-inflammatory corticosteroids

Anti-inflammatory corticosteroids, which are given systemically (orally or by injection) for the treatment of inflammatory diseases are prednisolone, dexamethasone, hydrocortisone, etc. In addition, a number of others are applied topically for treatment of local inflammatory reactions (Bowman and Rand, 1980). Corticosteroids block all the known pathways of eicosanoid synthesis, perhaps by stimulating the synthesis of several inhibitory proteins collectively called annexins or lipocortins. They inhibit phospholipase A_2 activity, probably by interfering with phospholipid binding and thus preventing the release of arachidonic acid (Foegh *et al.*, 1998). Thus the anti-inflammatory action of these agents may be related to the inhibition of prostaglandin and leukotriene synthesis (Vazquez *et al.*, 1996). Corticosteroids have powerful anti-inflammatory effect. Unfortunately, the toxicity associated with chronic corticosteroid therapy inhibits their use except in the control of acute flare-ups of joint diseases (Katzung and Furst, 1998). Long term use of glucocorticosteroids is associated with major adverse events in a dose dependent manner. Although some studies have shown the relative safety of long term low dose glucocorticosteroids, other studies highlight the cumulative toxicity that lead to osteoporosis, infections and peptic ulcer (Langenegger and Michel, 1999).

Disease-modifying antirheumatic drugs (DMARDs)

Members of the group DMARDs or slow-acting antirheumatic drugs (SAARDs) include methotrexate, azathioprine, penicillamine, hydroxychloroquin and chloroquin, organic gold compounds and sulfasalazine. The effects of DMARDs may take 6 weeks to 6 months to become evident. Very little is known about their mechanism of action, but

they may slow the bone damage associated with rheumatoid arthritis and are thought to affect more basic inflammatory mechanisms than do the NSAIDs. Considerable controversy surrounds the long-term efficacy of these drugs. The discovery that numerous cytokines are present in joints affected by the disease process suggests that one or more of these may be useful targets of disease-modifying drug therapy (Katzung and Furst, 1998).

Among the three groups of anti-inflammatory drugs, NSAIDs are the most used agents, since they can effectively relief the symptoms of inflammation and have less severe side effects when compared to the other two groups of drugs (Katzung and Furst, 1998).

EXPERIMENTAL MODELS USED IN THE PRESENT STUDY

1. Inflammatory models

1.1 Ethyl phenylpropiolate (EPP) and arachidonic acid (AA)-induced ear edema in rats: Edema is a useful parameter to look at when testing for agents which may be active in treating acute inflammation (Sedgwick and Willoughby, 1989).

EPP-induced ear edema is suggested to serve as a more useful model for the testing of anti-inflammatory activity (Yong *et al.*, 1984). This experiment seems to be a useful model for the rapid *in vivo* screening of agents since only a small amount of a test substance is needed. By using EPP, the mechanism involved can be suggested. Kinins, 5-HT and PGs are released in EPP induced ear edema (Brattsand *et al.*, 1982; Yong *et al.*, 1984).

AA-induced ear inflammation in mice has been reported to be sensitive in detecting the anti-inflammatory action of lipoxygenase inhibitors (Carlson *et al.*, 1985). Lipoxygenase metabolites, especially LTs, have an important role in producing vascular permeability and edema formation whereas COX products have low or no activity (Di Martino *et al.*, 1987).

1.2 Carrageenin-induced hind paw edema in rats: The hind paw edema induced in rat by subplantar injection of irritants including formalin, kaolin, dextran, carrageenin, arachidonic acid, etc., has long been known and used for testing substances for anti-inflammatory property. The most commonly used irritant is carrageenin (Winter *et al.*, 1962). Carrageenin is a sulphate polysaccharide which has been fractionated with potassium chloride into two separate components, kappa and lambda carrageenin (Di Rosa, 1972). The lambda carrageenin is more active in eliciting either acute or chronic inflammatory responses.

Carrageenin-induced hind paw edema in rats was first introduced by Winter *et al.* (1962). This model has become common as a test for anti-inflammatory activity. The advantage of carrageenin-induced edema in comparison with the edema elicited by other phlogistic agents is its responsiveness to doses of all clinical used anti-

inflammatory drugs at well below the toxic level, with the degree of edema inhibition being in a dose-related manner (Winter *et al.*, 1962).

1.3 Carrageenin-induced pleurisy in rats: Carrageenin-induced pleurisy in rats is an acute inflammatory model which is characterized by fluid and leukocyte accumulation associated with extravasation of plasma proteins (Capasso *et al.*, 1975; Lo *et al.*, 1981). It has been suggested that the pleural models can differentiate steroid and non-steroid activity (Sedgwick and Willoughby, 1989). The pleurisy model has been accepted as a reliable method to study inflammation allowing the determination of several parameters simultaneously including (1) measurement of exudate volume (2) measurement the white blood cell number in the exudate using a Coulter counter or a hematocytometer (3) determination of lysosomal enzyme activity (4) determination of PGE_2 (Vogel and Vogel, 1997a).

1.4 Cotton pellet-induced granuloma formation in rats: Meire *et al.* (1950) first introduced the method using cotton pellet to induce granuloma formation. This method has been widely employed to evaluate the transudative, exudative and proliferative phases of chronic inflammation. The response to a subcutaneously implanted cotton pellet in rat has been divided into three phases, namely (1) a transudative phase, a fluid that is low in protein and noninflammatory in origin, defined as the increase in wet weight of the pellet which has occurred during the first three hours after implantation (2) an exudative phase, defined as a leakage of fluid from the bloodstream around the granuloma and occurring between 3 and 72 hours after implanting the pellet, and (3) a proliferative phase, measured as the increase in dry weight of the granuloma which occurs between three and six days after implantation (Swingle and Shideman, 1972). The net dry weight of granuloma tissue indicates the intensity of the subchronic inflammation.

In the cotton pellet-induced granuloma formation model, serum alkaline phosphatase activity can also be assessed. Alkaline phosphatase is a lysosomal

enzyme. It is widely distributed in many tissues, including the osteoblasts (the bone-building cells), the cell lining the sinusoids and bile canaliculi in the liver. It is reported that the activity of alkaline phosphatase in serum was markedly increased during inflammation. Alkaline phosphatase activity in pouch wall was elevated during cotton pellet granuloma formation on the seventh day and decreased on the fourteenth day, when healing occurred. In rheumatoid arthritis the erosion of cartilage matrix appears to be due to the release of lysosomal enzymes from the inflammatory cells of the invading pannus (Weissmann, 1965a). Measurement of alkaline phosphatase activity in serum of rats implanted with cotton will indicate the activity of agents on the production and release of alkaline phosphatase in chronic inflammation (Nishikaze *et al.*, 1980; Ismail *et al.*, 1997).

2. Algesic models

2.1 Acetic acid-induced writhing response in mice: Most NSAIDs usually possess analgesic activity. Inhibition on PG biosynthesis is considered to be a shared mechanism of anti-inflammatory, analgesic and antipyretic actions of NSAIDs (Milton, 1982). Therefore, it is interesting to investigate the analgesic activity of test drugs possessing anti-inflammatory activity.

The writhing response induced in rat or mice by intraperitoneal injection of a noxious agent is commonly used as a basis for testing analgesic activity. The response consists of a wave of constriction and elongation passing caudally along the abdominal wall, sometimes accompanied by twisting of the trunk and followed by extension of the hind limbs. The latency and duration of writhing response depends on the characteristics of the challenge substances. The substance which has a long latency, such as acetic acid or phenylbenzoquinone, may be supposed to act indirectly by liberating an endogenous substance that excites pain nerve endings (Collier *et al.*, 1968). The inhibitory effect of a substance on writhing response in this test was found to be well correlated with clinical results in humans (Taber *et al.*, 1969).

2.2 **Formalin test:** The formalin test in mice has an advantage over other frequently used tests as it involves a biphasic response with an early and a late phase representing neurogenic and inflammatory pain and agents can be screened for activity in these two models of pain. The first phase response is believed to represent a direct irritant effect of formalin on sensory C fibers whilst the later phase response is most likely secondary to the development of an inflammatory response and the release of algesic mediators (Hunskarr and Hole, 1987).

3. Pyretic model

Yeast-induced hyperthermia in rats: The pyrexia induced in rat by brewers yeast subcutaneous injection, has been used to determine antipyretic activity of many compounds (Teotino, 1963). The pyrexia reaches its peak at 18 h after induction and the assessment is also made at this period. It has been postulated that many chemical neuromediators are involved in hypothalamic regulation of body temperature. PGE_2 is one of the most potent pyretic agents known and elevated concentration of PGE_2 has been found in cerebrospinal fluids taken from pyretic patients or animals. Drugs that inhibit prostaglandin biosynthesis, such as aspirin, can reduce the pyrexia (Milton, 1982).

4. Ulcerogenic models

Many plants with anti-inflammatory effect have been found to possess anti-ulcerogenic activity. It is therefore of interest to study this effect of substances with anti-inflammatory property. Various mechanisms may be associated with the formation of gastric mucosal damage in experimental models used to study anti-ulcer drugs in rats (Antonio and Souza Brito, 1998).

4.1 **Ethanol/hydrochloric acid induced-gastric lesions:** Ethanol/hydrochloric-induced lesions formation may be multifactorial, with stasis of gastric blood flow contributing significantly to the haemorrhagic as well as the necrotic aspects of the tissue injury (Guth *et al.*, 1984). Moreover, it is also suggested that the gastric wall

mucus depletion is one of the pathogenic mechanisms responsible for gastric lesions (Koo *et al.*, 1986).

4.2 **Indomethacin induced-gastric lesions:** NSAIDs, e.g. acetylsalicylic acid (ASA), phenylbutazone and indomethacin, are known to induce ulcers during the course of their anti-inflammatory action, by inhibition of prostaglandin synthetase through the cyclooxygenase pathway (Pal and Nag Chaudhuri, 1991). It has been reported that the reduction of gastric mucus is a possible cause of the lesion induced by indomethacin (Menguy and Desbaillets, 1967).

4.3 **Restraint water immersion stress-induced gastric lesions:** Stress-induced ulcers are probably mediated by histamine release with enhancement in acid secretion. The increase in gastric acid secretion is considered to be important factor in the genesis of stress ulcer and is often termed as the 'aggressive factor' (Goa and Monk, 1987).

4.4 **Pylorus ligation:** This model has been first described by Shay *et al.* (1945). This model has been shown to stimulate acid secretion via the vago-vagal reflex and cholinergic muscarinic mechanism causes accumulation of intraluminal HCl. Therefore, the total acidity can be measured (Shay *et al.*, 1945; Brodie, 1966; Hakanson *et al.*, 1980). The pylorus ligation model is used for evaluation of anti-secretory activity (Shay *et al.*, 1945).

5. Hippocratic screen

This test is commonly used in preliminaly screening of medicinal plants to detect interesting pharmacological activities (Malone and Robichaud,1962) The test reveals some pharmacological activities of compounds which can be observed from the drug-induced signs and symptoms. In this test one lethal dose, three effective doses and one ineffective dose are used. The animals are kept for 7 days. Changes in external

appearance and internal organs of animals dieing during this period and those surviving can be examined.

6. Acute toxicity study

The procedure is done according to the OECD guideline for testing of chemicals (1981). In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from a short term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. Acute oral toxicity is the adverse effect occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

7. Subacute toxicity study

In the assessment and evaluation of the toxic characteristics of a chemical the determination of oral toxicity using repeated doses may be carried out after initial information on toxicity has been obtained by acute testing. It provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. The test substance is administered orally in daily graduated doses to several groups of experimental animals, one dose per group for a period of 14 days.

HISTORICAL BACKGROUND OF *Ventilago harmandiana* Pierre

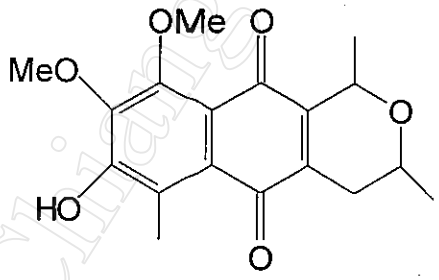
In recent years a wide-spread search has been launched to identify new anti-inflammatory drugs from synthetic and natural resources. Many studies on traditional plants have shown that most plants with anti-inflammatory property lack an ulcerogenic effect and some even possess anti-ulcerogenic activity, e.g. *Curcuma longa* Linn. (Merhra *et al.*, 1984; Ageel *et al.*, 1987), *Turnera ulmifolia* (Antonio and Souza Brito, 1988), *Zingiber officinale* Roscoe. (Manonmani *et al.*, 1994; Sharma *et al.*, 1994) and *Garcinia kola*, (Bradide, 1993; Ibriroke *et al.*, 1997).

Many compounds have been isolated from plants used in traditional medicine for the treatment of arthritis, muscle and joint pain as well as lower back pain. Some of these isolated compounds have been proved to possess anti-inflammatory, analgesic or antipyretic activities in many animal models. These compounds include mangostin from *Garcinia mangostana* Linn. (Chaikaeo, 1988), 5,7 dimethoxyflavone from *Boesenbergia pandurata* (Roxb.) Schltr. (Panthong *et al.*, 1989), diarylheptanoid compounds from *Curcuma xanthorrhiza* Roxb. (Claeson *et al.*, 1993; Claeson *et al.*, 1996), flavones and flavanones from three varieties of *Boesenbergia pandurata* Roxb. (Panthong *et al.*, 1994), compound D or {(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-2-ol}, from *Zingiber cassumunar* Roxb. (Panthong *et al.*, 1997) and bukittinggine from *Sapium baccatum* Roxb. (Panthong *et al.*, 1998).

Ventilago harmandiana Pierre, family Rhamnaceae, with local names of "Ngew dam" and "Khruea plok" (Smitinand, 1980) is a plant indigenous to Thailand and South East Asia. The heart wood of the plant is used in traditional medicine for the treatment of diabetic disease, chronic inflammation and wound (Pongboonroud, 1971; Wuttithamwate, 1999). The claimed effect, i.e. anti-inflammatory activity, of *V. harmandiana* Pierre was first studied by Phankummoon (1998). Four methanolic extracts from dry heart wood of dead tree, dry heart wood of fresh tree, dry stem bark of fresh tree and dry branch of fresh tree of "Ngew dam" supplied by Dr. Vichai Reutrakul were assessed using both acute and chronic inflammatory models. All extracts seem to possess strong activity on the acute phase of inflammation as seen in ethyl phenylpropiolate- and arachidonic acid-induced ear edema as well as carrageenin-induced hind paw edema in rat. In the chronic inflammatory model, it was found that all extracts elicited weak inhibitory activity on cotton pellet-induced granuloma formation whereas prednisolone, a steroidal drug, exerts profound inhibitory effect on this model. It is therefore unlikely that the methanolic extracts of "Ngew dam" possess a similar mechanism of anti-inflammatory action as a steroidal drug. Furthermore, they did not influence the thymus weight. It was concluded that the mechanism of action of methanolic extracts of "Ngew dam" was probably due to an inhibition of prostaglandin

biosynthesis. This conclusion was supported by the excellent antipyretic effect of all extracts in yeast-induced hyperthermia in rat, since prostaglandin production in the central nervous system is the final common pathway responsible for fever induction. However, the action of the extracts on the lipoxygenase pathway is also likely, since they exerted an inhibitory activity on arachidonic acid-induced ear edema, which was used as a model to screen for compounds showing *in vivo* lipoxygenase inhibitory activity. In the analgesic test, all extracts possessed strong inhibitory activity similar to that of aspirin on acetic acid-induced writhing response but elicited only weak effect in the tail-flick test when compared with morphine. Therefore, the peripheral mechanism of analgesic activity of the methanolic extracts from "Ngew dam" is likely.

PNQ-4482 or *cis*—8,9-dimethoxy—7-hydroxy—1,3,6-trimethyl—3,4-dihydro—1*H*-naphthol (2,3-*c*)pyran—5,10-quinone, a pyranonaphthoquinone compound, is the major compound isolated from the heart wood of *V. harmandiana* Pierre. The isolation and structure elucidation of PNQ-4482 have been performed by Prof. Dr. Vichai Reutrakul and coworkers, Department of Chemistry, Faculty of Science, Mahidol University. The structure of PNQ-4482 is illustrated below.



As the methanol extracts of *V. harmandiana* Pierre showed promising effects on many inflammatory models, it was therefore of interest to prove whether PNQ-4482 is responsible for the anti-inflammatory effect of the methanolic extract of this plant. Preliminary screening using carrageenin-induced paw edema in rats revealed an anti-inflammatory activity of the PNQ-4482. Preliminary toxicity testing of this compound showed that an oral single dose of 5,000 mg/kg did not show any sign or symptoms of

toxicity in rats. In addition, it has been found that PNQ-4482 at the dose of 300 mg/kg, given orally for 7 days, did not possess any ulcerogenic activity when compared with aspirin. And since some plants that possess anti-inflammatory activity also show anti-ulcerogenic effect as mentioned before, it was therefore reasonable to study concomitantly its anti-inflammatory and anti-ulcerogenic effects in detail.

PURPOSE OF THE STUDY

The purpose of the present study was to investigate and to evaluate anti-inflammatory effect and related activities, i.e., analgesic and antipyretic effects of PNQ-4482 using various animal models. Its effect was compared with various reference drugs, such as aspirin, morphine, phenylbutazone, phenidone and prednisolone. Anti-ulcerogenic activity of PNQ-4482 was also studied in many experimentally induced ulcers in rats in comparison with cimetidine, an H_2 antagonist, which is an important antiulcer drug. In order to evaluate the safety of PNQ-4482 as a potential anti-inflammatory drug, its effects on general behavior and toxicity in rats using the Hippocratic Screen and acute and subacute toxicities were included in the present study.

Figure 2. "Ngew dam" or "Khruea plok"

(*Ventilago harmandiana* Pierre, family Rhamnaceae)