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COMMENTARY

Multiple Controls in Inflammation

EXTRACELLULAR AND INTRACELLULAR PHOSPHOLIPASE A_2, INDUCIBLE AND CONSTITUTIVE CYCLOOXYGENASE, AND INDUCIBLE NITRIC OXIDE SYNTHASE

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ABSTRACT. Inflammation occurs as a defensive response to invasion of the host by foreign material, often of microbial nature. This response is normally a localized protective response that at the microscopic level involves a complex series of events including dilatation of arterioles, venules, and capillaries with increased vascular permeability, exudation of fluids including plasma proteins, and leukocyte migration into the inflammatory area. Since disease characterized by inflammation is an important cause of morbidity and mortality in humans, the processes involved in the host defense in inflammation have been and continue to be the object of several experimental studies. The role of several mediators such as histamine, serotonin, bradykinin, prostaglandins, and, more recently, cytokines and nitric oxide has been evaluated, and a contribution for each one of these mediators has been proposed. With the development of powerful molecular biology tools, it has become possible to study enzymes involved in this complex phenomenon by measuring the expression or evaluating the signaling pathways following a specific stimulus. These techniques have generated a proliferation of studies on the role of several enzymes and cytokines in inflammation. Most of these studies have been conducted *in vitro* on cell lines, and not many of the results have been confirmed by *in vivo* studies. This commentary does not pretend to analyze all of the studies and their possible incongruences, but endeavors to provoke in the reader a critical review of dogmas and current beliefs that most of the time are built on unilateral interpretation of the data. BIOCHEM PHARMACOL **55**;2:105–111, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cyclooxygenases; phospholipases; inducible nitric oxide synthase; inflammation

In the past, the role of mediators such as histamine, serotonin, bradykinin, prostaglandins, and, more recently, cytokines and NO† was the focus of studies of inflammation. However, in recent years, powerful molecular biology tools have made it possible to study enzymes involved in this complex phenomenon by measuring the expression or evaluating the signaling pathways following a specific stimulus. These techniques have generated a proliferation of studies on the role of several enzymes and cytokines in inflammation. Most of these studies have been conducted *in vitro* on cell lines, and not many of the results have been confirmed by *in vivo* studies. Until 2 years ago, most of the papers published in the "inflammation" field were focused on the role played by cytokines and the iNOS, leaving in the background the arachidonic acid cascade products and enzymes (Fig. 1). More recently, the discovery of an inducible form of COX (COX-2) has drawn new attention to the arachidonic acid cascade products, and, more importantly, to enzyme expression in chronic and acute inflammation. Due to the great complexity of the biochemical, pharmacological, immunological, and pathological processes that are involved in inflammation, there is a tendency to investigate in depth the pathogenic role played by one single agonist at a time rather than to analyze the effects of multiple factors in the initiation of tissue damage. Indeed, this approach is justified, since such complex models may be fraught with many technical and interpretational difficulties. However, there is the dilemma that many of the mediators produced, and the cytokines as well, operate in a network, and most of the time in an experimental system more than one enzyme at a time is induced. In several studies on the expression of COX-2 enzyme, researchers did not take into account that in their system it was most likely that other enzymes that were up-regulated by the same stimulus were also being expressed. For example, the role played by PLA_2, a rate-limiting enzyme in the arachidonic acid cascade, which exists in a secreted and cytoplasmic form, is often ignored in several *in vitro* protocols, where expression of these enzymes is certainly relevant. In addition, very rarely do researchers consider that cytokines such as IL-1, IL-6, and TNFα will induce extracellular and cytosolic PLA_2 as well as COX-2 and iNOS.

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† *Abbreviations:* NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL, interleukin; PLA_2, phospholipase A_2; COX, cyclooxygenase; LPS, lipopolysaccharide; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; and PG, prostaglandin.

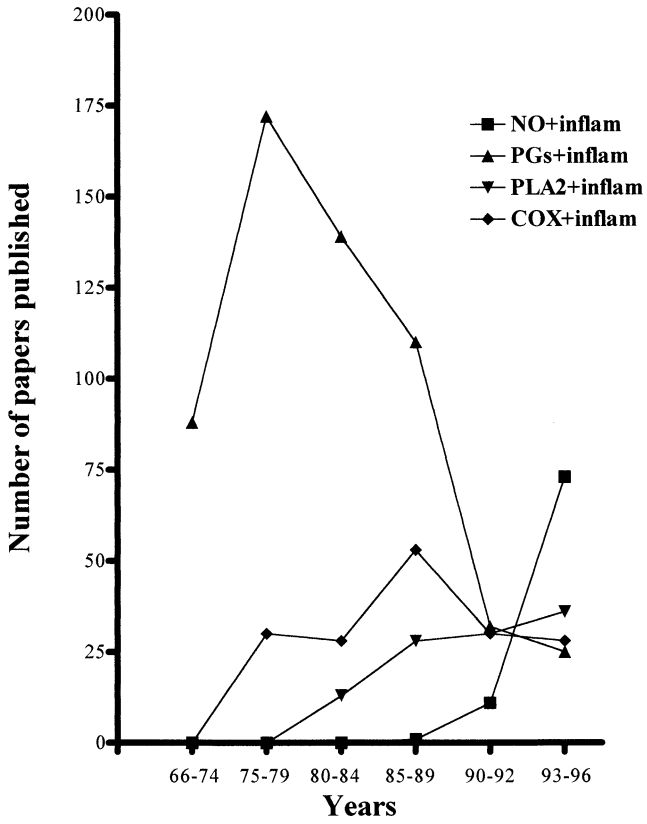

FIG. 1. Numbers of papers published from 1966 to 1996 based on a MEDLARS search. To perform this search the word inflammation has been combined with nitric oxide or prostaglandins or phospholipase A_2 or cyclooxygenase.

PROSTAGLANDIN H SYNTHASE (CYCLOOXYGENASE): INDUCIBLE AND CONSTITUTIVE

PGH (prostaglandin H synthase) is a bifunctional glycoprotein that catalyzes the biosynthesis of PGH_2, using arachidonic acid as substrate and leading to the formation of a class of mediators called prostaglandins. The responses to prostaglandins are often cell specific, and inflammatory responses are elicited by several exogenous stimuli or by products of the host defense response. Major sources of prostaglandins (PGE_2 and PGI_2) are endothelial cells, mononuclear phagocytes, platelets, mast cells, and polymorphonuclear leukocytes. Since their discovery, a large number of papers have been published on the role of prostaglandins in inflammation (Fig. 1). Prostaglandins exert their biological effect by binding to cell-surface receptors that will transduce the signal via G-protein, mainly through cyclic AMP. However, despite the similarities in the signal transduction pathway, eicosanoids have a variety of biological actions. PGE_2 is vasodilatory and also has a cytoprotective effect in the stomach, which it shares with PGI_2. PGI_2 also plays an important role in platelet aggregation, being a powerful natural antiaggregatory agent. In addition, prostaglandins are involved in plasma leakage as well as in pain by sensitizing pain receptors.

It is clear now that there are two isoforms of PGH_2 synthase, which are currently better known as COX type I (COX-1) and type II (COX-2). COX-1 is the constitutive enzyme and is thought to be the housekeeping enzyme responsible for the production of "good" (physiological) prostaglandins. COX-2 is the inducible form, mainly produced during inflammatory response, and it is thought to be largely responsible for the production of the "bad" (pathological) prostaglandins. Due to this discovery, research has been moving in two main directions, the first devoted to a better understanding of how the expression of these enzymes is regulated and the second to the development of new selective inhibitors of COX-2. COX-1 is constitutive, and its tissue distribution is almost ubiquitous. At the subcellular level it is located on the membrane endoplasmic reticulum, and its X-ray structure has been discovered recently. The gene is located on chromosome 9, and its structure is constituted by about 22 kb with 11 exons. The COX-2 enzyme is mainly inducible and is present for the most part in inflammatory cells. Apparently, it is most likely localized at a subcellular level in the nuclear membrane, and its gene structure is of 8 kb with 10 exons (for review, see Refs. 1–3).

EXTRACELLULAR AND INTRACELLULAR PHOSPHOLIPASES

The substrate for the COX enzymes is arachidonic acid, which is predominantly found esterified in the *sn*-2 position of membrane phospholipids. The enzyme that is responsible for its mobilization is PLA_2. Production of arachidonic acid through activation of PLA_2 is a rate-limiting step in the biosynthesis of prostaglandins. PLA_2s are generally divided into extracellular or low mass PLA_2 (14–18 kDa) and intracellular high molecular mass PLA_2s (80–110 kDa). Extracellular PLA_2 ($sPLA_2$) is the most studied form of the enzyme, since there are several sources of the enzyme in nature such as snake venoms and pancreatic juice.

In recent years, phospholipases have been classified into four groups. In group I there is the mammalian pancreatic PLA_2 and some cobra and sea snake venoms. The enzymes of this class are calcium dependent, express their optimal catalytic activity at acidic pH, and, as in the case of the pancreatic enzyme, can be expressed as pro-enzymes. In group II there are viper and rattlesnake venoms, human synovial fluid, human platelets, and inflammatory exudate PLA_2s. They are also calcium dependent, and their optimum activity is at alkaline pH. The human non-pancreatic gene is very similar to the group I pancreatic PLA_2 gene. The PLA_2 gene encodes for a 144 amino-acid protein containing a 20 amino-acid signal sequence for translocation, and it is not secreted as a pro-enzyme. The catalytic mechanism, originally proposed by Slotboom *et al.* [4], many years ago, has been confirmed recently by X-ray crystallographic studies showing that the catalytic domain involves histidine 48 and aspartic acid 99, calcium ions, and water in a proton relay system. The majority of

extracellular enzymes are sensitive to treatment with *p*-bromophenacyl-bromide, an alkylating agent acting on the His/Asp pair.

Cytosolic PLA_2s ($cPLA_2$s) are classified in group IV. In 1990, Clark *et al.* [5] identified a cytosolic form of PLA_2 from U937 cells, a human monocytic cell line. This form of PLA_2 was purified and the cDNA sequenced. The new protein having an apparent molecular mass of 100 kDa (SDS–PAGE) was named $cPLA_2$ and did not show any homology with the secreted form. The enzyme consists of 749 amino acids and contains 12 possible sites of serine/threonine phosphorylation, 4 possible sites of tyrosine phosphorylation, and no apparent disulfide bonds. The enzyme is active at micromolar concentrations of calcium and *in vitro* has been shown to selectively hydrolyze phospholipids containing arachidonic acid esterified at the *sn*-2 position. The $cPLA_2$ enzyme also has been characterized from the J774 macrophage line, and in this cell line it appears to be regulated by serine phosphorylation. Following cell stimulation, $cPLA_2$ is translocated to the cell membrane [6], and the calcium-lipid binding domain in the presence of calcium is involved in this translocation and association of the enzyme to phospholipids in the cell membrane. This enzyme is not affected by treatment with *p*-bromophenacyl-bromide, and the role played by the histidine/aspartic acid is unknown (for review, see Refs. 7–13).

iNOS

Although resting cells do not express iNOS, the capacity to express this enzyme is present in several tissues. Indeed, cells such as macrophages, hepatocytes, vascular smooth muscle cells, endothelial cells, kidney cells, chondrocytes, cardiac myocytes, pancreatic islets, and fibroblasts have the ability to express iNOS under an appropriate stimulus. Factors that will induce iNOS differ between cell types among the agents described thus far. The majority are microbes, microbial products, inflammatory cytokines, or a synergistic mixture of these agents. Murine macrophages express high levels of iNOS upon treatment with LPS, showing a strong synergy with IFNγ. IL-1 is a potent inducer in chondrocytes, smooth muscle cells, and hepatocytes. iNOS expression can be inhibited by dexamethasone in a number of cell types, and several cytokines such as TGFβ, IL-4, IL-8, and IL-10 have been shown to suppress iNOS in murine macrophages. The macrophage, being a cell involved in the immune response, has been widely studied, but while rodent cells respond well to LPS plus IFNγ, human cells exhibit variable responses and require prolonged exposure to these agents. The gene for iNOS was cloned from human tissues in 1994, and it is known now that it is about 37 kb in length and consists of 26 exons and 25 introns. It has been mapped to chromosome 17 at position 17 cen–q11.2. The gene also contains several putative binding sites for transcriptional factors and regulatory elements such as IFNγ, NF-κB, and NF-IL-6. Furthermore, since iNOS is not constitutively expressed, the enzyme does not require the addition of extracellular calcium for its activity, and its activity is mainly controlled at the transcriptional level (for review, see Refs. 2 and 14–16).

DO THESE ENZYMES INTERACT AMONG THEMSELVES DIRECTLY OR THROUGH MEDIATORS?

Several different stimuli such as cytokines can exert a positive or negative control on the induction of iNOS, COXs, and PLA_2s. To summarize all the experiments that have been performed thus far would be impossible; hence, I will concentrate in the rest of this paragraph on the more widely used inducers. The substances that have been most used in induction studies are the pro-inflammatory cytokines IL-1, TNF, and the lipopolysaccharide from *Escherichia coli* cell wall (commonly called LPS), on which there is a voluminous literature. The list of cells capable of expressing iNOS following these stimuli includes macrophages, neutrophils, keratinocytes, respiratory epithelium, retinal pigment epithelium, renal tubular epithelium, myoepithelium, adenocarcinomas, hepatocytes, pancreatic islet cells, uterine, fallopian tubes, endothelium, mesangial cells, endocardium, vascular smooth muscle cells, fibroblasts, osteoclasts, neurons, and astrocytes (for review, see Ref. 17). There is an equally long list of cells and tissues where $sPLA_2$ has been induced by these substances, such as astrocytes, vascular smooth muscle cells, osteoblasts, synovial cells, chondrocytes, platelets, neutrophils, mast cells, alveolar macrophages, spleen, placenta, amnion, liver, hepatoma cells, Kupffer cells, lung, kidney, renal mesangial cells, ileum, ascitic fluid, synovial fluid, pleural fluid, and heart (for review, see Ref. 7). The list of cells and tissues where COX-2 [3] and $cPLA_2$ [8–13] can be expressed by LPS, IL-1, or TNF is smaller, since the biochemical tools have been available for a shorter period of time. Induction of these enzymes has been studied in several papers where the results obtained emphasize one enzyme rather than another, even though it is quite well established that these enzymes function in a network. Rat mesangial cells constitute a cellular system that is widely studied. In rat mesangial cells, IL-1 induces expression of COX-2 [18]. At the same time, iNOS has been shown to be present in rat mesangial cells, and it has been shown to be regulated in a way similar to that described for macrophages [19–21]. IL-1 will induce both $cPLA_2$ [22, 23] and $sPLA_2$ [24, 25] contemporaneously in the same type of cell. All these papers document the importance of each enzyme in rat mesangial cells, but at the end the final output is not very clear. Indeed, by reading any single paper the impression is that each single enzyme is the key enzyme from which everything starts following the application of the stimulus (e.g. IL-1). Hence, from these papers it appears clear that IL-1 is responsible for the production of vasoactive prostanoids from mesangial cells, but the sequence of the events is not clear, even though the

stimulus is the same. For example, a paper by Martin *et al.* [18] demonstrates expression of COX-2 but not COX-1 in rat mesangial cells following treatment with IL-1, and the hypothesis has been proposed that PGE_2 production is driven mainly by COX-2. However, none of the other enzymes that are under the control of the same cytokine in the same cell, such as the PLA_2s, which surely play a role in the production of prostanoids by providing the substrate, are taken into account. Furthermore, most of these data have never been confirmed either *in vivo* or *ex vivo*, so their real relevance in the mesangial cell mechanism and in the related pathology is not clear.

Arthritis is a good example of how *in vitro* and *in vivo* data do not correlate and how difficult it can be to interpret the literature when, as in this case, even the data do not correlate between the animal model and the human disease. In arthritis, inflammatory mediators such as IL-1 and TNF induce COX activity in synovium and cartilage and induce degeneration of the articular interface [26, 27]. Chondrocytes isolated from human or experimental animal specimens have been widely studied, and several studies have been conducted to evaluate the enzymes and mediator involvement in this diffuse and disabling disease. It has been shown that while COX-1 is unaffected, COX-2 is strongly expressed in the same culture medium following stimulation with IL-1 [28]. However, $sPLA_2$ also is strongly expressed following the same stimulus. Half-maximal expression of $sPLA_2$ requires 0.15 ng/mL of IL-1, while COX-2 is estimated to require 1.2 ng/mL. Hence, it is possible that the response is under the control of these two enzymes, and prostaglandins are the distal mediators of this disease process. However, it is not so simple, since at the same time iNOS has been shown to be induced by IL-1 in the same cell following stimulation with IL-1 [29] as well as $cPLA_2$ [8, 13]. Now, the fact that all of these enzymes are expressed at the same time in a disease such as arthritis is not surprising. However, they must be regulated in some way, since it is unlikely that all of these mechanisms are activated all at the same time. In this regard, experimental protocols *in vitro* have been designed and based on these data; it has been proposed that there is an interaction between NO and COX pathways. However, there are conflicting data concerning this matter, since it has been shown that NO can either inhibit [30–32] or stimulate COX [33–35]. On the other hand, in all of these studies the contribution of $sPLA_2$ and $cPLA_2$ scarcely has been taken into account, even though in the presence of the stimuli applied in the above papers (e.g. LPS and IL-1) synthesis and formation of both enzymes will clearly occur [7, 8]. Furthermore, there are now data appearing in the literature studying the possible role played by lipoxygenase pathway products such as leukotrienes. Leukotrienes are synthesized by activated leukocytes during inflammatory diseases, and it has been demonstrated recently that $cPLA_2$ is colocalized with 5-lipoxygenase and 5-lipoxygenase-activating protein at the nuclear membrane of activated human neutrophils [36] and that lipoxygenase products modulate its activity [37], further complicating the interpretation of the sequence of the events involved in the inflammatory response. When it comes to *in vivo* and to human data, more confusion arises. In several *in vivo* experiments, amelioration of the pathological condition in arthritis has been stressed time after time to be linked to one enzyme rather than to another. $sPLA_2$ has been proposed to have a primary role in arthritis [38], and more recently several papers have appeared concerning the role of NO and iNOS in arthritis. By using the rat adjuvant arthritis model, it has been shown that inhibitors of NO are able to suppress arthritis development in this rat model [39, 40] as well as to reduce arthritis in MRL-*lpr*/*lpr* mice [41]. Furthermore, the NO precursor L-arginine, administered chronically, has been shown to exacerbate arthritis in the same model [42]. It has been determined recently in human synovial membranes of rheumatoid patients that synovial macrophages may express iNOS, but they are not the major producers of this mediator, supporting the hypothesis that NO levels are generated through a reciprocal pathway whereby NO from fibroblasts enhances pro-inflammatory cytokines from macrophages, which in turn could up-regulate iNOS expression [43]. However, NO up-regulates metalloprotease production in articular cartilage [44] and takes part in IL-1-mediated inhibition of proteoglycan synthesis [45]. Conversely, at the same time a chondroprotective role has been proposed for endogenous NO in bovine cartilage [46]. It appears that while in rats the NO has a detrimental effect, in humans its effect is not clear and could reflect a nonspecific synovial response to injury in inflammation with potential protective or pathological consequences.

THE FINAL PICTURE

This commentary is not the place to analyze all the studies and their possible incongruences, but endeavors to provoke in the reader a critical review of dogmas and current beliefs that most of the time are built on unilateral interpretation of the data. The main purpose of studies on the role of these enzymes should be to clarify the mechanisms involved in the inflammatory pathology examined, dissecting the phenomenon *in vitro* and trying to find a correlation between *in vivo* systems and human pathologies with the final goal of improving drug targeting. The final picture of the complex processes involved in host response to an inflammatory noxious stimulus is rather confusing (Fig. 2), and trying to be constantly up-to-date is very frustrating, since much new information is being produced, sometimes with divergent conclusions, and the field that goes under the generic name of inflammation has expanded enormously. Again this commentary does not pretend to show all the possible relations among these systems but only to highlight some of the possible and obvious interactions among the systems and to point out that most of the time researchers do not take into account that in their system they may not induce just the enzyme that they are analyzing at that moment, but also other enzymes, which most of the time are as relevant

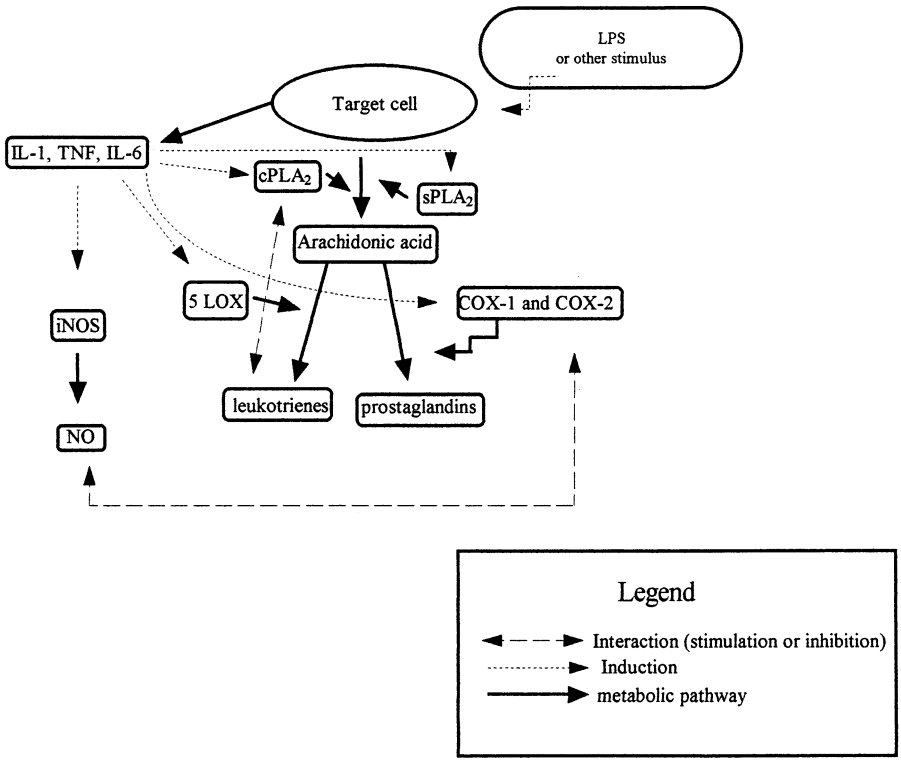

FIG. 2. Interactions between the different enzymes and mediators involved in the activation of the arachidonic acid cascade in inflammation. Abbreviations: 5-LOX, 5-lipoxygenase; COX-1 and -2, cyclooxygenase-1 and -2; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF, tumor necrosis factor; and PLA_2, phospholipase A_2.

to the process as the one analyzed. In some other cases they perform experiments using a cell line where an effect can be strongly amplified, but the effect may not play such a central role in an *in vivo* situation where there are several different cell populations that could come in contact with the stimulus applied. For this reason, researchers in this field sometimes look like the blind men in the fable "The blind men and the elephant" where all three men are touching the elephant, but one man sees a snake, another a bell rope, and a third a palm tree. Therefore, the biochemist looks for enzyme induction in many cell systems, sometimes losing contact with physiology and pathology, while on the other hand the pharmacologist studies the effect without looking into the single cell system, and the immunologists have their own approach to the same problem, with the final result that each scientist often has his or her own picture of the "elephant." Hence there are now special subspecialities dealing with platelets and inflammation, mast cells and inflammation, adhesion proteins and inflammation, and so on. It is very difficult to try to make some generalization and to assess the state of the art, since what is seen *in vitro* in a cell system is not always true in a human cell, or, worse, it can be true in both single cell systems, human and animal, but not play a very important role in disease processes where there are several other cells involved and interaction among them plays a crucial role. The final outcome is that the distance between what we know and what we do not know, instead of diminishing with time, increases. This is not a pessimistic view, but it is the only picture that a reader can obtain by analyzing some of the most recent papers dealing with inflammation cytokines, enzymes, protein induction, and signal transduction. There may be no possible alternative to this complexity since defense against an invading organism is the main function of our body's defense system, and it seems logical that the body can oppose a noxious stimulus and its following negative stimuli by using several different mechanisms.

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