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Regulation of cyclooxygenase enzymes by nitric oxide

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Abstract. Nitric oxide (NO), derived from L-arginine (L-Arg) by the enzyme nitric oxide synthase (NOS) is involved in the regulation of several important physiological and pathophysiological functions. The mechanisms by which NO exerts some of its beneficial or detrimental effects include activation of guanylate cyclase, formation of peroxynitrite, apoptosis, and regulation of cyclooxygenase (COX). Cyclooxygenase (COX) is the enzyme that converts arachidonic acid to prostaglandins (PG), prostacyclin (PGI_2) and thromboxane A_2. The role of NO in the regulation of COX and its importance in physiology, pathology and therapy will be reviewed. Evidence will be presented to suggest that COX enzymes are targets for the physiopathological roles of NO and that once activated in the presence of NO, they represent important transduction mechanisms for its multifaceted actions.
Key words. Nitric oxide; prostaglandins; cyclooxygenase; inflammation.

Introduction

The nitric oxide and cyclooxygenase pathways share a number of similarities. NO is the mediator generated from the nitric oxide synthase (NOS) pathway. Cyclooxygenase (COX) converts arachidonic acid to the prostaglandins (PG), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). Two major forms of NOS and COX have been identified to date. Under normal circumstances, the constitutive isoforms of these enzymes are found in virtually all organs. Their presence accounts for the regulation of several important physiological effects (e.g. antiplatelet activity, vasodilation, cytoprotection). On the other hand, in an inflammatory setting, these enzymes are induced in a variety of cells, resulting in the production of large amounts of proinflammatory and cytotoxic NO and PG. Release of NO and PG by these enzymes has been associated with the pathological roles of these mediators in several disease states. An important link between the NOS and COX pathways is that NO activates the COX enzymes resulting in an augmented production of prostaglandins. This article will not review the roles of NOS and COX in physiology and pathology, since a great body of information is already. Rather, I shall be discussing how the discovery that NO activates COX influences and furthers our understanding of these two pathways.

Biosynthesis of nitric oxide and prostaglandins

NO is generated via the oxidation of the terminal guanidino nitrogen atom of L-arginine by NOS. Three major isoforms of NOS have been identified. The three isoforms are distinct from each other based on their primary amino acid sequence (only 50–60% identity), tissue and cellular distribution, and mode of regulation. Two expressed constitutively, are calcium/calmodulin-dependent and are classified together as constitutive NOS isoforms (cNOS): endothelial-derived NOS (eNOS) and neuronal-derived NOS (nNOS). The third is a cytokine-inducible, calcium/calmodulin-independent isoform of NOS (iNOS) [1]. The distinct properties of each of the NOS isoforms have important implications since it is the magnitude, duration and cellular sites of NO production which determine the overall physiological or pathophysiological effect of NO. For example, release of NO from cNOS occurs in small amounts and for a short period of time. NO released under these circumstances plays a crucial role in the cardiovascular system where it controls organ blood flow distribution, inhibits the aggregation and adhesion of platelets to the vascular wall, and inhibits leukocyte adhesion and smooth muscle cell proliferation [1]. In contrast, the inducible NOS isoform is not continuously present but is expressed in a wide variety of cells in response to inflammatory stimuli such as cytokines and lipopolysaccharide (LPS). The net result is a delayed (typically 4–6 hours) but very prolonged synthesis of high levels of NO. NO released from iNOS is thus involved in several pathological events [1–3].

Prostaglandins are formed by the action of the prostaglandin synthase in a two-step conversion of arachidonic acid. First, the enzyme converts arachidonic acid to a cyclic endoperoxide (PGG_2) by the action of COX, which is then followed by a peroxidase that cleaves the peroxide to yield the endoperoxide (PGH_2) [4]. These unstable intermediate products of arachidonic acid metabolism by COX are then rapidly converted to the prostaglandins (e.g. PGE_2, PGF_2, $TxA_{2\alpha}$, PGI_2) by specific isomerase enzymes [4]. COX was

first purified from the sheep seminal vesicle, a prodigious source of the protein [5–8] as a homodimer of approximately 140 kD molecular mass and subsequently cloned from the same tissue [7, 8]. With the availability of the cDNA encoding the protein and specific antibodies, numerous studies were performed to evaluate the distribution, expression, and regulation of COX both in vitro and in vivo.

Initially, it was thought that COX was a single enzyme that produced prostaglandins in most tissues and cell types. However, several studies have illustrated that COX activity is increased in certain inflammatory states and is induced in cells by proinflammatory cytokines and growth factors in vitro [9–11]. Following these observations, extensive research in this field led to the discovery that two forms of COX exist. The constitutive isoform (COX-1) is present in tissues such as the stomach, gut or kidney, where PG production plays a cytoprotective role in maintaining normal physiological processes [12]. In inflammatory processes, the inducible isoform of cyclooxygenase (COX-2) is expressed in many cells including fibroblasts and macrophages, and accounts for the release of large quantities of proinflammatory PG at the site of inflammation [12]. Selective inhibition of COX-2 is anti-inflammatory and inhibits nociception [13, 14].

iNOS and COX-2 induction is blocked by anti-inflammatory steroids including dexamethasone and this property of steroids may explain their potent anti-inflammatory effect [12, 15].

In summary it is clear that the NOS and COX systems are often present together, share a number of similarities and play fundamental roles in similar physiopathological conditions. An additional feature that links the NOS and COX pathways is that NO can markedly enhance the production of PG [16]. This property of NO is attributable to its ability to activate the COX enzymes [16].

Nitric oxide activates COX enzymes

The discovery that NO regulates COX activity was originally made using cellular systems and purified enzymes. Microsomal sheep vesicles are a rich source of COX-1 and can thus be used to explore whether the exogenous application of NO can augment further COX-1 activity [17]. NO gas directly increases COX-1 activity of microsomal sheep seminal vesicles as well as murine recombinant COX-1; this leads to a remarkable seven-fold increase in PGE_2 formation [16, 18]. COX-2 is also activated by NO. COX-2 but not iNOS is induced in human fetal fibroblast by interleukin-1β (IL-1β). Therefore IL-1β-stimulated fibroblasts can be used as a cellular model to investigate the effects of exogenous NO on COX-2 activity. Exposure of IL-1β-stimulated fibroblasts to either NO gas or two NO-donors, sodium nitroprusside (SNP) and glyceryl trinitrate (GTN), increased COX-2 activity by at least four times; this resulted in increased production of PG. This phenomenon is independent of the known effects of NO on the soluble guanylate cyclase. Methylene blue, an inhibitor of the soluble guanylate cyclase, inhibited the increase in cGMP by NO in the fibroblast but did not prevent its ability to stimulate COX activity and hence PG production [16]. The ability of NO to activate COX-2 directly was supported by the finding that NO increases the activity of purified recombinant COX-2 enzymes. Having observed that NO activates COX-1 and COX-2 enzymes, we then asked whether COX-2 activity was affected by endogenously produced NO. The mouse macrophage cell line RAW-264.7 was stimulated with endotoxin so as to induce iNOS and COX-2 enzymes, resulting in the production of large amounts of NO and PG. Inhibition of iNOS activity by nonselective NOS inhibitors such L-NMMA or NO_2Arg, or more selective iNOS inhibitors such as L-NIL or aminoguanidine (AG) [19–22], attenuated as expected the release of NO from these cells. The remarkable finding was that when NO release was inhibited, there was a simultaneous inhibition of PG release [16]. The NOS inhibitors did not behave as nonsteroidal anti-inflammatory drugs (NSAIDs): they neither inhibited COX activity [16] nor affected the induction of COX-2 (unpublished observations). These results suggested that endogenously released NO from macro-phages exerted a stimulatory action on COX-2 activity, enhancing the production of PG. Thus, inhibition of NOS activity reduced the output of PG from COX-2.

The molecular mechanism by which NO activates COX remains to be identified but it is unlikely to combine with the ferric heme in COX [23]. A few possibilities can be put forward; these are depicted in figure 1. (1) Antioxidant effect: O_2^- is generated during COX activation and has been postulated to be involved in the auto-inactivation of the COX enzyme [24]. NO interacts with O_2^- and limits the amounts of the radical necessary for auto-inactivation [25]. It is therefore possible that NO augments COX activity by acting as an antioxidant (removal of O_2^-), preventing the auto-inactivation of COX. (2) Formation of nitrosothiols: NO nitrosylates cysteine residues in the catalytic domain of COX enzymes, leading to the formation of nitrosothiols; these can produce changes in the structure of the enzyme which results in increased catalytic efficiency [26]. (3) Generation of peroxynitrite: When the amounts of NO and O_2^- are increased, such as during an inflammatory response, NO and O_2^- interact to form the cytotoxic molecule peroxynitrite (ONOO–) which in turn can decompose to form the OH· [27]. Using purified COX-1· and COX-2 enzymes as well as sheep seminal vesicles, Landino and coworkers reported that ONOO– in-

creased COX-1 and COX-2 activity [28]. The participation of the hydroxyl radical in COX activation has recently been suggested by demonstrating that OH· relaxes isolated rabbit aorta via an indomethacin-sensitive pathway indicative of the participation of COX metabolites in this effect [29].

Physiological implications of NO-mediated COX activation

The activation of COX-1 by NO released from the constitutive form of NOS has important consequences under normal physiological conditions. This could indeed represent an important mechanism through which NO and PGs exert their beneficial cytoprotective effects in the cardiovascular and central nervous systems.

The production of small amounts of NO and PGs from the constitutive enzymes regulates various physiological processes including the inhibition of platelet aggregation and white blood cell adhesion, regulation of blood vessel tone, and cytoprotection in the kidney and intestinal mucosa. The main difference between the effects of NO and PGs in mediating these effects lies at the level of their respective intracellular transduction mechanisms. Thus, NO activates the soluble guanylate cyclase leading to increased cGMP, and PG activates the adenylate cyclase leading to increase in cAMP. This raises the possibility that the increased platelet aggregation, vasoconstriction and elevation of systemic blood pressure induced by inhibition of endogenous NO production by nonselective NOS inhibitors could be due not only to removal of endogenous NO but also to a concurrent reduction of antiplatelet and vasodilator COX products. Dual inhibition of NO and PG by nonselective NOS inhibitors may well explain the deleterious effects of these drugs observed in organs such as the kidneys and the gastrointestinal tract, where both NO and PG are cytoprotective.

An interesting effect of NO on COX-1 is perhaps in the regulation of neuropeptide release. Norepinephrine (NE) mediates the release of luteinizing hormone releasing hormone (LHRH) from LHRH terminals and it is known that LHRH release from these terminals requires increased release of PGE_2 [30]. In an elegant study, Rettori and colleagues demonstrated that the release of LHRH following NE stimulation of hypothalamic slices was prevented by NOS inhibition and subsequent blockade of NE-mediated PGE_2 release [30]. These results have opened the possibility that through COX activation, NO may mediate exocytosis of secretory granules not only for LHRH but also for other neuropeptides that are released by PGE_2. NO-driven COX-1 activation also seems to have a role in reproduction in that NO also modulates uterine motility [31] by activating COX-1 and releasing PGE_2. The potential interaction of NO and COX-1 and its implications in reproduction remain exciting areas for future investigation.

The discovery that NO directly activates COX-1 enzyme has given a new dimension to the usefulness of

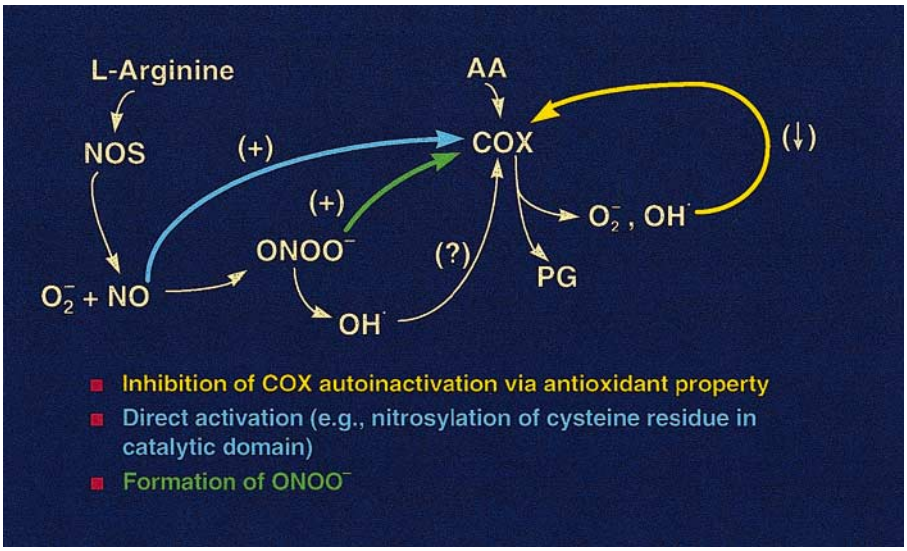

Figure 1. COX enzymes as potential "receptor" targets for NO. For simplicity the NOS and COX isoforms are not shown. COX enzymes are targets for the physiopathological action of NO and once activated in the presence of NO, represent important transduction mechanisms for the actions of nitric oxide. Possible mechanisms of activation are highlighted in different colors (color of arrows corresponds to color of text). The role of the hydroxyl radical is not yet known although it is known to increase PG release.

exogenous NO therapy and has uncovered additional mechanisms by which NO donors might exert their beneficial effects in the clinic. It is known that NO release from clinically useful NO donors such as GTN or SIN-1 accounts for their vasodilatory and antithrombotic actions, and that these properties are pertinent to the therapeutic effects of the NO donors in conditions such as myocardial ischemia, thrombosis and atherosclerosis that are associated with a failing endogenous NO pathway [32]. For instance, since NO donors do not require an intact endothelium in order to be effective [33] they can restore the desired vascular dilation and suppress platelet aggregation despite advanced atherosclerosis [34, 35]. The cardiovascular effects exerted by endogenously produced NO are often mediated in conjunction with prostacyclin (PGI_2), a potent vasodilator and platelet-inhibitory COX metabolite released from the endothelium. NO released from the NO donors activates COX-1 in vitro in endothelial cells as well as in vivo, promoting the release of PGI_2 [36, 37]. Activation of COX is a cGMP-independent process. Our work indicates that the vasodilator and antithrombotic effects of NO (and NO donors) occur via two steps: (1) direct NO-stimulated soluble guanylate cyclase activation and cGMP elevation, and (2) NO-mediated activation of cyclooxygenase in the endothelial cells leading to PGI_2 release and cAMP elevation [37]. During the progression of atherosclerosis or hypertension, defects at the level of the soluble guanylate cyclase have been observed. Theoretically this may hamper the effectiveness of NO donors in restoring their vasodilator and antithrombotic (cGMP-dependent) properties. However, since NO does not have to rely on the soluble guanylate cyclase to activate COX, NO donors will still be able to provide adequate vasodilation and antithrombotic effects.

Pathological implications of NO-mediated COX activation

In vivo studies revealed that the regulation of COX by NO is a powerful mechanism that is used to amplify the course of the inflammatory response. Indeed, we and others observed that iNOS and COX-2 are induced in a number of inflammatory models including rabbit hydronephrotic kidney [38], endotoxin-induced septic shock [39] and carrageenan-induced pouch and paw inflammation [40–42]. The prolonged release of large amounts of NO and PG may subsequently have deleterious effects. Thus, inhibition of either NOS or COX activity is protective in several diseases. The interesting observation was that inhibition of NO by selective iNOS inhibitors is associated with profound inhibition of not only NO but also of PG release; the anti-inflammatory potency of the iNOS inhibitors correlated with their respective ability to block both NO and PG (see all papers mentioned above). For instance, in an acute model of inflammation, namely carrageenan-induced paw edema in rats, inhibition of edema with L-NIL is associated with a dose-dependent inhibition of NO release and a clear inhibition of proinflammatory PG [41]. The proinflammatory roles of NO have a PG component, which has also been demonstrated by showing that the injection of SNP elicits edema in the footpad of rats and the formation of edema is blocked by NSAID [43]. Therefore, the effects of endogenously released NO on COX-2 are mimicked by exogenous NO. Inhibition of NO in lungs taken from endotoxin-treated rats resulted in an inhibition of PGI_2 release [44]. In acute pancreatitis, the increased production of PG was inhibited by NOS inhibitors, indicative of the participation of NO in this process [45].

Furthermore, injection of carrageenan into the preformed air pouch of a rat induces an inflammatory response characterized by iNOS and COX-2 induction, white blood cell infiltration, edema and protein leakage into the pouch [40]. Selective inhibition of iNOS by L-NIL and AG inhibited not only NO but also PG production [40]. All other parameters of inflammation were attenuated and histological examination of the pouch lining taken from animals treated with the iNOS inhibitor revealed a lack of inflammation [40]. In this model, we were able to demonstrate that in the presence of L-NIL, COX-2 was activated at least seven-fold by exogenous injection of NO donors such as sodium nitroprusside or nitroglycerin; this activation resulted in a profound increase in PGE_2 release [40]. Similar results have been observed in the hydronephrotic rabbit model of inflammation [38]. Nonselective NOS inhibitors such as L-NMMA are also anti-inflammatory in these models and block both NO and PG. Nevertheless, when compared to a more selective iNOS inhibitor their anti-inflammatory profile is weaker. This may stem from the fact that nonselective NOS inhibitors also block release of NO and PG from the constitutive enzymes which are known to play key cytoprotective roles. This would mask to some degree protective effects that would arise from their inhibitory effects on iNOS. It is therefore imperative to preserve cNOS and COX-1 activity in an inflammatory setting and this can be achieved by the use of glucocorticoids. The potent anti-inflammatory glucocorticoid dexamethasone is a good example of an agent able to inhibit the induction of both iNOS [1] and COX-2 [15]. Unfortunately, serious side effects of steroids, independent of their ability to block iNOS and COX-2 expression, limit their clinical usage. Selective inhibition of iNOS or COX-2 is also anti-inflammatory in a number of models. Besides its proinflammatory role, a feature of NO that is not shared by PG is its potent cytotoxic effect. Although there is some evidence that massive amounts of PGE_2 inhibit the formation of NO [46, 47] the relevance of these observations is ques-

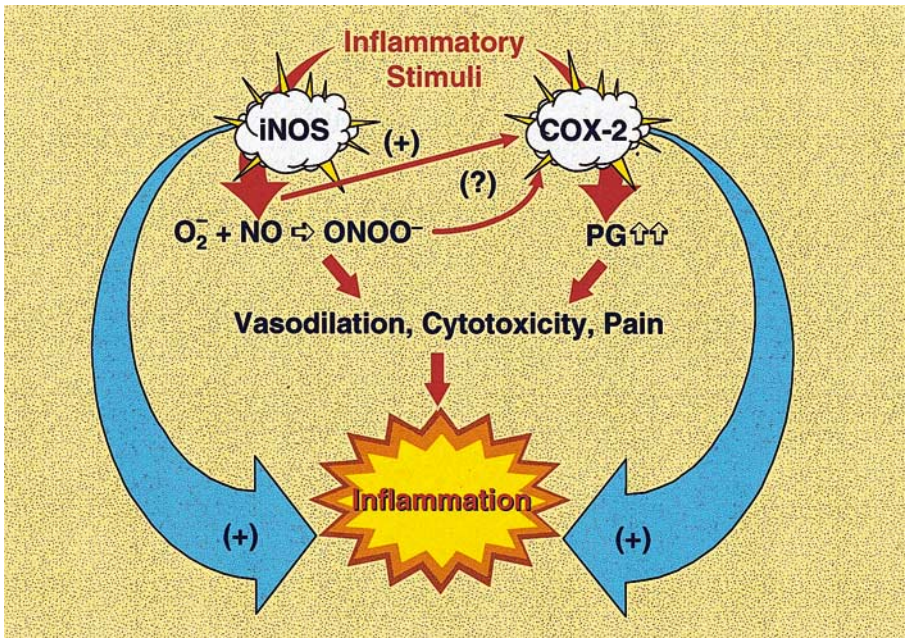

Figure 2. NO-driven COX-2 activation in inflammation. In those conditions where both the iNOS and COX-2 systems are induced, there is an NO-mediated increase in the production of proinflammatory PG that may result in an exacerbated inflammatory response. Regulation of COX-2 by NO itself or by a product formed from its interaction with other radicals is a powerful mechanism which amplifies the course of the inflammatory response. In this model it is easy to see how, by inhibiting not only NO but also PG, NOS inhibitors exert their anti-inflammatory effects.

tionable. We and others have found that in vivo inhibition of PG release by selective inhibition of COX-2 in inflammation has only minimal effects on NO production, indicating that products of the COX pathway do not play a major role in the regulation of NOS activity. This could explain why in arthritis a NSAID such as indomethacin, by blocking PG but not NO production, alleviates the symptoms associated with the inflammatory insult but does not modify the course of the disease [48]. Since selective iNOS inhibitors have the potential of reducing both NO and PG, it is exciting to imagine that the use of these selective iNOS inhibitors may have the advantage of not only alleviating inflammatory symptoms through dual inhibition of NO and NO-driven COX-2 activation, but may also eliminate further cytotoxic damage of NO, and may prove to be important disease modifying drugs in chronic inflammatory diseases such rheumatoid arthritis. Amplification of the inflammatory response by NO-driven COX-2 activation is depicted in figure 2.

NO has also been reported to modulate COX-2 activity in other models. Rat islet cells or vascular smooth muscle cells stimulated with IL-1β release both NO and PG; inhibition of NO production with NOS inhibitors blocked the production of PGE_2 from immunostimulated cells [49, 50]. Recent studies also indicate that the release of PG following induction of COX-2 in astroglial cells stimulated with NMDA is inhibited by iNOS inhibition [51]. Stimulation of RAW 264.7 macrophages with conditioned medium of activated encephalitogenic lymphoid cells is associated with induction of both iNOS and COX-2 [52]. Inhibition of iNOS activity by the selective iNOS inhibitor aminoguanidine inhibited not only NO but also the increased production of PGE_2 [52]. Aminoguanidine had no effects on the induction of COX-2 enzyme [52]. The interactions between the iNOS and COX-2 system may play an important modulatory role in pathophysiological mechanisms such as cerebral ischemia, stroke and multiple sclerosis [51, 52].

Inhibition of PG by nitric oxide

The above in vitro as well as in vivo data indicate that NO activates COX enzymes. A few investigators have however reported an inhibitory effect of NO on both the expression and the activity of COX in cells including mouse macrophage cell line J774.2, rat Kupffer cells and rat peritoneal macrophages [53–55]. In endotoxin-stimulated J774.2 macrophages sodium nitroprusside inhibits expression of COX-2 as well as its activity and the inhibition of endogenous NO by NOS inhibitor increased PGI_2 release and COX-2 expression [53]. Nevertheless, when these cells were treated first with a NOS inhibitor and then stimulated with an NO donor, an increased release of PGI_2 was observed, indicative of the ability of NO to increase COX activity [53]. Most of the data collected have come from in vitro cells or tissue. Overall, the experimental data obtained so far on an inhibitory effect of NO on the COX pathway are lim-

ited and controversial. Thus the in vivo significance of a possible negative effect of NO on COX needs to be assessed in future studies.

Modulation of NO pathway by COX products

Products of the COX pathway may inhibit or increase the release of NO. Large amounts of PGI_2 inhibit LPS-stimulated iNOS induction in J774.2 macrophages or murine peritoneal macrophages without affecting the enzyme once expressed [46, 47, 56, 57].

A role for PG as enhancers of NO production has been reported. In rat Kupffer cells stimulated with endotoxin, inhibition of endogenously produced PG by indomethacin resulted in a concomitant inhibition of NO release [58]. On the other hand, indomethacin did not inhibit NO release in mouse RAW 264.7 cells [16] nor in J774.2 macrophages [53].

The mechanism of action of the PG on the NOS pathway has been attributed in most instances to activation of adenylate cyclase system with subsequent increase in cyclic AMP (cAMP) levels. Increases in intracellular cAMP have been suggested as mediating either the inhibitory role of PG on the NOS pathway [56, 57] or the stimulatory effect of PG on the NOS pathway. For instance, in rat vascular smooth muscle, agents such as forskolin or dibutyryl-cAMP increase iNOS mRNA as well as the formation of NO [59, 60].

Again, the experimental data obtained so far on the inhibitory/stimulatory effects of PG on the NOS pathways is limited and controversial. The in vivo significance of possible effects of PG on the NOS system both in physiology and pathology needs to be evaluated. We and others have found that in vivo inhibition of PG release by selective inhibition of COX-2 in inflammation has only minimal effects on NO production, indicating that products of the COX pathway do not play a major role in the regulation of NOS activity.

Conclusions

This review highlights the importance of the discovery that cyclooxygenase enzymes are activated by NO. The molecular mechanisms involved in this activation require further investigation. Further understanding of how these two critical systems interact will undoubtedly provide us with a better understanding of the complex roles of NO in physiopathological events. The discovery that NO may regulate physiological and pathological effects via the activation of the COX enzymes raises the novel possibility that the COX enzymes are important endogenous transducers of the physiological and pathological effects of NO.

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