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Modulation of Prostaglandin Biosynthesis by Nitric Oxide and Nitric Oxide Donors

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***Abstract*——The biosynthesis and release of nitric oxide (NO) and prostaglandins (PGs) share a number of similarities. Two major forms of nitric-oxide synthase (NOS) and cyclooxygenase (COX) enzymes have been identified to date. Under normal circumstances, the constitutive isoforms of these enzymes (constitutive NOS and COX-1) are found in virtually all organs. Their presence accounts for the regulation of several important physiological effects (e.g. antiplatelet activity, vasodilation, and cytoprotection). On the other hand, in inflammatory setting, the inducible isoforms of these enzymes (inducible NOS and COX-2) are detected in a variety of cells, resulting in the production of large amounts of proinflammatory and cytotoxic NO and PGs. The release of NO and PGs by the inducible isoforms of NOS and COX has been associated with the pathological roles of these mediators in disease states as evidenced by the use of selective inhibitors. An important link between the NOS and COX pathways was made in 1993 by Salvemini and coworkers when they demonstrated that the enhanced release of PGs, which follows inflammatory mechanisms, was nearly entirely driven by NO. Such studies raised the possibility that COX enzymes represent important endogenous "receptor" targets for modulating the multifaceted roles of NO. Since then, numerous papers have been published extending the observation across various cellular systems and animal models of disease. Furthermore, other studies have highlighted the importance of such interaction in physiology as well as in the mechanism of action of drugs such as organic nitrates. More importantly, mechanistic**

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studies of how NO switches on/off the PG/COX pathway have been undertaken and additional pathways through which NO modulates prostaglandin production unraveled. On the other hand, NO donors conjugated with COX inhibitors have recently found new interest in the understanding of NO/COX reciprocal interaction and potential clinical use. The purpose of this article is to cover the advances which have occurred over the years, and in particular, to summarize experimental data that outline how the discovery that NO modulates prostaglandin production has impacted and extended our understanding of these two systems in physiopathological events.

I. Introduction

Prostaglandins (PGs[1]) and nitric oxide (NO) represent some of the most relevant local mediators that participate, under basal conditions, in the modulation of many cellular functions. This occurs as a consequence of constitutive biosynthesis and release of both mediators via well identified bioenzymatic complexes [cyclooxygenases (COX) for PGs and nitric-oxide synthases (NOS) for NO]. These highly regulated biosynthetic pathways act on specific substrates [arachidonic acid (AA) and L-arginine, respectively], leading to pulsed release of nanomolar concentrations of both mediators. The basal release of NO and PGs has been shown to exert a protective role in many physiopathological conditions, such as vascular diseases (via enhanced vasodilatation and antiplatelet activity), gastric lesions (via activation of gastroprotective processes), erectile dysfunction, and learning and memory processes (via potentiation of neuronal plasticity). Therefore, the use of PG derivatives and NO donors has been proposed in the treatment of such disorders due to their ability of restoring basal levels of PGs and NO (e.g., organic nitrate esters, which release NO after enzymatic bioconversion, have been used for more than a century in the treatment of myocardial ischemia).

Under inflammatory states or in early stages of many diseases characterized by the occurrence of inflammatory processes, NO and PGs are released simultaneously in large amounts; this effect is mainly due to the activation of inducible enzymes, which release NO and PGs in micromolar concentrations. The release of PGs and NO in large amounts and the evidence that both PGs and NO overproduction may be detrimental for cell survival, suggested experimental work over the last two decades to identify the possible participation of both mediators in the pathogenesis of many disease states. In particular, overt production of PGs and NO has been shown to occur in the damaged tissue accompanying the inflammatory processes involved in rheumatic diseases, chronic degenerative disorders, and, in the brain, neurodegenerative processes associated with brain ischemia as well as in neuroinflammatory diseases (such as multiple sclerosis, demyelinization, HIV-related brain disorders, Alzheimer's disease). In addition, the development of novel and more selective COX and NOS inhibitors suggested their potential use for the protection of inflamed tissues.

Recently, evidence has been accumulated indicating that there is a constant cross talk between NO and PG release that occurs at many levels, but having its central feature in the modulation of molecular mechanisms that regulate PG-generating pathway. The final effect of this modulatory activity is not univocal, since endogenous NO as well as NO donors have been found to switch on/off the COX pathway, depending on the basal levels of NO released, by the cell type in which PG biosynthesis is generated and by the intensity of the stimulus employed for PG release. In addition, NO conjugates with superoxide anions, leading to the formation of peroxynitrite, which modulates COX enzymes. Finally, recent evidence has been accumulated that NO donors conjugated with COX inhibitors (e.g., nitroaspirin and nitroflurbiprofen) may have a potential use in the treatment of inflammatory as well as noninflammatory disorders, suggesting that NO/COX reciprocal interaction are relevant in the pathophysiological mechanisms underlying many disease states. Taken together, these findings seem to give credence to an active reciprocal modulation of NO and

[1]Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; NOS, nitric-oxide synthase; AA, arachidonic acid; HIV, human immunodeficiency virus; LPS, lipopolysaccharide; NSAID, nonsteroidal anti-inflammatory drug; MK-801, dizocilpine maleate; CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; CGRP, calcitonin gene-related peptide; SC560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; TNF-α, tumor necrosis factor-α; NS-398, *N*-[2-(cyclohexyloxyl)-4-nitrophenyl]-methane sulfonamide; AD, Alzheimer's disease; IL, interleukin; INF-γ, interferon-γ; APP, amyloid precursor protein; JTE-522, 4-(4-cyclohexyl-2-methyl-oxazol-5-yl)-2-fluorobenzenesulfonamide; iNOS, inducible NOS; MAPK, mitogen-activated protein kinase; Aβ, amyloid-β; FK-506, tacrolimus; PLA2, phospholipase A2; sPLA2, secretory PLA2; NF-κB, nuclear factor-κB; SC-58125, 1-[(4-methysulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl) pyrazole; EDRF, endothelium-derived relaxing factor; eNOS, endothelial NOS; nNOS, neuronal NOS; A23187, calcimycin; $ONOO^-$, peroxynitrite; BH_4, tetrahydrobiopterin; HNO, nitroxyl; L-NMMA, N^G-monomethyl-L-arginine; ARL 17477, *N*-[4-(2-{[(3-chlorophenyl)methyl]amino}ethyl)phenyl]-2-thiophenecarboximidamide; PBI-TU, *S*,*S*′-[1,3-phenylene-bis(1,2-ethanediyl)]-bis-isothiourea; 1400W, *N*-3-aminomethyl-benzylacetamidine; SNP, sodium nitroprusside; GTN, glyceryl trinitrate; ISDN, isosorbide dinitrate; NAC, *N*-acetylcysteine; TSA, thiosalicylic acid; L-NIO, L-N^5-(1-iminoethyl)-ornithine; GW274150, *S*-2-amino-1-ethylamino-5-thioheptanoic acid; PD98059, 2′-amino-3′-methoxyflavone; cNOS, constitutive NOS; NCX-4016, 2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl ester; NCX-4215, 2-acetoxybenzoate 2-(2-nitroxy)-butyl ester; VSMC, vascular smooth muscle cell; ASA, acetylsalicylic acid; SC-58236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide; L-745,337, 5-methanesulfonamido-6-(2,4-difluorothiophenyl)-1-indanone; GW273629, 3-[[2-[(1-iminoethyl)amino]ethyl]sulphonyl]-L-alanine; SB202190,4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole.HCl; S-2474, (*E*)-(5)-(3,5-di-*tert*-butyl-4-hydroxybenzylidence)-2-ethyl-1,2-isothiazolidine-1,1-dioxide.

PG-generating systems which represent the basis for a rationale use of traditional NO donors and for the development of novel and more suitable NO donors and COX inhibitors.

II. The Prostaglandin Biosynthetic Pathway

PGs and related compounds are some of the most prevalent autacoids detected in every tissue and body fluid except for the red blood cells. As local hormones, they produce in minute concentrations an incredibly broad spectrum of effects that modulate almost every biological function. They mostly derive from the 20-carbon fatty acid, arachidonic acid, by the action of the PG synthase in a two-step conversion (Fig. 1). First, arachidonic acid is converted into a cyclic and unstable endoperoxide (PGG_2) by PG endoperoxide synthase (PGHS) or COX, which is then followed by a peroxidase that cleaves the peroxide to yield the endoperoxide (PGH_2). These unstable intermediate products of arachidonic acid metabolism are then rapidly converted to the prostanoids (PGD_2, $PGF_{2\alpha}$ PGE_2, thromboxane A_2, PGI_2) by specific isomerase enzymes (Flower and Vane, 1972; Smith and Lands, 1972; Needleman et al., 1986; Smith et al., 1991) (Fig. 1). In 1976, COX was first purified from sheep seminal vesicles, a prodigious source of the protein (Hemler and Lands, 1976; Miyamoto et al., 1976; Van der Ouderaa et al., 1977), as a homodimer with a molecular mass of 71 kDa and subsequently cloned from the same tissue (DeWitt and Smith, 1988; Merlie et al., 1988) (Fig. 2). The enzyme exhibits both COX and hydroperoxidase activities. 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.

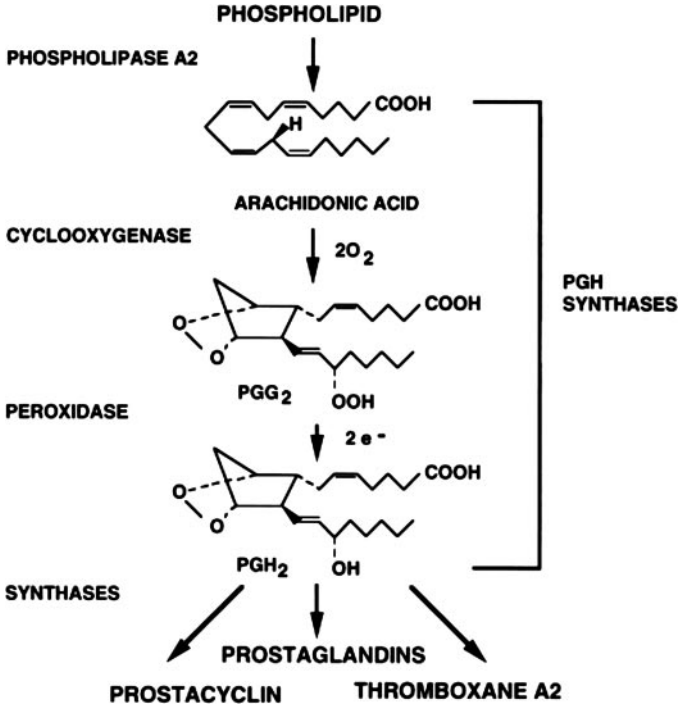

FIG. 1. The membrane phospholipids—arachidonic acid cascade.

Over the years, there were various suggestions that there was a second COX enzyme. As early as 1972, Smith and Lands (1972) and Flower and Vane (1972) speculated on the existence of isoenzymes. Later, Lin et al. (1989) hypothesized that a different COX enzyme could be induced by platelet-derived growth factor, and as often happens before a new discovery is properly defined, many pharmacologists and biochemists reported an inducible COX without knowing that they were working with a different enzyme. Needleman and his colleagues (Morrison et al., 1978) demonstrated a progressive increase in PG release dependent on de novo synthesis of COX enzyme in a inflammatory model of ureter-obstructed kidney.

In the following years, a number of 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 (Bailey et al., 1985; Sano et al., 1992). Needleman and his group continued their earlier work and reported that bacterial lipopolysaccharide (LPS) increased the synthesis of PGs in human monocytes in vitro and in mouse peritoneal macrophages in vivo. This increase, but not the basal level of enzyme, was inhibited by dexamethasone and associated with de novo synthesis of a new COX protein. They reinforced the concept of "multiple COX pools, constitutive and stimulated, possibly under different regulatory controls" (Seibert et al., 1991). The breakthrough discovery of a different COX came from molecular biologists outside the field of PGs. Simmons and his colleagues (Simmons et al., 1989; Xie et al., 1991) were studying early response genes and discovered an inducible form of COX in chicken embryo cells. It was encoded by a 4.1-kb mRNA similar in size to that reported by other authors (Rosen et al., 1989). They cloned the gene, deduced the protein structure, and found it homologous to COX, but to no other known protein (Fig. 2). Independently, other groups found similar results in different animal species (O'Banion et al., 1991; Sirois and Richards, 1992). Thus, there are two distinct enzymes, COX-1, the constitutive isoform, and COX-2 (the inducible one). Both enzymes have a molecular weight of 70 to 71 kDa and are almost identical in length, with just over 600 amino acids, of which 63% are in an identical sequence. However, the human COX-2 gene of 8.3 kb is a small immediate early gene, whereas human COX-1 originates from a much longer 22-kb gene. The gene products also differ, with the mRNA for the inducible enzyme being approximately 4.5 kb and that of the constitutive enzyme being 2.8 kb (Otto and Smith, 1995; Herschman, 1996). Garavito and his colleagues (Picot et al., 1994) have determined the three-dimensional structure of COX-1, which consists of three independent folding units: an epidermal growth factor-like domain, a

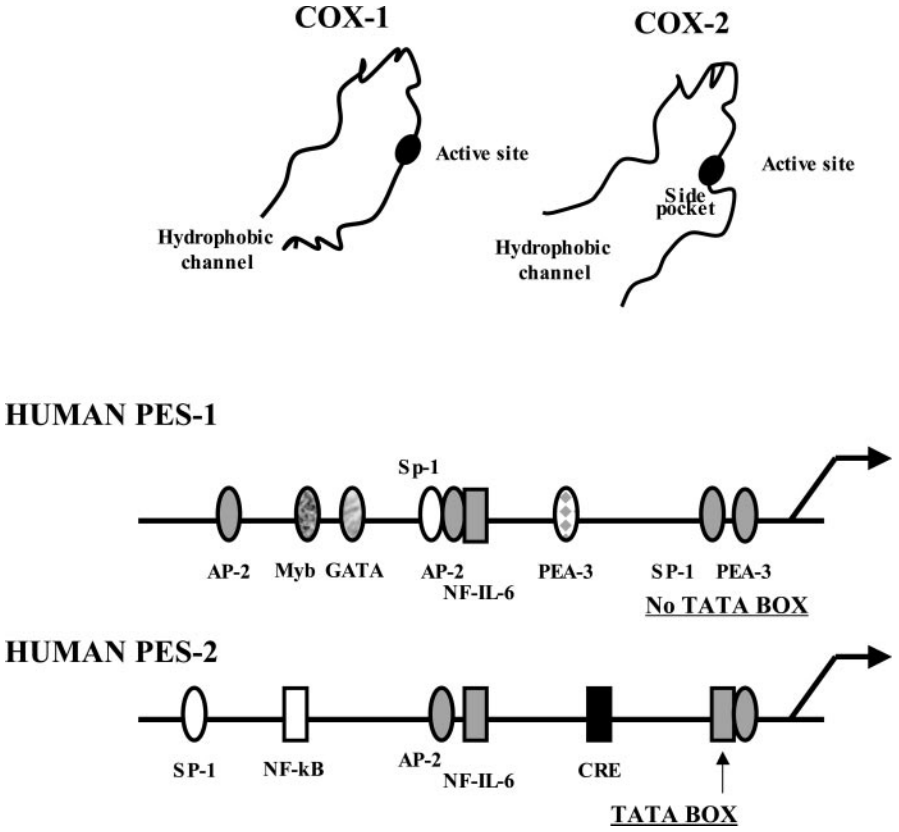

FIG. 2. Representation of COX isoforms and pattern of molecular modulation of both enzymes.

membrane binding section, and an enzymatic domain. The three-dimensional X-ray crystal structure of human or murine COX-2 can be superimposed on that of COX-1; the residues that form the substrate binding channel, the catalytic sites, and the residues immediately adjacent are all identical except for two small variations. In these two positions, the same substitutions occur: isoleucine in COX-1 is exchanged for valine in COX-2 at position 434 and 523 (Fig. 2). Most nonsteroidal antiinflammatory drugs (NSAID) compete with arachidonic acid for binding to the active site; uniquely, aspirin irreversibly inhibits COX-1 by acetylation of serine 530, thereby, excluding access to the endogenous substrate (Figs. 2 and 3) (Roth et al., 1975). COX-1 is ubiquitous and has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin (PGI_2) which, when released into blood vessels, produces vasodilation and antithrombogenic activity and is cytoprotective when released by the gastric mucosa (Moncada et al., 1976; Whittle et al., 1978). Maintenance of kidney function both in animal models of diseases and in patients with congestive heart failure, liver cirrhosis, and renal insufficiency is dependent on vasodilator PGs (PGE_2 and PGI_2) mainly by COX-1, although low levels of mRNA for COX-2 have been reported (Harris et al., 1994), and up-regulation of COX-2 expression has been observed in the macula densa following salt deprivation (Harris et al., 1994). In platelets, the only isoform detectable is COX-1, and loss of arachidonic acid-induced platelet aggregation is not only a well established side effect of NSAID treatment, but also the therapeutic aim of the "half an aspirin a day" prophylaxis against thromboembolic disease. This prophylaxis is achieved through inhibition of COX-1, which leads to decreased production of thromboxane A_2.

An intense biosynthesis of AA metabolites has also been shown to occur in the brain in almost all periods of life, participating in many physiological changes and being strongly involved in many disease states. High levels of PGs, especially PGE_2, are detected in the blood and brain of the neonate (Mitchell et al., 1978; Jones et al., 1993; Li et al., 1995). In the retina, both increased COX-1 and COX-2 activities contribute to the augmented production of neonatal PGs (Hardy et al., 1998). In the brain, however, this mostly arises from increased expression and activity of the COX-2 pathway in brain vasculature, as opposed to adult brain, where prostanoid formation is catalyzed mainly by COX-1 (Peri et al., 1995). The rapid drop in PG levels in brain within the first 48 to 72 h after birth (Jones et al., 1993) is associated with a relative decrease in COX-2 expression, which seems to increase again thereafter (Parfenova et al., 2000).

COX-1 and COX-2 are expressed in discrete brain regions under basal conditions (for a review, see Yermakova and O'Banion, 2000; Hurley et al., 2002). No evi-

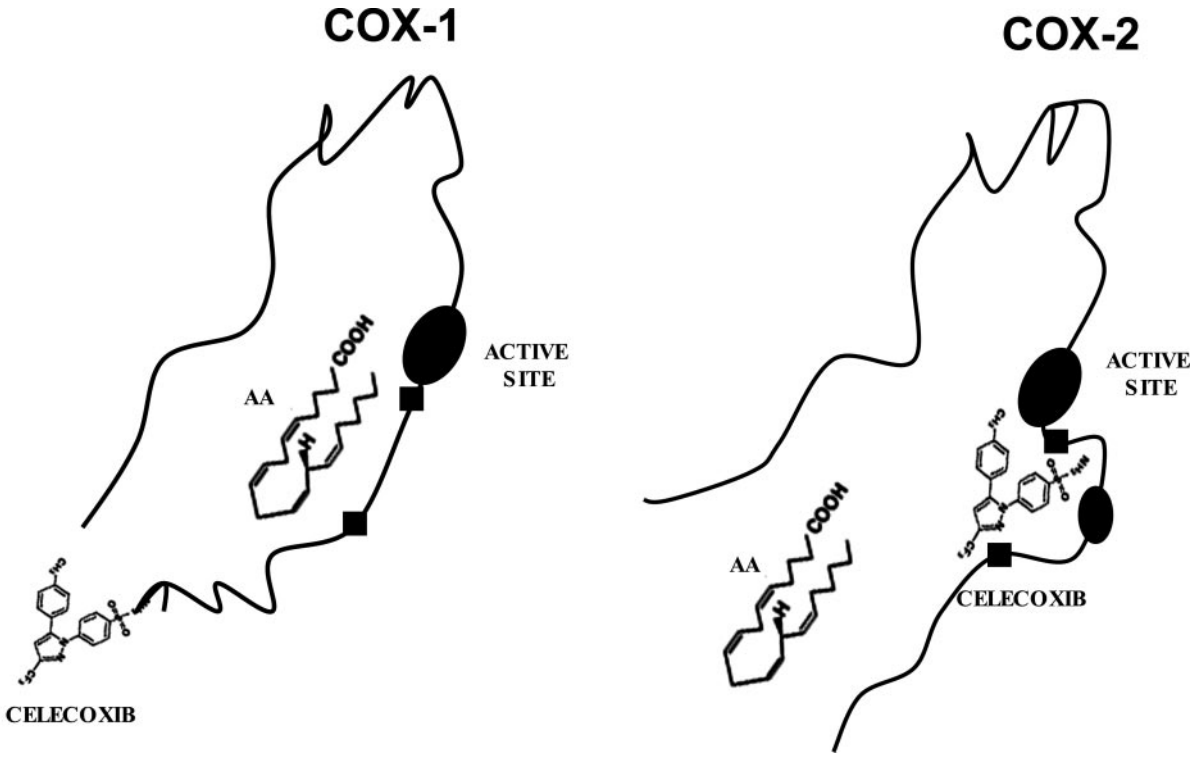

FIG. 3. Most NSAIDs compete with arachidonic acid for binding to the active site. COX-2 isoform structure has been targeted to design a new class of a more selective compound.

dence has been collected yet on the possible localization of COX-3 in the human brain, unless the putative target for the antipyretic activity of the COX-3 inhibitor acetaminophen is represented by the thermoregulatory regions of the hypothalamus (for a review, see Warner and Mitchell, 2002). In particular, evidence has been accumulated indicating that the central nervous system (CNS) represents one of the few tissues that contain detectable concentrations of COX-2 mRNA expressed constitutively (Seibert et al., 1994), although this point still remains to be elucidated. Indeed, according to the variability in the experimental procedures used by different groups, the constitutive expression of COX-2 isoenzyme within CNS still represents a controversial point. Basically, COX-2 immunoreactivity was found within the hippocampus (CA3 and CA4 areas in the rat's brain), piriform cortex, amygdala, and in layers II and III of the neocortex. The distribution of COX-2 immunoreactive neurons in the CNS suggests that this isoform may be involved in the processing of visceral and special sensory input and in elaboration of the autonomic, endocrine, and behavioral responses (Breder et al., 1995). In particular, COX-2 localization seems to strictly correlate with the glutamatergic neurotransmission. Indeed, COX-2 is mainly expressed by glutamatergic neurons that are immunolocalized in the cell body, proximal and distal dendrites, and dendritic spines (Kaufmann et al., 1996). Both basal expression of COX-2 and seizure-induced COX-2 overexpression are blocked by MK-801, a noncompetitive antagonist acting at the *N*-methyl-D-aspartate (NMDA) receptors for the excitatory amino acid L-glutamate, thus suggesting the possible involvement of constitutive COX-2 in glutamatergic mechanisms, mainly within the hippocampus (Yamagata et al., 1993). In addition, the possible link between COX-2 expression and excitotoxic mechanisms driven by exaggerated release of glutamate has also been described in many injury states (see below). Thus, the neuronal localization of COX-2 seems to correlate with the critical period of activity-dependent synaptic remodeling (Kaufmann et al., 1996) and strongly correlates with areas of CNS (such as the hippocampus) where synaptic plasticity may occur. COX is also expressed by cell types other than neurons including astrocytes, oligodendrocytes, and microglia (see Minghetti et al., 1998). In particular, COX-1 positive neurons are detectable in pyramidal neurons of CA3 and CA4 of human hippocampus, unless the concentrations of COX-2 seem to be predominant in these areas. However, COX-1 immunoreactivity prevails on COX-2 within the midbrain, pons, and medulla.

Although the central localization of COX-2, even controversial, seems to correlate with restricted populations of neurons, COX-1 is mainly located on microglial cells where the most relevant immunohistochemical labeling in the CNS is detectable (Yermakova et al., 1999). The possible role of COX-1 is still controversial. However, in contrast to the intense induction of COX-2 by different stimuli applied to microglial cells, the total COX-1 concentration seems to remain unchanged. Until recently, intense proliferation of COX-1 positive microglial cells

has been described in many injury states including perilesional areas of cerebral infarct, allergic encephalomyelitis, and experimental glioma (Deininger and Schluesener, 1999; Schwab et al., 2000a,b).

Expression of COX-1 is much greater than COX-2 in fetal hearts, kidneys, lungs, and brains as well as in the decidual lining of the uterus (Gibb and Sun, 1996). Constitutive COX-1 activity in the amnion could also contribute in the maintenance of a healthy pregnancy (Trautman et al., 1996). Furthermore, the so-called "cytoprotective" action of PGs in preventing gastric erosions and ulcerations has mainly brought about endogenously produced prostacyclin and PGE_2, which reduce gastric acid secretion and exert direct vasodilator action on the vessels of the gastric mucosa; prostanoids also stimulate the secretion of gastric mucus and duodenal bicarbonate. Knockout mice, in which the COX-1 gene was deleted, do not develop gastric ulcers spontaneously and show a decreased sensitivity to the damaging effects of indomethacin (Langenbach et al., 1995). In inflammatory processes, COX-2 is expressed in many cells (e.g., macrophages, monocytes, fibroblasts, and synoviocytes) and accounts for the synthesis of prostanoids involved in pathological processes, such as acute and chronic inflammatory states (Wu, 1995).

A. The Contribution of Prostaglandin Biosynthesis in Disease States

1. Inflammation and Pain. PGs are important mediators in spinal nociceptive processing. Basal release of PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2 occurs in the spinal cord and dorsal root ganglia. PGs then bind to G-protein-coupled receptors located in intrinsic spinal neurons (receptor types DP and EP_2) and primary afferent neurons (receptors EP_1, EP_3, EP_4, and IP) (Oida et al., 1995; Kawamura et al., 1997; Beiche et al., 1998; Rowlands et al., 2001; Vanegas and Schaible, 2001). Acute and chronic peripheral inflammation, interleukins, and spinal cord injury increase the expression of COX-2 and the release of PGE_2 and PGI_2. By activating the cAMP and protein kinase A pathway, PGs enhance tetrodotoxin-resistant sodium currents, inhibit voltage-dependent potassium currents, and increase voltage-dependent calcium inflow in nociceptive afferents. These events decrease firing threshold, increase firing rate, and induce release of excitatory amino acids, substance P, calcitonin gene-related peptide (CGRP), and NO. Conversely, glutamate, substance P, and CGRP increase PG release. PGs also facilitate membrane currents and release of substance P and CGRP induced by low pH, bradykinin, and capsaicin. All this should enhance elicitation and synaptic transfer of pain signals in the spinal cord. Direct administration of PGs to the spinal cord causes hyperalgesia and allodynia, and some studies have shown an association between induction of COX-2, increased PG release, and enhanced nociception by facilitating transmission of the nociceptive input (Yamamoto and Nozaki-Taguchi, 1996). The localization of PG biosynthetic pathway in the spinal cord is relevant to understand the contribution of COX enzymes in inflammatory pain. In particular, immunohistochemical studies detailed, in the last few years, the localization and regulation of COX-1 and COX-2 and neuronal NOS in lumbar spinal cord under basal conditions as well as following induction of painful peripheral stimuli. COX-1 immunoreactivity was found in glial cells of the dorsal and ventral horns, but not in neurons (Maihofner et al., 2001). In unstimulated mice, COX-2 immunoreactivity was found in the motorneurons of the ventral horns and in lamina X, but not in dorsal horn neurons. Inflammation causes an increased synthesis of COX-2-dependent PGs, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity (Beiche et al., 1996). In addition, after induction of a paw inflammation with zymosan, COX-2 immunoreactivity increased dramatically in dorsal horn neurons of laminae I–VI and X, paralleled by a significant increase in PGE_2 release from lumbar spinal cord (Maihofner et al., 2001). In particular, COX-2 was colocalized with neuronal NOS immunoreactivity in several neurons in superficial laminae of the dorsal horns and in the area surrounding the central canal. Under the same experimental conditions, NOS was distributed in the cytoplasm and extended to processes of some neurons. In contrast, electron microscopy revealed that COX-2 immunoreactivity was restricted to the nuclear membrane and rough endoplasmic reticulum, suggesting that inflammatory processes modulate NOS and COX enzymes in different subcellular compartments of spinal cord neurons involved in pain sensitivity (Maihofner et al., 2001).

Another important role of inflammatory PGs is represented by the induction of swelling. In this case, PGs are thought to cause plasma exudation in a synergistic fashion with other mediators such as complement factor 5a (Williams and Peck, 1977).

In addition, in animal models of arthritis, COX-2 is induced and thought to be responsible for the associated increase in PG production (Anderson et al., 1996). In particular, COX-2 expression has been identified in human osteoarthritis-affected cartilage (Amin et al., 1997) as well as in synovial tissue taken from patients with rheumatoid arthritis (Kang et al., 1996); moreover, PGE_2 appears to "sensitize" peripheral sensory nerve endings located at the site of inflammation (Bley et al., 1998). However, despite a clear role for COX-2 in causing inflammatory swelling in animal models (Chan and Rodger, 1997), the relative role of the two isoforms in pain is more complex. Clearly the perception of acute pain is more likely to be modulated by COX-1 because time for induction must elapse for COX-2. However, a number of studies have strongly implicated a role for COX-2 in inflammatory pain. For instance, highly selective COX-2 inhibitors inhibit hyperalgesia in rats (Riendeau et al., 1997), and the process of sensing pain may

lead to the induction of COX-2 in spinal cord (Beiche et al., 1996). Importantly, selective inhibitors of COX-2, e.g., rofecoxib, have been shown to be analgesic in humans when used for postdental surgery pain (Morrison et al., 1999).

Prostanoid-related neurochemical mechanisms other than inflammatory response are also involved in pain sensitivity. Indeed, bicuculline, an antagonist acting at GABA-A receptors when applied to the dorsal surface of the spinal cord, induces highly localized allodynia, an effect sustained with repeated bicuculline application and evoked by NMDA-dependent afferent input. This response is related to spinal PGs overproduction via constitutive COX-2 and depends on spinal PGs contribution to the abnormal processing of tactile input via spinal EP_1 receptors.

On the basis of these evidences, spinal application of specific COX inhibitors sometimes diminishes behavioral responses to persistent nociception. In particular, evidence has recently shown that some COX-2 inhibitors may be useful in many pathological conditions in which an altered pain sensitivity should occur. In particular, it has been shown that meloxicam, a selective COX-2 inhibitor, given peripherally, reduces the prolonged stimulation-evoked afterdischarge of dorsal horn neurons, suggesting that COX-2 may be involved in mediating and/or modulating excitatory effects of nociceptive inputs to dorsal horn neurons (Pitcher and Henry, 2002). In addition, acute administration of a selective COX-2 inhibitor attenuated both ongoing and movement-evoked bone cancer pain, whereas chronic inhibition of COX-2 significantly reduced ongoing and movement-evoked pain behaviors and reduced tumor burden, osteoclastogenesis, and bone destruction by >50% (Sabino et al., 2003). On the other hand, it has been reported that the specific COX-2 inhibitor celecoxib suppressed inflammation-induced PG levels in cerebrospinal fluid, whereas the selective COX-1 inhibitor SC560 was inactive in this regard (Smith et al., 1997). Moreover, the intraspinal administration of a COX-2 inhibitor was accompanied by a decrease in central PGE_2 levels and mechanical hyperalgesia (Samad et al., 2001).

The effect of COX-1 and COX-2 inhibitors depends upon different pathophysiological conditions and by noxious modalities by which nociception is activated. Indeed, it has been shown that the selective COX-1 inhibitor SC560 significantly reduced the formalin-evoked nociceptive response and completely abolished the formalin-evoked PGE_2 raise. In contrast, celecoxib, a selective COX-2 inhibitor, was ineffective in both regards. Indeed, the flinching behavior was largely unaltered, and the formalin-induced PGE_2 raise as assessed using microdialysis was only slightly, not significantly, reduced (Tegeder et al., 2001). This suggests that the formalin-evoked rapid PG release was primarily caused by COX-1 and was independent on COX-2. COX-2 mRNA and protein expression in the spinal cord were not affected by microdialysis alone, but the mRNA rapidly increased following formalin injection and reached a maximum at 2 h. COX-2 protein was unaltered up to 4 h after formalin injection. The time course of COX-2 upregulation suggests that the formalin-induced nociceptive response precedes COX-2 protein de novo synthesis and may therefore be unresponsive to COX-2 inhibition. Thus, it may be hypothesized that the efficacy of celecoxib in early injury-evoked pain may be less than that of unselective NSAIDs (Tegeder et al., 2001). Thus, on the basis of these evidences, it may be argued that unless clear evidence exists in favor of the benefit of selective and nonselective NSAIDs in acute pain, the relative contribution of COX-1 and COX-2 in chronic pain (i.e., associated with rheumatoid or osteoarthritis) remains to be established.

2. Cancer. One of the exciting observations correlated with the use of NSAIDs is the association with a reduction in the incidence of colon cancer. Indeed, a retrospective study revealed the findings that patients taking relatively low doses of aspirin (a maximum effect being seen at four to six tablets per week for long periods of time) had substantially reduced risks of developing colon cancers (Thun et al., 1991; Giovannucci et al., 1995). The NSAID sulindac can reduce the number and size of polyps in patients with familiar adenomatous polyposis (Giardiello et al., 1993; Giovannucci et al., 1994). It is not entirely clear how this protective effect of NSAIDs is exerted. However, adenocarcinomas in human subjects appear associated with marked increases in COX-2 expression (Smalley and Dubois, 1997), and evidence from studies with isolated cells in culture (Dubois et al., 1998) or animal models (Williams et al., 1997) similarly points to COX- 2 being the level at which the beneficial effects of NSAIDs are exerted. Several hypotheses have been postulated to clarify the mechanisms by which the overexpression of COX-2 may contribute to colorectal carcinogenesis. The overexpression of COX-2 in rat intestinal epithelial cells and the addition of PGE_2 to human colon cells are associated with increased levels of Bcl-2 and thus, with resistance to apoptosis (Tsujii and Dubois, 1995; Sheng et al., 1998). Moreover, the growth of tumors is associated with immune suppression; PGE_2 inhibits, in vitro, the production of tumor necrosis factor-α (TNF-α) and induces the production of interleukin-10 (Kambayashi et al., 1995), and this response is reduced by exposure to NSAIDs of the colon cancer cells (Dubois et al., 1998). These results suggest that PGE_2 can suppress the cell-mediated antitumor immune response. COX-2 activity is also associated with the promotion of tumor angiogenesis in colon cancer cell lines (Tsujii et al., 1998) and in human colorectal cancer (Cianchi et al., 2001). The effect of selective COX-2 inhibitors on colon cancer in man is currently under investigation. However, Tsujii and colleagues (1998) have also shown that COX-1 in endothelial cells plays an important role in the modulation of angiogen-

esis. PGs produced by COX-1 in endothelial cells could be important in regulating genes required for endothelial tube formation, and it could be a relevant target for cancer prevention or treatment in tumors lacking COX-2 expression. As such, NSAID could inhibit angiogenesis by inhibition of COX-2 activity in colon carcinoma cells and down-regulating production of angiogenic factors by induction of apoptosis and by inhibiting COX-1 activity in endothelial cells. Recent studies have further indicated COX-2 overexpression in various other malignancies, such as lung (Hida et al., 1998), breast (Ristimaki et al., 2002), esophageal (Zimmermann et al., 1999), gastric (Murata et al., 1999), pancreatic (Tucker et al., 1999), prostate (Klotz et al., 1998), head and neck (Gallo et al., 2000; Gupta et al., 2000), and hepatocellular carcinoma (Bae et al., 2001; Fantappiè et al., 2002). On the basis of these data, it is conceivable that specific COX-2 inhibitors might be used as adjuvants in the treatment of tumors, as well as in cancer prevention.

3. Neuronal Excitotoxicity. The contribution of PG biosynthesis and release in the pathophysiological mechanisms underlying brain disorders has been investigated in the last three decades in almost all brain injury experimental models. In particular, excitotoxic mechanisms associated with acute exposure of brain tissue to noxious endogenous as well as exogenous neurotoxins and chronic neuroinflammatory/neurodegenerative disorders have widely been explored to evaluate the potential neuroprotective activity of compounds known to modulate PG biosynthesis. Indeed, direct administration in brain tissues of excitatory stimuli such as NMDA and kainate (which stimulate ionotropic channels reactive to the endogenous excitatory amino acid L-glutamate) has been associated to prostanoid-mediated neuronal cell death (Chen et al., 2002). This effect is associated with NMDA-related activation of COX-2, which as an immediate-early gene, is dramatically and transiently induced in these neurons. In addition, administration of kainic acid, an analog of the excitotoxin glutamate, results in hippocampal cell death and seizures in mice. The hippocampal lesions were associated with a high level of COX-2 production as well as astrogliosis (Chen et al., 2002).

Besides these evidences, the possible effect of COX inhibitors in such mechanisms leading to excitotoxic brain damage is still to be clarified. Indeed, in primary cortical neurons, both indomethacin (COX-1/-2 nonselective inhibitor) and aspirin (COX-1 preferential inhibitor) significantly reduced basal and kainic acid-induced PGE_2 production and prevented neuronal cell death after kainic acid treatment. In contrast, NS-398 (COX-2 selective inhibitor) had no effect on kainic acid-induced neuronal cell death. In hippocampal neurons, however, COX-2 inhibitors prevented both kainic acid-induced neuronal death and PGE_2 production (Kim et al., 2001). COX-2 expression was remarkably up-regulated by kainic acid in hippocampal neurons, whereas in cortical neurons, COX-2 expression was comparatively less significant. Astrocytes were unresponsive to kainic acid in terms of PGE_2 production and cell death. Thus, the release of PGE_2 induced by kainic acid seems to occur through COX-1 activity rather than COX-2 in cortical neurons (Kim et al., 2001). The inhibition of PGE_2 release by COX-1 inhibitors prevented kainic acid-induced cortical neuronal death, whereas in the hippocampal neurons, COX-2 inhibitors prevented kainic acid-induced PGE_2 release and hippocampal neuronal death, thus suggesting that excitotoxic mechanisms may involve differentially COX-1 and COX-2 according to the area of the brain undergoing excitotoxic lesion (Kim et al., 2001).

The reasons of such controversial effects of COX inhibitors in excitatory neurotransmission is unknown. However, recent studies indicate that AA metabolites interfere in discrete brain regions with L-glutamate turnover acting at many levels, including the reuptake and storage of excessive L-glutamate by astrocytes, the modulation of Ca^{2+}-linked glutamate receptors, and the release of free radicals (including NO and peroxynitrite) by postsynaptic neurons which, in turn, results in neuronal and astroglial damage (Volterra et al., 1992, 1994). In particular, it has been shown that AA itself is able to inhibit glutamate reuptake by glial cells, a key event in the regulation of glutamate concentration in the synaptic wall. Since the neurotoxic activity of glutamate is closely related to its relative concentration across L-glutamate receptors, it is likely that COX inhibitors, by regulating back AA concentrations, may directly or indirectly interfere with L-glutamate neurotoxicity (Volterra et al., 1992, 1994).

Mechanisms other than excitotoxicity have also been taken into account for detecting the possible role of prostanoid biosynthesis in brain injury. In particular, apoptotic cell death, occurring mainly via the chronic activation of cytokine/free radical-mediated mechanisms, strongly involves PG release. In particular, evidence exists that most of the inflammatory processes occurring in the early stages of some neuroimmune diseases such as allergic encephalomyelitis, multiple sclerosis, Alzheimer's disease (AD), and HIV-related brain disorders are associated with COX-1 and mainly COX-2 overexpression, an effect which suggested the potential benefit when using COX inhibitors in the treatment of such diseases (see below). The exact mechanisms involved in prostanoid-mediated activation of apoptotic machinery is unclear. However, evidence exists that apoptosis is mainly mediated by the release of PGE_2 which would activate the programmed death of neurons via an EP_2-like receptor (Takadera et al., 2002).

Besides these evidences, the possible use of COX-2 inhibitors in the treatment of such and other diseases characterized by apoptotic neuronal cell death is still controversial. Indeed, treatment of male Sprague-Dawley rats with kainic acid, which triggers limbic

seizures in 60% of the animals and induces COX-2 mRNA expression in the pyramidal cells of the hippocampus, induces cell loss via apoptotic mechanisms in the amygdala and the piriform cortex. Treatment with rofecoxib selectively attenuated the number of apoptotic cells in the hippocampus, whereas the cells of the thalamus, amygdala, and piriform cortex were not protected, thus suggesting that COX-2 inhibitors may exert an antiapoptotic effect against excitotoxic stimuli only in very selected areas of the brain (Kunz and Oliw, 2001).

The role of COX-2 expression in apoptotic phenomena seem to be associated with intense cooperation with neurotrophin-mediated mechanisms occurring in insulted brain cells. Indeed, it has been demonstrated that activation of COX-2 inhibits nerve growth factor withdrawal apoptosis in differentiated PC12 cells (Chang et al., 2000). The inhibition of apoptosis by COX-2 was concomitant with prevention of caspase 3 activation and was associated with increased expression of prosurvival genes coupled to inhibition of NO- and superoxide-mediated apoptosis. This indicates that an active cooperation exists between the COX pathway and neurotrophin/free radical-mediated mechanisms which regulate both necrosis and apoptotic cell death according to the cell type and the intensity of the injuring stimuli applied to brain tissues.

4. Neuroinflammatory Processes. An altered biosynthesis of prostanoids is clearly involved in neuroinflammatory processes (Genis et al., 1992; Cao et al., 1996). Indeed, acute inflammation following spinal cord injury results in secondary injury and pathological reorganization of the CNS architecture associated with substantial changes in COX expression (Schwab et al., 2000a,b). In particular, in injured spinal cord, COX-1 positive microglia/macrophages accumulated significantly at perilesional areas and in the developing necrotic core early after injury, being the number of COX-1 positive cells persistently elevated up to 4 weeks following injury. Furthermore, COX-1 positive cells were located in perivascular spaces, between spared axons, and in areas of Wallerian degeneration (Schwab et al., 2000a,b).

On the other hand, overexpression of both COX-1 and COX-2 and subsequent abnormal release of prostanoids has been described after exposure of brain cells to many inflammatory agents such as endotoxin and cytokines. Astroglial cells and, in a most relevant extent, microglial cells release large amounts of PGE_2 and PGD_2 after exposure to *Escherichia coli* LPS (see Minghetti and Levi, 1998). This effect seems to correlate directly with the expression of COX-2 enzyme in microglial cell culture. A nearly similar effect has been described when cytokines were incubated with glial cells. In particular, IL-1β alone or in combination with interferon-γ (IFN-γ) has been found to release large amounts of PGE_2 (Mollace et al., 1995, 1998; Janabi et al., 1996; O'Banion et al., 1996; Slepko and Levi, 1996) from cultured astrocytes as well as microglial cells, an effect mainly due to COX-2 expression.

Many substances able to produce inflammatory responses in brain tissues other than LPS or cytokines have been found to also act via prostanoid formation. In particular, evidence exists that several components of HIV envelope, such as gp120 glycoprotein, may account for some inflammatory aspects of neurodegenerative disorders accompanying AIDS-dementia complex by activating both NO and prostanoid formation (Genis et al., 1992; Mollace et al., 1993, 1994; Bagetta et al., 1998; Pereira et al., 2000). This effect occurred via expression of COX-2 isoenzyme in HIV-infected monocyte-derived macrophages, human brain microvascular endothelial cells in vitro (Pereira et al., 2000), and in neocortical neurons in vivo in which COX-2 overexpression was accompanied by apoptotic phenomena (Bagetta et al., 1998).

COX-2 protein is also up-regulated in macrophages causing active demyelination. Indeed, by means of in situ hybridization it has been described that COX-2 mRNA signals were strongly expressed on macrophages adhering to the demyelinating nerve fibers at the endoneurium. This observation has suggested a rationale for the application of neuroprotective strategies employing COX-2 inhibitors in inflammatory demyelinating neuropathies (Kawasaki et al., 2001). This is further demonstrated by the evidence that progression of myelopathies, which involve spinal motorneurons such as amyotrophic lateral sclerosis, is driven by inflammatory-related events associated with altered prostanoid biosynthesis. Indeed, in both early symptomatic and end-stage transgenic mice undergoing amyotrophic lateral sclerosis, neurons and, to a lesser extent, glial cells in the anterior horn of the spinal cord exhibit robust COX-2 immunoreactivity (Almer et al., 2001).

On the other hand, other aspects of inflammation of brain tissues are related to COX-2 up-regulation and the subsequent prostanoid formation. Indeed, it has recently been shown that in mice expressing transgenic COX-2 in anterior hypothalamus, the febrile response was significantly potentiated in transgenic compared with non-transgenic mice, with an accelerated onset of fever by 1 to 2 h after LPS administration, suggesting a role for neuronally derived COX-2 in the fever response (Vidensky et al., 2003). In addition, overexpression of COX-2 in the brain of a transgenic mouse line leads to selective induction of endogenous complement component C1qB expression in neurons, suggesting that neuronal COX-2 may influence inflammatory responses in the brain, in part, through the modulation of complement gene expression (Spielman et al., 2002). Finally, it has recently been shown that COX-2, in part, through TNF-α-related mechanisms, contributes to LPS-induced neuronal death, since this effect was antagonized by selective COX-2 inhibitors. COX-2, in addition to its role in glutamate excitotoxicity, participates in the cytotoxicity as-

sociated with neuroinflammatory processes (Araki et al., 2001).

5. Alzheimer's Disease. Evidence exists that neuroinflammatory mechanisms associated with abnormal modulation of COX pathway represent the central feature of AD. In AD, signs of an inflammatory activation of microglia and astroglia are present inside and outside amyloid deposits. In addition, studies carried out be means of cell culture and animal models of AD suggest an interactive relationship between inflammatory activation, reduced neuronal functioning, and deposition of amyloid (see Pasinetti, 2001).

In particular, it has been shown that COX-2 is upregulated in the brain with AD, an effect which has been shown to be injurious to neurons (Pasinetti, 2001). The immunointensity of COX-2 signal in the CA3 and CA2 but not CA1 subdivisions of the pyramidal layers of the hippocampal formation of the AD brain increased as the disease progressed. COX-2 signal was increased in all three regions examined among cases characterized by severe dementia, indicating that neuronal COX-2 content in subsets of hippocampal pyramidal neurons may be an indicator of progression of dementia in early AD. This has also been shown in transgenic mice overexpressing constitutively COX-2 in neurons and producing elevated levels of PGs in brain. Those animals developed an age-dependent deficit in spatial memory at 12 and 20 months but not at 7 months and a deficit in aversive behavior at 20 months of age. These behavioral changes were associated with a parallel age-dependent increase in neuronal apoptosis occurring at 14 and 22 months but not at 8 months of age and astrocytic activation at 24 months of age. These findings suggest that neuronal COX-2 may contribute to the pathophysiology of age-related diseases such as AD by promoting memory dysfunction, neuronal apoptosis, and astrocytic activation in an age-dependent manner (Andreasson et al., 2001).

Many factors have been identified in AD brain which are known to promote and sustain inflammatory responses and subsequently altered biosynthesis and release of PGs. They include β-amyloid protein, the pentraxins C-reactive protein and amyloid P, complement proteins, the inflammatory cytokines interleukin-1, interleukin-6, and TNF-α, the protease inhibitors α-2-macroglobulin, and α-1-antichymotrypsin (for a review, see McGeer and McGeer, 2001). In particular, in AD brains, COX-1 positive microglial cells were primarily associated with amyloid β plaques, while the number of COX-2 positive neurons was increased compared with that in control brains. The different distribution patterns of COX-1 and COX-2 in AD could implicate that these enzymes are involved in different cellular processes in the pathogenesis of AD (Hoozemans et al., 2001).

The possible interactions between prostanoid formation and inflammatory effects of β-amyloid in AD has been better assessed in the past few years as a potential target for the treatment of AD with COX inhibitors. Recent studies demonstrated that COX-2 expression is closely related to the expression of high levels of mRNA for the amyloid precursor protein (APP). Amyloid β-peptide and a secreted form of APP, both derived from APP by proteolysis, were also increased, since both effects were inhibited by a selective COX-2 inhibitor (JTE-522) and by nonselective COX inhibition using indomethacin, thus suggesting that COX pathways may play important roles in the β-amyloid-related neurodegenerative processes of AD (Kadoyama et al., 2001). In addition, injection into the nucleus basalis of the rat of preaggregated amyloid-β [Aβ(1–42)] segments produced a congophylic deposit and microglial and astrocyte activation and infiltration and caused a strong inflammatory reaction characterized by IL-1β production, increased COX-2, and inducible NOS (iNOS) expression. Rofecoxib, a COX-2 inhibitor, reduced microglia and astrocyte activation, iNOS induction, and p38 mitogen-activated protein kinase (MAPK) activation to control levels showing that COX-2 overexpression by β-amyloid is a crucial event in the inflammatory cascade occurring in AD (Giovannini et al., 2002). Moreover, human COX-2 expression in APP/COX-2-expressing mutant mice induces potentiation of brain parenchymal amyloid plaque formation and a greater than 2-fold increase in PGE_2 production suggesting that COX-2 influences APP processing and promotes amyloidosis in the brain (Xiang et al., 2002). On the other hand, it has been shown that both the synthesis of the APP and its processing (i.e., to amyloidogenic Aβ peptides, soluble nonamyloidogenic APPs, and other APP fragments) are regulated by mediators, including prostanoids, able to elevate cAMP levels into brain cells. In addition, evidence exists that the neurotoxic and proinflammatory actions of the Alzheimer peptide Aβ are dependent on its aggregation and β-sheet conformation. Chronic use of NSAIDs, such as aspirin for arthritis, decreases the risk of developing AD by unknown mechanisms (Pasinetti 2001). Recently, it has been found that aspirin prevented enhanced Aβ aggregation by aluminum, an environmental risk factor for AD. This antiaggregatory effect was restricted to NSAIDs and was not exhibited by other drugs used in AD therapy (Thomas et al., 2001). Furthermore, S-2474, a selective COX-2 inhibitor, showed a protective effect on Aβ-induced cell death in primary cultures of rat cortical neurons. In particular, S-2474 ameliorated Aβ-induced apoptotic features, such as the condensation of chromatin and the fragmentation of DNA completely, indicating that COX-2 inhibitors may possess therapeutic potential for AD via ameliorating degeneration in neurons as well as by suppressing chronic inflammation in non-neuronal cells (Yagami et al., 2001). Finally, it has been demonstrated that neuroimmunophilin ligands [like cyclosporin A or FK-506 (tacrolimus)] and NSAIDs, including COX-2 inhibitors, can also prevent APP overexpression and the overproduction of amyloidogenic pep-

tides. Indeed, APP overexpression by PGE_2 is inhibited by neuroimmunophilin ligands like cyclosporin A or FK-506. In addition, NSAIDs, which reduce PG synthesis by inhibiting COX-1 and -2 enzymes, might also be expected to lower APP levels and increased levels of soluble APPs in the media of cultured astrocytes and neurons, perhaps acting by inhibition of PG production. Since APP haloprotein can be amyloidogenic, whereas APPs may be neurotrophic, it can be suggested that some neuroimmunophilin ligands, NSAIDs, and COX-2 inhibitors might suppress amyloid formation and enhance neuronal regeneration in Alzheimer's disease (Lee and Wurtman, 2000).

The expression of COX-2 in the brain of patients with AD, however, is strictly related to the inflammatory injury which characterizes the early stages of the disease. Indeed, COX-2 occurs simultaneously to the activation of inflammation-associated enzymes such as p38 MAPK and is not restricted to glial cells, but may also be found in neurons and may contribute to intraneuronal damage (Hull et al., 2002). In contrast, neuronal COX-2 expression is decreased in AD subjects with dementia compared with nondemented subjects in different hippocampal subfields (Yermakova and O'Banion, 2001). These changes also occurred in subjects with other dementia and thus may not be disease specific. The proportion of COX-2 positive neurons decreased in subjects with clinical dementia rating 5 but not clinical dementia rating 4, suggesting that this was a late event in the course of the disease. Furthermore, COX-2 was not preferentially associated with paired helical filament immunoreactivity, a marker of neuronal pathology. COX-2 immunoreactivity was also observed in astrocytes and cerebrovasculature. Indeed, the density of COX-2 immunopositive astrocytes was increased in AD temporal cortex. Thus, it is unlikely that neuronal COX-2 contributes to pathology in end-stage AD (Yermakova and O'Banion, 2001).

6. Brain Ischemia. PGs and other arachidonic acid metabolites are subjected to altered synthesis or relocation after an ischemic insult. This effect is mainly due to changes occurring in many steps of prostanoid formation, including phospholipase A2 (PLA2) and COX isoforms. In addition, many COX inhibitors have shown to possess protective effects when used in experimental models of brain ischemia.

In particular, expression of group IIA secretory PLA2 (sPLA2-IIA) is documented in the cerebral cortex after ischemia, suggesting that sPLA2-IIA is associated with neurodegeneration. Indeed, after mean cerebral artery occlusion, sPLA2 activity was increased in the cortex and associated to a neurodegenerative effect, since both responses were prevented by the sPLA2 inhibitor, indoxam (Yagami et al., 2002). The neuroprotective effect of indoxam was observed even when it was administered after occlusion. In addition, in primary cultures, sPLA2-IIA caused marked neuronal cell death. Morphologic and ultrastructural characteristics of neuronal cell death by sPLA2-IIA were apoptotic, as evidenced by condensed chromatin and fragmented DNA. Before apoptosis, sPLA2-IIA liberated AA and generated PGD2 from neurons. Indoxam significantly suppressed not only AA release, but also PGD2 generation. Indoxam also protected neurons from sPLA2-IIA-induced cell death, since this effect was found even when it was administered after sPLA2-IIA treatment. Furthermore, the use of COX-2 inhibitors significantly prevented neurons from sPLA2-IIA-induced PGD2 generation and subsequent cell death (Yagami et al., 2002). This suggests that sPLA2-IIA induces neuronal cell death via apoptosis, which might be associated with overproduction of AA metabolites, especially PGD2 in ischemic brain tissues.

An increase of both COX-1 and COX-2 have also been found in brain ischemia. In particular, focal ischemia induced in the frontoparietal region of rat brain is accompanied by formation of PGD2 peaking 60 to 90 min postischemia and declining thereafter. This effect is due to increased COX-2 and is characterized by morphological alterations with necrosis of neurons, glial cells, and blood vessels surrounded by a halo with pyknotic cells with cytoplasm swelling and vacuolization (Govoni et al., 2001). Many cell types within ischemic brain tissue have been shown to overexpress COX enzymes; however, in focal ischemic damage, the elevation of COX-2 was restricted to microglial cells, suggesting that the isozymes of COX are differentially regulated depending on the cellular source and the types of ischemic damage (Tomimoto et al., 2002).

The administration of COX inhibitors before as well as soon after the ischemic insult reduces the extension of cerebral damage in rats. In particular, either selective inhibition of COX-2 with rofecoxib or inhibition of COX-1 with valeryl salicylate significantly increased the number of healthy neurons in the hippocampal CA1 sector even when the treatment began 6 h after global ischemia, suggesting that both COX isoforms are involved in the progression of neuronal damage following global cerebral ischemia and have important implications for the potential therapeutic use of COX inhibitors in cerebral ischemia (Candelario-Jalil et al., 2003). In the same way, SC-58236, a selective COX-2 inhibitor, dose-dependently prevented ischemia-induced eicosanoid formation and caused significant reduction of the damaged area, suggesting that selective inhibitors of COX-2 are neuroprotective (Govoni et al., 2001). Moreover, anoxic stress attenuates NMDA-induced pial arteriolar dilation via a mechanism involving actions of COX-derived reactive oxygen species, an effect protected by the selective COX-2 inhibitor NS-398 (Domoki et al., 2001). Finally, the specific long-acting COX-2 inhibitor SC-58236 also showed a protective effect in a reversible rabbit spinal cord ischemia model (Lapchak et al., 2001).

The effects of COX antagonism are relevant not only for the protection of ischemic tissue but also in the area

surrounding the ischemic core. Indeed, the marginal area surrounding a region of ischemic brain tissue, designated as the penumbra, is of interest as a potential area for the rescue of neurons from cell death. In the penumbra and surrounding cortex, it has been shown that up-regulation of c-Fos, brain-derived neurotrophic factor, and COX-2 mRNAs was observed, whereas expression of HSP70 mRNA was restricted to the penumbra. This spatial discrepancy of mRNA expression suggests that different mechanisms are involved in the regulation of c-Fos/brain-derived neurotrophic factor/COX-2 and HSP70 expression (Kinoshita et al., 2001).

In addition to these evidences, inhibition of COX does not seem to represent the only mechanism involved in the neuroprotection that follows the treatment of ischemic injury by means of NSAIDs. Indeed, both piroxicam and NS-398 protect neurons against hypoxia/reperfusion. However, evidence exists that their protective effect is independent of COX activity and not solely explained by modulation of NF-κB and AP-1 binding activity. Instead, piroxicam- and NS-398-induced phosphorylation through extracellular signal-regulated kinase pathway may contribute to the increased neuronal survival (Vartiainen et al., 2002). Furthermore, COX-2 is an important modulator in the enhancement of proliferation of neural progenitor cells after ischemia (Sasaki et al., 2003). Due to these latter evidences, the use of COX-2 inhibitors is restricted to the early stages of ischemic insult (when the neurochemical changes of injured tissues relevant to neuronal and glial dysfunction are mainly driven by inflammatory processes). These have to be further investigated in the phases of repair mechanisms which occur later in the course of the disease.

B. Selective Nonsteroidal Anti-Inflammatory Drugs

NSAIDs is a "catch all" name for a large number of chemically distinct drugs. Together they represent the single most important group of self-prescribed pharmaceuticals and the most widely used drug class. Sales of NSAIDs are now estimated at 5.8 billion dollars a year with the United States accounting for 1.8 billion dollars (Vane and Botting, 1996). The therapeutic benefits of all NSAIDs include inhibition of swelling and/or pain at the site of inflammation. In addition, aspirin also offers protection against stroke and thrombosis, Alzheimer's disease, and cancer (see above). There are, however, side effects of NSAIDs that limit their use in some patients. Most common among these side effects is irritation and damage to the gastrointestinal mucosa, particularly at the gastric level. Each member of the NSAIDs family has some individual effects, however, the unifying common mechanism of action of all is inhibition of COX (Vane, 1971). Together with the identification of two distinct isoforms of COX, a new hypothesis has been formulated to explain the effects of this class of drugs; COX-2 inhibition accounts for the therapeutic benefits and inhibition of COX-1 for the side effects of NSAIDs (Mitchell et al., 1994). The following explains why this hypothesis was presented and how its validity was proven. As a class, the NSAIDs represent a major risk for morbidity and mortality from gastrointestinal damage, perforation, ulcers, and bleeding. As reported by Mitchell and Warner (1999), in the United States the number of deaths per year due to NSAIDs is approximately 16,500 with 107,000 hospitalizations in the same period (Fries, 1998). In the United Kingdom, it is estimated that 12,000 ulcer complications and 1200 deaths per year are directly linked to NSAID intake (Hawkey, 1996). However, some NSAIDs cause more gastrointestinal side effects than others (Henry et al., 1996).

A number of different experimental assays have been used to compare the potencies of NSAIDs on COX-1 and COX-2. The results from these assays are used to calculate a measure of COX-2 selectivity, and then NSAIDs are compared with each other by ranking their COX-2 selectivity. The many test systems developed have resulted in different COX-1/COX-2 ratios, sometimes for the same drug, thus confusing comparisons. However, on the whole the in vitro studies have shown that the ability of a given NSAID to inhibit COX-1 correlates with the degree of side effects it causes. Moreover, the use of COX screens in vitro has been remarkably successful and has led to the development of a number of COX-2 selective compounds. These include from Merck Frosst (Kirkland, QC, Canada) L-745,337 (Chan et al., 1995), DFU [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2-(5*H*)-furanone] (Riendeau et al., 1997), and DFP [3-(2-propyloxy)-4-(4-methylsulphonylphenyl)-5,5-dimethylfuranone] (Black et al., 1999) and from Monsanto Searle (St. Louis, MO) SC-58125 (Guo et al., 1996). Furthermore, this approach has led to the development of two COX-2 selective NSAIDs with the approval of the Food and Drug Administration that are currently available in the United States and Europe. These are rofecoxib (Vioxx; Chan et al., 1999) from Merck Frosst and celecoxib (Celebrex; Geis, 1999) from Pfizer (St. Louis, MO). In addition to the development of new compounds by these screens, it has been possible to identify currently prescribed NSAIDs that are COX-2 selective inhibitors. For example, using whole blood assay compounds displaying more than 5-fold selectivity for COX-2 would include etodolac, meloxicam, and nimesulide (Warner et al., 1999).

However, two other important questions are still ongoing: are COX-2 selective inhibitors really safe for the gastrointestinal tract, and is the selective inhibition of cyclooxygenase-2 sufficient for a full anti-inflammatory efficacy? A large amount of studies have been published showing different responses to the above questions. Recently, Wallace (1999) tried to comment on these two different aspects in a very interesting article published in *Trends in Pharmacological Science*. There is large

evidence to support the claim that selective inhibitors of COX-2 produce less gastric damage than standard NSAIDs when administered acutely to healthy animals (Seibert et al., 1994; Chan et al., 1995; Futaki et al., 1993). However, it has also been demonstrated that most patients taking standard NSAIDs do not develop clinically significant gastric injury; rather, it occurs in a subset of patients who are more susceptible to the gastric-damaging actions of these drugs (Wolfe et al., 1999). Patients infected with *Helicobacter pylori* are more likely than those not infected to develop endoscopic ulcers when taking NSAIDs. Other influences on endoscopically detected ulcers are less well established. Potential factors that have been reported as associated with NSAID-induced ulceration are smoking, male sex for duodenal lesions, apparently independent of *H. pylori* status, age, and dose of drug assumed, particularly for gastric ulcers (Hawkey et al., 1998). We still have much to learn about the potential risks of the inhibition of COX-2 in the gastrointestinal tract; for example, COX-2 inhibitors impair tolerance of dietary antigen (Newberry et al., 1999) and exacerbate experimental colitis in rodents (Morteau et al., 2000).

COX-2 also appears to play an important role in promoting the healing of ulcers in the stomach. It has been recognized for many years that NSAIDs interfere with the healing of peptic ulcers in humans (Armstrong and Blower, 1987; Stadler et al., 1991), whereas administration of PGs accelerate their healing (Jaszewski et al., 1992). Mizuno and coworkers (1997) recently showed that COX-2 mRNA and protein were strongly induced in the mouse stomach in which an ulcer had been induced, and a parallel increase in mucosal PG synthesis was found. Although COX-2 expression in the healthy stomach is low and expression around a site of ulceration is considerably higher, it should be noted that rapid induction of COX-2 can occur, even in response to quite subtle mucosal irritation. A further functional role for COX-2 in mediating gastric epithelial proliferation has been demonstrated by Sawaoka et al. (1997), whereas Nakatsugi et a1. (1996) have provided evidence that COX-2 contributes to mucosal defense in a rat model of stress ulcer. Central to the "selective COX-2 inhibitor" thesis is the assumption that COX-2 is the major form responsible for the production of PGs at sites of inflammation. However, if COX-1 contributes significantly to the production of PGs at such sites, selective block of COX-2 will not produce anti-inflammatory effects to the same extent as drugs that inhibit both isoforms. There is excellent evidence for marked up-regulation of COX-2 at sites of inflammations. In some models, there is also evidence for anti-inflammatory effects of COX-2 inhibitors at doses that do not significantly affect gastrointestinal PG synthesis or COX-1 activity. COX-2 has been constitutively localized in the renal vasculature, the cortical macula densa, and the medullary interstitial cells of the kidney, and its content in these areas increases with age. COX-1 is found in the vasculature, the collecting ducts, and in the thin loops of Henle (Nantel et al., 1999). The presence of both isoforms in the vasculature raises the question of which is the predominant source of the increased production of vasodilator PGs that are critical to the preservation of renal blood flow in the presence of volume depletion. Inhibition of this homeostatic response accounts for the most common renal side effects associated with nonselective NSAID therapy (Nantel et al., 1999). Little information on the renal pharmacology of COX-2 inhibitors in humans is available at this moment. An analysis of the postmarketing data for celecoxib revealed that edema occurred in 2.1% of patients, hypertension in 0.8%, and exacerbation of preexisting hypertension in 0.6%, a profile similar to those of nonselective NSAIDs (Whelton et al., 2001); similarly, post hoc analysis of the rofecoxib database revealed that peripheral edema occurred in 3.8% of patients treated daily with 25 mg of rofecoxib (Whelton, 2001). Controlled studies are necessary to assess the risk of hypertension. However, there are also examples of studies in which anti-inflammatory efficacy was not observed unless doses of the COX-2 inhibitors were used that were well above those necessary for COX-2 inhibition. Indeed, in this particular case, suppression of PG synthesis at the site of inflammation correlated significantly with the suppression of COX-1, but not with the suppression of COX-2 (Wallace et al., 1998).

III. Nitric Oxide and Nitric Oxide Donors

NO is a free radical molecule, which was discovered to be a potent vasodilator (Vallance et al., 1989) as well as a novel type of retrograde neurotransmitter (Snyder and Bredt, 1992). At the vascular level, NO appears to mediate endothelium-dependent relaxation of vascular smooth muscle (Ignarro et al., 1987; Palmer et al., 1987) and to be chemically equivalent to endothelium-derived relaxing factor (EDRF) discovered by Furchgott and Zawadzki (1980). NO is a double-edged sword, serving as a key signal molecule in both physiological and pathological processes. NO and its reaction products, reactive nitrogen oxide species, has been found to modulate all facets of physiology and pathology in species as evolutionarily distant as plants and humans. Additionally, the chemical literature regarding the role of NO, especially as a pollutant, has been with us for over a century. The naming of NO as "Molecule of the Year" by Science magazine in 1992 and the award in 1998 of the Nobel Prize in Medicine for the role of NO in the cardiovascular system reflects the importance given to this molecule by the general scientific community.

NO was described as an effector product of activated macrophages (Tayeh and Marletta, 1989). As is often the tendency in science, the ability of NO to cause cytostasis and cytotoxicity in tumor cells and certain pathogens resulted in its initial perception as a beneficial mecha-

nism to the host. Furthermore, the finding that NO derived from the endothelial NOS (eNOS) could inhibit platelet aggregation and the adhesion of platelets (Radomski et al., 1987; Sneddon and Vane, 1988) and of activated neutrophils (Kubes et al., 1991) suggested a beneficial role for NO in ischemia/reperfusion injury. Under physiological conditions, in all parts of the body, the concentrations of NO are believed to be fluctuating continuously at rather low levels. These levels are controlled by constitutively expressed neuronal and endothelial types of NO synthase [nNOS (NOS-I) and eNOS (NOS-III), respectively], which are widely distributed in the body. However, realization that concentration of NO can rapidly increase by the massive expression of the iNOS (NOS-II) in sepsis (Szabò and Thiemermann, 1995) or by hyperactivation of nNOS in glutamate-mediated neurodegenerative processes in the central nervous system, led to a change in this perception (Dawson, 1995). Rapidly, data were published which implicated the aberrant expression of iNOS in numerous inflammatory conditions, such as inflammatory bowel disease, Crohn's disease, Alzheimer's disease, and hemorrhagic shock, to name a few (Boughton-Smith et al., 1993; Clancy and Abramson, 1995). These studies and numerous others began to paint a darker picture of NO as a toxic byproduct of inflammation that should be inhibited to restore homeostasis. Thus, the great effort of several groups and in particular of the Moncada group was to develop selective inhibitors of the inducible form of the enzyme as novel therapeutics for disease (Moncada and Higgs, 1995; Ho and Pasinetti, 2001). These tools provided not only invaluable information as to the proinflammatory roles of NO but started to highlight its potential role as an anti-inflammatory molecule. Thus, it is now becoming more and more evident that NO can be considered a double-edged sword. On one hand, it can exert beneficial effects on the body by acting as an antibacterial, antiparasital, antiviral agent, or as a tumoricidal agent; on the other hand, high levels of NO, if uncontrolled, can be detrimental. Such negative effects are produced because persistent high amounts of NO can react with concomitantly produced superoxide anions, which thereby generates highly toxic compounds, such as peroxynitrite and hydroxyl radicals. Recently, NO has been reinvented as a positive modulator of the inflammatory process. For example, NO was found to suppress the proliferation of lymphocytes and cause their apoptosis (Moulian et al., 2001). It is therefore not surprising that emphasis has been given to the development of NO donors as potential anti-inflammatory agents (Cena et al., 2003; Kankuri et al., 2003). Simultaneously, NO was described to inhibit apoptosis of various cell types, notably hepatocytes, through mechanisms involving the nitrosative suppression of caspases (Martin-Sanz et al., 2002).

A. Biosynthesis of Nitric Oxide

NO is an inorganic, colorless gas with good solubility in water (Butler and Williams, 1993). NO is one of the simplest odd electron species. The half-life of NO in water is considerably longer, about 3 s, than would be expected for other free radicals. This is, in part, due to the reluctance of NO to dimerize and to the third-order kinetics of its reactions with oxygen. However, NO reacts rapidly with superoxide anion to form peroxynitrite, a relatively stable product (Beckman et al., 1990). In the gas phase, NO reacts with oxygen to form nitrogen dioxide ($\cdot NO_2$); under aqueous, aerobic conditions NO spontaneously oxidizes to its inactive, stable products nitrite and nitrate at a half-life of 6 to 10 s. This property explains the previously puzzling observation that mammals excrete more nitrate than they ingest, especially when inflammation is present (Wagner et al., 1983). In vivo, nitrite reacts with various biologically active species (e.g., oxyhemoglobin), and the stable end product that can be measured in plasma is nitrate (Butler and Williams, 1993; Feldman et al., 1993; Ignarro et al., 1993). NO can react with thiols to form *S*-nitrosothiols, such as *S*-nitrosocysteine and *S*-nitrosoglutathione. Some *S*-nitrosothiols and protein thiols may have a significant stability (Nathan, 1992; Stamler et al., 1992; Feldman et al., 1993), and it is possible that NO circulates in plasma as an *S*-nitroso adduct of serum albumin (Stamler et al., 1992). NO has a high affinity for both heme and nonheme iron atoms present in the prosthetic groups of proteins (Lancaster and Hibbs, 1990), and it can form complexes with hemoglobin, myoglobin, cytochrome *c*, and guanylyl cyclase. Furthermore, NO can interact with iron-sulfur centers in various enzymes, such as aconitase and complexes I and II of the mitochondrial respiratory chain altering their biological activity (Nathan, 1992; Feldman et al., 1993). Ribonucleotide reductase is another iron-containing enzyme inhibited by NO (Lepoivre et al., 1990). By blocking this enzyme, NO impairs DNA synthesis and cell division; it damages DNA through nitrosylative and deaminative reactions causing strand breaks (Wink et al., 1991). Peroxynitrite, but not NO, nitrosylates tyrosine residues of several proteins such as iron, manganese, and copper-zinc superoxide dismutases, as well as on other copper-containing proteins (Ischiropoulos et al., 1992).

The role of NO in normal physiologic homeostasis became apparent when two groups of researchers identified it as the EDRF, which helps to regulate blood pressure (Ignarro et al., 1987; Palmer et al., 1987). In response to vasodilating stimuli, such as acetylcholine, histamine, and bradykinin, the endothelial cells secrete EDRF/NO.

Over the past decade, following the discovery that a variety of cell types synthesize the free radical NO, research focusing on this simple diatomic molecule has led to a formidable amount of publications, determining

that NO plays significant roles in most fields of life sciences (Nathan and Xie, 1994). However, at the beginning of the new millennium, a number of questions regarding NO biology still remain unanswered, the most challenging and confusing problem being set by the ambivalent character of NO. Although being a critical signaling messenger involved in the regulation of a wide range of physiologic processes, NO also has the ability to turn into a major cytotoxic effector molecule that is involved in a number of pathophysiologic conditions and in the pathogenesis of a growing list of human diseases (Jeremy et al., 2002; Szabo, 2003; Walia et al., 2003). On a clinical viewpoint, such a paradoxical fate of NO is particularly troublesome when one considers manipulating NO availability as a potential therapeutic option in different pathologic conditions. Reducing or increasing NO availability in a given circumstance may inevitably be associated with both beneficial effects and deleterious consequences.

In addition, further adding to an already complex situation, some theoretical misconceptions have also contributed to the confusion surrounding the perplexing biological functions of NO. For instance, the proper effects of NO have often and abusively been assimilated to those of a family of NO-derived molecules, collectively termed reactive nitrogen species, which all possess their unique biochemical characteristics (Patel et al., 1999). Another frequent misconception is that NO, as a free radical, is a highly reactive molecule with a very short lifetime. Although the free radical nature of NO constitutes the chemical basis of its biological activity, its reactivity is relatively weak, and basically, NO interacts only with transition metals, oxygen, and other free radicals (Beckman and Koppenol, 1996). This low reactivity, combined with a high lipophilicity, confers to NO the potential to diffuse away from the point of origin, and thereby to carry out its function as a messenger molecule (Wink and Mitchell, 1998). As a general rule, the direct effects of NO prevail in conditions of low and brief NO production and mainly support protective and signaling functions, which are consistent with the chemical biology of NO encountered under normal, physiologic conditions (Wink and Mitchell, 1998). In contrast, indirect effects will occur under a high and sustained flux of NO, as noted under pathophysiologic circumstances and will essentially result in toxic consequences, which include oxidation, nitrosation (adjunction of NO^+), and nitration (adjunction of NO_2^+) reactions (Wink and Mitchell, 1998; Grisham et al., 1999). It appears that the type of NO chemistry prevailing at a particular moment in time is the key feature that determines its biological actions.

1. Endothelial Nitric-Oxide Synthase. Vascular eNOS is a calcium/calmodulin-dependent enzyme with a monomeric molecular weight of 133 kDa that is bound to the cell membrane with a myristoylate bridge linked to the N-terminal glycine of the enzyme (Nathan, 1992; Sessa et al., 1992; Dinerman et al., 1993). It is now well appreciated that eNOS is important for cardiovascular homeostasis, vessel remodeling, and angiogenesis (Nadaud et al., 2000; Chiou, 2001). In contrast to nNOS and iNOS, which are cytosolic, eNOS is particulate, and cytosolic post-translational modification at these sites is not necessary for its membrane association, which has been shown by the elegant work of Sessa's group to occur preferentially at the caveoli (Fulton et al., 2001). Such a localization favors high local concentrations of NO in the vicinity of circulating blood cells and vascular smooth muscle. Little is known about the turnover and the regulation of eNOS, although it has been established that TNF-α down-regulates eNOS by enhancing the degradation of its mRNA (Yoshizumi et al., 1993) and that eNOS activity in platelets is, at least in part, modulated by thromboxane A_2 that functions via calcium as a second messenger (Chen et al., 1997). Agents that increase intracellular calcium by activating influx of extracellular calcium or releasing calcium from intracellular stores cause endothelium and NO-dependent relaxation in many blood vessels in vitro and in vivo. Such endothelium-dependent vasodilators include acetylcholine, angiotensin, substance P, bradykinin, 5-hydroxytryptamine, histamine, norepinephrine, calcium ionophore A23187, adenine nucleotides, thrombin, arachidonic acid, leukotrienes, and platelet-activating factor (Gryglewski et al., 1988; Vane et al., 1990; Calver et al., 1993). Important from a physiologic point of view is the finding that pulsatile pressure, visible light, and electrical field stimulation also release this labile mediator with a half-life counted in seconds (Pohl et al., 1986; Rubanyi et al., 1986). Veins seem to produce less NO than arteries, both basally and upon stimulation (Luscher et al., 1988).

Furthermore, NO derived from eNOS has numerous effects on the vessel wall, including vasodilation (Rees et al., 1989b; Vallance et al., 1989), inhibition of platelet aggregation (Radomski et al., 1987; Sneddon and Vane, 1988), inhibition of the production of monocyte chemoattractant protein-1 and macrophage-colony stimulating factor (De Caterina et al., 1995; Zeiher et al., 1995), changes in vascular permeability (Kurose et al., 1993), inhibition of smooth muscle cell proliferation (Garg and Hassid, 1989), and inhibition of leukocyte-endothelium interaction (Kubes et al., 1991; Kurose et al., 1993). Release of NO from eNOS has also been shown to account for the immediate fall in blood pressure induced by *E. coli* lipopolysaccharide (Salvemini et al., 1989, 1990a; Hallemeesch et al., 2003). NO is also involved in the pentagastrin-induced stimulation of gastric blood flow (Walder et al., 1991), and loss of endothelium-derived NO contributes to the pathogenesis of acute coronary syndromes (Okumura et al., 1992; Bogaty et al., 1994). In particular, aggregating platelets from patients with acute coronary syndromes produce less NO, and impaired platelet-derived NO production contributes to the

development of acute coronary syndromes in patients suffering from unstable angina and myocardial infarction (Freedman et al., 1998). NO is therefore cytoprotective and beneficial in various experimental models of vascular pathology. Platelet aggregation is enhanced by incubation with inhibitors of NOS and inhibited by incubation with the NOS substrate L-arginine (Chen and Mehta, 1996), and it has been shown that inhibition of the basal release of NO by NOS inhibitors results in a rapid, prolonged, and L-arginine reversible increase in blood pressure (Rees et al., 1989a). Administration of NOS inhibitors produces a reduction in blood flow to most organs, including the brain, heart, lung, and kidney (Gardiner et al., 1990; Benyo et al., 1991; Kovach et al., 1992). NO synthase inhibitors reduce the hypotensive effect of the "endothelium-dependent vasodilator substances" (Aisaka et al., 1989; Whittle et al., 1989). Moreover, eNOS knockout mice are hypertensive (Huang et al., 1995) and show impaired wound healing and angiogenesis (Lee et al., 1999). Acute release of NO from the endothelium may be involved in some forms of reactive hyperemia (Kostic and Schrader, 1992), such as postischemic or exercise-induced hyperemic response (Gilligan et al., 1994; Tagawa et al., 1994). In this scenario, eNOS is responsible for moment-to-moment changes in vascular tone that form the basis for responses to ischemia and to exercise.

Vasodilation is an important component of angiogenesis. Endothelial NOS contributes to the proangiogenic program of capillary endothelium by triggering and transducing cell growth and proliferation throughout a cyclic GMP-dependent protein kinase and MAPK cascade (Hood and Granger, 1998; Parenti et al., 1998) leading to a membrane linked signal to the nuclear level. Moreover, Ziche and collaborators (Ziche and Morbidelli, 2000) have demonstrated that the eNOS pathway controls the balance between metalloprotease (MMP-2 and MMP-9) and their inhibitors.

Long-term inhibition of eNOS may lead to prolonged hypertension that is triggered, at least in part, by the renin-angiotensin system (Ribeiro et al., 1992). Chronic inhibition of eNOS causes renal morphologic changes such as glomerular damage (Baylis et al., 1992). Endothelial NOS is also expressed in cardiac myocytes (Balligand et al., 1993; Feron et al., 1996), where NO$^{\bullet}$ has a paracrine action on cardiac contractility and oxygen consumption (Brady et al., 1993; Shen et al., 1995; Loke et al., 1999).

2. Neuronal Nitric-Oxide Synthase. There is no doubt that NO from the neuronal enzyme plays a critical role as a neurotransmitter and neuromodulator in the nervous system. However, unlike classical neurotransmitters, NO is not stored in synaptic vesicles, nor is it released by exocytosis, nor is its activity terminated by reuptake or enzymatic degradation; rather, it is synthetized on demand, diffuses from nerve terminals, and its actions terminated postinactivation with a substrate.

The NOS isoform present in the central and peripheral nervous system (nNOS) is a Ca^{2+}-calmodulin-dependent enzyme with a molecular weight of 166 kDa that is present in dimeric form (Calver et al., 1993). The primary structure of neuronal NOS, revealed by molecular cloning (Bredt et al., 1991), indicates that the protein has an α helical, calmodulin-binding consensus sequence and a cAMP-dependent protein kinase phosphorylation sequence (referred to as nNOSα). Four splice variants have been identified to date: nNOSβ, nNOSγ, nNOSμ, and nNOS-2 (Nakane et al., 1993; Silvagno et al., 1996; Alderton et al., 2001). They each appear to have distinct cellular and tissue locations. All nNOS positive neurons have the α-nicotinamide-adenine dinucleotide phosphate (NADPH) diaphorase activity which has been used as a histochemical marker (Wolf, 1997) of nitrergic neurons. Although the main NOS found in the nervous system is nNOS, eNOS and iNOS have been found.

Neural NOS is expressed in neurons in many parts of the brain, primarily in the cerebellum and hippocampus (Bredt et al., 1990, 1991); in other areas such as the cerebral cortex or the stratum, NOS-I positive neurons comprise about 1 to 2% of the total neuronal population. The modulation of various physiological functions by NO has been proposed, among them, pain perception, sleep, feeding behavior or thermoregulation, and, in particular, regulation of microcirculation (Szabo, 1996). Alternatively spliced forms of the enzyme appear to dominate in discrete brain regions (Eliasson et al., 1997). Neural NOS is targeted to proteins of the postsynaptic density in close association with the NMDA receptor (Garthwaite and Boulton, 1995). Calcium-dependent stimulation of NOS-I activity has been implicated in excitatory glutamatergic neurotransmission, long-term potentiation, and long-term depression (Garthwaite, 1991; Shibuki and Okada, 1991; Boje and Arora, 1992; Holscher and Rose, 1992; Endoh et al., 1993; Iadecola et al., 1993). Thus, NOS-I may be involved in phenomena based on synaptic plasticity such as learning and memory formation, tasks that are supported, and in NOS-I-deficient neurons may be taken over, by NOS-III (Dinerman et al., 1994). In turn, NO has been implicated in excitotoxicity after excessive stimulation of neurons by glutamate, which occurs in stroke, although the underlying mechanisms of neuronal cell death as well as of the resistance of NOS-I-containing neurons to NO are still a matter of debate. Nevertheless, excessive generation of neuronal NO appears to be responsible for ischemia-reperfusion and traumatic injury of the brain which can only, in part, be overcome by the beneficial effect of endothelial cell-derived NO on blood flow.

In excess, NO is toxic to neurons; this toxicity is mediated largely by an interaction with superoxide and subsequent generation of peroxynitrite. The toxic effect of NO may occur through a variety of mechanisms, including inhibition of the mitochondrial electron trans-

port chain, inhibition of ribonucleotide reductase, inhibition of *cis*-aconitase, and enhanced adenosine diphosphate (ADP) ribosylation of glutaryl-aldehyde-phosphate-dehydrogenase. Recent studies indicate that NO or peroxynitrite-mediated neuronal injury involves damage of DNA with the subsequent activation of the nuclear protein, poly(ADP-ribose)synthetase (Zhang et al., 1994). Evidence is also growing that nNOS is also present in skeletal muscle where it is involved in the regulation of metabolism and muscle contractility (Stamler and Meissner, 2001).

3. Inducible Nitric-Oxide Synthase. Compared with constitutive NOS isozymes, it is iNOS which is thought to mediate the vast majority of pathophysiological effects attributed to NO. Consequently, this isoform is believed to be of fundamental importance to the inflammatory process.

Under physiologic conditions, unlike eNOS and nNOS, iNOS is not expressed constitutively in mammalian cells, but rather is induced by proinflammatory stimuli such as bacterial LPS or the cytokines TNF-α, IL-1β, or IFN-γ, individually, or in combination (Sanders et al., 1999; Wahl et al., 2003). Inducible NOS possesses tightly bound calmodulin in a noncovalent manner and is calcium independent. Therefore, once expressed, iNOS continues to synthesize NO$^{\bullet}$ in large amounts for a prolonged period of time (Nathan, 1992). iNOS activity is regulated by protein expression rather than functional modulation (Lee et al., 2003).

Inducible NOS is active as a homodimer of approximately 260 kDa, and only the dimeric forms exhibit catalytic activity. The expression of iNOS is regulated at the level of transcription and at the level of iNOS mRNA stability. The mechanism of iNOS induction involves de novo transcription and biosynthesis of new protein. The 5′-flanking region of murine and human iNOS share a 66% homology and contain conserved consensus sequences for NF-κB as well as containing both γ-interferon/tumor necrosis factor responsive elements (Chartrain et al., 1994). Despite their tight similarities, the transcriptional control of iNOS in murine and humans differs because in the former, but not in the latter, a 1.6-kb 5′-flanking region has the necessary promoter sequences to induce full gene expression. These differences might help shed light as to why it has been so difficult in inducing iNOS in human cells (Weinberg et al., 1995).

In fact, agents known to interfere with NF-κB activity seem to modulate the induction of iNOS. Many antioxidants, including pyrrolidine-dithiocarbamate and diethydithiocarbamate, inhibit iNOS expression in cultured cells (Mulsch et al., 1993; Sherman et al., 1993) in addition to nonselective protease inhibitors. Several other distinct classes of agents have been demonstrated to prevent expression of iNOS via inhibition of the NF-κB transduction system (Griscavage et al., 1996). Glucocorticoids such as dexamethasone interfere with iNOS expression in many cell types (Di Rosa et al., 1990); moreover thrombin, macrophage deactivation factor, tumor growth factor-β, platelet-derived growth factor, IL-4, IL-8, and IL-10 inhibit iNOS induction. The same immunological stimuli that induce iNOS also induce guanosine triphosphate cyclohydrolase, an enzyme that produces tetrahydrobiopterin (BH_4) and thus supplies iNOS with its cofactor (Salvemini and Masferrer, 1996). Induction of iNOS is associated with the induction of arginosuccinate synthetase, which may supply iNOS with its substrate from intracellular sources by turning on the "recycling" of L-arginine from L-citrulline (Salvemini et al., 1995). In some cell type, the same stimuli that induce iNOS also up-regulate the membrane transport system for L-arginine, thereby supplementing intracellular L-arginine from extracellular sources. Some NOS inhibitors (e.g., N^G-monomethyl-L-arginine) also inhibit the Y^+ cationic transporter system responsible for L-arginine, and other cationic amino acid transport into the cells, whereas others (e.g., nitro-L-arginine) do not affect it (Bogle et al., 1992).

Induction of iNOS may have either toxic or protective effects. Factors that appear to dictate the consequences of iNOS expression include the type of insult, the tissue type, the level and duration of NOS expression, and probably the redox status of the tissue. Much attention has focused on the toxic effects of iNOS. For example, induction of iNOS in endothelial cells produces endothelial injury (Corbett et al., 1992). Induction of iNOS has been shown to inhibit cellular respiration in macrophages and vascular smooth muscle cells; these processes can lead to cell dysfunction and cell death. Such processes, when occurring within vascular smooth muscle cells, play a key role in the same cell where the activation of iNOS (Misko et al., 1993), in turn, can act as NO donors, activating guanylyl cyclase (Moore et al., 1994; Connor et al., 1995). Such a mechanism has been proposed in relation to bradykinin-induced cell signaling in pulmonary blood vessels (Zingarelli et al., 1997). The generation of large amounts of NO following iNOS induction in activated macrophages accounts for the antimicrobial effects of these cells. In various pathophysiological conditions, iNOS not only is expressed in macrophages, but can also be induced in various other cells, including fibroblasts, Kupffer's cells, hepatocytes, neutrophils, mesangial cells, chondrocytes, pancreatic islet cells, microglia, epithelial and endothelial cells, smooth muscle cells, cardiac myocytes, megakaryocytes, and various tumor cells where a positive correlation between iNOS expression and tumor progression has been extensively reported (Moncada and Palmer, 1991; Nathan, 1992; Green et al., 1993; Nussler and Billiar, 1993; Vane et al., 1994; Gallo et al., 1998; Thomsen et al., 1998; Fantappiè et al., 2002). Since NO is a radical, it reacts with other radicals; this is exemplified by the interaction of NO with superoxide yielding peroxynitrite ($ONOO^-$). Indeed, this reaction proceeds three times more rapidly than the disproportion of superoxide by

superoxide dismutase. The combination between these two radicals to give peroxynitrite has received considerable attention over the past few years, since it has been suggested that peroxynitrite may represent an important mediator of cytotoxicity and cytostasis (Radi et al., 1991). Currently, little information is available regarding the "physiological" roles of peroxynitrite, although the evidence for its roles in pathophysiological conditions is expanding.

Although there are a number of experimental difficulties related to delineation of the actual role of peroxynitrite in shock and other pathophysiological conditions, theoretical considerations strongly favor the production of peroxynitrite when NO and superoxide are produced simultaneously, because the reaction of these two species is nearly diffusion-controlled. In fact, the reaction of superoxide with NO is the only reaction that outcompetes the reaction of superoxide with superoxide dismutase (Corbett et al., 1993; Perkins and Kniss, 1999). The finding that peroxynitrite is produced during inflammation and shock is not surprising, in light of the previous evidence for the overproduction of oxygen-derived free radicals. Nitrotyrosine formation, and its detection by immunostaining, was initially proposed as a relatively specific means for detecting the "footprint" of peroxynitrite (Von Knethen and Brune, 1997). Recent evidence, however, indicates that certain other reactions can also induce tyrosine nitration; for example, the reaction of nitrite with hypochlorous acid and also the reaction of myeloperoxidase and other peroxidases with hydrogen peroxide can lead to the formation of nitrotyrosine (Tsai et al., 1994; Egan et al., 1976). The physiopathological relevance of this reaction remains to be further elucidated. More recent reviews take an increased nitrotyrosine staining as an indication of "increased nitrosative stress" rather than a specific marker of peroxynitrite (Jijon et al., 2000). The formation of nitrotyrosine has recently been demonstrated in inflammation, ischemia-reperfusion, neurodegeneration, and shock; the staining was abolished by treatment of the animals with iNOS inhibitors and peroxynitrite scavengers (Cuzzocrea et al., 1998, 2000, 2001; Muscoli et al., 2003). Several evidences strongly suggest that peroxynitrite is produced in shock and inflammation. Specific peroxynitrite scavengers that could help to further elucidate the role of peroxynitrite in pathological situations are not available. Glutathione, melatonin, and uric acid, a putative scavenger of peroxynitrite, are sometimes used as a probe for peroxynitrite (Inoue et al., 1993; Landino et al., 1996; Upmacis et al., 1999); however, uric acid can interfere with a number of other oxidants (Alvarez et al., 1999; Notoya et al., 2000), and caution should therefore be applied in the interpretation of these results. Therefore, the evidence implicating the role of peroxynitrite in a given pathophysiological condition can only be indirect.

A simultaneous protective effect of superoxide neutralizing strategies and NO synthesis inhibition, coupled with the demonstration of peroxynitrite in the particular pathophysiological condition, can be taken as a strong indication for the role of peroxynitrite. However, it is likely that additional interactions of oxygen- and nitrogen-derived free radicals also contribute to the inflammatory cell injury.

B. Contribution of Nitric Oxide Biosynthesis and Release in Disease States

NO is generated by a family of enzymes termed NOS, via a five-electron oxidation of the terminal guanidinum nitrogen of the amino acid L-arginine. The reaction is both oxygen- and NADPH-dependent and yields L-citrulline in addition to NO in a 1:1 stoichiometry; molecular oxygen serves as an electron acceptor (Kwon et al., 1990). The enzyme is stereospecific for the L-isomer of arginine, since D-arginine is not a substrate. The distribution of different isoforms of NOS is largely related to their respective functions. Although the L-arginine/NO pathway has been identified in many species, including fish, birds, and bacteria, NOS has been best studied in mammalian systems (Nathan, 1992; Knowles and Moncada, 1994). Based upon several criteria including cellular localization, regulation of activity, and substrate/inhibitor profiles, three isoforms of NOS enzymes have been described and subsequently cloned. Molecular cloning has shown these to share 50 to 60% homology. The first is nNOS (or type I) a constitutive form whose activity is regulated by Ca^{2+} and calmodulin and was found in neuronal tissue, both centrally and peripherally. Neuronal NOS is also present in the epithelium of rat trachea and human bronchi (Kobzik et al., 1993) and in human skeletal muscle (Nakane et al., 1993). The reader is referred to a recent detailed review by Esplugues covering the multifaceted roles of NO from nNOS (Esplugues, 2002). A second Ca^{2+}/calmodulin requiring constitutive enzyme was found in vascular endothelial cells and human platelets (Mehta et al., 1995), eNOS or type III, and an inducible Ca^{2+}/independent isoform, iNOS or type II, which can be isolated from macrophages and from a number of cell types following induction with inflammatory mediators and bacterial products. Depending upon the source, these enzymes are active as monomers or homodimers with monomeric molecular weights of 125 to 155 kDa; only the dimeric forms exhibit catalytic activity. All forms of NOS characterized thus far are flavoproteins with the rare property of containing both flavin mononucleotide and flavin adenine dinucleotide; the reduced form of NADPH and BH_4 are required as cofactors. The C-terminal half of the NOS protein bears remarkable resemblance to only one other mammalian protein, cytochrome P450 reductase and appears to possess the same cofactor binding sites, and this is often referred to as the reductase domain. NO synthase, unlike cytochrome P450 reductase, is a self-sufficient

enzyme in that the oxygenation of substrate, L-arginine, occurs at the heme site in the N-terminal portion (oxygenase domain) of the protein. Stoichiometric amounts of heme are present in NOS and are required for the catalytic activity (Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992). Heme coordination is thought to be provided by Cys-415 (nNOS) based upon homology to cytochrome P450 and heme incorporation following site-directed mutagenesis (McMillan and Masters, 1995). Indeed, this Cys residue is conserved in all NOS isoforms across differing species and corresponds to Cys-200 in human iNOS and Cys-184 in human eNOS. Close to the heme, catalytic site is the binding site for the substrate L-arginine. Neuronal NOS possesses at its N terminus a PDZ domain, also known as DHR domain or GLGF repeats (Morais Cabral et al., 1996), that facilitates binding to specific protein bearing a similar motif. All forms of NOS contain four prosthetic groups: flavin adenine dinucleotide, flavin mononucleotide, BH_4, and a heme complex iron protoporphyrin IX. Recently, zinc was identified as an additional prosthetic group of NOS.

The mechanism of NOS-catalyzed oxidation of L-arginine to NO proceeds in at least two distinct steps. The initial reaction involves *N*-hydroxylation of the guanidinium nitrogen to form *N*-hydroxy-L-arginine, which is the only intermediate identified to date (Schmidt et al., 1996). The reaction utilizes one equivalent of NADPH and O_2 to conduct a simple two-electron oxidation of nitrogen. This reaction mimics classical P450-like hydroxylation (Poulos, 1988). The subsequent step in the conversion of *N*-hydroxy-L-arginine to NO and L-citrulline is unclear. Recent studies have shown that nitroxyl (HNO) and not NO is the preferred nitrogen-oxide product (Fukuto et al., 1992, 1993). If NO were to be the product, NO synthase has to facilitate an odd-electron oxidation, but such reaction is difficult to reconcile with P450 chemistry ($2e^-$ transfer). In this way, HNO would be the expected two-electron oxidation product from *N*-hydroxyl guanidines. Therefore, NO synthase generates HNO from L-arginine in a four-electron process consistent with the enzymology of cytochrome P450, and a subsequent one-electron oxidation of this product yields NO as proposed by Hobbs and coworkers (1994).

All of the NOS isoforms can be inhibited to a variable degree with N^G-substituted L-arginine analogs, e.g., N^G-monomethyl-L-arginine (L-NMMA) (Hobbs et al., 1999). For the biochemistry and regulation of NO synthases, the reader is referred to specialized reviews (Clancy and Abramson, 1995; Moncada and Higgs, 1995; Hobbs et al., 1999).

C. Nitric-Oxide Synthase Inhibitors

There are a large number of NOS inhibitors described in the literature and in use as pharmacological tools. The guanidino-substituted arginine, L-NMMA, has been the first NOS inhibitor described and has been used to identify many of the physiological actions of NO and to investigate its role in some pathophysiological processes (Rees et al., 1989a). In general, substitution of guanidino moiety of L-arginine yields compounds that are potent NOS inhibitors in vitro and in vivo. Esterification of the α-carboxyl group, as in N^G-nitro-L-arginine methyl ester, alters the potency of the original inhibitor, N^G-nitro-L-arginine, by requiring cleavage by esterases to generate the active compound (Pfeiffer et al., 1996). This changes the water solubility of the compound and provides the basis for inhibition of NOS only in dose tissues possessing a specific esterase. The NOS inhibition by various guanidino-substituted L-arginine analogs differs considerably; in fact, L-NMMA can be metabolized by NOS yielding *N*-methyl-*N*-hydroxyl-L-arginine, which can inactivate the enzyme in an irreversible way (Feldman et al., 1993). However, L-NMMA has been shown to metabolize to L-arginine, acting as a substrate. Guanidino-substituted L-arginine molecules also appear to occur endogenously and may be important in regulating NO production in vivo. Asymmetric dimethylarginine is a potent inhibitor of all NOS isoforms, and it was found in many organs and tissues in several disease states (Vallance et al., 1992; Komori et al., 1994; Yu et al., 1994).

Regarding the selectivity of NOS inhibitors, there are many misleading statements in the literature. As described by Alderton et al. (2001), this has been a consequence of differing criteria for what constitutes selectivity and how selectivity is defined or determined: effects on an isolated enzyme or in isolated tissues/organs or in vivo pharmacological properties. For the purposes of this review, we define levels of selectivity on pragmatic grounds and primarily on the basis of relating their potency under identical conditions in the physiological range (L-arginine concentration, etc.). Inhibitors with less than 10-fold selectivity are unlikely to be useful as selective agents because of the difficulties inherent in attempting to use them in such a way that only one isoform is affected and should be regarded as nonselective inhibitors. Agents which have 10- to 50-fold selectivity can be useful as "partially selective" inhibitors, as long as great care is taken over the necessary controls and concentration/dose used. With compounds of over 50- or 100-fold selectivity, inhibitors become much simpler to use to inhibit the activity of a single isoform without affecting others and also start to have potential as selective therapeutic agents without the potential for side effects that might arise from inhibiting the other isoforms. Several in vivo pharmacological effects of NOS inhibition have been associated with the functioning of one or the other of the three isoforms, and effects or lack of them on these is sometimes used to infer isoform selectivity of action.

Frequently, a misunderstanding is the definition of an inhibitor as selective for, e.g., iNOS versus eNOS, and then ignoring its nonselectivity for nNOS or completely distinct enzyme targets. An interesting example is ami-

noguanidine that is partially selective for iNOS versus eNOS (Alderton et al., 2001), whereas the selectivity over nNOS is minimal. Moreover, it has a wide range of other effects, inhibiting advanced glycosylation end-product formation, diamine oxidase and polyamine metabolism (Bieganski et al., 1983; Nilsson et al., 1996), catalase (Ou and Wolff, 1993), and having antioxidant effects (Giardino et al., 1998; Yildiz et al., 1998). For this reason, aminoguanidine should not be described as a selective inhibitor. In the more subtle instances of agents that have selectivity versus only one NOS isoform, this needs to be made explicit when describing and using them. Furthermore, another difficulty that arises in assessing efficacy and selectivity of NOS inhibitors and of comparing such data from different groups in the literature is the frequent finding of time-dependent inhibition which may vary significantly between isoforms as described by Bryk and Wolff (1999). Inhibitors of NOS have been described which interact with the NOS enzymes in a variety of ways, such as different sites, as well as differing time and substrate dependence, and mechanism of inhibition.

Identification of selective inhibitors of iNOS and nNOS has been a goal of both academic and pharmaceutical scientists for some years now, given that several inhibitors of more than 100-fold selectivity for iNOS versus eNOS have already been identified. A large number of patents claiming a range of structural classes as selective NOS inhibitors have been published (Mete and Connolly, 2003).

In the search for selective nNOS inhibitors (Babu and Griffith, 1998a), some amino acids have been described which are partially selective for nNOS versus eNOS and iNOS. For example, *S*-ethyl- and *S*-methyl-L-thiocitrulline (Furfine et al., 1994; Babu and Griffith, 1998b) and N^5-(1-imino-3-butenyl)-L-ornitine (vinyl-L-NIO) (Babu and Griffith, 1998b) all show time-dependent inhibition of nNOS with significant selectivities versus isolated eNOS and iNOS enzymes. However, *S*-ethyl- and *S*-methyl-L-thiocitrulline appeared less selective in intact rat tissues and in vivo, raising questions over their usefulness as pharmacological tools. The non-amino acid ARL 17477 [*N*-[4-(2-{[(3-chlorophenyl)methyl]amino}ethyl)phenyl]-2-thiophenecarboximidamide] has been reported to be both a selective nNOS inhibitor in vitro and effective in vivo in animal models of brain damage in stroke (Zhang et al., 1996; O'Neill et al., 2000); however, these articles do not report whether this compound inhibits iNOS. Recently, Alderton and colleagues (2001) have demonstrated that this compound is partially selective for nNOS versus eNOS (23-fold) but only 5-fold versus iNOS, so that "selective" in this context is primarily relative to eNOS only. Given the potential role of iNOS in stroke (Iadecola et al., 1997; Nagayama et al., 1998), this may be significant. Other selective inhibitors of nNOS have been described, such as 7-nitroindazole and 3-bromo-7-nitroindazole (Gorlach et al., 2000; Kobayashi et al., 2003) and 6-nitroindazole and its water soluble sodium salt. Among the guanidine substitute arginine, N^G-propyl-L-arginine has been indicated as a highly selective inhibitor of nNOS (Table 1).

The first "highly selective" iNOS inhibitor versus eNOS were the bis-isothiourea compounds reported by Garvey et al. (1994). Of these, *S*,*S*′-[1,3-phenylene-bis(1,2-ethanediyl)]-bis-isothiourea (PBI-TU) is an L-arginine-competitive, rapidly reversible inhibitor of human iNOS with a selectivity of 190-fold versus eNOS, although it is only 5-fold selective versus nNOS. This compound is a clear demonstration that the substrate-binding sites of full-length human iNOS and eNOS in solution are, in fact, significantly different from each

TABLE 1
Nonselective inhibitors of nitric-oxide synthase isoforms
The values of IC_{50} and K_i are calculated on binding studies to the relative isotype selectivity of the inhibitor. These values depend upon the exact system used for the measurement (see *References*).

		NOS-I	NOS-II	NOS-III	References
Inhibitors of NOS-I (nNOS)					
5-Ethyl-*N*-[4-trifluoro(methyl)phenyl]-isothiourea	K_i	0.32 μM	37 μM	9.4 μM	Wolff et al., 1994
α-Fuanidinoglutaric acid					Shearer et al., 1997
L-N^5-(1-Iminoethyl)-ornithine (L-NIO)	K_i	1.7 μM	3.9 μM	3.9 μM	Yokoi et al., 1994
N^5-(1-Imino-3-butenyl)-L-ornitine	K_i	0.1 μM	60 μM	12 μM	Furfine et al., 1994
7-Nitroindazole	IC_{50}	0.71 μM		0.80 μM	Rees et al., 1990
3-Bromo-7-nitroindazole	IC_{50}	170 nM	≈200 nM		Babbedge et al., 1993
N^{ω}[ρ]-Propyl-L-arginine	K_i	0.057 μM	180 μM	8.5 μM	Zhang et al., 1997
L-Thiocitrulline	K_i	0.06 μM	3.6 μM		Furfine et al., 1994
S-Methyl-L-thiocitrulline	IC_{50}	0.3 μM		5.4 μM	Handy et al., 1936
1-(2-Trifluoromethylphenyl)imidazole	IC_{50}	28.2 μM	27.0 μM	1.06 mM	Babu and Griffith, 1998a
Inhibitors of NOS-II (iNOS)					
S-Methyl-isothiourea	K_i		6 μM		Webber et al., 1998
S-(2-Aminoethyl)-isothiourea	K_i		6 μM		Garvey et al., 1994
2-Imino-4-methylpiperidine	IC_{50}	0.2 μM	0.1 μM	1.1 μM	Griffiths et al., 1993
Aminoguanidine	K_i	157 μM	75 μM		Garvey et al., 1994
L-N^5-(1-Iminoethyl)-ornithine (L-NIO)	K_i	1.7 μM	3.9 μM	3.9 μM	Rees et al., 1990
L-Thiocitrulline	K_i	0.06 μM	3.6 μM		Furfine et al., 1994
Inhibitors of NOS-III (eNOS)					
S-Ethyl-isothiourea	K_i	0.029 μM	0.017 μM	0.036 μM	Nakane et al., 1995
S-Isopropyl-ITU	K_i		0.01 μM		Rees et al., 1990
L-N^5-(1-Iminoethyl)-ornithine (L-NIO)	K_i	1.7 μM	3.9 μM	3.9 μM	Southan et al., 1995

other and poses a challenge to the structural biologists to explain the basis of this. It may be revealing to study NOS crystal structures with this compound bound. Unfortunately, the utility of this series of compounds as pharmacological agents is limited by the poor cellular and tissue penetration of the more selective compounds, as well as by significant acute toxicity.

The compound *N*-3-aminomethyl-benzylacetamidine (1400W), also identified by Garvey et al. (1997), has proven to be a further step forward, since it is not only highly selective as an iNOS inhibitor versus both eNOS and nNOS, but also penetrates cells and tissues. Inhibition of human iNOS by 1400W was competitive with L-arginine, $NADPH^+$-dependent and developed rather slowly, and no significant reversal of this inhibition was observed after 2 h. Efficacy and selectivity were maintained in vivo in the stringent test of its differential effects on vascular leakage; unlike L-NIO [L-N^5-(1-iminoethyl)-ornithine] and aminoguanidine, it suppressed the late, iNOS-driven phase of endotoxin-provoked leakage with no exacerbation of the early phase as it has been observed when eNOS and nNOS are inhibited (Garvey et al., 1997; Laszlo and Whittle, 1997). Unfortunately, this compound exhibits an acute toxicity at high doses that is likely to prevent its safe therapeutic use in humans, but there is a significant therapeutic window so that it can be used as a pharmacological tool in a variety of animal models (Garvey et al., 1997; Laszlo and Whittle, 1997; Thomsen et al., 1997; Kankuri et al., 2001).

Two novel NOS inhibitors, the sulfone GW273629 and the sulfide GW274150, of acetamidine derivatives of lysine and homolysine have been identified from a series of acetamidine amino acids, which like 1400W have a very high degree of selectivity for iNOS versus both eNOS and nNOS (Young et al., 2000). Both are sulfur-substituted amino acids acting in competition with L-arginine. Like 1400W, the inhibition of human NOS is $NADPH^+$-dependent and develops rather slowly, whereas the inhibition of human eNOS and nNOS is rapidly reversible. Again, like 1400W, this efficacy and selectivity is maintained in intact cells and tissues, but there was no indication of the acute toxicity seen with 1400W and several other non-amino acid NOS inhibitors. The two inhibitors, GW273629 and GW274150, have been used to probe the role of iNOS in several animal models of diseases in which iNOS has been implicated (Alderton et al., 2000; Knowles et al., 2000) (Table 2).

The heme-binding substituted pyrimidine imidazoles discussed in the previous section are not direct inhibitors, but they inhibit the assembly of active dimeric iNOS during its synthesis. Because of this, it is not straightforward to determine their isoform selectivity, but studies with transient transfection of the three isoforms suggested that high selectivity was achievable (McMillan et al., 2000). It will be interesting to see what the pharmacology and utility of such compounds will be and whether other compound series are discovered with this unusual mechanism of action. Therefore, genetic or pharmacological inhibition of iNOS was associated with a dramatic reduction of liver, intestine, and lung injury measured by plasma transaminase levels (Needleman and Manning, 1999). Moreover, recent results indicate that in patients with compensated cirrhosis, portal hypertension, and hyperdynamic circulation, a condition where iNOS is up-regulated (Laffi et al., 1995), acute inhibition of NO synthase corrects the altered systemic hemodynamic and improves renal function and sodium excretion (La Villa et al., 2001). Subsequent studies have revealed a similar protective effect of iNOS inhibition on bowel damage and maintenance of small bowel motility as well as preventing the gut permeability following zymosan shock (Nogawa et al., 1998). Similar protective effects were also observed using antioxidants (Cheng et al., 2000). However, a potentially unique aspect of NO-mediated signaling in zymosan shock is the associated redox stress. It is possible under conditions of reduced antioxidant capacity that NO or a reaction product, such as peroxynitrite, activates intracellular redox-sensitive signaling pathways. Furthermore, nitrogen-based radicals most likely represent only one of the oxidant species that participate in oxidant signaling in shock.

The role of iNOS-derived NO or peroxynitrite as an amplifier of the inflammatory response is now also supported by previous observations: 1) inhibition of iNOS suppresses TNF-α production in the delayed phase of allergic encephalomyelitis (Anggard, 1991); 2) inhibition of iNOS suppresses interleukin-1, collagen, and strome-

TABLE 2

High selective inhibitors of nitric-oxide synthase isoforms

The values of IC_{50} and K_i are calculated as reported in Table 1. In the case of mechanism-based inactivators (e.g. 1400W) that bind to all isoforms, irreversible inhibition only occurs with iNOS. In this case, a K_i value alone does not indicate selectivity, and the selectivity ratio has been calculated (see *References*).

		NOS-I	NOS-II	NOS-III	References
2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-tiazine	IC_{50}		0.036 μM		Nakane et al., 1995
L-N^6-(1-Iminoethyl)-lysine	IC_{50}	92 μM	3.3 μM		Moore et al., 1994
S-2-Amino-1-ethylamino-5-thioheptanoic acid (GW274150)	K_i	8 μM	100 nM	25 μM	Young et al., 2000
S,*S*-[1,3-Phenylene-bis(1,2-ethanedyl)]-bis-isothiourea (1,3-PB- isothiourea)	K_i	0.25 μM	0.047 μM	9 μM	Garvey et al., 1994
1,4-PB-Isothiourea	Ratio	25	5000	1	Garvey et al., 1997

lysin production in arthritis (Parker, 1987; Luscher, 1990); 3) inhibition of iNOS suppresses INF-γ production in a murine model of leishmaniasis (Stamler and Loscalzo, 1991); and 4) the expression of certain chemokines is suppressed in the absence of iNOS in zymosan-induced peritonitis (Davidge et al., 1995). Thus, a picture of a pathway is evolving that contributes to tissue damage both directly via the formation of peroxynitrite with its associated toxicities and indirectly through the amplification of the inflammatory response.

D. Nitric Oxide Donors

The NO donors represent a very heterogenous class of drugs that exert their actions by a similar final step of bioactivation, namely the release of NO (see Feelisch, 1991). The physiological roles played by NO are numerous and range from the control of blood pressure and platelet function to the effects on genitourinary and respiratory function and to the killing of tumor cells, bacteria, and parasites. NO donors are crucial pharmacological tools in those diseases of the cardiovascular system that are associated with a failing endogenous L-Arg/NO pathway. Nitrosothiols such as *S*-nitrosopenicillamine and sodium nitroprusside (SNP) release NO spontaneously. Similarly, nitrosamines such as 3-morpholinosydnonimine (the main metabolite of the prodrug molsidomine) do not need an enzyme or the presence of a thiol for delivering NO (Feelisch, 1991). The spontaneous release of NO is a process that basically depends on the presence of oxygen as electron acceptor from the sydnonimine molecule. On the other hand, organic nitrites such as amyl nitrite require interaction with a mercapto group to form an *S*-nitrosothiol intermediate from which the NO radical is eventually released (Feelisch, 1991). Organic nitrates such as glyceryl trinitrate (GTN) or isosorbide dinitrate (ISDN) require two distinct bioactivation pathways for the conversion of organic nitrates to NO. One is enzymatic and the other is nonenzymatic and relies on the presence of thiol-containing molecules such as cysteine. Smooth muscle cells, endothelial cells, and inflammatory cells such as macrophages are capable of converting organic nitrates to NO (Benjamin et al., 1991; Feelisch and Kelm, 1991; Salvemini et al., 1992a,b). In mammals, denitration and clearance of the drug are mainly carried out by cytosolic hepatic glutathione *S*-transferase enzymes and glutathione reductase (Needleman and Johnson, 1973). Hepatic first-pass metabolism of organic nitrates, which is generally considered an inactivation pathway, also leads to the formation of NO. Recently, it has been shown that cytosolic hepatic glutathione *S*-transferase forms NO from the organic nitrates, and an additional two distinct microsomal pathways exist for NO formation from the organic nitrates; on the basis of these observations, it has been proposed that this metabolic route may be exploited in the treatment of certain liver diseases (Spahr et al., 1994). Evidence arising from the use of hepatic and renal cells has implicated a role for the cytochrome P450 enzyme system in the formation of NO from organic nitrates (Servent et al., 1989; Schroder and Schror, 1992). NO is also formed endogenously from L-Arg in the same cells capable of metabolizing organic nitrates to NO. Inhibitors of NO biosynthesis do not affect the biological properties of organic nitrates (Ahlner et al., 1991). This indicates that the organic nitrate-to-NO pathway is distinct from the L-Arg/NO pathway. Inhibitor of the nitrate-to-NO system has been identified. Nitroblue tetrazolium, a well known scavenger of superoxide anions and an inhibitor of NADPH-dependent oxidations, inhibits the biological activity of organic nitrates in vitro and in vivo, but has no effects on those NO donors (e.g., SNP, 3-morpholinosydnonimine) that release NO spontaneously (Pistelli et al., 1994).

Organic nitrates are rapidly and nonenzymatically degraded if certain sulfhydryl (SH) groups containing molecules such as *N*-acetylcysteine or cysteine are present (Ahlner et al., 1991). It may be due to a nucleophilic attack by a thiolate anion on the nitrogen atom of the ester group of GTN (Feelisch, 1991). The generation of NO increases with the number of nitrate ester substitutes in the molecule (Feelisch et al., 1987; Feelisch, 1991). Thus, GTN is more active than ISDN and in turn ISDN is more active than isosorbide-5-mononitrate. Metabolites of GTN such as 1,2- and 1,3-glyceril dinitrate also form NO through enzymatic and nonenzymatic pathways (Salvemini et al., 1993b,c). Feelisch and coworkers demonstrated that depending upon the structure of the thiol used, two distinct pathways are involved in the formation of either NO or nitrite from the molecule of the organic nitrate (Feelisch, 1991). Thus, although 54 different thiols were capable of realizing nitrite from organic nitrate in aqueous solution, *N*-acetylcysteine (NAC), cysteine, and thiosalicylic acid (TSA) were able to release NO as well (Feelisch et al., 1987; Feelisch, 1991). Chong and Fung (1991) also reported that among aliphatic and aromatic thiols tested, NAC, mercaptosuccinic acid, and TSA were the most potent generators of NO from GTN in buffer with an order of potency of NAC < mercaptosuccinic acid < TSA. Later studies then showed that this correlated with their efficacy in increasing the antiplatelet effects of GTN (Salvemini et al., 1993b) and in potentiating the in vivo hemodynamic effects of organic nitrates (Lawson et al., 1991; Salvemini et al., 1993b,c). Glutathione does not release NO and releases only nitrite, which is devoid of biological effects (except perhaps at very high doses). Thus, glutathione does not potentiate the pharmacological actions of organic nitrates (Feelisch, 1991; Salvemini et al., 1993b).

Under normal circumstances and in the presence of an intact endothelium, antiplatelet NO keeps blood vessels in a dilated state and in conjunction with PGI_2 maintains the nonthrombogenic nature of the endothelium.

Disease states that damage the endothelium or that alter the normal properties of the endothelial cells lead to a decrease in the production of NO, tipping the balance in favor of constriction, thrombosis, and vasoocclusion. Disease processes associated with localized impairment of the release of NO include coronary artery spasms that occur either spontaneously (Prinzmetal angina) or in the context of atherosclerosis, myocardial ischemia, and stroke (Vane et al., 1990).

This imbalance can be theoretically restored by exogenous NO donors, and these should be uniquely beneficial in conditions with endothelial dysfunction because they substitute for a failing endogenous system. Since NO donors do not require an intact endothelium to be effective, they will restore vascular dilation and suppress the tendency to platelet aggregation.

Although the vascular effects of NO donors have been well documented, these compounds are also endowed with antiplatelet effects and antithrombotic properties. NO donors inhibit platelet adhesion and aggregation in vitro and in vivo (De Caterina et al., 1984, 1990; Salvemini et al., 1990b; Mollace et al., 1991), increase bleeding time in humans (Lichtenthal et al., 1985), and synergize with PGI_2 (Korbut et al., 1990; Mollace et al., 1991). Furthermore, NO donors potentiate the activity of thrombolytic factors in providing further protection against vascular occlusion (Korbut et al., 1990). NO donors that release NO spontaneously are far more potent inhibitors of platelet function in vitro when compared with those requiring bioconversion. Platelets do not have the enzyme that converts organic nitrates to NO, and therefore, in vitro they will respond only to very high doses of nitrates (Ahlner et al., 1991). The fact that endothelial cells can metabolize organic nitrates such as GTN efficiently, as do smooth muscle cells (Benjamin et al., 1991; Feelisch and Kelm, 1991; Salvemini et al., 1992a), is important because it provides the key for understanding why organic nitrates inhibit platelet function in vivo, whereas they generally display weak antiplatelet effects in vitro (Schafer et al., 1980; Loscalzo, 1981; Benjamin et al., 1991; Salvemini et al., 1992a). The findings that smooth muscle cells and endothelial cells significantly increase the in vitro antiplatelet effects of GTN (where the dose of GTN required to inhibit platelet aggregation in vitro approximates that used in in vivo studies) led to the proposal that the increased effectiveness of organic nitrates in vivo as antiplatelet agents is due to conversion to NO by cells of the vascular tree, such as smooth muscle cells and endothelial cells (Benjamin et al., 1991; Feelisch and Kelm, 1991; Salvemini et al., 1992a). In this respect, the endothelial cells may be more important because they come into contact with platelets.

NO donors also promote the release of PGI_2 in vivo and in vitro from endothelial cells (Levine et al., 1981; Schror et al., 1981, 1984; Mehta et al., 1983, 1993; De Caterina et al., 1985). The latter effect presumably reflects the ability of NO donors to directly activate the cyclooxygenase pathway, a phenomenon associated with a marked release of prostaglandins (Salvemini et al., 1993a). Release of vasodilator prostaglandins by NO donors does not account for the vasodilator effects of the NO donors, but the release of antiplatelet COX products does not account, at least in part, for their antithrombotic actions (Ahlner et al., 1991). This gives a new dimension to the usefulness of exogenous NO therapy, since regulation of cyclooxygenase activity by NO may be an additional pathway used by NO donors to confer protective effects. Activation of COX is a cGMP-independent process (Salvemini et al., 1993a). During the progression of atherosclerosis or hypertension, defects at the level of the soluble guanylate cyclase transduction system have been observed. This may hamper the effectiveness of NO donors in restoring their vasodilator and antithrombotic (cGMP-dependent) properties. Not having to rely on the soluble guanylate cyclase to activate COX, NO donors will still be able to release antiplatelet PGI_2. The therapeutic clinical usefulness of organic nitrates is limited by the development of tolerance in patients after continuous treatment for either chronic stable angina (Parker and Fung, 1984; Parker et al., 1987) or congestive heart failure (Jordan et al., 1985), a phenomenon not seen with those NO donors that release NO spontaneously. Extensive work by several groups has demonstrated that NO is the active biological component released by a variety of NO donors but that the pathways of bioactivation differ substantially depending on the individual chemistry of the NO donor. Combined therapy of an organic nitrate and a thiol-containing compound may be a useful approach to limit the extent of tolerance.

IV. Interaction between Nitric Oxide and Prostaglandin Biosynthesis

A. Nitric Oxide/Cyclooxygenase Reciprocal Interactions

The simultaneous biosynthesis and release of both NO and PGs in many tissues has widely been explored in the last few years. Due to their role in the pathophysiological mechanisms underlying some relevant inflammatory disorders, the possible interaction between NO and PG biosynthetic pathways has been studied to verify a possible combined approach in the treatment of many disease states (Fig. 4) (Abramson et al., 2001; Mebazaa et al., 2001; Beierwaltes, 2002; Davel et al., 2002; Gallo et al., 2002; Naoki et al., 2002; Sanchez et al., 2002; Shinmura et al., 2002; Takeuchi et al., 2002; Vayssettes-Courchay et al., 2002; Kawabe et al., 2003; Slomiany and Slomiany, 2003).

In particular, increasing evidence suggest that there is considerable "cross talk" between NO and PG biosynthetic pathways involving an active back modulation operated by reaction end products, including NO, PGs, and cyclic nucleotides. However, the final effect of these

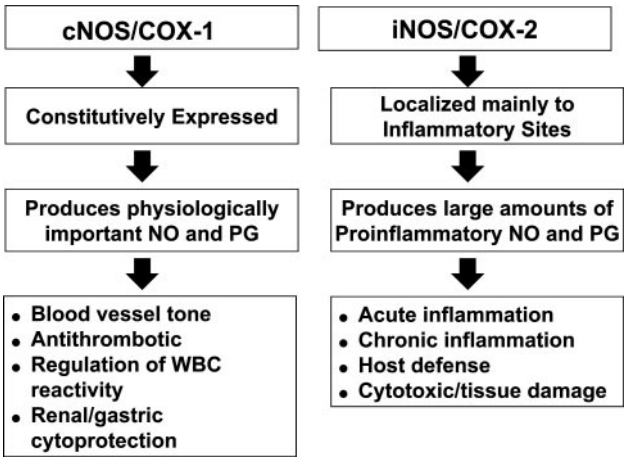

FIG. 4. Homology of NO- and PG-generating system.

interactions is often unclear, and the effect of NO and NO donors on PG biosynthesis and release still remains to be better elucidated.

The interaction between NO- and PG-generating machinery occurs at multilevels. Indeed, NO may interfere directly with COX expression and PG biosynthesis (Fig. 5). On the other hand, AA and its metabolites generated by COX isoforms may also interfere with NO biosynthesis (Fig. 5). Furthermore, expression of NOS seems to correlate with PG receptors and intracellular messengers, such as cAMP, generated by activation of G-coupled PG receptors. Moreover, both NO and PGs interact with their own respective biosynthetic pathway by modulating molecular events underlying NOS and COX expression. Finally, some NO donors combined with several NSAIDs (e.g., nitroaspirin and nitroflurbiprofen) have recently been synthesized and used in the treatment of many inflammatory and noninflammatory disorders, suggesting the possible beneficial effect of drugs acting on NO and PGs simultaneously. The discovery that NO regulates COX activity was originally made using cellular systems and purified enzymes (Salvemini et al., 1993a). Microsomal sheep vesicles are a rich source of COX-1 and can be used to explore whether the exogenous application of NO can augment further COX-1 activity (Salvemini et al., 1993a). NO gas directly increases COX-1 activity of microsomal sheep seminal vesicles as well as murine recombinant COX-1; this leads to a remarkable 7-fold increase in PGE_2 formation (Salvemini et al., 1993a). COX-2 is also activated by NO. Indeed, evidence exists that COX-2, but not inducible NO synthase, is induced in human fetal fibroblast by 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 (Salvemini et al., 1993a). Exposure of IL-1β-stimulated fibroblasts to either NO gas or two NO donors SNP and GTN increased COX-2 activity by at least 4-fold; this resulted in increased production of PGs (Fig. 6). More recently, NO was shown to enhance the release of PGI_2 from endothelial cells, and by this mechanism, NO and NO-stimulated PGI_2 contributed to the marked antiplatelet effects associated with the administration of nitrovasodilators (Salvemini et al., 1996). This observation is also in keeping with earlier work illustrating synergies between the vascular action of NO and PGI_2. The ability of NO to directly activate COX-2 was supported by the evidence 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 the question whether COX-2 activity was affected by endogenously produced NO. In this respect, the mouse macrophage cell line RAW-264.7 was stimulated with endotoxin to induce iNOS and COX-2 enzymes. This results in the production of large amounts of NO and PGs. Inhibition of iNOS activity by nonselective NOS inhibitors such as L-NMMA methyl ester or N^G-nitro-L-arginine (NO_2Arg) or more selective iNOS inhibitors such as L-N^6-(1-iminoethyl)lysine or aminoguanidine (Corbett et al., 1992; Misko et al., 1993; Moore et al., 1994; Connor et al., 1995) attenuated, as expected, the release of NO from these cells. The remarkable finding was that when NO release was inhibited, there was a simultaneous inhibi-

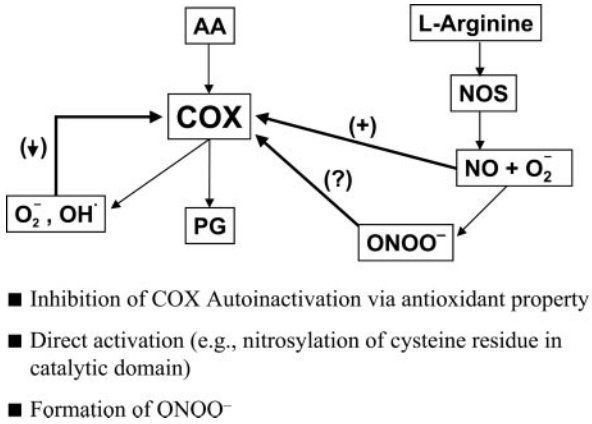

FIG. 5. Possible molecular mechanism by which NO contributes to the modulation of COX.

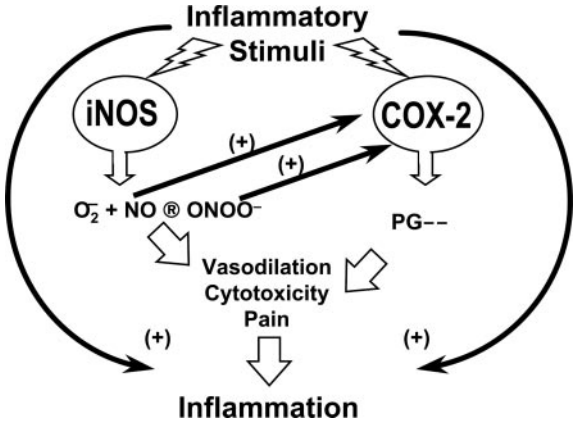

FIG. 6. NO formation occurring after inflammatory conditions further potentiates COX-2 activity.

tion of PG release (Salvemini et al., 1993a). The NOS inhibitors do not behave as NSAIDs, for they do not inhibit COX activity (Salvemini et al., 1993a, 1995a,b; Zingarelli et al., 1997; Perkins and Kniss, 1999). These results suggested that endogenously released NO from the macrophages exerted a stimulatory action on COX-2 activity enhancing the production of PGs. Activation of the enzyme has been observed in various cellular systems (Corbett et al., 1993; Inoue et al., 1993; Misko et al., 1995; Mollace et al., 1995; Von Knethen and Brune, 1997; Watkins et al., 1997) and seems to be independent of the known effects of NO on the soluble guanylyl cyclase. Thus, methylene blue, an inhibitor of the soluble guanylyl cyclase, inhibited the increase in cGMP induced by NO in the fibroblast but did not prevent its ability to stimulate COX activity and therefore PG production.

This has also been confirmed in CNS cells, such as astrocytes, which are currently involved in the modulation of neuronal activity under basal conditions as well as in neuroimmune disorders. In particular, it has been demonstrated that the release of PGE_2 by astroglial cells pretreated with NMDA is driven by activation of the L-arginine-NO pathway, an effect relevant in the pathophysiological mechanisms where glutamatergic neurotransmission is involved (Mollace et al., 1995). In addition, spontaneous release of PGE_2 by hypoxic astrocytes occurs mainly via IL-1β-dependent activation of iNOS (Mollace et al., 1997). Finally, the release of PGE_2 by astroglial cells pretreated with IL-1β and TNF-α is due to enhanced COX-2 expression via activation of iNOS, and this may be relevant to better understand the pathophysiological mechanisms underlying neuroimmune disorders (Mollace et al., 1998). This is also confirmed by substantial evidence showing that iNOS and COX-2 are induced concurrently in a number of models of inflammation, including rabbit hydronephrotic kidney, endotoxin-induced septic shock, and carrageenan-induced pouch and paw inflammation (Mitchell et al., 1995; Salvemini and Masferrer, 1996; Salvemini et al., 1994, 1995a,b) and that NO not only stimulated PG synthase, but also up-regulated the expression of COX-2 protein (Hughes et al., 1999).

Besides these evidences, a mass of experimental results have shown that NO, under certain conditions, down-regulates PG biosynthetic pathway, mainly via inhibition of COX-2. Indeed, Minghetti et al. (1996) showed that LPS-induced COX-2 expression in microglial cells and the subsequent PG release are enhanced by coincubation with NOS inhibitors. On the other hand, these results were confirmed by evidence that NO donors added exogenously to LPS-treated microglial cells inhibit PG release, an effect driven by reduced expression of COX-2 (Guastadisegni et al., 1997). This has also been found in other cell types, including vascular endothelial cells (Doni et al., 1988), rat Kupffer cells (Stadler et al., 1993), and the J774 macrophage cell line (Swierkosz et al., 1995) in which NO has been found to inhibit COX pathway either in vitro or in vivo. In addition, in some cases, COX activation by inducible stimuli is driven by mechanisms that do not involve NO formation. This has been shown to occur, for instance, in astroglial cells treated with IFN-γ, in which the elevation of PG levels and the expression of COX-2 have been found to not be affected by NOS activation (Hewett, 1999).

On the other hand, COX activation, in turn, modulates L-arginine-NO pathway and it has been shown that COX inhibition decreases NOS activity in human platelets (Chen et al., 1997). Both COX and NOS pathways regulate platelet function; thromboxane A_2 induces platelet aggregation, whereas NO inhibits it. Inhibition of COX by two different agents, aspirin and indomethacin, markedly attenuates NOS activity as determined directly by the measurement of L-citrulline and nitrite formation and indirectly by the measurement of cGMP accumulation in platelets. The inhibitory effects of aspirin and indomethacin on NOS activity were reversed by exogenous Ca^{2+} as well as by a functional thromboxane A_2 mimetic, whereas a selective thromboxane A_2 synthase inhibitor and the thromboxane A_2 endoperoxide receptor blocker exerted qualitatively similar effects as COX inhibitors on NOS activity. These effects were not associated with a change in NOS protein expression in platelets suggesting that the effects of COX inhibitors are mediated, at least in part, via thromboxane A_2 inhibition and Ca^{2+} mobilization in platelets (Chen et al., 1997). Because Ca^{2+} is a key regulator of NOS activity, it is not surprising that a reduction in intracellular Ca^{2+} by aspirin, indomethacin, or a selective thromboxane A_2 synthase inhibitor results in decreased NOS activity (Chen et al., 1997). Thus, mobilization of intracellular Ca^{2+} is a key step in the regulation of platelet function, and the inhibition of NOS by COX inhibitors may serve as a regulatory step in the interaction between the COX and NOS pathways. These results indicate that the modulatory effect of NO on the PG biosynthetic pathway may differ according to the cell type used for experimental procedures and to the nature and intensity of the stimulus which leads to activation of PG biosynthesis.

B. Molecular Basis of Nitric Oxide/Cyclooxygenase Reciprocal Modulation

The reason for discrepancies found by several observers in the evaluation of the cross talk between NO and PG biosynthesis is still unclear. However, the net amount of NO released by constitutive or inducible NOS activity and the redox state of stimulated or unstimulated cells seem to play a relevant role in the modulation of COX enzymes leading to possible opposite responses. Thus, the molecular mechanisms involved in both NOS and COX activation may have a crucial role in the understanding of reciprocal interactions between NO and COX.

In early observations, NO has been suggested to activate COX enzymes directly. However, it is unlikely that NO activates COX by binding directly to its heme prosthetic group (Tsai et al., 1994), and other mechanisms have been suggested in this respect. A few possibilities can be put forward; these are depicted in Fig. 7. The first possibility is that NO acts as an antioxidant. Indeed, COX activity also provides a source of superoxide anion, and it has been postulated that superoxide could be involved in the autoinactivation of COX enzymes (Egan et al., 1976). NO interacts with superoxide and limits the amounts of the radical necessary for autoinactivation (Gryglewski et al., 1986). Therefore, one possibility could be that NO augments COX activity by removing superoxide and acting as an antioxidant preventing the autoinactivation of COX. The second possibility is the 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 (Hajjar et al., 1995). The third possibility is the generation of $ONOO^-$, the coupling product of superoxide and NO. Using purified COX-1 and COX-2 enzymes, as well as sheep seminal vesicles, Landino and coworkers (1996) reported that $ONOO^-$ increased COX-1 and COX-2 activity. Peroxynitrite stimulated COX-1 activity in aortic smooth muscle cells (Upmacis et al., 1999). The mechanisms of action by which peroxynitrite can activate the enzymes remain to be defined but could involve either oxidative inactivation (Markey et al., 1987) or modification of key amino acid residues in the polypeptide backbone (Alvarez et al., 1999). Peroxynitrite can also oxidatively modify the COX substrate arachidonic acid, yielding F_2-isoprostanes (Lynch et al., 1994; Moore et al., 1995) which have been described as novel bioactive PGF_2-like compounds and exert powerful vasoconstrictor effects. Peroxynitrite could block the activity of prostacyclin synthase and thereby attenuate the production of prostacyclin (Zou and Ullrich, 1996). At this stage, the relative role of NO and peroxynitrite on COX activation remain to be defined, although there is no doubt that both species are involved at least in vitro. The relevance for peroxynitrite as an activator of the COX enzymes in vivo has not yet been addressed, mainly because selective inhibitors for peroxynitrite have not been available.

In addition to effects on COX-2 enzyme activity, NO has been shown to increase the production of PGs from macrophages by acting at a post-transcriptional or translational level to increase COX-2 protein (Von Knethen and Brune, 1997). In particular, subsequent studies using human osteoarticular chondrocytes revealed that NO released from sodium nitroprusside was able to induce cell death, an event associated with DNA fragmentation, caspase-3 activation, down-regulation of Bcl-2, overexpression of COX-2, and increased release of PGE_2 (Notoya et al., 2000). These events were abolished by blocking the MAPK pathway with the MAPK inhibitor PD98059, the p38 kinase inhibitor SB202190, and the COX-2 selective inhibitor NS-398. Interestingly, although PGE_2 alone had no effect on cell death, it did sensitize sodium nitroprusside-mediated death. These results suggest that NO activates the extracellular signal-related protein kinase and p38 kinase pathway which in turn induce COX-2 and subsequent PGE_2 release. The latter, in turn, may sensitize human osteoarticular chondrocytes to the cell death induced by NO (Notoya et al., 2000). NO is also necessary for maintaining prolonged COX-2 gene expression (Perkins and Kniss, 1999) and sustained PGE_2 biosynthesis. The NO-induced alterations in COX-2 gene expression were not related to COX-2 mRNA (Tetsuka et al., 1996; Perkins

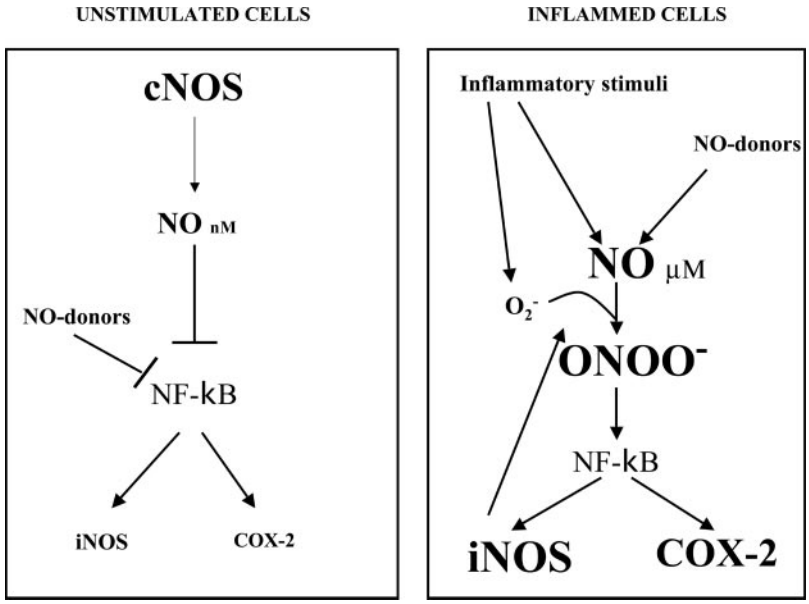

FIG. 7. Reciprocal NO/COX interaction in resting and inflamed cells. NF-κB seems to play a crucial role in both conditions.

and Kniss, 1999), and the mechanism through which NO influences COX gene expression is not yet known.

In the last few years, increasing evidence suggest that intracellular concentrations of NO and PGs may be relevant in switching on/off inflammatory cells by modulating their own biosynthesis and NOS/COX enzymes. Indeed, in human fetal microglial cells, very low concentrations of constitutive NO released by cNOS seem to be required to activate COX expression (Janabi et al., 1996). Furthermore, low amounts of NO enhance prostanoid formation, whereas higher concentrations downregulate COX pathway (Swierkosz et al., 1995). Thus, the concentration of endogenous or exogenous NO plays a crucial role in the regulation of COX activity, although the mechanism of this effect is still unclear.

Evidence exists that in cells possessing both cNOS and iNOS, such as astroglial cells as well as in cells expressing only inducible NOS as a consequence of inflammatory stimuli such as microglial cells, NO and NO donors are able to regulate, according to the cell type and to the amount of NO released, the expression of both constitutive and inducible enzymes (for a review, see Colasanti and Persichini, 2000; Colasanti and Suzuki, 2000). In particular, evidence has been provided that NO released under basal conditions by cNOS keeps iNOS under a nonactivated state through the inhibition of the NF-κB signaling, which in turn regulates the iNOS transcriptional mechanisms. In particular, it is known that NF-κB represents one of the most relevant signaling pathways that mediates the expression of iNOS following many extracellular mediators, including endotoxin and inflammatory cytokines (Bonaiuto et al., 1997; Chao et al., 1997; Massa and Wu, 1998; Hartlage-Rubsamen et al., 1999). When low concentration of endogenous as well as exogenous NO is released, NF-κB is kept in an inactive state (Togashi et al., 1997). Many factors may contribute to NO-related inhibition of NF-κB, including the direct effect of NO at the level of the binding of NF-κB to its promoter response element without affecting the activation and translocation of NF-κB (Matthews et al., 1996; DelaTorre et al., 1997). Moreover, NO has been shown to inhibit NF-κB DNA binding through *S*-nitrosylation of the Cys 62 residue of p50 subunit (DelaTorre et al., 1998). However, further data have recently shown that NO interacts with NF-κB via stabilization of its endogenous inhibitor IκB. In particular, evidence has been collected indicating that NO and NO donors stabilize IκB by preventing its degradation from NF-κB and increase the mRNA expression of IκB-a without affecting the mRNA expression of NF-κB subunits p65 or p50 (Peng et al., 1995). Thus, in cells in which iNOS is activated by extracellular inducers of NF-κB, NO may exert an early inhibitory role. When increasing concentrations of NO are released, NF-κB is not suppressed by NO and may contribute to iNOS activation, possibly via a combination with superoxide anions and peroxynitrite formation (Fig. 7). Because NF-κB is also a potent inducer of COX-2 and since the inhibitory role of NO on COX-2 was mediated by the inhibition of NF-κB in mesangial cells (Diaz-Cazorla et al., 1999), it is likely that these mechanisms may underlie some of the inhibitory effects of NO in PG biosynthesis.

A similar relationship seems to occur between PG and NOS and COX expression. Indeed, evidence exists that increasing amounts of PGE_2 inhibits iNOS mRNA expression and nitrite production in LPS-stimulated microglial cells (Minghetti et al., 1997a). In contrast, exogenous addition of PGE_2 in LPS-activated microglial cells induced COX-2 expression (Minghetti et al., 1997b). Both effects were mediated by elevation on cAMP levels, suggesting that activation of adenylate cyclase by stimulation of G-coupled PG receptors led to modulation of both iNOS and COX-2 expression. This has been confirmed by using drugs, such as isoproterenol, which elevates cAMP levels. In contrast, the use of COX inhibitors, such as indomethacin, enhanced PG-induced inhibition on NO production and up-regulated COX-2 expression (see Minghetti and Levi, 1998). This suggests that constitutive release of PG via COX-1 modulates, in an opposite way, NOS and COX inducible enzymes (see Minghetti and Levi, 1998). Thus, basal levels of NO and PGs modulate their own release by affecting constitutive NOS and COX.

C. Drugs Acting Simultaneously on Nitric Oxide and Cyclooxygenase

Based on the reciprocal interactions between NO and COX, many compounds possessing both NO donor and NSAID activity have been synthetized and proposed, in the last few years, in the treatment of many inflammatory and noninflammatory disorders, including cardiovascular diseases, arthritis, and brain injury (Ignarro et al., 2002; Wallace and Del Soldato, 2003). In particular, since the major limitation for the long-term use of NSAIDs is their ability to cause gastrointestinal toxicity and according to the gastroprotective effect of NO released constitutively, the use of such compounds has been suggested to minimize the gastrolesive effect of NSAIDs (Wallace and Cirino, 1994). One of the most relevant problems in making NO donors conjugate with NSAIDs was represented by the possible effect of this combination on the kinetics of NO release. In particular, many efforts have been made looking for a pharmaceutical formulation able to ensure a stable and durable release of NO at a concentration near physiological levels generated by the constitutive enzyme. This combination may be particularly useful since COX inhibitors such as acetylsalicylic acid (ASA) can decrease endogenous NOS activity. A combination of ASA and NO donors may be particularly useful, since each individually is tissue-protective.

Nitroaspirins are nitrate ester compounds which include 2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl

ester (NCX-4016) and 2-acetoxybenzoate 2-(2-nitroxy)-butyl ester (NCX-4215). NCX-4016 represents a stable compound, requires enzymatic hydrolysis to liberate NO, and possesses a kinetic of its metabolic processing which makes NO at a constant rate from the site of metabolism (Wallace and Cirino, 1994; Minuz et al., 1998; Del Soldato et al., 1999). NCX-4016 has been shown to prevent gastric damage in a rat model of shock (Wallace et al., 1997).

On the other hand, nitroaspirin is more efficient than aspirin at inhibiting platelet activation (aggregation and adhesion) induced by thrombin, and it also dose dependently inhibited the thrombin-induced aggregation of platelets pretreated with ASA (Lechi et al., 1996a,b). Since this effect is reversed by oxyhemoglobin and methylene blue, which traps NO and inhibits its activity at the guanylate cyclase level, respectively, it is likely that the release of NO may have the major role in producing these effects. The antithrombotic activity of this nitroaspirin has also been demonstrated in vivo in a rodent model of thrombosis (Wallace et al., 1999). Nitroaspirin also induces dose-dependent relaxation of both intact and endothelium-denuded epinephrine-precontracted arteries (Minuz et al., 1995) and has been shown to possess greater protective activity than aspirin in a model of focal cerebral ischemia (Fredduzzi et al., 2001).

In hypercholesterolemic mice, nitroaspirin has been shown to produce significant protective effects against restenosis occurring after vascular injury in rat arteries. Indeed, NCX-4016 reduced vascular smooth muscle cell (VSMC) proliferation and macrophage infiltration at the site of arterial injury (Napoli et al., 2001). Reduction of VSMC proliferation by NCX-4016 is comparable with the powerful inhibitory action of endogenous NO on rat VSMC proliferation in vitro (Ignarro et al., 2001). Thus, nitroaspirin derivatives may be effective drugs for reducing restenosis, especially in the concomitant presence of hypercholesterolemia or in the setting of an increased risk of gastrointestinal injury or hemorrhage.

Some other NO donors conjugated with NSAIDs have also been investigated. In particular, the combination of *S*-nitroso-NSAIDs has recently been used. In particular, a prototype of the *S*-nitroso ester class of NSAIDs that releases NO, such as *S*-nitroso-diclofenac, has recently been shown to possess unique properties (Bandarage et al., 2000). This agent is orally bioavailable as a prodrug, producing significant levels of diclofenac in plasma within 15 min after oral administration to mice. In addition, *S*-nitroso-diclofenac has equipotent anti-inflammatory and analgesic properties as diclofenac but is gastric-sparing compared with the parent NSAID. Thus, *S*-nitrosothiol esters of diclofenac and other NSAIDs constitute a novel class of NO-donating compounds with uncompromised anti-inflammatory and analgesic properties but a markedly enhanced gastric safety profile. In addition, in line with the combined effect of NO on PG biosynthesis described in the previous paragraphs, it is likely that more selective compounds acting on both pathways may be really useful in the future in the treatment of many disease states.

V. Perspectives and Concluding Remarks

Since the initial observation that NO activated COX (Salvemini et al., 1993a), compelling data has been generated over the last decade supporting the concept that NO is crucially involved in the regulation of COX pathway. NO can thus modulate eicosanoid production by acting at several levels. Thus, in inflammatory conditions where both the iNOS and COX-2 systems are induced, there is a NO-mediated induction of COX-2 leading to increased formation of proinflammatory PG resulting in an exacerbated inflammatory response. Under these circumstances, dual inhibition of NO and PGs account for the anti-inflammatory effects of NO synthase inhibitors. On the other hand, the inhibitory effect of some NO donors on COX-2 expression suggest that the inhibition of NO formation must be graded according to the time of onset of the disease and to the cellular substrate we are working on.

Data are also emerging to suggest that peroxynitrite, the product of the reaction between NO and superoxide, is accounted to play an important role in regulating the COX enzymes. Since peroxynitrite is known to be a potent inducer of NF-κB, the transcription factor which is generated by NO and superoxide anions released in inflammatory states, it is likely that NO may contribute to the activation of both iNOS and COX-2 by using this way. Under these conditions, NOS inhibitors and peroxynitrite decomposition catalysts may have a beneficial effect on COX-2 modulation.

Thus, COX enzymes are clearly important "receptor" targets for the action of NO and other reactive oxygen species such as peroxynitrite. Once activated or induced, these represent important transduction mechanisms for their multifaceted actions. The challenge in the future will be to understand the molecular mechanisms used by such species in modifying key steps of the COX pathway because this will undoubtedly elucidate important molecular targets for future pharmacological intervention.

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