The role of nitric oxide in prostaglandin biology; update

Sangwon F. Kim

Department of Psychiatry, Center for Neurobiology and Behavior, University of Pennsylvania School of Medicine, 125 S 31st St. TRL Rm 2207, Philadelphia, PA 19104

Abstract

The biosynthesis of nitric oxide (NO) and prostaglandin share many similarities. Two major forms of nitric oxide synthase (NOS) and cyclooxygenase (COX) have been identified: constitutive vs inducible. In general, the constitutive form functions in housekeeping and physiologic roles whereas the inducible form is up-regulated by mitogenic or inflammatory stimuli and is responsible for pathophysiological responses. The cross talk between the COX and NOS pathways was initially reported 1993 and since then, numerous studies have been undertaken to delineate the functional consequences of this interaction as well as the potential mechanism by which each pathway interacts. This review will focus in particular on recent advances in this field that extend our understanding of these two pathways under various systems.

Keywords

nitric oxide; nitric oxide synthase; cyclooxygenase; prostaglandin; s-nitrosylation; peroxynitrite

Generation of Nitric Oxide and its action

Redox Species of NO

NO is a highly reactive diatomic molecule that is produced within mammalian cells mostly by the enzyme NO synthase [1], which catalyzes the stepwise conversion of L-arginine to L-citrulline, releasing NO as a byproduct [2; 3]. In biological systems, NO exists as three redox-related species, and NO is used as a generic term to encompass all these species. Redox-related species of NO include $NO^{\bullet}$, which can modulate iron (Fe)-containing proteins by direct coordination to Fe centers (see below) and can have a cytotoxic effect by reacting with superoxide anion ($O_2^{\bullet-}$) to produce peroxynitrite ($ONOO^-$). Further oxidation of $NO^{\bullet}$ species can generate nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), nitrite (NO_2^-), nitrate (NO_3^-) and others [4]. The second important species of NO, the nitrosonium ion (NO^+), can nitrosylate thiol groups of proteins, a modification that may have important regulatory functions [5]. Nitrosonium ion has an extremely short half-life ($\approx 10^{-10}$ s) in solution at physiological pH [6], and binds extremely rapidly to thiol groups. Following such binding, the resulting – SNO compounds maintains a "nitrosonium" characteristic and can transfer NO^+ to other thiols, in a process called trans-nitrosylation [7–9]. The final redox-related species of NO is the nitronyl (NO^-) anion, which

To whom correspondence should be addressed: Sangwon F. Kim (sangwonk@mail.med.upenn.edu).

was reported to have a similar reactivity to NO^+ [10], leading to S-nitrosylation, however, the physiological relevance of this species is unclear.

Functions of NO

NO has two principal yet divergent functions in cells: homeostasis and cytotoxicity. For regulatory functions, NO is produced in small amounts under physiological conditions and mediates vasorelaxation, controls the adhesion and aggregation of platelets and neutrophils, and is involved in neurotransmission [1; 11]. Most of these actions are mediated through the binding of NO to Fe in the heme prosthetic group of soluble guanylate cyclase which catalyzes the conversion of GTP to cyclic GMP [12]. The importance of Fe in mediating the functions of NO is also apparent when examining the cytotoxic effects of the molecule, which are observed when NO produced in much larger quantities by macrophages, hepatocytes and other cells following their exposure to cytokines or microbial products. NO produced *via* such high-output systems inhibits proliferation of intracellular pathogens and tumor cells. These effects can be explained by the reactivity of $NO^\bullet$ with Fe in the [Fe-S] centers of several important macromolecules, including aconitase, and Complex I and Complex II of the electron transport chain [13; 14]. The high affinity of $NO^\bullet$ for iron probably results in both the removal of Fe from [Fe-S] centers and the formation of nitrosyl Fe species within [Fe-S] proteins. Thus $NO^\bullet$ exerts its function through its interaction with the Fe of heme and non-heme Fe proteins.

S-nitrosylation

The reactivity of NO with iron containing moieties, such as heme or Fe-S clusters, adequately explains its immediate bioactivity but does not provide an explanation about the effects on cellular signaling pathways after post-translational modification of proteins by NO. In 1993, it was demonstrated that NO^+, can interact with thiol groups of proteins to form S-nitrosothiols [7]. Importantly, S-nitrosylation of cysteine residues in proteins by the addition of NO^+ results in the modification of their activities. S-nitrosylation can be achieved in a test tube by a reaction between the acidified nitrite and thiol groups (equations 1 and 2, in which RSH represents a thiol and RSNO an *S*-nitrosylated product).

$$HNO_2 + H^+ \rightarrow NO^+ + H_2O \tag{1}$$

$$RSH + NO^+ \rightarrow RSNO + H^+ \tag{2}$$

It is highly unlikely that NO^+ production can occur by this mechanism in biological systems because of the requirement for low pH. It is likely that biological production of NO^+ requires iron-catalyzed oxidation of $NO^\bullet$ which is generated by NOS [15; 16].

$$NO^\bullet + Fe^{3+} \rightarrow Fe^{2+}NO \tag{3}$$

$$Fe^{2+}NO + RSH \rightarrow Fe^{2+} + RSNO + H^+ \tag{4}$$

Support for this argument has been found in the detection of iron-nitrosyl complexes and an increase of *S*-nitrosothiols in LPS/IFN-γ treated macrophages, which produce a large quantity of NO [17].

As described above, simple *S*-nitrosylation is a result of a non-enzymatically catalyzed chemical reaction. Nevertheless, there appears to be some specificity in this reaction. First, not all proteins containing free cysteines are *S*-nitrosylated. Moreover, not every available cysteine in a given target is *S*-nitrosylated. For example, the ryanodine receptor contains 87 cysteines but only 13 of them are *S*-nitrosylated [18], suggesting a certain specificity of *S*-nitrosylation. Furthermore, it has been postulated that there is a primary consensus sequence for *S*-nitrosylation requiring the presence of acidic or basic residues adjacent to a target cysteines [19]. However, this acid-base consensus motif can also arise as a result of three-dimensional protein structure, as shown by analysis of the crystal structure of caspase-3 whose active cysteine is *S*-nitrosylated by NO *in vivo* [20]. Hence, the conditions for *S*-nitrosylation appear to be far more complex than what may be suggested by a primary sequence or availability of a target cysteine.

More than a hundred proteins have been identified as targets of *S*-nitrosylation which is associated with modification of their functions or structures [5; 8]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is among the first proteins shown to be *S*-nitrosylated. It has been reported that NO production is correlated with inhibition of GAPDH in rat liver during chronic inflammation [21]; the *S*-nitrosylation of a cysteine residue in the active site of GAPDH causes reversible inactivation of the enzyme [22] as well as its translocation to nucleus leading to cell death [23]. Moreover, nitrosylated GAPDH functions as a carrier of NO and transnitrosylate nuclear proteins such as sirtuin-1 (SIRT1), histone deacetylase-2 (HDAC2) and DNA-activated protein kinase (DNA-PK) [9].

S-nitrosylation of hemoglobin was extensively studied and Cys93 has been shown to be a site for this modification. The binding of NO to hemoglobin is dependent on the allosteric state of hemoglobin [24]. When oxygenated, hemoglobin is *S*-nitrosylated and the NO group is released during arterial-venous transit [25]. This report concluded that NO released from hemoglobin plays an essential but previously unexpected role in the respiratory cycle by ensuring adequate blood flow to tissues.

S-nitrosylation of other proteins such as the N-methyl-D-aspartate (NMDA) receptor, p21[ras], caspase-3, AMPA receptor and RASD1 affects their regulation or localization [26–29] and the list of S-nitrosylated proteins continues to grow [5]. Interestingly, p21[ras] can be *S*-nitrosylated and activated by NO [26], with the concentration of NO required to obtain maximal activation of this protein *in vitro* being 1000 fold higher than that required for activation using intact cells, suggesting that the intracellular environment is more favorable for nitrosothiol formation [30].

Peroxynitrite

Superoxide is generated by various sources under physiological conditions and its production is tightly regulated by superoxide dismutases (SODs) [31]. However, during acute and chronic inflammation, superoxide production exceeds the capacity of endogenous SODs to remove it [32; 33]. Under the same conditions, NO production is also enhanced. Excess superoxide scavenges NO, generating peroxynitrite, thus destroying the biological activity of NO [34]. Peroxynitrite is highly reactive and exerts its action *via* various mechanisms, however, it is the interaction with tyrosine residues in proteins forming nitrotyrosine that has received the most attention. So far, numerous proteins have been identified as targets for nitrotyrosine modification but the specificity is not well understood. Nevertheless, nitrotyrosine formation within proteins leads to either gain or loss of function [35–37]. MnSOD is a good example of an enzyme whose activity was lost after tyrosine nitration *in vivo* [31; 38]. The functional consequence of nitrotyrosine formation of MnSOD is quite severe as loss of activity enhances the production of superoxide, leading to an increase in peroxynitriate, followed by additional modifications on other proteins such as

aconitase, ATPase, and cytochrome c, further disrupting mitochondria respiratory function and consequently resulting in apoptosis [36; 39; 40]. This nitrotyrosine modification has been detected in various pathophysiological conditions such as ischemia and reperfusion injury, Alzheimer's disease, and organ transplant [41–43]. Thus, peroxynitrite functions as a potent cytotoxic and anti-inflammatory molecule [37; 42; 44]. Nitrotyrosine formation is similar to S-nitrosyaltion in that the NO moiety modifies a specific residue in the target proteins but many differences also exist [5; 37]. Unlike the labile S-nitrosylation modification, once a tyrosine residue is modified by peroxynitrite, it appears to be long-lasting. One way to remove the nitrotyrosine is by protein degradation [37; 42; 44]. A yet unidentified protein, termed "denitrase", appears to be able to remove the nitro group without protein degradation and has been observed in tissues [45].

Cycolooxygenases and the role of prostaglandin in biology

COX1 and 2

Cyclooxygenase (COX) was first purified in 1976 from sheep seminal vesicles a homodimer of molecular mass of 71kDa containing both cyclooxygenase and hydroperoxidase activities [46; 47]. Subsequently, several laboratories speculated an existence of COX isoenzymes as certain stimuli could induce the production of prostaglandin [48; 49]. Later Needleman's group reported production of prostaglandin generated from *de novo* synthesis of new COX protein in mouse peritoneal macrophage *in vivo* [50]. The following year, cyclooxygenase was shown indeed to have two isoforms initially called prostaglandin H synthase-1 and -2 and now commonly referred to as COX1 and COX2 [51; 52]. These are encoded by two different genes; the human COX2 gene of 8.3kb is a small immediate early gene on chromosome 1 while COX1 is a much longer 22kb gene on chromosome 9. Human COX1 and COX2 are homodimers sharing 60% identity in sequence [53; 54]. Both isoforms are composed of 3 domains; the epidermal growth factor domain, the membrane bound domain and the catalytic domain containing cyclooxygenase and peroxygenase active sites on the either side of heme prosthetic group which is important for both activities. Peroxidase active site, on the opposite side of membrane binding domain, consists of heme prosthetic group [54; 55]. Interestingly, sequences of sidechain between the isoforms near peroxidase site are less conserved than any other catalytic domain area and yet hemes from both isoforms have comparable affinities for cyanide as the sixth axial ligand [56]. The cyclooxygenase active site is located on the top of a long hydrophobic channel originating from the membrane binding domain near the heme. The binding site for fatty acids as well as competitive COX inhibitors resides above this channel, and is the most strongly conserved region in COX isoforms [54; 57]. When arachidonic acid binds to the cyclooxygenase site, it sits at the end of channel and carbon-13 of arachidonic acid is placed near Tyr-385, which is a crucial amino acid for cyclooxygenase reaction. However, mutation of the Tyr-385 does not impact the midpoint potential of heme in COX2 further suggesting that peroxidase site is independent of cyclooxygenase site. Moreover, crystallographic as well as biochemical data showed that two active sites are physically separated as fatty acids do not interfere peroxidase catalysis [57].

The conversion of arachidonic acid to the hydroperoxyendoperxoide, PGG2 is initiated by abstraction of the pro-S hydrogen atom from carbon-13 and then dioxygen traps the pentadienyl radical at carbon 11. This reaction is followed by a series of radical reaction which is similar to polyunsaturated fatty acid autoxidation producing prostaglandinG2 (PGG2). The enzyme activation, which is dependent of peroxidase activity, is required for this cyclooxygenase reaction. In this process, an electron is transferred from heme to Tyr-385 generating tyrosl radical in the cylooxygenase active site and initiating a reaction. Moreover, tyrosl radical is regenerated by reducing perxyl radical to the hydroperoxide to from PGG2. After cyclooxygenase reaction, peroxidasereduces the 15-hyroperoxy of pGG2

to the corresponding alcohol of PGH2 (Fig. 1) [54; 57–59]. Even though the two isoforms appear to follow the same basic mechanism and their 3 dimensional structures can be almost superimposed [54], there is a minor difference within the substrate binding site in catalytic domain which explains the specificity of non-steroid anti-inflammatory drugs (NSAIDs) [55].

Despite the structural similarity of the COX isoforms, their expression patterns and localization are very distinct. COX1 is widely distributed and constitutively expressed in most tissues while COX2 is activated by various inflammatory and proliferative stimuli [54]. Due to this obvious difference in expression patterns, it had been speculated that COX1 produces prostaglandins which are required for homeostatic functions such as gastric protection and hemostasis whereas COX2 participates in pathophysiological process including inflammation and tumorigenesis. This idea drove pharmaceutical companies to invest massive resources to develop COX2 specific inhibitors as anti-inflammatory drugs to avoid common side effects of traditional NSAIDs such as gastrointestinal bleeding [60; 61]. However, continued research has revealed that this path may not be ideal. Firstly, deletion of COX1 in mice did not show an increased susceptibility of gastric ulcer [62], while COX2 inhibitors have been shown to attenuate the healing process [63]. Secondly, COX1 is also expressed in inflammatory cells [64] and COX2 is constitutively expressed in certain tissues including brain, and kidney [62; 65–67]. Interestingly, it has been showed that COX1 knockout rather than COX2 deletion reduced the LPS-mediated inflammatory response in the brain [68]. Moreover, COX2 knockout mice showed unexpected results: disruption of postnatal kidney development, infertility in female mice due to failure to ovulate and embryo implantation [66; 67; 69; 70]. Hence, to ascribe such a dichotomy in function between the COX isoforms may be considered too general, though, there is clearly some differential functions between them. An elegant study performed by Funk's group showed that replacement of COX2 with COX1 cannot compensate for a loss of function in COX2 [71] strongly suggesting that there are specific roles assigned to each isoform.

Function of prostaglandin signaling in biology

Virtually all cells except for red blood cells can produce prostaglandin from arachindonic acid which is prevalent at high concentrations in phospholipids of the cell membrane. Prostaglandins are members of a lipid family which are enzymatically derived from polyunsaturated fatty acids with 20-carbon chains and 4 cis-double bonds (20:4) *via* the cyclooxygenase pathway. Prostaglandins are also known as "prostanoids," which includes classical prostaglandin as well as specialized species such as thromboxane and prostacyclin [72; 73].

The first report of the existence of prostaglandins was made in 1930 showing that fresh semen led to rhythmic contraction of human myometrium *in vitro* [74]. A few years later, a couple of groups independently confirmed that presence of an active substance responsible for muscle contraction and named it "prostaglandin," as an homage to the origin being the prostate gland [73; 75; 76]. Continued research has revealed that prostaglandins are a mixture of biologically active substances such as prostaglandin E or prostaglandin F, but that ironically, the actual origin of prostaglandins is the seminal vesicle and not the prostate gland [77]. One of the most important discoveries in the field was when Vane showed that anti-inflammatory activity of aspirin and other NSAIDs was due to an inhibition of cyclooxygenase [78], proposing that major pharmacological effects of NSAIDs were attributable to an inhibition of this enzyme.

Nervous System

The presence of prostaglandin in the brain was first reported in 1964 [79]. It has been postulated that prostaglandin production from COX2 plays a prominent role in fever, pain or any pathological conditions [80]. Moreover, COX2 is up-regulated in the brains of patients with Alzheimer's disease (AD) suggesting abnormal modulation of the COX pathway [81]. Several clinical trials were performed to evaluate the effects of NSAIDs or COX2 specific inhibitors but the outcomes have been inconclusive [82; 83].

Dramatic increases in COX2 mRNA and protein levels have been observed under excitotoxic conditions, defined as a process by which high levels of glutamate or its analogs excite neurons and induce neuronal cell death [84]. Glutamate enhances calcium influx, which can initiate multiple cascades of events including an increase in NO production by neuronal NOS (nNOS) and activation of enzymes involved in generation of arachidonic acids and its metabolism, such as phospholipase A2, COX2 and lipoxygenase [85; 86]. Therefore, it is not surprising that glutamate-mediated excitotoxic conditions enhance prostaglandin production. Indeed, our laboratory has shown that N-methyl D-aspartate (NMDA)-mediated activation of COX2 requires nNOS and plays a crucial role in NMDA-excitotoxicity which can be attenuated by COX2 specific inhibitors [87].

Ischemic insults also influence synthesis or relocation of prostaglandins and other arachidonic acid metabolites [88]. Interestingly, both COX1 and COX2 are increased in brain ischemia. Furthermore, either selective inhibition of COX1 or COX2 significantly reduces neuronal cell death, suggesting that both COX isoforms participate in the progression of neuronal damage following global cerebral ischemia and identifies a potential therapeutic use of COX inhibitors in cerebral ischemia [89].

One interesting aspect of neuronal COX2 is that unlike in other tissues, COX2 is constitutively expressed [65], thus, we can speculate that COX2 may play a role in not only pathological progression as described above but also in physiological neuronal function such as synaptic plasticity. Worley's group has shown that the basal expression of COX2 is regulated by NMDA receptor-dependent synaptic activity and especially up-regulated by long term potentiation (LTP) suggesting that the expression and activity of COX2 may be associated with long-term synaptic plasticity [65; 90]. This idea was further confirmed by showing that COX2 specific inhibitors or suppression of COX2 gene reduced high-frequency stimulation (HFS) induced LTP implying that constitutively expressed COX2 plays a crucial role in synaptic plasticity [65; 84]. However, hippocampal slices prepared from COX2-deleted mice did not show any significant difference on HFS-induced LTP [91], implying that COX1 may be able to compensate for the loss of COX2 and its own role in synaptic plasticity [92].

Cardiovascular

Prostaglandin E series are generally potent vasodilators of the vascular bed [93]. They usually affect small vessels including arterioles and pre/postcapillary venules [94]. Thromboxane and prostacyclin are more specialized prostaglandins that exert major impacts on the cardiovascular system [94; 95]. Thromboxane is normally produced by platelets, acting as a very potent vasoconstrictor, and plays an important role on platelet aggregation and hemostasis. Its effect is counterbalanced by prostacyclin, which is a powerful vasodilator and inhibitor of platelet aggregation. Prostacyclin is the main product in the vascular system [96] and is produced in endothelial cells [97; 98]. As prostaglandin production in the endothelial cell is dependent on COX2 induction [99], the usage of COX2 specific inhibitors under chronic inflammatory conditions has raised concerns [100; 101],

leading to some COX2 specific inhibitors being withdrawn from the market due to an increased cardiovascular risk.

Cancer

The potential link between prostaglandin and tumorigenesis was initially proposed several decades ago [102; 103]. Further studies have shown COX2 elevation particularly in colon cancers, while epidemiological studies have indicated that NSAIDs indeed reduce the incidence of colon cancer [104; 105]. The effects of prostaglandins on tumors is not limited to colon cancer and extends to cancers in other tissues such as breast [106], pancreas [107], esophagus [108], lung [109] and gastric [110]. Hence, COX2 expression is dysregulated in many types of cancer and COX2-derived prostaglandin E2 (PGE2) has been shown to elicit multiple oncogenic signals to promote carcinogenesis [111; 112]. Significant research efforts are currently being undertaken to identify more selective targets and elucidate the molecular pathway of prostaglandin-mediated oncogenesis. As the rate-limiting enzyme for this pathway, COX2 is garnering considerable attention in hopes that COX2 specific inhibitors may be used as anti-cancer drugs or for cancer prevention, however, further studies are necessary.

Metabolism

While prostaglandins are produced in virtually every system in the human body and play a role in cellular homeostasis, recent studies have uncovered an unexpected role for COX2-produced prostaglandins. Kristiensan's group first reported that uncoupling protein 1 (UCP1), a hallmark protein in brown adipocytes crucial for cold- or diet-induced thermogenesis, is regulated by COX2 [113]. Moreover, Herzig's group demonstrated that overexpression of COX2 in white adipose tissue, where excess fat is stored, can convert these cells to become brown adipose tissue, which burns fat to produce heat [114]. This brown fat is usually prominent in infants who require more energy to maintain their body temperature and continuously regress with age. A considerable amount of this brown fat still remains in adulthood and is correlated to leanness [115; 116]. Hence, increasing brown fat content in humans has become an attractive target for anti-obesity drugs and manipulation of COX2 activity may be a new therapeutic strategy, though a strong note of caution is warranted, as the triggering of the inflammatory response is a potential side-effect of enhanced prostaglandin production.

Crosstalk between the NO and Prostaglandin pathways

NO and prostaglandin pathways share numerous similarities and the two molecules can be produced simultaneously in the same tissues, as described in a number of models of inflammation such as endotoxin induced septic shock, carrageenan-induced pouch or paw inflammation (Fig. 2) [117]. Hence numerous studies have explored the potential crosstalk between NO and prostaglandin pathways and increasing evidence supports this idea. However, the detailed mechanisms by which NO regulates prostaglandin production or vice versa is still controversial, partly because the interaction between these two pathways occurs at multiple levels along with the complexity of NO redox chemistry [117; 118]. An initial report detailing the cross talk between NO and prostaglandin was made by Needleman's group showing that NO can activate cyclooxygenase [119]. In this study, NOS and COX2 activity were induced in a macrophage cell line, RAW264.7, treated with LPS to produce both NO and prostaglandin. The production of both was attenuated by NOS inhibitors. As the NOS inhibitors used in this study do not have any NSAID characteristics, the inhibition of prostaglandin production was likely due to the decrease in NO production and not as a direct effect on COX2 [119]. This study was further corroborated by *in vitro* studies showing that even exogenous NO gas or chemical donors can induce COX1 activity [120;

121]. Moreover, NO-mediated activation of COX does not seem to be limited to its enzyme activity. Lie *et al* showed that NO can increase COX2 mRNA levels via the β-catenin/TCF pathway leading to activation of the polyoma enhancer activator 3 (PEA3) transcription factor [122]. Furthermore, NO also interacts with various other pathways which can influence COX expression such as the cAMP/PKA/CRE and JNK/Jun/ATF2 signaling cascades [122; 123].

In addition to this body of evidence showing an up-regulation of COX activity by NO, a large number of reports supporting the idea that NO can inactivate COX under certain conditions also exists. Levi's group showed that LPS-induced COX2 expression and production of prostaglandin in microglia cells are augmented by NOS inhibitors [124]. Indeed, similar findings were reported in various other cells types including a J774 macrophage cell line and the rat Kupper cells [125; 126]. Interestingly, the interaction between NO and prostaglandin is not uni-directional, as it has been reported that NSAIDs such as aspirin and indomethacin can significantly reduced NOS activity [127].

The mechanism by which the NOS and COX pathways interact is complicated by conflicting reports in the field. For instance, while most of the actions by NO are mediated by its interaction with Fe in heme prosthetic group of soluble guanylate cyclase (sGC), Salvemini *et al* has shown that inhibition of sGC with the sGC-specific inhibitor, methylene blue, does not attenuate NO-mediated activation of prostaglandin biosynthesis [119] and has proposed that NO may directly activate COX enzyme. This theory is further supported by numerous reports demonstrating that exogenous NO donors can activate recombinant COX. However, Tsai *et al* reported that it is unlikely that NO directly binds to heme group in COX to activate under anaerobic condition [128]. One reason for this disagreement may be due to the complexity of NO chemistry with the result that various NO species are generated, as described earlier.

Currently, there are several proposed mechanisms by which NO mediates prostaglandin synthesis (Fig. 3). One is that NO and superoxide can be produced simultaneously and can react together to form peroxynitrite, which can feedback to modify COX. Another possible mechanism is the direct S-nitrosylation of COX enzymes. These two post-translational modifications can alter the function of these enzymes and are further discussed below.

Peroxynitrite-mediated regulation of prostaglandin synthesis

Superoxide and nitric oxide are readily produced in the cells under both physiologic and pathological conditions [37]. Upon production these two compounds, peroxynitrite can be easily formed. Many laboratories have reported that peroxynitrite can interact with COX enzymes but the consequence of the interaction differs depending on where this interaction occurs. So far, two peroxynitrite binding sites in COX enzymes have been identified: within the endogenous heme moiety and at tyrosine residues on the protein.

Peroxynitrite has been shown to activate both COX1 and COX2 by several groups. In particular, treatment of cells with SIN-1, an exogenous peroxynitrite generator, enhances prostaglandin synthesis and this activation is inhibited by oxygen radical scavengers, strongly implicating peroxynitrite as an activator of COX [129]. The mechanism by which peroxynitrite activates COX activity is still unclear, however, it has been speculated that peroxynitrite may be interacting with Fe in the heme group of COX and forms a radical intermediate product which can accelerate the enzyme reaction [130–132]. This hypothesis is supported by the fact that peroxynitrite is known to interact with the heme group of certain proteins such as myeloperoxidase and horseradish peroxidase to generate an intermediate product [133].

Most of the studies about peroxynitrite and COX enzymes have been focused on tyrosine residue modification in the enzyme and the formation of a nitrotyrosine residue. It appears that this modification, especially at Tyr385, leads to an inactivation of enzyme [134–137], as demonstrated by inhibition of COX1 enzyme activity when either purified enzyme COX1 or smooth muscle cells are treated with NO donors and arachidonic acid simultaneously [138]. It is interesting to note that pure NO donors such as NOC-7 does not lead to formation of nitrotyrosine in the absence of arachidonic acid, while peroxynitrite, SIN-1 or tetranitromethane (TMN) can readily carry out this reaction.

It appears that peroxynitrite has dual actions on COX depending on where it interacts in the enzyme. Even though nitrotyrosine modification of COXs is detected in many pathological conditions and clearly inactivates its enzyme activity *in vitro,* the exact mechanism of nitration remains elusive. The levels of peroxynitrite markedly increase under pathological conditions, as inflammation induces NO production from iNOS and superoxide production from by NAD(P)H and xanthine oxidase and correlates to formation of nitrotyrosine in COXs. However, further investigation is required to identify different conditions that determine the target selectivity of peroxynitrite: to choose between interacting with tyrosine to inactivate an enzyme versus interaction with heme in COXs to activate it. Interestingly, Deeb *et al* showed that holoCOX1 (heme-containing) can be nitrated at Tyr 385 by peroxynitrite, even in the absence of arachidonic acid, while apoCOX1 (heme-deficient) is modified at the different site, thus indicating a heme requirement for peroxynitrite-mediated Tyr385 nitration [139]. The identity of the peroxynitrated tyrosine residue in apoCOX1 and its downstream biological function are yet to be determined.

It has been proposed that peroxynitrite may interact with Fe in the heme moiety of COX to generate a short-lived intermediate which can facilitate the formation of nitrotyrosine [140]. While heme is essential for this reaction within COX, the role of this co-factor is unclear, as there are other proteins which can be nitrated without heme moiety in the protein [141]. It is possible that Fe binding in the heme molecule in COX may expose the Tyr385 residue, which resides just below the heme moiety, making it more readily accessible to peroxynitrite. It will be interesting to examine whether recombinant COX with non-Fe protoporphyrin such as Zn-protoporphyrin or Sn-protoporphyrin can be nitrated by peroxynitrite. This theory, however, conflicts in part with the aforementioned evidence that peroxynitrite binding to the heme moiety in COX leads to activation of the enzyme while nitration of Tyr385 leads to enzyme inactivation. This may be partially explained by differing concentrations of arachidonic acid, which can be oxidized by peroxynitrite, with this oxidized substrate having different impacts on COX activities. Deeb *et al* has indeed shown that higher arachidonic acid reduces formation nitrotyrosine in COX1 [142].

Most importantly, this peroxynitration modification of COX enzymes has been detected in many pathological or chronic inflammatory conditions such as atherosclerosis, Parkinson's disease or Alzheimer's disease. However, it is not clear whether an inactivation of this enzyme by nitrotyrosine formation contributes to the pathology of these diseases or simply an indication of nitrossative stress.

Finally, COXs are not the only target which can be modified by Peroxynitrite. Prostacyclin synthase (PGCI) is selectively inhibited by peroxynitrite at low concentration (IC50=50 nM) while thromboxane A2 (TxA2)-synthase is activated [143; 144]. In particular, PGCI contains one Cys at position 469 which is involved in heme binding and spectrophotometry study showed that this thiolate ligand at the fifth coordination position of the heme iron is not affected by peroxynitrite. It appears that nitration of tyrosine residue 430 in PGCI interferes the metal center of the active site and inhibits its activity [144; 145].

S-nitrosylation of COX

The recent development of a simple method to detect S-nitrosylation flourished identification of new targets and its functional consequence since this modification was first reported with albumin by Stamler *et al.* COX has also been identified as a target of this modification [5; 6; 146]. It was first proposed by Hajjar *et al* showing that S-nitrosothiol may be responsible for NO-mediated activation of COX as it occurs in an heme-independent manner [147]. On the other hand, Marnett's group investigated the role of free cysteines in COX1 and showed that Cys313 and Cys540 mutation into serine alone inhibited its enzyme activity implying that NO-mediated Cys modification may interfere with COX1 activity [148]. Interestingly, Marnett's and Hajjar's groups independently reported that SNAP, an NO chemical donor, cannot activate COX1 [138; 149], thus confusion exists over whether S-nitrosylation of COX1 leads to its activation.

Our group has shown that iNOS can specifically bind to COX2, leading to direct S-nitrosylation of cysteine residues and activate COX2 [150]. This interaction and modification is specific for COX2 as iNOS does not interact with COX1. In particular, we observed more than one S-nitrosylated cysteine residues in LPS/IFN-γ activated RAW264.7 cells and individually mutated each cysteine to serine. We have identified Cys523, located near the catalytic domain, as the only cysteine which does not affect base level enzyme activity and when mutated, NO can no longer activate COX2. This cysteine is located in near the catalytic site of COX2 and we speculated that this modification leads to a slight conformational change in COX2 to affect its enzyme kinetics. Indeed, we performed viscosity studies examining enzyme activity in various concentrations of sucrose and showed that release of product is accelerated in the presence of NO donor. However, it is not possible to exclude a potential involvement of any other cysteines for NO-mediated activation of COX2 as mutation of other cysteines alone affected the basal level enzyme activity of COX2. Given that NO has a high affinity for heme, it is unclear how NO can specifically S-nitrosylate cysteines of COX2 rather than interacting with heme moiety. This may be partially explained by the fact that S-nitrosylation of COX2 by iNOS requires physical interaction between the two proteins, specifically between the subunit binding site of iNOS and catalytic domain of COX2. Hence, we speculated that NO generated from iNOS is simply in direct proximity of the S-nitrosylation target residue in COX2 for immediate delivery and modification. Indeed, disruption of iNOS/COX2 binding diminishes S-nitrosylation and activation of COX2 in LPS-induced RAW264.7 cells. Interestingly, Goodwin *et al* demonstrated that removal of superoxide in LPS-activated RAW264.7 cells inhibited prostaglandin formation to the same extent as NOS inhibitors [137]. While the immediate speculation will be peroxynitrite-mediated nitrotyrosine formation of COX2, Cross' group showed that peroxynitrite can also generate S-nitrosothiol [151] and therefore, further studies are necessary to identify the form of NO-mediated modification of COX2 in activated macrophages.

iNOS-mediated regulation of prostaglandin production is not limited to COX enzymes. Xu *et al* showed that iNOS activates cytolosic phospholipase A2a (cPLA2α) by S-nitrosylation [152]. In this case, cPLA2α does not directly interact with iNOS, but rather forms ternary complex with COX2 and iNOS, and hence induction of COX2 is a crucial step for S-nitroyslation and activation of cPLA2α [152].

S-nitrosylation of COX2 does not seem to be limited to macrophages. Unlike other tissues, COX2 in the brain is constitutively expressed. We showed that COX2 is S-nitrosylated in neuronal cells by nNOS [87]. To our surprise, nNOS binding is mediated by its PDZ domain, differing from the domain near the catalytic region that mediates iNOS binding to COX-2. We also showed that a physical interaction between nNOS and COX2 is required

for S-nitrosylation of COX2 and that disruption of binding attenuated NMDA-mediated excitotoxicity, as did a selective COX2 inhibitor.

Perspective/Conclusion

The prostaglandin pathway has been the center of attention as a main target to treat numerous pathological conditions such as pain, neuroexcitotoxicity, ischemia, neurodegeneration, cancer, and metabolic deficiencies. There is no doubt that NO signaling is tightly coupled to prostaglandin pathway and new evidence also supports that NO-conjugated COX inhibitors (e.g. nitroaspirin) may have a great potential for the treatment of both inflammatory as well as non-inflammatory conditions. Yet we do not have clear understanding of the molecular mechanism by which the pathways interact. Efforts from many outstanding laboratories have proposed multiple potential mechanisms but some of them are quite contradictory. It may be partially due to a lack in sophisticated enough tools to examine the exact identity of NO, both spatially and temporally *in vivo,* as it appears that the total amount and ratio of NO and superoxide produced in the cells may play a key role in explaining the discrepancies among many reports. Moreover, there is a considerable difference depending on which cell type or tissue is examined and the surrounding microenvironment may also be a crucial factor. Undoubtedly, further studies are necessary to understand the molecular basis of interaction between NO and prostaglandin and that these will unveil a crucial target for intervention in numerous pathological conditions.

Acknowledgments

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Research highlight

- Nitric oxide regulates prostaglandin synthesis.

- Nitric oxide activates cyclooxygenase2 activity via S-nitrosylation.

- Peroxinitrite inactivates cyclooxygenase1/2 via Tyrosine nitration.

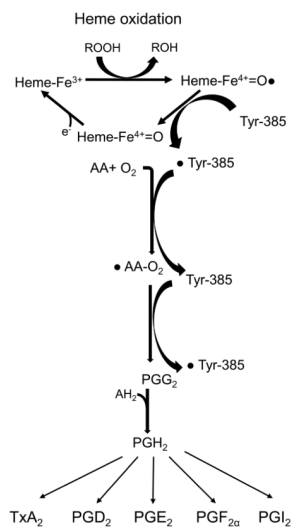- Interaction of Peroxinitrite and heme in cyclooxygenase can activate their activity.

Figure 1. The scheme of COXs enzyme reaction mechanism
Cox enzymes produce PGH2 by two step chemical reactions; cyclooxygenase and
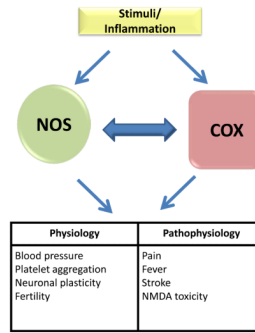peroxidase. PGH2 is further converted to other prostanoid by tissue specific enzymes.

Figure 2. The role of Nitric oxide Synthase (NOS) and Cyclooxygenase
Both enzymes share the similar pathway for activation and their physiological as well as pathophysiological implication.

Figure 3. Proposed mechanisms of NO-mediated regulation of prostaglandin production
Nitric oxide produced by NOS can directly modify cysteine(s) in the COXs and activate it. Moreover, superoxide can be simultaneously generated under the same environment which can enhance NO production. Superoxide rapidly interacts with NO producing peroxynitrite which can interact with either heme or tyrosine residue(s) in COX2.