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Forum Review

Regulation of Cyclooxygenase-2 Expression by Nitric Oxide in Cells

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ABSTRACT

Two different cyclooxygenases (COXs) are functional in mammals: COX-1 and COX-2. COX-2 is mainly an inducible isoform that shares significant features with inducible nitric oxide synthase (iNOS) in terms of its tissue distribution and participation in pathophysiological phenomena. Furthermore, the product of iNOS catalysis, nitric oxide (NO), is an important regulator of COX-2 activity and expression, and the products of COX-1 and COX-2 (diverse prostanoids) may also influence iNOS expression. Both positive and negative effects of NO on COX-2 expression have been encountered in experimental systems, showing that the outcome of the NO–COX-2 interaction is exquisitely dependent upon the temporal frame and the cell type studied. The pathophysiological significance of NO–COX cross-talk also arises from *in vivo* studies, in which most evidence points to a positive effect of NO on COX-2 activity and/or expression. This emphasizes the need to understand the underlying mechanisms. Among these, the capacity of NO and its effector cyclic GMP to modulate the function of several target proteins, including transcription factors such as nuclear factor-κB and activator protein-1, appears as the key pathway by which NO may regulate COX-2 expression. Given the capacity of some prostanoids to modulate the inflammatory response, the interplay between NO synthase and COX pathways stands at the center of the pathophysiological basis of inflammatory diseases. Antioxid. Redox Signal. 3, 231–248.

INTRODUCTION

Cyclooxygenases (COXs): a tale of two isoforms

SINCE THE DISCOVERY OF A SECOND COX ISOFORM, COX-2, more than a decade ago (134), an important body of information has emerged concerning its biological significance and pathophysiological role in several contexts. Whereas COX-1 is assumed to be constitutively expressed and was considered the classical target of aspirin, COX-2 is inducible in many tissues, and the desire to obtain specific inhibitors for it has fostered one of the most important efforts developed by pharmaceutical companies ever known. Both COXs share in common their capacity to transform arachidonic acid into the cyclic endoperoxide prostaglandin H_2 (PGH_2) in a two-step process involving peroxidation and reduction (114). This is a critical step in the generation of several eicosanoids including PGI_2, PGE_2, PGD_2, $PGF_{2\alpha}$, and thromboxane A_2. Both enzymes are membrane-bound through unique hydrophobic regions, and both can be localized at the lumenal surface of the endoplasmic reticulum and inner membrane of the nuclear envelope. Although they share important structural features based on a 65% amino acid identity, their most important differences are related to their pattern of tissular

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presence, regulation of gene expression, and biological function. COX-1 is widely expressed in most tissues in a constitutive manner without substantial variation along the cell cycle. In contrast, COX-2 behaves as an inducible gene in most tissues and cell types (133), although it is constitutively expressed in a few tissues such as the kidney, placenta, and brain. Among the stimuli capable of inducing COX-2, proinflammatory cytokines, lipopolysaccharide (LPS), and growth factors are the best known (see below and 132 for review). In general, COX-2 expression under inductive stimuli follows the pattern of the so-called early genes with a rapid increase after 2–4 h and a gradual fade after 24–48 h.

The central question of why nature has provided humans and other mammals with two different isoforms with almost exact biochemical functions and catalytic properties has not yet been solved. Nevertheless, partial light has been shed with the approach of knockout mice where the suppression of one or the other isoform has been related to the presence of concrete phenotypes. In the case of COX-1, the mice were viable and exhibited no gross abnormalities (65). A more detailed study showed diminished platelet aggregation in response to arachidonic acid and, surprisingly, reduced ulceration after treatment with antiinflammatory agents. This was striking because it was assumed that COX-1 was gastroprotective and its inhibition by nonsteroidal antiinflammatory drugs (NSAIDS), the main cause of gastric and duodenal ulcer (125), this concept precisely underlying the search for selective COX-2 inhibitors. Phenotypes corresponding to COX-2 null mice were, on the contrary, much more conspicuous: the animals developed severe renal pathology in the first weeks of life, including subcapsular hypoplasia, progressive glomerulosclerosis, and tubulointerstitial atrophy (83). Of interest, alterations in every phase of the reproductive cycle were found, and these animals were infertile in a very high proportion. Again, no gastrointestinal pathology was found. These findings support the existence of alternative routes in gastric protection that do not involve PGs, and also add unexpected functions to COX-2 as an essential regulator of reproduction. In fact, recent studies add a word of caution about the clinical use of selective inhibitors of COX-2 (130). It is highly probable that COX-1 is also involved in inflammation, and there is clinical evidence that the use of nonselective inhibitors like indomethacin may confer a more complete antiinflammatory response. Another very interesting and unique feature of COX-2 has been learned from clinical studies reporting a reduced risk of colon cancer in patients receiving NSAIDs (see 105 and 132). There is evidence now that COX-2 is expressed in mesenchymal cells of adenomas where it may contribute to tumor formation and growth. Whereas the mechanisms underlying these observations await to be elucidated, it is now clear that a differential fate in the downstream mediators generated by COX-2 may profoundly influence cell cycle and other nuclear events, in contrast with the theoretically similar eicosanoids synthesized after COX-1 activation.

A well known context where there is proof for COX-2 induction is inflammation, where COX-2 expression is elicited by inflammatory mediators. Initial studies have documented the presence of COX-2 in several inflammatory models, such as the carrageenin-induced pleurisy (124). However, it is plausible that the role of COX-2 in inflammation is a complex and dual one, as in the latter model, cyclopentenone PGs may actually favor resolution of inflammation (30). It has been suggested that COX-2 may behave as proinflammatory in the first 24 h, but that it promotes a shift to antiinflammatory effectors after 48 h, this being related to a change in the type of PG generated—from PGE_2 to 15-deoxy-Δ^{12-14}-PGJ_2 (15-d-PGJ_2). COX-2 has also been described in atheromatous plaques, but not in normal arteries (111). However, the relative contribution of COX-1 and COX-2 to eicosanoid production in the clinical setting of atherosclerosis is not well known and has only started to be addressed (10).

Inducible nitric oxide synthase (iNOS) and COX-2: closely related enzymes

The demonstration of the L-arginine-dependent synthesis of nitric oxide (NO) in the mid 1980s in several cell types and under different scenarios led to the purification and cloning of

a set of enzymes capable of supporting the catalysis of the guanidino group of L-arginine into NO and citrulline, through a series of complex intermediate steps (72). Three different NO synthase (NOS) isoforms are now recognized in humans, each with unique features of expression and catalytic requirements (see 119 for review). The three of them share many features, among which are cofactor requirements and conjoint oxidoreductase properties which have their basis on common structural motifs. In general, it is accepted that endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutive isoforms, even when both can experiment a modest degree of regulation after specific stimuli (63). In contrast, iNOS is inducible and is most relevant for the discussion in the context of COX-2, as both enzymes seem to be coregulated in many ways (Fig. 1). iNOS and COX-2 are coexpressed in proinflammatory contexts as a result of their induction in archetypal cells such as polymorphonuclear cells, and macrophages (133). Immunohistochemical colocalization has been documented in animal experimental models (126), and it is believed that the concurrent production of eicosanoids such as PGE_2 and NO strongly contributes to the inflammatory response by triggering the production of their downstream cyclic nucleotide effectors, cyclic AMP (cAMP) and cyclic GMP (cGMP), respectively. Very likely, the oxidative capacities of both enzymes generate toxic amounts of reactive oxygen and nitrogen intermediates, which have pleiotropic effects on multiple signaling pathways, thus contributing to amplify and perpetuate the inflammatory setting (85). For example, COX-2 and iNOS are coexpressed in native and transplant atherosclerosis, possibly allowing for interaction between the enzymes and suggesting an alternative mechanism for the benefits of aspirin via inhibition of COX-2 activity (6). The interaction between COX-2 and iNOS may also occur through their metabolites. Numerous studies indicate that NO augments COX activity both *in vitro* and *in vivo*, possibly through the interaction with its heme group (107, 108). In fact, PG production has been proposed to mediate, at least in part, some of the biological effects of NO, such as cervical ripening during

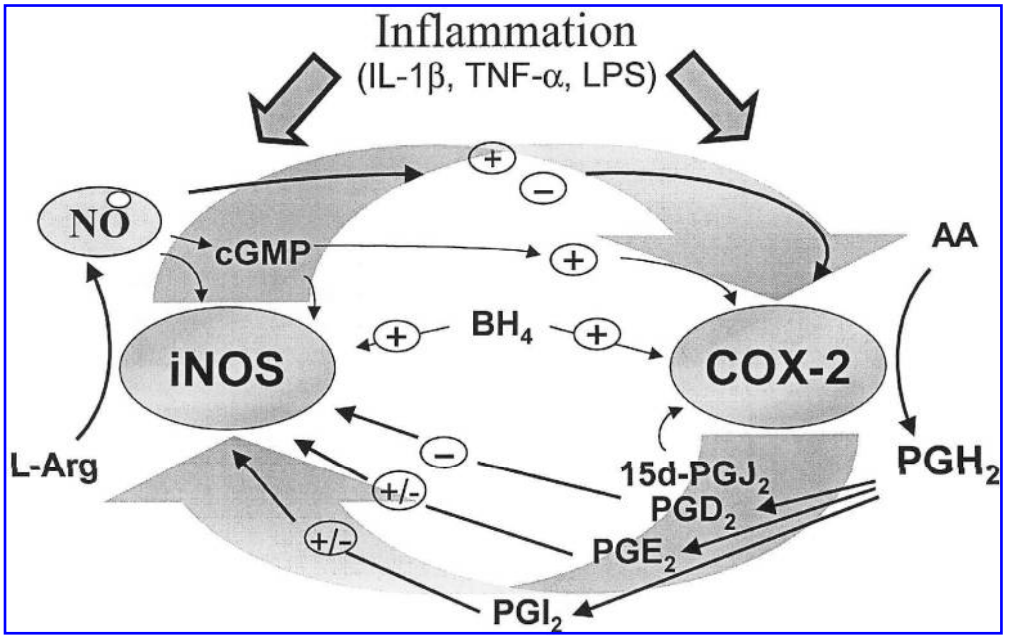

FIG. 1. Cross-talk between NOS and COX pathways. An intricate relationship exists between COX and NOS pathways. In inflammation, the inducible isoforms of these enzymes are expressed in response to similar stimuli. In addition, the products of the two pathways, NO and PGs, can regulate the activity and/or expression of iNOS and COX-2. The effect of NO on COX-2 expression can be positive or negative depending on the system under study, and it can take place through cGMP-dependent and -independent mechanisms. In turn, PGs can modulate iNOS expression through multiple mechanisms that may result in a positive or negative regulation of iNOS levels. AA, arachidonic acid; BH_4, tetrahydrobiopterin; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

pregnancy (67). The interaction between iNOS and COX-2 has also been shown in a model of cerebral ischemia in rats, where iNOS may increase COX-2 activity (88). Interestingly, not only does NO influence the activity and expression of COX-2 (see below), but it has also been reported that COX products may modulate iNOS expression. For instance, in macrophages, PGE_2 and a prostacyclin analogue (Iloprost) were found to inhibit iNOS expression (74). Finally, it is important to emphasize that, even when a coordinated induction of iNOS and COX-2 has been confirmed in many cases, a differential pattern of response to several agents such as indomethacin or p38 mitogen-activated protein kinase (MAPK) inhibitors has been described (31).

NO AS A REGULATOR OF GENE EXPRESSION: THE TIP OF AN ICEBERG?

Although the physiological functions of NO have been clearly established in the vascular, immune, and nervous systems (82), there is increasing evidence that it may also govern other important intracellular processes, among which gene expression is one of the most studied ones. There is now abundant literature reporting multiple genes that may be directly or indirectly modulated by NO. A list, which is not necessarily comprehensive, is provided in Table 1. Because of the relationship of NO to inflammation, the interest immediately was focused on the possibility of its interaction with transcription factors involved in the inflammatory response. Among these, the one that has received most attention is nuclear factor-κB (NF-κB) (see below). Of interest, it is now known that the expression of iNOS and COX-2 can be modulated by NO, possibly through the modification of transcription factors (NF-κB, c-*fos*, junB) that play an important role in the regulation of both genes.

An important facet that has not yet been completely clarified is the comprehension of the molecular mechanisms by which NO may modify the function of proteins in general and transcription factors in particular. In theory, and as shown in Fig. 2, there are several molecular targets for NO, most of which are related to the direct modification of specific moieties within proteins, such as heme groups and zinc-sulfur and iron-sulfur clusters (19). Besides, a very important property of NO is its capacity to react with specific amino acid residues of proteins. Two major modifications have occupied this picture, nitrosylation of thiol groups in cysteine residues of proteins (117), and nitration of tyrosyl groups (7), the latter reaction being specifically promoted by the reactive nitrogen intermediate peroxynitrite. However, although more examples are emerging concerning the functional relevance of these modifications, it is yet unclear how these changes may affect DNA binding or transcription factor activation. Of interest, NO has been shown to induce the glutathionylation (mixed disulfide formation) of specific cysteine residues in transcription factor subunits (see 59 for review), and this confers a new mechanistic resource by which nitrosative stress may be transduced into gene expression changes. Proteins that are involved in the regulation of mRNA translation have also been shown to be targets for NO. A very elegant example was described by Hentze and Kuhn, who showed that two RNA binding proteins, IRP-1 and IRP-2 (iron-regulatory proteins) are regulated by NO and redox agents, which induce a conformational change from the state of aconitase to the Apo-IRPs conformation, in which these proteins have RNA binding capacity (41). In fact, NO has been shown to regulate mRNA stability of transferrin (91) and soluble guanylate cyclase (25). In these examples, the biochemical modification of heme groups or Fe-S or Zn-S clusters are likely involved in the functional changes promoted by NO. In other examples, NO may modulate gene expression and act as a gene silencer by promoting specific chemical modifications of DNA, such as methylation (43).

However, it is important to keep in mind that in some cases it is the downstream effector, cGMP, that is the main protagonist of the gene expression changes mediated by NO. It has been shown that genes containing activator protein-1 (AP-1) *cis*-regulatory elements are susceptible of being regulated by the NO–cGMP pathway (103) (see below). The cyclic nucleotide may activate cGMP-dependent protein kinases (PKGs), which in turn may phosphorylate a series of proteins involved in the regulation of gene expression (71, 102). Other tar-

TABLE 1. EFFECTS OF NO ON GENE EXPRESSION

Induction or potentiation				*Inhibition*			
*Gene**	*Cell type†*	*Reference*		*Gene**	*Cell type†*	*Reference*	
iNOS	RMC	Mühl and Pfeilschifter, 1995	(84)	iNOS	AGC	Park et al., 1994	(93)
COX-2	RMC	Tetsuka et al., 1996	(123)	COX-2	RPM	Habib et al., 1997	(37)
TNF-α	U937	Wang et al., 1997	(131)	TNF-α	Raw 264.7	Eigler et al., 1995	(24)
IκB-α	EC	Peng et al., 1995	(98)	VCAM-1	EC	Spiecker et al., 1997	(116)
Hsp70	SMC	Xu, 1997	(135)	ICAM-1	RMC	Ikeda et al., 1996	(46)
EGFR	OGC	Hattori et al., 1996	(39)	MCP-1	EC	Zeiher et al., 1995	(139)
c-*fos*	PC12	Peunova and Enikolopov, 1993	(101)	MCSF	EC	Peng et al., 1995	(98)
c-*fos*	REF52	Pilz et al., 1995	(103)	sGC	SMC	Filippov et al., 1997	(25)
*jun*B	REF52	Pilz et al., 1995	(103)	PKG-I	SMC	Soff et al., 1997	(115)
TGF-β	A549	Vodovotz et al., 1999	(127)	GnRH	GT1	Belsham et al., 1996	(9)
Cu/Zn SOD	HK	Frank et al., 2000	(27)	IL-8	EC	Fowler et al., 1999	(26)
VEGF	GC	Kimura et al., 2000	(57)	IL-2	ML	Berendji et al., 1999	(11)
HIF-1α	GC	Kimura et al., 2000	(57)	Thioredoxin	PPAEC	Zhang et al., 1999	(140)

This table provides some examples of genes that are modulated by NO in a positive or in a negative manner. In some cases, including iNOS, COX-2, and tumor necrosis factor-α (TNF-α), both a positive and a negative modulation of gene expression by NO have been reported.

*IκB, inhibitory protein of NF-κB; Hsp70, heat shock protein 70; EGFR, epidermal growth factor receptor; TGF-β, transforming growth factor-β; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia inducible factor-1α; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; MCSF, macrophage colony stimulating factor; sGC, soluble guanylate cyclase; PKG-I, cGMP-dependent protein kinase I; GnRH, gonadotropin releasing hormone; IL, interleukin.

†RMC, rat mesangial cells; EC, endothelial cells; SMC, smooth muscle cells; OGC, ovarian granulosa cells; PC12, pheochromocytoma cells; REF, rat embryo fibroblasts; HK, human keratinocytes; GC, glioblastoma cells; AGC, astroglial cells; RPM, rat peritoneal macrophages; GT1, hypothalamic gonadotropin-releasing hormone-secreting neuronal cell line; ML, murine lymphocytes; PPAEC, porcine pulmonary artery endothelial cells.

gets of cGMP include cGMP-regulated phosphodiesterases and cGMP-gated ion channels. Identification of the downstream mediators of the NO–cGMP pathway that may regulate gene expression is a very active field of research, in which several gaps of information remain to be filled. It has recently been shown that PKG-II may also act as a transcriptional regulator in cells of neuronal and glial origin (36). However, the fact that the expression of PKGs is lost rapidly in many cells in culture makes necessary the use of transitory transfections as tools for the study of PKG function, a circumstance that adds obscurity to the interpretation of the results in a physiological context.

NO AS A REGULATOR OF COX-2 EXPRESSION

As outlined above, one of the components of the coordination that exists between COX and NOS pathways is represented by the ability of NO to regulate COX-2 expression. In recent years, numerous experimental results have documented this interaction, although the effects encountered are varied and complex. NO has been reported either to induce COX-2 expression *per se* or to potentiate the expression of COX-2 elicited by other stimuli. In some settings, the effects of NO on the expression of COX-2 induced by various agents are inhibitory. The mechanisms underlying the diversity of NO effects on COX-2 expression may be related to the levels of NO, the cell type, and/or the state of activation of the cells.

The effect of NO may depend on NO concentration, with low levels being stimulatory and high levels being inhibitory for COX-2 activity and induction (121). In murine macrophages stimulated with LPS, a threshold effect of NO generation has been observed, with an inhibitory effect of endogenous NO generation on COX-2 protein levels that is unveiled only when LPS-induced nitrite accumulation is reduced to $<$15% by the use of NOS inhibitors (95).

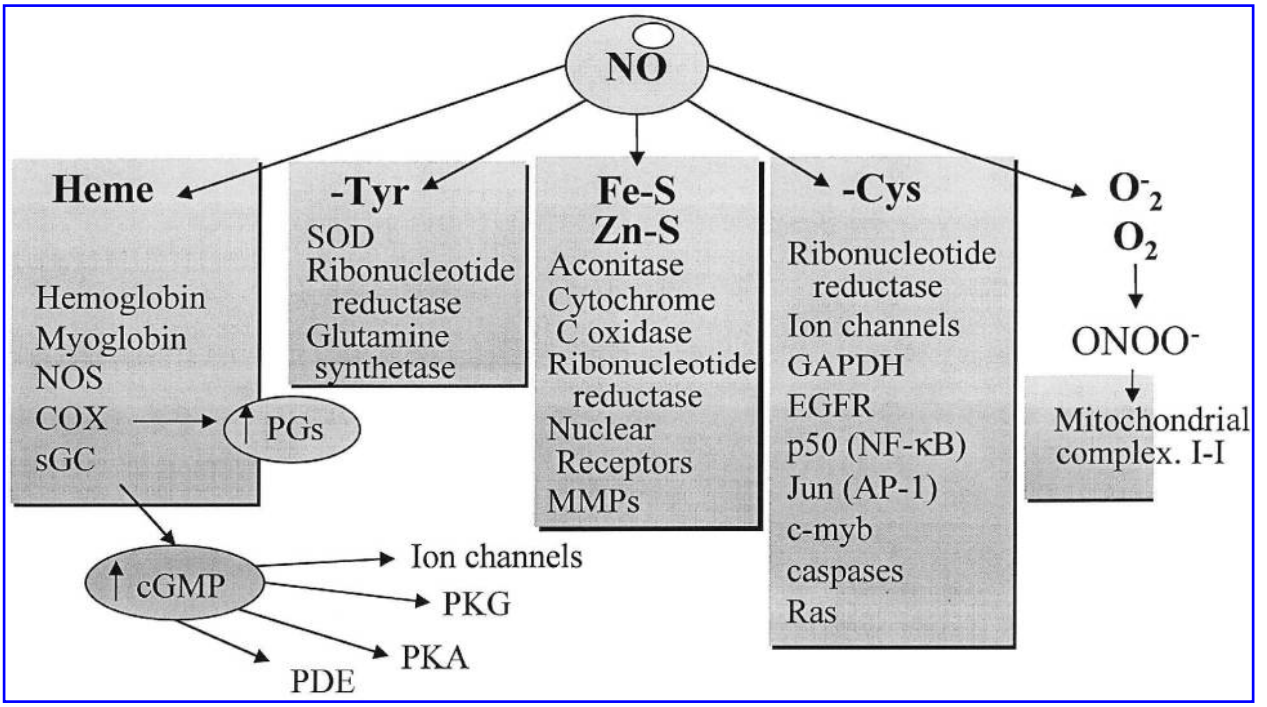

FIG. 2. Mechanisms of NO signaling. NO can interact with functional groups in proteins, such as heme or Fe-S or Zn-S clusters, with amino acid residues, such as tyrosine or cysteine, and with other free radicals. Provided are some examples of proteins, the function of which can be modulated by these various interactions. The multiplicity of the potential targets of NO in cells is at the basis of the pleiotropic effects of this simple molecule. sGC, soluble guanylate cyclase; PDE, phosphodiesterase; SOD, superoxide dismutase; MMPs, matrix metalloproteases; GAPDH, glyceraldehyde 6-phosphate dehydrogenase; EGFR, epidermal growth factor receptor; AP-1, activator protein-1; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.

The effect of NO or NO donors depends on the experimental system under study. In a comparative study, using RAW 264.7 macrophages and primary rat microglial cells, it has been observed that different NO donors can act at multiple levels to modulate prostanoid production and COX-2 expression, exerting different effects in the two cell types and also in the same cell type, depending on the state of activation of the cells. In RAW macrophages, several NO donors have been found to stimulate basal COX-2 expression (33, 128), but slightly down-regulate LPS-elicited COX-2 (33). Meanwhile, in murine microglial cells, NO donors or iNOS-inducing agents down-regulate COX-2 expression and prostanoid production, whereas negative regulators of iNOS up-regulate COX-2 (33, 80). In rat peritoneal macrophages stimulated with LPS, generation of NO displays inhibitory effects on COX-2 expression, because NOS inhibitors additionally enhanced LPS-induced COX-2 expression and activity, whereas NO donors reversed this effect (37). However, in rat mesangial cells, NO was found to play a positive role in interleukin-1 (IL-1)-elicited COX-2 induction (123), exemplifying once more the variability of the response depending on the cell type studied.

NO effects on COX-2 expression may be stimulus-dependent. In rat osteoblasts, interferon-γ-induced COX-2 expression was blocked by NOS inhibitors, and although exogenous NO donors elicited COX-2 expression, IL-1- and tumor necrosis factor-α (TNF-α)-induced COX-2 expression occurred independently of NO synthesis (45). These results suggest the existence of both NO-dependent and -independent pathways for the regulation of COX-2 expression in this cellular system. In addition, in human mesangial cells, NO donors appear to amplify COX-2 induction when the stimulation of these cells is submaximal, but not when a maximal stimulation of the cells is achieved by the simultaneous addition of several proinflammatory stimuli (Díaz-Cazorla et al., unpublished results).

The effects of NO on COX-2 expression may also be time-dependent. In the murine macrophage cell line ANA-1, endogenous NO is not required for the onset of COX-2 mRNA and protein expression; however, NO is necessary for maintaining prolonged COX-2 gene expression (100). The opposite effect is observed in human mesangial cells that express iNOS and COX-2 in response to cytokine stimulation. In these cells, endogenous generation of NO is necessary for COX-2 induction. However, NO also plays a role in limiting the response of these cells, because at late times of induction it reduces the levels of NF-κB activity. This dual effect of NO can also be evidenced by supplementing mesangial cells with NO donors, which exert an amplification of COX-2 induction at the initial stages of cell activation, but in the long-term reduce the extent of COX-2 induction (21). The time dependence of the effects of NO may be related to changes in the expression of NO targets that occur with time of exposure to NO. In this regard, prolonged exposure to NO and cGMP analogues has been shown to down-regulate the activity and expression of soluble guanylate cyclase (25) and PKG (115). A rapid desensitization of soluble guanylate cyclase has been recently proposed to underlie the diversity of cellular cGMP responses (8). Some of these mechanisms could be involved in the time-dependent effects of NO on cytokine-elicited COX-2 expression in mesangial cells, which appear to be mediated, at least in part, by activation of soluble guanylate cyclase (123). In these cells, the interaction between NOS and COX pathways takes place at several levels. The availability of tetrahydrobiopterin (BH_4), an essential cofactor for NOS activity, the synthesis of which is increased by proinflammatory stimuli in endothelial and smooth muscle cells, has been reported to modulate COX-2 expression in human mesangial cells (99). The effects of BH_4 may be due in part to improved generation of NO by iNOS or constitutive NOS in BH_4-supplemented cells, although NO-independent effects of BH_4 have also been encountered.

The interactions between COX and NOS pathways have been explored in intact animals. Again, it is evident that an important cross-talk between the two pathways occurs *in vivo*, and although the nature of this interplay is complex, most evidence points to a positive role of NO on COX-2 activity and/or expression. Inhibition of NOS activity during LPS administration to rats limited the increase in PG production attributable to COX-2 activity without interfering with the activity of the enzyme *in vitro*, thus suggesting a positive role for NO generation in the induction of COX-2 (109). The importance of NO generation for PG synthesis has been confirmed in experiments using iNOS knockout mice. Upon stimulation by proinflammatory agents, PGE_2 formation by cells from iNOS-deficient animals was severely reduced compared with that by cells from control animals, although COX-2 protein expression was not significantly different in both types of cells (73).

Meanwhile, in an *in vivo* model of osteoarthritis, selective inhibition of iNOS activity resulted in a significant decrease in the production of catabolic factors, such as matrix metalloproteases, IL-1β and peroxynitrite, and of COX-2 expression in the cartilage, synovial lining, and mononuclear infiltrate of the osteoarthritic joints (97). In rheumatoid synovial cells in culture, exposure to a NO donor was sufficient to induce the expression of COX-2, and this effect could be reduced by therapeutic concentrations of dexamethasone (44).

In endotoxin-challenged pregnant mice, COX-2 and iNOS are expressed in a coordinated manner in the uterus, where their enzymatic products may contribute to the signaling of uterine activity or cervical changes culminating in expulsion of the fetus. In this *in vivo* system, NO synthesis appears to be a prerequisite for subsequent stimulation of COX-2 and iNOS gene expression, because in mice treated with NOS inhibitors before LPS administration, the accumulation of the transcripts for both enzymes was prevented (120).

An interesting interaction between NO production and COX-2 expression may take place in the macula densa of kidney where nNOS is present and COX-2 expression occurs in basal conditions and increases with salt depletion. Administration of a nNOS inhibitor to rats results in lower expression of COX-2 in control rats and in rats fed a low-salt diet with or without captopril, and it is suggested that NO me-

diates increased renal cortical COX-2 expression seen in volume depletion. These studies envisage important interactions between the NO and COX-2 systems in the regulation of arteriolar tone and the renin–angiotensin system by the macula densa (38).

In contrast, chronic inhibition of NO synthesis in rats has been reported to lead to enhanced expression of COX-2 in mesenteric arteries, and this has been interpreted as a compensatory mechanism to preserve vasodilation through the generation of vasodilatory PG (40).

Therefore, from the above-mentioned studies, it can be inferred that the modulatory role of NO on COX-2 expression and/or activity is key in many systems, but the nature of this interaction may depend on the system or on the pathophysiological situation under study. Although, from a mechanistic point of view, much can be learned from cell culture models about the effects of NO on COX-2 expression, the confirmation of the physiological relevance of these studies awaits further characterization in *in vivo* models or clinical studies.

MODULATION OF SIGNAL-TRANSDUCTION CASCADES LEADING TO COX-2 EXPRESSION

The precise cascade of intracellular events that leads to the expression of COX-2 is not completely understood. Tyrosine phosphorylation, activation of protein kinase C (PKC), and MAPK cascades have been identified as important events in the induction of COX-2 in response to cytokines. Inhibitors of tyrosine kinases block COX-2 induction by various stimuli (1, 20, 106). NO has been shown to modulate tyrosine phosphorylation and/or tyrosine phosphatase activity. Therefore, this could represent a potential mechanism for the modulation of COX-2 expression by NO. Activation of PKC has also been suggested to play a role in the induction of COX-2 in several experimental systems (17, 112). Overexpression of the ζ isoform of PKC in rat mesangial cells markedly increases COX-2 expression (78). In murine astrocytes, PKC has been reported to mediate COX-2 induction by IL-1β acting upstream of the activation of the MAPKs p42/44 [extracellular signal-regulated kinase 1/2 (ERK1/2)] (81). PKC-dependent expression of COX-2 also requires mainly activation of p42/44 in human endothelial cells (42).

The involvement of the MAPK p38 and/or p42/44 in COX-2 induction has been assessed in numerous studies. Induction of COX-2 by LPS requires the activation of p38 MAPK, which in turn activates AP-1 and NF-κB in J774 macrophages (15), whereas involvement of both p42/44 and p38 MAPK has been reported in LPS-elicited COX-2 induction in RAW 264.7 macrophages (96). IL-1β regulation of COX-2 involves the p42/44 and p38 MAPK signaling pathways in cardiac myocytes (66), and p38 MAPK has been also reported to mediate IL-1β-induced COX-2 expression in mesangial cells (31). Both types of MAPK are important targets for regulation by NO. NO has been reported to modulate the activity of p42/44 by cGMP-dependent and -independent mechanisms. In mesangial cells, endogenous iNOS-derived NO or NO donors can activate p42/44 through multiple mechanisms. Activation by cytokines and by NO donors is biphasic with a rapid and transient and a late and prolonged phase. In cytokine-treated cells, the early phase of p42/44 activation is cGMP-independent, whereas the late phase is NO-dependent. Activation by NO donors is cGMP-dependent in the early phase, whereas in the late phase it appears to be cGMP-independent and related to inhibition of tyrosine phosphatase activity (13). In other systems, the activation of p42/44 by NO appears to require cGMP generation (92) and PKG activation (62). PKG appears to be involved in the activation of p38 by NO as well (12). NO can also modulate the activity of c-Jun N-terminal kinase (JNK) (55), which is an important mediator of inflammatory responses (50) and has been involved in the induction of COX-2 by several stimuli, including cytokine stimulation (32) and treatment with phosphatase inhibitors (79), and in tonicity-stimulated COX-2 expression in mouse collecting duct cells (138). In cultured rat aortic smooth muscle cells, NO–cGMP-mediated activation of JNK is mediated by PKG (62). Although a correlation with COX-2 expression

has not been established in all cases, the identified interactions of NO with the signaling cascades leading to COX-2 induction provide a basis to explore the mechanisms underlying the diverse effects of NO on COX-2 levels.

MODULATION OF TRANSCRIPTION FACTORS THAT REGULATE THE COX-2 PROMOTER

Although the regulation of the COX-2 promoter has been studied in some detail, it is far from being completely understood. A number of consensus sites for the binding of transcription factors appear in the analysis of the DNA sequence of the human and murine promoters (3). The functional relevance of some of these sites has been established experimentally. Among the transcription factors important for either human or murine COX-2 expression are NF-κB (110), nuclear factor of activated T cells (NF-AT) (47), cAMP response element binding protein (CREB) (48), CAAT enhancer binding protein (C/EBP) (56), AP-1 (2), AP-2 (58), nuclear factor of IL-6 (NF-IL6) (49, 137), specificity protein-1 (SP-1) (136), and peroxisome proliferator activating receptors (PPARs (76) (Fig. 3). From the consideration of the general mechanisms of NO action discussed above, it is evident that many of these factors are potential targets for modulation by NO. A functional link between NO modulation of transcription factor activity and COX-2 transcriptional regulation has been ascertained in some instances.

Potential interactions of NO with NF-κB and their importance in the regulation of COX-2

NF-κB is a key transcription factor for the expression of proinflammatory genes (please see 4 and 5 for excellent reviews on NF-κB regulation). The active form of NF-κB is a dimeric protein integrated by two members of the NF-κB/Rel family of proteins (29). Its efficacy in the rapid activation of transcriptional events in response to proinflammatory stimuli relies on its characteristic of being presynthesized in the cytoplasm of cells, but kept inactive by the binding of the inhibitory subunit, IκB (inhibitory protein of NF-κB). In a very simplified view, binding of proinflammatory stimuli such as IL-1 or TNF to their cellular receptors triggers a cascade of events that leads to the activation of a kinase complex known as IKK, or IκB kinase. Phosphorylation of IκB by IKK occurs very rapidly after receptor stimulation and determines the ubiquitination and subsequent degradation of IκB by the proteasome. The NF-κB active dimer is thus free to translocate to the nucleus where it activates transcription.

The human COX-2 promoter contains two NF-κB sites located at −446 and −223 bp from the transcriptional start site. The downstream site is involved in COX-2 induction by LPS, phorbol esters, and hypoxia (48, 110). The upstream site is involved in IL-1β- and TNF-α-elicited COX-2 induction (86, 137).

In NO-producing cells or in cells exposed to NO by paracrine mechanisms or treatment with NO donors, the activity of NF-κB may be

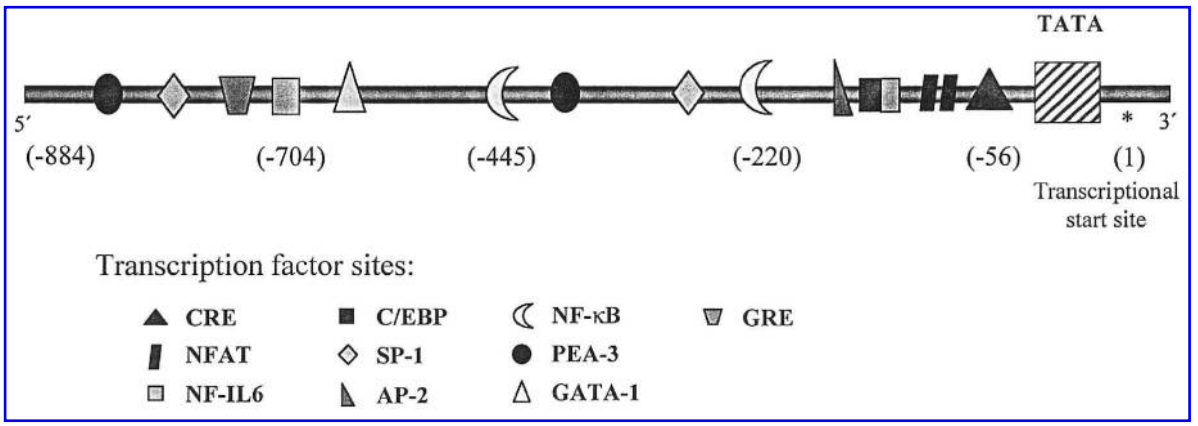

FIG. 3. Schematic representation of the human COX-2 promoter showing putative sites for transcription factor binding. CRE, cAMP response element; PEA-3, polyomavirus enhancer activator-3; GRE, glucocorticoid response element.

modulated in multiple ways. A schematic view of some of the points of interaction between NO and NF-κB identified to date is shown in Fig. 4. Interestingly, NO can have both positive and negative effects on NF-κB activity. The predominance of either effect may depend on the activation of interacting signaling pathways, on the generation of scavengers of NO such as superoxide anion, or on the presence of precise NO targets in a given cell type. Therefore, the effects of NO on NF-κB activation and their consequences on COX-2 expression may be cell- and time-dependent, as well as influenced by the redox status (51).

It has been known for some years that NO can directly inhibit NF-κB DNA binding. This phenomenon has been claimed to be at the basis of the inhibition of proinflammatory signaling by NO in murine macrophages (94). The structural basis of this inhibition may in turn be multiple. By means of mass spectrometry analysis, *S*-nitrosylation of a cysteine residue in the p50 subunit that is important for the regulation of DNA binding, Cys62 in human p50, was initially identified (75). More recent *in vitro* studies, performed in the presence of physiological glutathione concentrations, have shown the NO-induced formation of a mixed disulfide between glutathione and purified p50 that involves Cys62. This modification correlates with an inhibition of p50 DNA binding (61). In addition, NO may induce other oxidative modifications of this or other cysteine residues in the p50 molecule that may account for part of its inhibitory activity (Pineda-Molina et al., unpublished results).

Inhibition of NF-κB activity by NO can occur in some systems as a result of the amplification of IκB expression. This effect is independent of cGMP generation and occurs through the transcriptional activation of the IκB promoter by as yet undefined mechanisms. This results in increased levels of IκB mRNA and protein that cause retention of the NF-κB dimer in the cytoplasm (98). Increased expression of IκB has been shown to correlate with NO-elicited inhibition of NF-κB activity and COX-2 expression at the late stages of human mesangial cell activation by cytokines (21). Although these effects appear to occur by cGMP-independent mechanisms, a cGMP-dependent mechanism for NF-κB inhibition has also been proposed in macrophages (54). NO-mediated pathways can also interfere with the phosphorylation and degradation of IκB, thus impairing NF-κB activation (53).

In spite of the ability of NO to regulate NF-κB activity negatively, the result of NO gen-

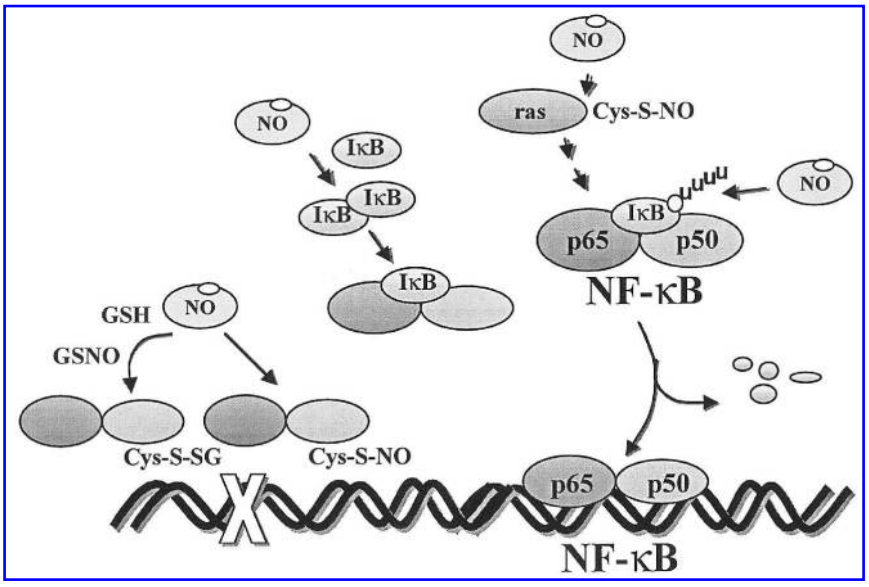

FIG. 4. Potential interactions of NO with NF-κB. The paradigmatic proinflammatory transcription factor NF-κB is an important target for NO action on the transcription of numerous genes, including COX-2. Some of the potential effects of NO on NF-κB include inhibition of its DNA-bindinag activity and modulation of IκB levels, either through the regulation of IκB mRNA expression or by the interference with IκB degradation in a positive or in a negative way. GSH, glutathione; GSND, nitroso-*S*-glutathione.

eration or NO donor administration in some experimental systems is an activation of NF-κB. NO donors can either elicit or amplify NF-κB activation. NO has been reported to activate NF-κB by a Ras-dependent mechanism. NO directly modifies Ras by single *S*-nitrosylation at Cys118. This results in stimulation of guanine nucleotide exchange, possibly by destabilization of the interaction between residues in the GDP-binding pocket and the nucleotide, facilitating GTP binding and Ras activation (64). Among the signaling pathways leading to NF-κB activation, MEKK1 and JNK appear to be required in rat lung epithelial cells (52). Activation of NF-κB by NO contributes to COX-2 expression and protection from apoptosis in RAW 264.7 macrophages (128). NO donors can also amplify NF-κB activation by proinflammatory stimuli. In human mesangial cells, a rapid, short-term amplification of cytokine-elicited NF-κB nuclear translocation, in association with increased COX-2 expression, is observed when cells are stimulated in the presence of NO donors (21). These effects appear to be due to a delay in the resynthesis of IκB. Thus, these cells offer an interesting model in which positive and negative effects of NO on NF-κB and COX-2 expression can be encountered in a time-dependent fashion.

NO could also modulate NF-κB activation through the generation of second messenger molecules such as cGMP and cAMP. cGMP analogues have been reported to inhibit NF-κB activity in hypoxic/reoxygenated HUVEC (human umbilical vein endothelial cells) (18) and in macrophages (54), although a link between these effects and COX-2 expression has not been established. Cellular cAMP levels can be modulated by NO (131). It is known that cAMP can modulate NF-κB activation by several mechanisms, including phosphorylation of IκB (113) and of p65 (141) by cAMP-dependent protein kinase, both resulting in increased activation. An induction of the mRNA for the NF-κB subunit p65 associated with PGE_2-driven increases in cAMP content has been observed in rat mesangial cells in correlation with COX-2 induction (89). From these results, it appears that the transcription factor NF-κB may be one of the main targets for the regulatory effects of NO on COX-2 expression.

Potential interactions of NO with AP-1 and their importance in the regulation of COX-2

The importance of the transcription factor AP-1 as a mediator of the transcriptional effects of NO was first suggested by the studies of Peunova and Enikolopov, who reported that NO and cGMP can increase c-*fos* expression (101). It has now been shown that genes containing AP-1 *cis*-regulatory elements are susceptible of being regulated by NO (103). In some cell types, the NO–cGMP pathway induces c-*fos* mRNA in the presence of an increased intracellular calcium concentration (101). In fibroblast cell lines, PKG-I activates the c-*fos* promoter after translocation into the nucleus through the concourse of a nuclear localization signal domain within the protein that is itself regulated by cGMP (34, 35). With respect to COX-2, studies in murine macrophages using a dominant negative Jun mutant have established an essential role for AP-1 in the superinduction of COX-2 expression elicited by the combination of proinflammatory stimuli and NO donors (129). However, the participation of PKGs in this effect has not been ascertained.

It is interesting to note that the interactions of NO with AP-1 could also result in inhibitory effects. In the presence of high concentrations of NO donors, Jun and Fos proteins may be modified by NO at specific cysteine residues, which results in inhibition of DNA binding (87). In the presence of glutathione concentrations in the physiological range, Jun may suffer nitroso-*S*-glutathione-induced *S*-glutathionylation, a reversible modification that occurs at the cysteine located in the DNA binding domain of the protein and also correlates with inhibition of the ability of the Jun homodimer to bind DNA (60). However, at present it is not known whether inhibition of AP-1 activity is at the basis of the negative effects of high-dose NO donors on COX-2 expression observed in some experimental systems.

COX-2–iNOS INTERACTION

When analyzing the cross-talk between the NOS and COX pathways, it is important to consider that the products of these enzymes may exert a feedback and a reciprocal regulation of both pathways, as shown schematically

in Fig. 1. Although the interaction between COX-2 and iNOS has received the greatest attention, the cross-talk between the PG and NO biosynthetic pathways may also affect eNOS and nNOS. PGE_2 has been reported to regulate nNOS in the perinatal period in rats and pigs (22), whereas PGD_2 regulates eNOS in the developing pig choroid (23). PGs may exert their effects through interaction with membrane receptors that results in activation of adenylate cyclase and increased cAMP content. cAMP in turn is an important regulator of iNOS expression in many cellular systems (see 28 for review). In the murine macrophage cell line J774, PGs of the E and I series down-regulate LPS-elicited COX-2 and iNOS expression by a PG-driven cAMP-mediated process (90). In murine microglial cells, PGE_2 down-regulates iNOS expression through its interaction with EP2 receptors and the generation of cAMP (68). In rat mesangial cells, PGE_2, which is the main product of the COX pathway in this cell type, has been reported to down-regulate iNOS expression, apparently in a cAMP-independent fashion, whereas stable analogues of PGI_2 and cAMP-elevating agents such as forskolin display a positive effect (122). A similar, but in this case, positive feedback mechanism has been proposed in RAW 264.7 macrophages where treatment with LPS increases intracellular cAMP levels via COX-2 induction and PG E_2 production, resulting in cAMP-dependent protein kinase activation, that in turn plays a positive role in NF-κB activation, iNOS expression, and NO production (16). Interestingly, in J774 macrophages, the effects of PGE_2 on iNOS induction appear to be dose-dependent, with low doses of the PG exerting a positive effect on LPS-induced iNOS expression, and high doses being inhibitory (77). These effects could be related to the ability of PGE_2 to modulate TNF-α-expression and secretion by these cells.

The COX metabolite 15-d-PGJ_2 is a negative modulator of cell activation by proinflammatory stimuli that down-regulates cytokine-elicited iNOS induction in several experimental models. This PG may exert its effects through multiple mechanisms. 15-d-PGJ_2 acts as a ligand for the nuclear receptor PPARγ, which in turn may inhibit NF-κB by sequestering coactivators needed for the transcription of iNOS (69). In addition, 15-d-PGJ_2 can directly inhibit NF-κB activation by blocking IKK activity (14) through covalent modifications of critical cysteine residues in IKK and also by modifying cysteine residues in the DNA-binding domains of NF-κB subunits (118). These mechanisms act in combination to inhibit transactivation of the NF-κB target genes COX-2 and iNOS, thus constituting a feedback loop that may contribute to negative regulation of inflammation (49).

The cross-regulation of iNOS and COX-2 expression has been evidenced in *in vivo* situations. In a model of murine inflammation, selective COX-2 inhibitors reduce iNOS activity and expression, whereas inhibition of iNOS activity exerts a negative effect on the COX pathway (104). In an *in vivo* model of glomerular immune injury, inhibition of iNOS activity reduced glomerular synthesis of PGE_2 and PGI_2, although it had no effect on thromboxane A_2 production (70). In turn, inhibition of glomerular PG synthesis increased iNOS mRNA levels in glomeruli, thus suggesting that during glomerular inflammation the COX pathway generates negative modulators of iNOS expression. However, in the light of recent findings, certain NSAIDs can directly inhibit NF-κB activation or activate certain PPAR subtypes; thus, the possibility exists that some of the effects observed with the use of COX-2 inhibitors are due to direct transcriptional effects of these compounds, more than to the limitation of PG production. Therefore, the results obtained from the use of these inhibitors should be interpreted cautiously.

SUMMARY AND FUTURE DIRECTIONS

We believe that it is now clear that there is a strong relationship between NO, COX-2, and the COX-2 products. However, one idea that should emerge from this review is that the cellular context and temporal frame of interaction are critical points that regulate the final effect of NO on COX-2 expression. Besides, it will be important to define in each case which is the precise molecular mechanism of the effects observed. An important effort needs to be de-

voted to identify the COX-2 products (new or already known) present in each response, as well as their potential influence on iNOS and the other NOSs. The proximity of the COXs to the nuclear topology, where transcriptional events take place, makes it attractive to speculate that several prostanoids play a role in transcription. Finally, it is highly likely that the interactions herein described may affect other pathophysiological scenarios besides inflammation or vascular pathology, some of which may have not yet been envisioned.

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ABBREVIATIONS

AP-1 and AP-2, activator proteins 1 and 2; BH_4, tetrahydrobiopterin; cAMP, cyclic AMP; C/EBP, CAAT enhancer binding protein; cGMP, cyclic GMP; COX, cyclooxygenase; CREB, cyclic AMP response element binding protein; 15-d-PGJ_2, 15-deoxy-Δ^{12-14}-prostaglandin J_2; eNOS, endothelial nitric oxide synthase; IκB, inhibitory protein of nuclear factor-κB; IKK, IκB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; IRP, iron regulatory protein; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-AT, nuclear factor of activated T cells; NF-IL6, nuclear factor of interleukin-6; NF-κB, nuclear factor κB; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NSAID, non-steroidal antiinflammatory drug; PG, prostaglandin; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; PPAR, peroxisome proliferator activating receptor; SP-1, specificity protein-1; TNF, tumor necrosis factor.

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