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Forum Review

Cross-Talk Between Constitutive and Inducible NO Synthase: An Update

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ABSTRACT

Inducible nitric oxide synthase (iNOS) is expressed upon exposure of some cell types to bacterial lipopolysaccharides (LPS) and/or a variety of proinflammatory cytokines. The authors present an overview of some of the recent findings further supporting the notion that this response takes place after an early decline in constitutive nitric oxide (NO) levels (i.e., NO released by constitutive NOS, cNOS). This response is indeed critical for allowing activation of the transcription factor NF-κB. Thus, generation of NO by cNOS represents a limiting factor for iNOS expression. Some of the physiological and pathological implications of the cross-talk between these two NOS isoforms are discussed. In addition, the results of recent studies are summarized, suggesting possible mechanisms whereby LPS and/or proinflammatory cytokines may cause inhibition of cNOS. *Antioxid. Redox Signal.* 8, 949–954.

INTRODUCTION

Nitric oxide (NO) is generated in different cell types by the conversion of L-arginine into citrulline, mediated by at least three distinct isoforms of the enzyme NO synthase (NOS). Two enzymes, the neuronal (nNOS or NOS-I) and the endothelial (eNOS or NOS-III) isoforms, are Ca^{2+}-dependent and constitutively expressed (also termed cNOS). The third enzyme is an inducible Ca^{2+}-independent isoform (iNOS or NOS-II), expressed in some cell types after stimulation with *Escherichia coli* lipopolysaccharide (LPS) and/or different cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), or tumor necrosis factor-α (TNF-α) (36). iNOS induction occurs at the transcriptional level and is mediated by the early activation of some nuclear transcriptional factors, including nuclear factor-κB (NF-κB) (18).

An extensive literature documents that low and high concentrations of NO produce remarkably different effects. Low concentrations of the gaseous messenger (putatively n*M*) are released by cNOS activation and are thought to be mainly involved in the regulation of physiological events (13). However, conditions promoting iNOS expression often lead to spatial and temporal formation of high NO levels (μ*M*), critically involved in important functions (e.g., host defense) but nevertheless associated with the onset of deleterious effects (13).

Thus, cNOS and iNOS are both differently regulated and involved in the regulation of different processes. Although this notion may suggest that these enzymes function independently, growing experimental evidence has led to the development of a theory on a cross-talk between cNOS and iNOS (13). This review will summarize the most recent findings on this topic.

CROSS-TALK BETWEEN cNOS AND iNOS

Constitutive NO prevents NF-κB-dependent iNOS expression

About 10 years ago, Mariotto *et al.* (29–31) produced results suggesting that iNOS expression is a NO-regulated

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process. They showed that two NO-releasing agents, sodium nitroprusside and nitroflurbiprofen, prevent iNOS expression in neutrophils from rats treated with LPS. These results were later on followed by the demonstration that low amounts of exogenous NO inhibit NF-κB activation and suppress iNOS expression in human microglia stimulated with LPS/TNF-α (10). Interestingly, the effects of endogenous NO were also found to be critically involved in preventing NF-κB activation and iNOS expression in cell types that express cNOS (8, 41).

Thus, it may be suggested that low amounts of NO play a protective role by keeping suppressed the accidental activation of NF-κB and the successive, potentially harmful, induction of iNOS expression. Generation of NO by cNOS may then represent a limiting factor for iNOS expression in the same cell (i.e., astrocytes) or in cells localized in the close vicinity of cNOS expressing cells. In this perspective, proinflammatory cytokines and LPS rapidly decrease the levels of NO at the early phase of the inflammatory response to then allow the prompt activation of NF-κB and the ensuing induction of iNOS expression (8, 13).

The notion that cNOS and iNOS are inversely regulated either in a cell or in tissues has been validated by the experimental work performed in different laboratories (Table 1). In this respect, it is interesting to note that many compounds that either stimulate the catalytic activity of cNOS, or that upregulate the expression of the enzyme, are reported to act as downregulators, or suppressors, of iNOS expression (13, 32). They include dexamethasone, erythropoietin, estrogens, ethanol, glucose, insulin, insulin-like growth factor (IGF), angiotensin converting enzyme inhibitors, and flavonoids (Table 1). In contrast, LDL-cholesterol, metals, drugs, endorphin, hypoxia, and LPS/proinflammatory cytokines on the one hand upregulate iNOS expression, whereas, on the other hand, inhibit cNOS-dependent NO production (Table 1).

Table 1. Compounds Modulating cNOS Activity and iNOS Expression

Compounds	*cNOS activity*	*iNOS expression*	*References*
β-Amyloid[a]	↓	↑	17, 52
Cholesterol	↓	↑	19
Clonidine[a]	↓	↑	53
Copper[a]	↓	↑	12
Dexamethasone	↑	↓	3
Endorphin	↓	↑	2
Erythropoietin	↑	↓	25
Estrogen	↑	↓	46
Ethanol	↑	↓	55
Flavonoids	↑	↓	22
Gabexate mesylate[a]	↓	↑	11
Glucose	↑	↓	38
Hypoxia	↓	↑	37
IGF[b]	↑	↓	47
Insulin	↑	↓	4
LPS + IFN-γ[c]	↓	↑	7

[a]These compounds caused a significant, dose-dependent iNOS mRNA overexpression only in cells incubated with a suboptimal concentration of LPS plus IFN-γ.

[b]IGF, Insulin-like growth factor;

[c]LPS + IFN-γ, *E. coli* lipopolysaccharide plus interferon-γ.

Numerous *in vivo* and *in vitro* studies have provided growing experimental evidence for the cross-talk between cNOS and iNOS in a variety of inflammatory-correlated pathologies. As an example, a deficiency of cNOS-dependent NO release was shown to take place, in parallel with an excessive production of NO by iNOS, in allergen-induced airway hyperresponsiveness (35). In the pregnant rat uterus, estradiol administration caused a dose-dependent increase in eNOS expression associated with the inhibition of iNOS expression (56). Decreased cNOS and increased iNOS were also observed in aortic endothelial cells of donryu rats fed with a cholesterol-enriched diet (1). During aging, NO production in the brain faces drastic changes associated with a continuous decline in eNOS and nNOS activities and with the spontaneous expression of iNOS (33).

The notion that NF-κB is critically involved in the cross-talk between cNOS and iNOS has been confirmed in a variety of biological systems (21, 28, 42–44, 50, 51). Recent work has provided further *in vivo* experimental evidence that physiologically produced NO may regulate NF-κB activation and iNOS expression. It was reported that repeated administration of the NOS inhibitor L-NAME enhances NF-κB activation in the heart of rats subjected to ischemia/reperfusion (24). Similarly, ischemia/reperfusion induced a super-induction of iNOS in the heart of $eNOS^{-/-}$ KO mice (20).

Mechanism(s) of cNOS inhibition

Although inhibition of cNOS activity is known to take place via a number of phosphorylation reactions (5, 6, 9, 14, 16, 23, 45), the upstream events leading to kinase activation are in general poorly defined. Furthermore, it is easy to predict that different mechanisms are involved in different cell types and biological systems. We will therefore focus on a specific cell system in which nNOS is expressed and iNOS can be expressed upon stimulation (i.e., primary astrocytes and glial cell lines). Early reports indicated that in a human astrocytoma cell line, LPS and IFN-γ cause rapid inhibition of nNOS activity (7) by shifting the equilibrium between native and tyrosine-phosphorylated nNOS to the latter (9). Since, tyrosine-phosphorylated nNOS is less active than native nNOS, this shift may directly lead to the inhibition of nNOS activity. The upstream events leading to tyrosine kinase activation are described below.

A recent study (40) showed that inhibition of nNOS in PC12 (rat pheochromocytoma), N1E-115 (mouse neuroblastoma), and C6 (rat glioma) cells can be achieved via activation of cytosolic phospholipase A_2 ($cPLA_2$), a large molecular mass member of the family of PLA_2 enzymes. The activities of nNOS and $cPLA_2$ are both regulated by increases in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) (27) and, not surprisingly, enhancing the $[Ca^{2+}]_i$ was found to cause a parallel increase in both activities and in the accumulation of the respective products, NO and arachidonic acid (AA). Interestingly, however, critical levels of AA were eventually reached that reduced or even suppressed formation of NO (40). In particular, it was observed that exposure of the cells to concen-

trations of the calcium ionophore A23187 in the 0.5–2.5 μ*M* range leads to a progressive increase in NO formation and that this response is abolished when the ionophore is utilized at 7.5 μ*M*. It was then demonstrated that high concentrations of A23187 cause an extensive $cPLA_2$-dependent release of AA, which then promotes a nNOS inhibitory signaling pathway. Consistently, exogenous AA suppressed formation of NO mediated by lower concentrations of A23187.

Thus, sustained activation of $cPLA_2$ is expected to promote a rapid inactivation of nNOS via the AA-dependent inhibitory signaling. It is therefore intriguing that both LPS and IFN-γ potently stimulate the activity of $cPLA_2$ (49, 54). This consideration led to the hypothesis that AA may represent the upstream signal triggering tyrosine kinase-dependent phosphorylation, and inactivation of nNOS after stimulation with LPS/IFN-γ. We also made the additional consideration that AA would have been a very good candidate since its ability to promote tyrosine kinase activation had already been described (39) and, in our opinion, it made sense that an inflammatory response is triggered by a product largely available at the inflammatory sites. Using a variety of experimental approaches, it was found that indeed AA promotes inhibition of nNOS activity (41). In addition, experimental evidence that LPS/IFN-γ itself is a potential stimulus for nNOS was also provided, since formation of NO was readily detected in cells in which $cPLA_2$ was pharmacologically inhibited or genetically depleted. This response was prevented by nanomolar levels of exogenous AA. Under normal conditions, LPS/IFN-γ fails to generate NO via nNOS activation simply because the parallel stimulation of $cPLA_2$ triggers the AA-dependent nNOS inhibitory signaling.

Because of the well-established notion that suppression of nNOS activity is an early necessary event for cytokine-induced NF-κB activation and iNOS expression, the role of AA in these responses was also investigated. As expected, inactivation of nNOS by AA provided optimal conditions for the LPS/IFN-γ-induced NF-κB activation and subsequent iNOS expression. Furthermore inhibition of $cPLA_2$ activity, while reducing the availability of AA, consistently inhibited NF-κB activation and iNOS mRNA induction and delayed NO formation. These responses were promptly re-established by addition of exogenous AA.

As described above, the LPS/IFN-γ-induced inhibition of nNOS activity is mediated by tyrosine phosphorylation (9). Further results showed that the LPS/IFN-γ-dependent tyrosine phosphorylation of nNOS was indeed triggered by endogenous AA (41). Thus, the mechanism whereby LPS and IFN-γ impair nNOS activity in glial cell lines as well as rat primary astrocytes involves an AA-dependent tyrosine phosphorylation of the enzyme, and this event is causally-linked to NF-κB activation and iNOS expression.

Pathological implications

The discovery of the cross-talk between cNOS and iNOS has also led to the development of a novel hypothesis for the neurotoxic effects mediated by high levels of NO, produced by iNOS, in Alzheimer's disease (AD) (26). This hypothesis can be summarized as follows: basal levels of NO maintained by cNOS (i.e., nNOS and/or eNOS) play a critical role in neuro- and vascular protection (15). At the early stages of AD, an underlying β-amyloid-driven process may contribute to the development of the pathology by lowering basal NO levels (i.e., constitutive NO) (15, 34, 48) a condition that would then facilitate the expression of NF-κB-dependent genes, including iNOS.

The mechanism whereby the β-amyloid (Aβ) peptide inhibits cNOS activity is still poorly understood. Recent evidence indicates that soluble Aβ (25–35) interacts with NADPH *in vitro* and the occurrence of this event *in vivo* might well cause inhibition of cNOS activity (52). According to the cross-talk between cNOS and iNOS, the repression of cNOS activity by Aβ (25–35) might then result in enhanced iNOS expression, an event that was indeed observed upon supplementation of suboptimal concentrations of the cocktail LPS/IFN-γ. It is, however, unlikely that the Aβ peptide impairs cNOS activity only by lowering the availability of the redox cofactor and additional/alternative mechanisms are likely to be involved. Since the Aβ peptides can affect a variety of cellular processes and signal transduction pathways, inhibition of cNOS activity may take place as a consequence of changes in the phosphorylation of the protein. Soluble globular Aβ (1–40) localized in the vascular endothelium was indeed reported to cause inhibition of eNOS enzymatic activity via a mechanism involving an increase in $[Ca^{2+}]_i$ associated with PKC-dependent phosphorylation of the protein (17). Future studies should determine the generality of this mechanism (e.g., in other cell types including astrocytes) and whether additional Ca^{2+}-dependent signalling pathways (e.g., PLA_2/tyrosine kinase) mediate, or contribute to, the inhibition of cNOS activity caused by β-amyloid peptides.

As indicated throughout this review, it is likely that the cross-talk between cNOS and iNOS or, more generally, proteins expressed under the transcriptional regulation mediated by NF-κB is critically involved in a variety of pathological conditions associated with inflammation. Hence, all the treatments that either enhance constitutive NO formation or decrease cNOS inhibition mediated by proinflammatory cytokines are expected to inhibit the expression of NF-κB-dependent genes. This event would obviously mitigate the underlying inflammatory response and thus mediate beneficial effects for the host.

CONCLUDING REMARKS

The theory of the cross-talk between cNOS and iNOS has been challenged for several years, and the notion that an early decline in constitutive NO is critically involved in the inflammatory response is now widely accepted. This event is indeed required to allow the activation of NF-κB and the ensuing expression of iNOS stimulated by inflammatory cytokines. It would make sense that some product available at the inflammatory sites is responsible for the inhibition of cNOS activity and recent data indicates that this effect may be mediated by AA (40, 41). AA is extensively released during inflammation and, under these conditions, exogenous and endogenous AA may cause profound effects on cytokine-induced NF-κB activation and iNOS expression (Fig. 1). As a consequence, inhibition of PLA_2 may represent an effective strategy to prevent, or mini-

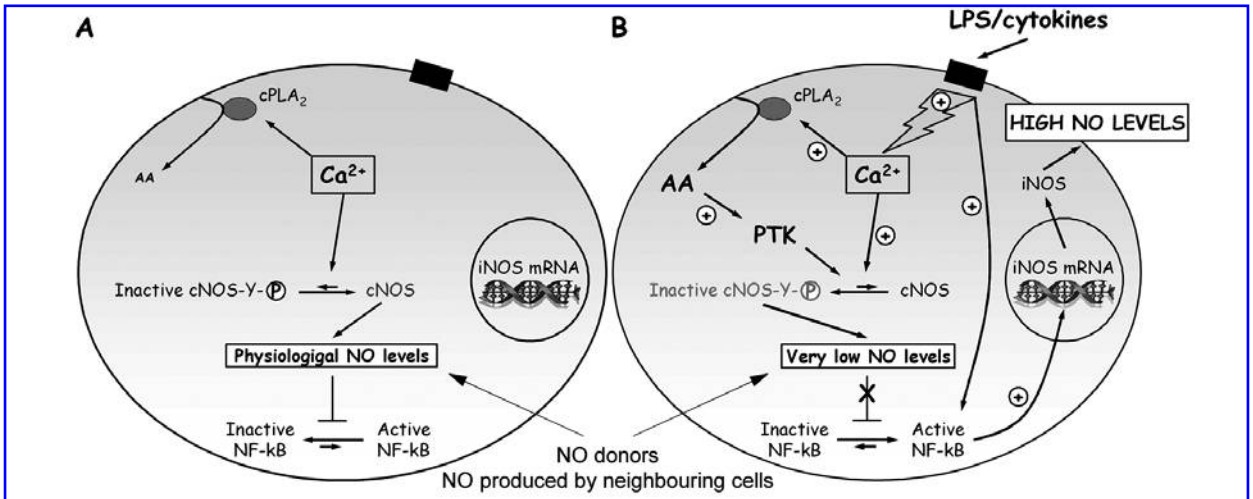

FIG. 1. The cross-talk between nNOS and iNOS. (**A**) Under normal conditions, low amounts of NO (presumably n*M*) generated by cNOS within the same, or from surrounding, cells, or released by exogenous donors, inhibits NF-κB activity. Under these conditions, $cPLA_2$ releases low levels of AA, as a consequence of membrane lipids turnover. (**B**) At the early phase of the inflammatory response, LPS/proinflammatory cytokines promote a sudden increase in the intracellular Ca^{2+} concentration, resulting in sustained activation of $cPLA_2$ and extensive release of AA. Under these conditions, the lipid messenger stimulates the activity of a protein tyrosine kinase (PTK) that causes phosphorylation and inactivation of cNOS, thereby lowering the intracellular NO concentration. LPS/proinflammatory cytokines can thus activate NF-κB and promote iNOS expression, an event associated with the release of large amounts of NO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mize, the expression of NF-κB-dependent proteins. The identification of additional signaling pathways leading to cNOS inhibition after stimulation by proinflammatory cytokines may provide the bases for the development of novel pharmacological strategies to reduce the impact of the inflammatory response.

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ABBREVIATIONS

Aβ, β-amyloid; AA, arachidonic acid; AD, Alzheimer's disease; cNOS, constitutive NOS; cPLA2, cytosolic phospholipase A_2; eNOS, endothelial NOS; IFN-γ, interferon-γ; IGF, insulin-like growth factor; IL-1β, interleukin-1β; iNOS, inducible NOS; L-NAME, nitro-L-arginine methyl ester; LPS, *Escherichia coli* lipopolysaccharide; NF-κB, nuclear factor-κB; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; NOS-I, neuronal NOS; NOS-II, inducible NOS; NOS-III, endothelial NOS; PKC, protein kinase C; PTK, protein tyrosine kinase; TNF-α, tumor necrosis factor-α.

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