S-nitrosylation: Physiological regulation of NF-κB

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A spate of recent discoveries indicates that the study of protein S-nitrosylation is rapidly leaving its salad days behind. S-nitrosylation, the formation of *S*-nitrosothiol (SNO) by covalent addition to cysteine (Cys) residues of a nitric oxide (NO) moiety (formally as NO^+), has been shown to regulate in intact cells the function of a broad spectrum of proteins (1). Important recent findings include demonstrations of major roles for S-nitrosylation in vesicle-mediated insulin release (2), in protein processing associated with the neurodegeneration of Parkinson's disease (3), and in the essential mechanisms of vectorial membrane trafficking (4). In this issue of PNAS, Reynaert *et al.* (5) lend a hand in the unveiling of S-nitrosylation as it operates in cellular context by elucidating a role in regulating NF-κB. NF-κB denotes a ubiquitous family of transcription factors that transduce a wide range of noxious or inflammatory stimuli into the coordinated activation of multiple genes, including those coding for cytokines, cytokine receptors, adhesion molecules, and antiapoptotic proteins (6). NF-κB thus serves as a critical element in immune and inflammatory responses and in cell survival and proliferation, with central roles in host defense and in acute and chronic disorders of immune function. NF-κB can upregulate the expression of all major NO synthases (nNOS, iNOS, and eNOS).

It is well established that NF-κB is complexed with and sequestered in the cytoplasm by inhibitory IκB (inhibitor of NF-κB) proteins and that many activating stimuli induce phosphorylation of IκB by the IκB-kinase complex (IKKα, IKKβ, and IKKγ), initiate IκB ubiquitinylation and degradation by means of the 26S proteasome, and allow translocation of NF-κB to the nucleus (6). The molecular cascades that run through NF-κB present multiple loci at which oxidative/nitrosative modification could potentially modulate signal transduction, but the role of NF-κB and its attendant proteins in physiological redox responsivity has remained controversial, principally because of the lack of evidence for their direct redox-based modification in the context of physiological signal transduction. The prototype of the NF-κB family is the p50/p65 heterodimer expressed constitutively in most mammalian cells. S-nitrosylation of NF-κB *in vitro* or in intact cells, either with exogenous NO or consequent upon induction of iNOS, inhibits NF-κB-dependent DNA binding, promoter activity, and gene transcription (7, 8). Analysis *in vitro* indicated that p50 is S-nitrosylated at Cys-62, which is located in the N-terminal DNA binding loop within the Rel-homology domain. Cys-62 is conserved in other Rel-homology domain-containing proteins that can serve as NF-κB subunits, including p65, p52, p100, p105, and c-Rel. In addition, it was shown that treatment of intact cells with either NO or SNO significantly enhances tumor necrosis factor (TNF)-α-induced apoptosis in a cGMP-independent fashion and that this facilitation may reflect not only reduced DNA-binding affinity of NF-κB but also decreased IκB degradation, thereby preventing the nuclear translocation of NF-κB (9). Thus, it appeared that S-nitrosylation (of as yet unidentified elements) might also regulate the phosphorylation-dependent proteasomal targeting of IκB. The central finding of Reynaert *et al.* (5) is that S-nitrosylation of the catalytic IKKβ subunit of the IKK complex inhibits IκB phosphorylation. It is further shown that TNF-α activation of IKKβ is coordinated with denitrosylation.

S-nitrosylation of IKKβ Inhibits IκB Phosphorylation

These investigators first demonstrated that phosphorylation of IκB *in vitro* by activated IKK was suppressed by exposure of IKK to the endogenous or synthetic NO^+ donors, *S*-nitroso-glutathione, or *S*-nitroso-*N*-acetyl-D,L-penicillamine. Both of these S-nitrosylating agents also inhibited TNF-α-induced IKK activation and IκB phosphorylation in intact C10 and Jurkat T cells, although L-cysteine was a potent cofactor, evidently serving to facilitate cellular uptake of S-nitrosylating equivalents, inasmuch as L-Cys-NO (but not D-Cys-NO) was a particularly effective inhibitor. It should be noted that L-Cys-NO was effective at doses (e.g., 10 μM) that would predictably yield levels of cellular SNO well within the physiological range (9).

IKKβ was identified directly as a cellular substrate for S-nitrosylation by chemiluminescent analysis of the SNO content of IKKβ immunoprecipitates from cells treated with nitrosothiols and by the labeling of SNO proteins in cell lysates with the biotin-switch method followed by immunoprecipitation of IKKβ. Importantly, application of the biotin-switch method revealed basal S-nitrosylation of IKKβ in Jurkat T cells, which decreased in response to treatment with TNF-α. In addition, pharmacologic inhibition of endogenous NOS reduced IKKβ S-nitrosylation and enhanced basal and TNF-α-induced IKK activity. Although the source of endogenous NO was not examined, mononuclear cells may express nNOS or eNOS in addition to iNOS (5, 10).

Phosphorylation of serines 177 and 181 is required for activation of IKKβ by TNF-α, and covalent modification (e.g., arsenite) of the interjacent Cys-179, conserved in IKKα, is known to inhibit IKK activity (11). Reynaert *et al.* (5) found that mutation of Cys-179 to alanine substantially reduced both inhibition of TNF-α-induced activation of IKKβ and S-nitrosylation of IKKβ after treatment with NO^+ donors of cells transfected with wild-type or mutant IKKβ. Residual effects may indicate the presence of additional sites within IKKβ susceptible to S-nitrosylation. Although the molecular mechanism of inhibition of IKKβ kinase activity by S-nitrosylation of Cys-179 is unknown, it is significant that treatment with SNO had no effect on TNF-α-induced phosphorylation of IKK itself and that IKKβ activity could be inhibited by S-nitrosylation subsequent to activation by TNF-α. Thus, intercalation of an NO group at Cys-179 within the "activation loop" of IKKβ is apparently sufficient to modulate kinase function.

A number of previous studies have reported oxidative activation of IKK; most have measured phosphorylation of IκBα without specifying the IKK isoform involved or the mechanism of activation. There is evidence that both IKKα and IKKβ can be activated by H_2O_2 *in situ* and use IκBα as substrate (12). In combination with the finding that NO/oxidative modification (e.g., arsenite and cyclopentenone) of Cys-179 inhibits IKKβ (11, 13), these observations suggest that redox activation is indirect (perhaps through inhibition of protein phosphatases). However, the possibility remains that IKKβ contains additional redox-sensitive Cys or that different redox modifications of Cys-179 (e.g., S-glutathionylation and S-hydroxylation versus S-nitrosylation) can exert different effects on kinase activity by analogy to the bacterial transcriptional activator OxyR (14).

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Multifaceted Regulation of NF-κB by S-nitrosylation

The findings of Reynaert *et al.* (5) contribute to a more nuanced view of the role of NO in regulating NF-κB activity. In combination with prior descriptions of S-nitrosylation of NF-κB p50 and of multiple elements upstream of the NF-κB–IκB–IKK complex, their results highlight the fact that transduction through signaling pathways is regulated coordinately by S-nitrosylation at multiple steps.

NO can activate NF-κB through S-nitrosylation and activation of the small G protein $p21^{ras}$ (15). Stimulation of $p21^{ras}$ guanine nucleotide exchange activates downstream effectors (including NF-κB) through the PI3K-Akt pathway. Thioredoxin is also activated by S-nitrosylation (16). Cytokine stimulation of NF-κB induces nuclear translocation of thioredoxin, where it reduces Cys-62 of NF-κB p50 through an interaction with the nuclear protein Ref-1 (17). Reduction of Cys-62 of p50 allows for p50–p65 DNA binding and NF-κB-dependent transcription. Interestingly, cytoplasmic overexpression of thioredoxin has been shown to inhibit NF-κB (18), perhaps because the denitrosylation and consequent activation of NOS promotes S-nitrosylation of IKKβ (19).

Several proteins identified as active in the NF-κB pathway and subject to S-nitrosylation are, like p50 and IKKβ, inhibited by the modification. Apoptosis-related signaling kinase 1 (ASK1), a mitogen-activated protein kinase kinase, is known to activate NF-κB by phosphorylation of IKBα (22), and more recent data points to direct inhibition of ASK1 by S-nitrosylation (21). c-Jun N-terminal kinase (JNK)1 is another mitogen-activated protein kinase family member reportedly regulated by S-nitrosylation. Cytokine-stimulated iNOS activity has been shown to S-nitrosylate and inactivate JNK1 in macrophages (22). Interestingly, Raynaert *et al.* (5) did not detect inhibition of JNK1 activity by SNO treatment *in vitro*. These discrepant results may reflect differences in cell type (i.e., respiratory epithelium versus macrophage) and activating stimulus (TNF-α versus IFN-γ). Cell-type-specific effects of NOS on kinase regulation have been shown for Tyk2 kinase (natural killer cell versus T cell) (23).

Other proven or likely targets for nitrosylation in the NF-κB pathway include the epidermal growth factor and src tyrosine kinases (24, 25), which activate NF-κB through $p21^{ras}$; tyrosine phosphatases (26); and NADPH oxidase (27), known to activate NF-κB through IKK (presumably by means of the oxidative inactivation of phosphatase). Given the multiple loci of S-nitrosylation in NF-κB-related pathways, the effects of S-nitrosylation may be considered analogous to those of phosphorylation, with a large number of regulatory permutations that combine ultimately to optimize cellular responses.

Emerging Tenets of S-nitrosylation

An additional theme reinforced by the results of Reynaert *et al.* (5) is the emerging understanding that regulation by S-nitrosylation is often exerted through control of protein stability via modulation of ubiquitinylation and proteasomal degradation. S-nitrosylation has been found to regulate the activity of hypoxia-inducible factor, tumor suppressor p53, iron-response proteins, as well as IKK–IκB–NF-κB by regulating the degradation of the S-nitrosylated protein or a regulatory partner. Thus, although the mechanism and locus of action of S-nitrosylation differ from case to case, the influence of S-nitrosylation is, in a growing number of cases, reflected in altered proteasomal targeting. Very recently, ubiquitin ligases themselves were identified as targets for S-nitrosylation. In particular, parkin, an E3 ubiquitin ligase, is inhibited by S-nitrosylation in neuronal tissue after activation of either nNOS or iNOS (3).

A number of additional major themes, emerging from the burgeoning analyses of S-nitrosylation *in situ*, are highlighted by the findings of Reynaert *et al.* (5). First, the critical role of Cys-179 in IKKβ emphasizes that regulation of protein function by S-nitrosylation is consistently found to involve one or a very few Cys residues, which reflects specific targeting that is subserved by multiple aspects of protein structure and protein–protein interaction. In the case of IKKβ, a role for phosphorylation of Ser-177 and Ser-181 in modulating S-nitrosylation of Cys-179 remains an intriguing possibility. In addition, the results of Reynaert *et al.* (5) reinforce previous findings, which suggest that nucleotide-binding proteins, including a broad spectrum of kinases, G proteins, and ATPases, comprise one prominent set of substrates.

Finally, the finding that IKKβ is S-nitrosylated constitutively and that activation by TNF-α is associated with denitrosylation reinforces the emerging understanding that, as for other precisely regulated posttranslational modifications, the influence of S-nitrosylation on protein function will be subserved by mechanisms that govern both addition and removal of the NO group from Cys thiol. In particular, apoptosis triggered by Fas stimulation is associated with activating denitrosylation of some caspase isoforms (10), and it should be noted that TNF-α stimulation has been shown to trigger caspase and NF-κB denitrosylation as well (8, 28). The mechanism(s) of regulated denitrosylation remains an outstanding issue.

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