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# Forum Review

# Zinc Finger Proteins as Molecular Targets for Nitric Oxide-Mediated Gene Regulation

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## ABSTRACT

**A multiplicity of biological functions have been ascribed to nitric oxide (NO). It plays a significant role as a signal as well as a cytotoxic effector molecule. NO may, however, also play regulatory and/or modulatory roles in biology. A growing body of evidence suggests that posttranslational modifications of transcription factors serve a regulating role on gene transcription, particularly after changes of the redox state of the cell. Zinc fingers are the most prevalent transcription factor DNA-binding motif. As NO is able to *S*-nitrosate thiols of zinc-sulfur clusters leading to reversible disruption of zinc finger structures, this provides a molecular mechanism to regulate the transcription of genes. Current knowledge about effects of NO on the cellular zinc homeostasis and on the gene-regulating activity of zinc finger transcription factors is reviewed.** Antioxid. Redox Signal. 3, 565–575.

## INTRODUCTION

DURING THE LAST 15 YEARS, THE RADICAL NITRIC OXIDE (NO), previously known as an air pollutant only, has been found to be an important signal molecule in mammalian, plant, and microbial cells (for reviews, see 7, 29, 39, 40). The signal molecule NO is synthesized on demand in a tightly regulated fashion for short periods of time by two constitutively expressed NO synthases (endothelial and neuronal NO synthases, respectively). However, after activation by inflammatory mediators like proinflammatory cytokines [*e.g.*, interleukin-1$\beta$ (IL-1$\beta$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interferon-$\gamma$] and/or bacterial products (*e.g.*, lipopolysaccharide), an inducible NO synthase (iNOS) is expressed. Once expressed, the iNOS is active as long as the enzyme is functionally intact, substrate and essential cofactors are available, and the effector cell is viable. The consequence of an active iNOS thus is a prolonged (hours to days) synthesis of NO.

Although NO is a radical, its reactivity in biological systems is relatively low. Due to its small size and its lipophilicity, NO easily crosses cell membranes. However, NO will react with oxygen, thus explaining its oxidation under physiological conditions. This reaction is of third order, with two molecules of NO reacting with one molecule of $O_2$ (Fig. 1). Thus, the half-life of NO depends almost exclusively on the concentration of NO. In other words, the higher the concentrations of NO, the more likely its reactions with oxygen. In addition, hydrophobic milieus like the cell membrane can

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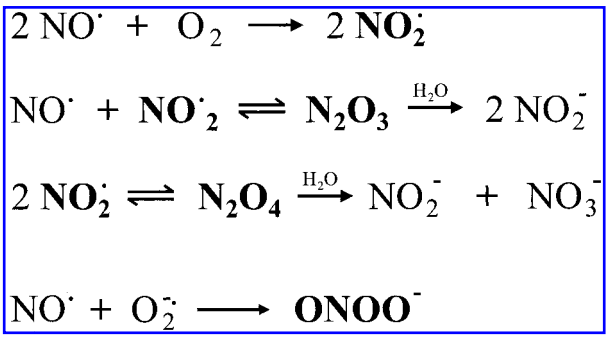


**FIG. 1. Reaction products of NO with oxygen and the superoxide anion radical.** The multiple chemical reactions are highly dependent on the local NO concentrations. Stable oxidation products of NO are nitrite ($NO_2^-$) and nitrate ($NO_3^-$). NO may also react with the superoxide anion ($O_2^{-\cdot}$), yielding the strong oxidant peroxynitrite anion ($ONOO^-$). Unstable and highly reactive intermediate products are shown in bold.

accelerate the reaction of NO with $O_2$ several hundredfold (21). Products of this reaction are the so-called reactive nitrogen oxide intermediates (RNOI), also termed higher nitrogen oxides ($NO_x$) like $NO_2$, $N_2O_3$, and $N_2O_4$ (Fig. 1) (for reviews, see 34, 44). These RNOI are highly reactive and short-lived species and exhibit a much broader reaction spectrum toward biomolecules than NO itself. RNOI may induce "nitrosative stress," a term introduced by J. Stamler (11). Thus, it is primarily the concentration of NO that determines whether NO acts as a signal molecule or whether nitrosative stress is induced by $NO_x$. This may explain the signaling functions of NO produced by the constitutive NO synthase, resulting in low local concentrations of NO for short periods of time. On the other hand, unregulated iNOS activity results in highly increased local NO concentrations (probably in the low micromolar range) and thus elevated levels of RNOI for extended periods of time. In addition, NO may also react with the superoxide anion radical ($O_2^{-\cdot}$) yielding the strong oxidant peroxynitrite (Fig. 1). It is this complex chemistry of reactive oxygen and reactive nitrogen oxide species that plays a key role in regulating the cellular redox status (for review, see 32). For simplicity, the term NO is used in the following, although the exact nitrogen oxide species involved is often not defined.

In the early 1990s, it was found that activated macrophages are able to kill not only parasites via NO (for reviews, see 4, 30), but also certain

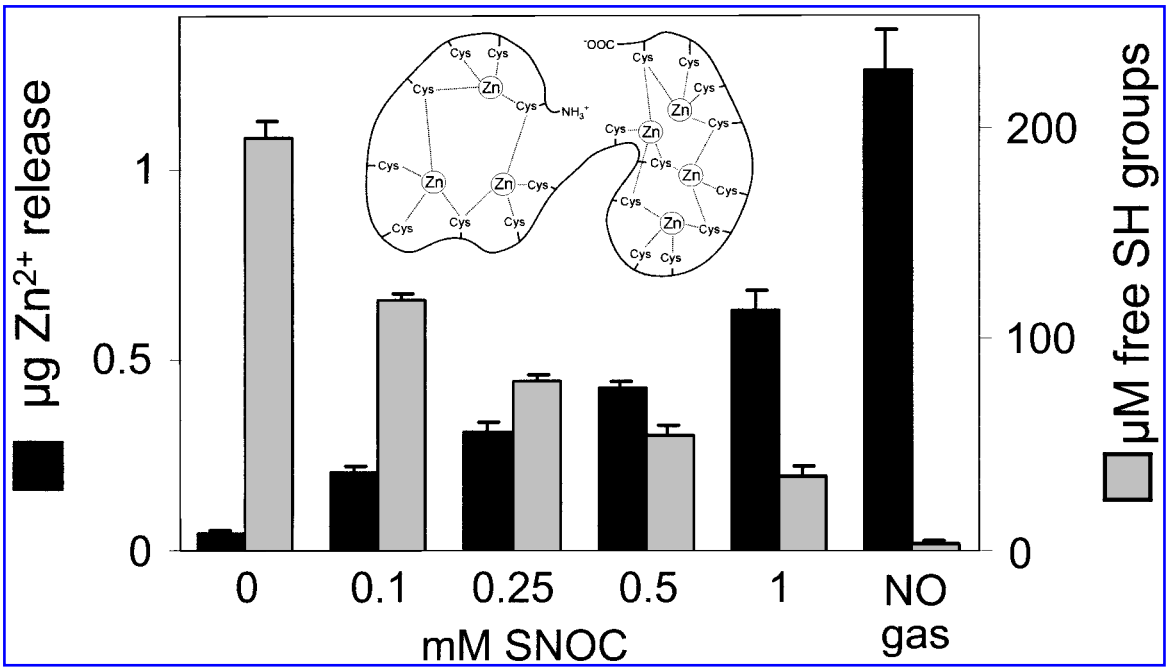


**FIG. 2. NO mediates $Zn^{2+}$ release from metallothionein concomitant with a decrease of free SH groups.** Metallothionein (50 $\mu$g; see **inset** drawn according to 42) was incubated under aerobic conditions for 1 h at 37°C with graded concentrations of SNOC or with NO gas. Subsequently, the $Zn^{2+}$ concentrations in the supernatant, as well as the free thiol content of metallothionein, were determined as described (18).

types of susceptible mammalian cells, for instance, pancreatic islet cells (16). At that time, iNOS-derived NO was thought to act as a cytotoxic molecule only. However, it was soon noticed that not all mammalian cell types show the same sensitivity toward NO (17). In the last few years, our understanding of the role of iNOS-mediated NO synthesis has changed considerably, mostly due to recent findings demonstrating protective effects for iNOS-derived NO (9, 36, 37, 41), as well as observations on altered gene expression exerted by high-output NO synthesis (for reviews, see 20, 23). As the importance of zinc fingers as protein domains mediating DNA and RNA binding was increasingly recognized, we hypothesized that in addition to well known intracellular molecular targets of NO like heme groups, protein iron-sulfur clusters, and thiols, protein zinc-sulfur clusters might also represent molecular targets for NO.

## WORKING WITH NO

To analyze NO-mediated effects, NO-releasing compounds provide a controllable and easy way of exposing targets to NO. The two main and commonly used classes of NO donor compounds are the *S*-nitrosothiols and the diazeniumdiolates (for reviews, see 12, 19, 25). *S*-Nitrosothiols like *S*-nitrosocysteine (SNOC) or *S*-nitrosoglutathione can be detected in cells treated with NO and may thus be regarded as "physiological" NO donors. Their decomposition is accelerated by free thiols probably via nucleophilic sulfur attack and subsequent transfer of their NO moiety (transnitrosation). In addition, $Cu^{1+}$ and $Fe^{2+}$, as well as light, have been shown to induce NO release from *S*-nitrosothiols. Thus, degradation and NO generation rates of *S*-nitrosothiols are not predictable and strongly depend on the conditions used. Diazeniumdiolates (also known as NONOates or polyamine/NO complexes) are currently those NO donors that release NO in the most predictable and controllable way. They spontaneously release pure NO under physiological conditions with defined NO generation rates (for review, see 13).

## NO MEDIATES ZINC RELEASE FROM METALLOTHIONEIN

To investigate effects of NO on zinc finger structures, we used the zinc-sulfur cluster protein metallothionein as a model protein. Metallothionein is thought to have at least two functions, firstly to sequester $Zn^{2+}$ and secondly to release it by events signaling $Zn^{2+}$ requirements (22). When we investigated

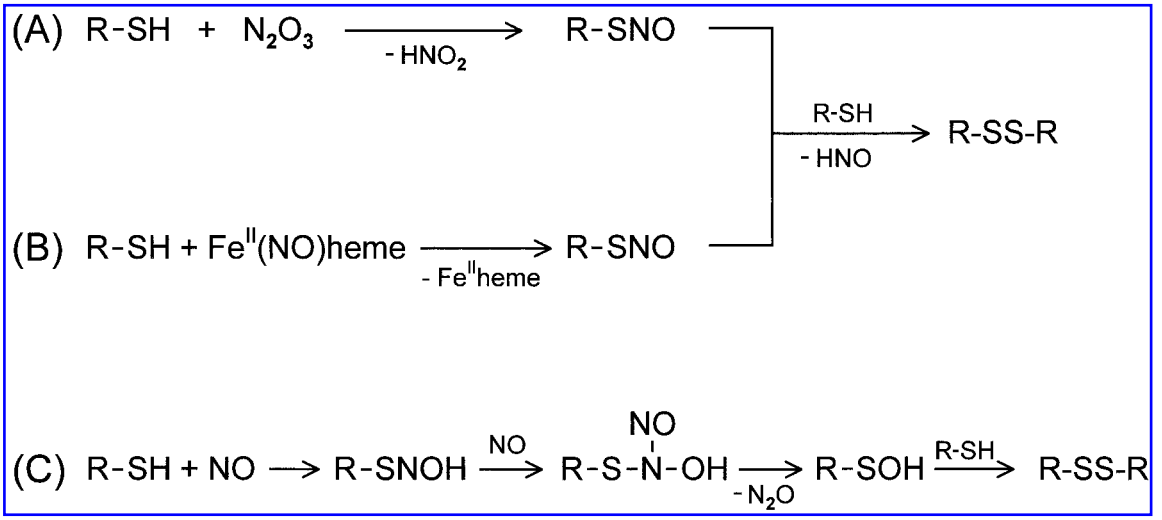


**FIG. 3. Oxidation of thiols by NO.** Under aerobic conditions, NO *S*-nitrosates thiols RNOI such as $N_2O_3$ (**A**). NO bound to heme may also lead to *S*-nitrosothiol formation (**B**). Reaction of *S*-nitrosothiols with thiols leads to disulfide formation. Under anaerobic conditions, pathway (**C**) has been identified. Thus, NO directly or indirectly oxidizes thiols to the corresponding disulfides.

whether NO will affect the zinc-complexing activity of metallothionein, we found that under aerobic conditions NO in a concentration-dependent manner mediates zinc release from this protein and diminishes the thiol content of metallothionein (Fig. 2) (18). This has now been confirmed by several other groups (1, 26, 27). As NO may *S*-nitrosate thiols under physiological conditions (Fig. 3) (for review, see 8), we investigated whether *S*-nitrosation of metallothionein cysteine residues and subsequent disulfide formation might be the molecular mechanism for the NO-mediated zinc release from metallothionein. Indeed, under aerobic conditions, a temporary absorption with a maximum at ~350 nm indicative for *S*-nitrosothiol formation can be observed (1, 18, 27). Analysis by raman spectroscopy showed an absorption with a maximum at 541 $cm^{-1}$ suggesting disulfides (18). Anaerobic conditions lead to NO-mediated $Zn^{2+}$ release from metallothionein also (1), probably occurring via reaction C shown in Fig. 3. These results show that indeed zinc-sulfur clusters are molecular targets for NO.

## NITROSATIVE STRESS CAUSES ZINC RELEASE IN WHOLE CELLS

Having found that NO mediates $Zn^{2+}$ release from the main zinc-storing protein *in vitro*, we next wanted to learn whether NO may influence the zinc homeostasis in whole cells. Several cell types were therefore cultured in the presence of nontoxic concentrations of various NO donors (*S*-nitrosothiols and diazeniumdiolates). By using $Zn^{2+}$-specific fluorophores, it could be demonstrated that NO-mediated nitrosative stress indeed induces $Zn^{2+}$ release

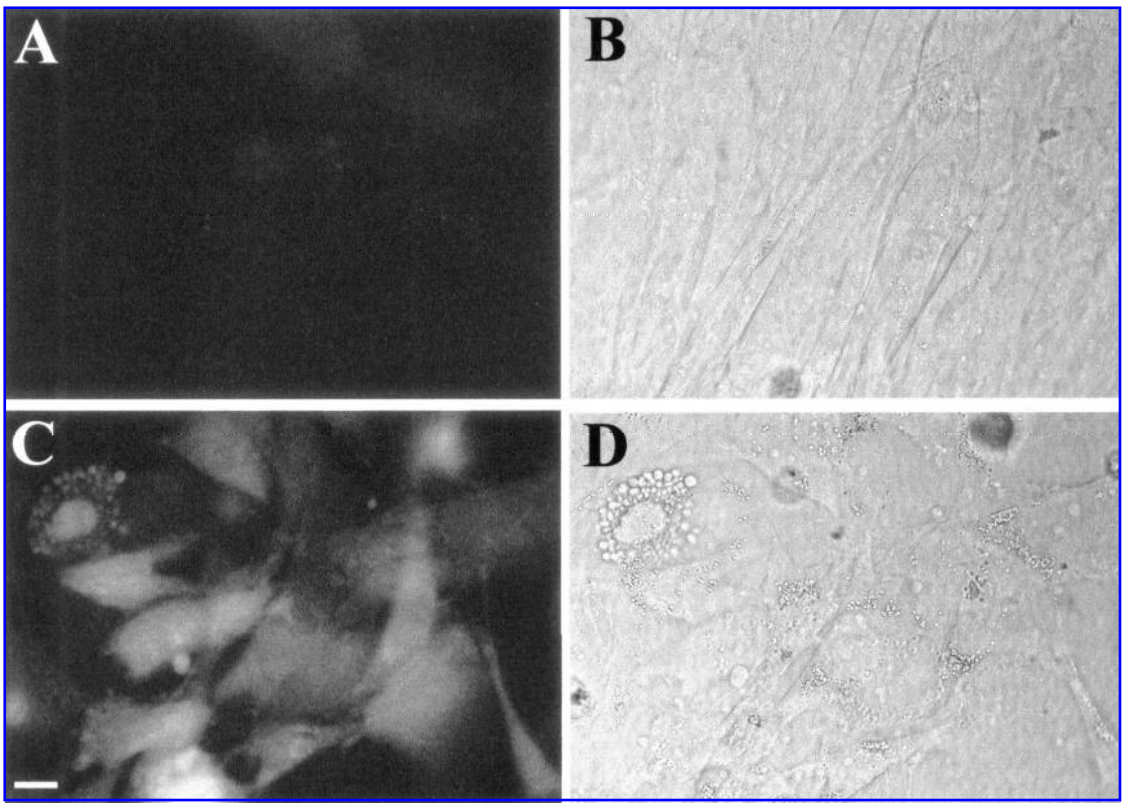


**FIG. 4. Exogenously added NO mediates cytoplasmic and nuclear $Zn^{2+}$ release in rat aortic endothelial cells.** Cells were cultured in the absence (**A** and **B**) or presence of 1 m*M* of the slowly NO-releasing diazeniumdiolate DETA/NO (**C** and **D**) for 24 h in RPMI/20% fetal calf serum. Subsequently, live cells were stained with the $Zn^{2+}$-specific fluorophore Zinquin ethyl ester and investigated under the fluorescence microscope (2). Whereas control cells show only low amounts of free intracellular $Zn^{2+}$, NO-mediated nitrosative stress induces a massive increase of labile intracellular $Zn^{2+}$. Bar = 8 $\mu$m.

within the cytoplasm, as well as sometimes also in the nuclei of lymphocytes, fibroblasts, and endothelial cells (Fig. 4) (2) and in glioma cells (10). In addition, administration of NO donors into the dorsal hippocampus of rats results in mobilization of $Zn^{2+}$ in neuronal perikarya (6). In a very elegant study, Pearce et al. (33) used cells transfected with metallothionein fused to green fluorescent protein and the fluorescent resonance energy transfer technique. They demonstrated that NO mediates $Zn^{2+}$ release from metallothionein in live endothelial cells (33). All these results show that NO-mediated nitrosative stress will alter the $Zn^{2+}$ homeostasis in cells.

Whether the intracellular zinc remains in a free pool available to rebind to zinc fingers in proteins has to be investigated. To show whether oxidative stress induces $Zn^{2+}$ release in cells also, fibroblasts were incubated with 0.5–5 m*M* $H_2O_2$ for 1 h at 37°C. However, at no $H_2O_2$ concentration could we see increased intracellular $Zn^{2+}$ release compared with that from untreated cells (Berendji and Kröncke, unpublished observation).

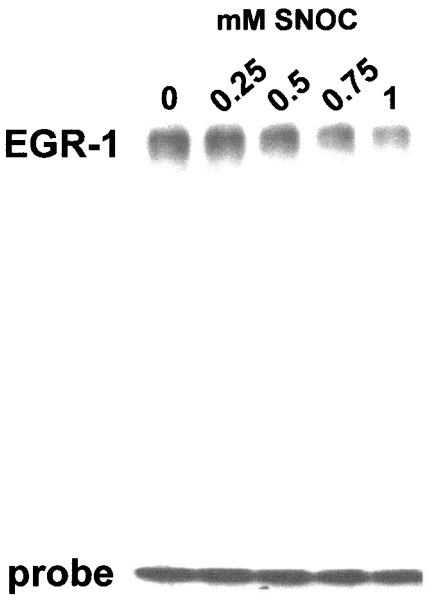


**FIG. 5. SNOC in a concentration dependent manner inhibits the DNA-binding activity of recombinant EGR-1.** Recombinant EGR-1 was incubated with increasing concentrations of SNOC for 30 min at room temperature. Subsequently, gel shift experiments were performed using a $GC_7$ EGR consensus site as described (38). Results show that SNOC inhibits the DNA-binding activity of EGR-1 in a concentration-dependent manner.

## NO MEDIATES REVERSIBLE INHIBITION OF THE DNA-BINDING ACTIVITY OF ZINC FINGER TRANSCRIPTION FACTORS *IN VITRO*

A first demonstration for a possible regulatory role of NO in cellular gene expression was the finding that NO under aerobic conditions abrogated the DNA-binding activity of the yeast transcriptional activator LAC9 containing a zinc finger indispensable for its activity (18). Indeed, we now know that NO can inhibit the DNA-binding activities of transcription factors containing one or several cysteines within or near their DNA-binding domains (DBD), *i.e.*, of activator protein-1 (AP-1) (31), of nuclear factor-$\kappa$B (24, 28) or of c-Myb (5).

Sp1 and early growth response factor-1 (EGR-1) are widely distributed zinc finger transcription factors that are involved in regulating the transcription of numerous mammalian constitutively expressed or inducible genes. Using these as NO targets, we saw that both recombinant Sp1 (4) and recombinant EGR-1 (Fig. 5) will progressively lose their DNA-binding activity upon treatment with increasing concentrations of SNOC. In addition, incubation of lymphocyte nuclear extracts with SNOC also resulted in inhibition of the Sp1 DNA-binding activity (3). These results suggest that NO, comparable to the effects toward metallothionein, mediates zinc release from the zinc finger domains of Sp1 and EGR-1. This results in conformational changes of the DBD of the protein and thus loss of their capacity to bind to their specific DNA response elements (Fig. 6). To investigate whether NO might have effects on other transcription factors as well, we also incubated nuclear factor of activated T cell (NFAT), a transcription factor lacking zinc fingers or cysteines in the DBD, with SNOC. However, even relatively high SNOC concentrations did not affect the DNA-binding activity of NFAT (3), indicating a selective action of NO toward redox-sensitive transcription factors.

Another very important family of zinc finger transcription factors is the nuclear receptor superfamily, the members of which contain a highly conserved DBD in their amino-terminal

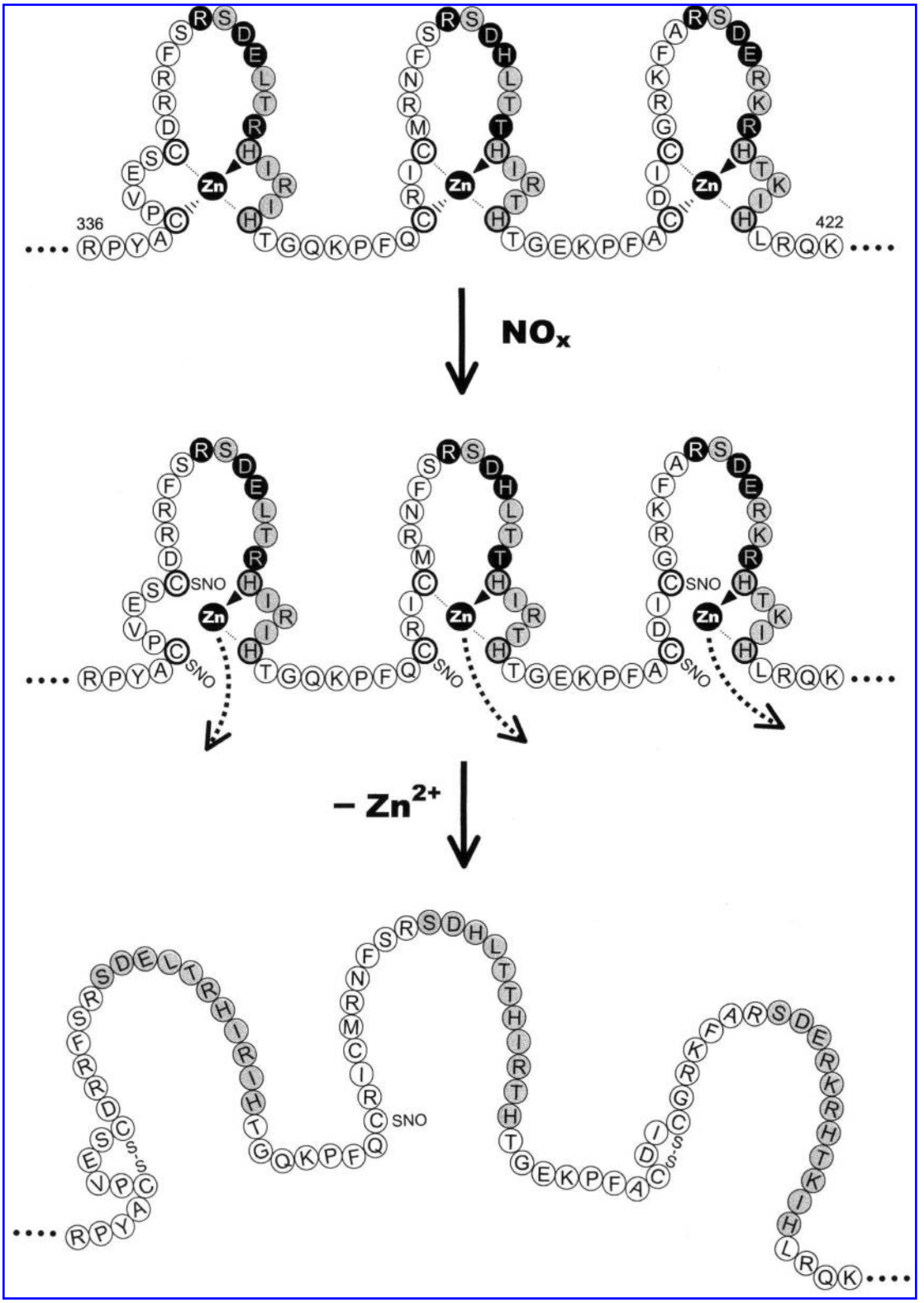


**FIG. 6. Molecular mechanism of NO-mediated disruption of protein zinc finger domains.** Shown are the three zinc fingers of EGR-1 according to (14). Amino acid residues that directly bind to the DNA are highlighted in black, and $\alpha$-helices are marked in gray. NO under aerobic conditions *S*-nitrosates cysteine thiols, thus leading to $Zn^{2+}$ ejection and subsequent changes of the zinc finger structures. The structure of EGR-1 after NO treatment is not yet known and thus is hypothetical.

repeat formed by two zinc fingers containing four cysteines each. The vitamin $D_3$ receptor (VDR) and the retinoid X receptor (RXR) are representative members of this superfamily. VDR and RXR form heterodimeric complexes that bind to specific sequences in the promoter region of vitamin $D_3$ (VD) target genes, commonly referred to as VD response elements (VDREs). Deletion studies have shown that both zinc fingers of VDR and RXR are directly involved in DNA-binding and additionally are essential for functional protein dimerization domains.

Using these well characterized nuclear receptors, we found that both SNOC and the diazeniumdiolate MAMA/NO inhibited the DNA-binding activity of *in vitro* translated VDR-RXR heterodimeric complexes in a concentration-dependent manner with similar $IC_{50}$ values (15). The DNA-binding activity can be restored by subsequent incubation with dithiothreitol (DTT) (Fig. 7), suggesting that the zinc fingers refolded properly and rebound zinc. This indicates that cells principally are able to repair zinc finger structures disrupted by NO. As neither VDR nor RXR binds to VDRE as monomers, both were individually pretreated with SNOC prior to addition of the respective counterpart. Interestingly, NO inhibited subsequent dimerization of VDR with RXR. This indicates that both heterodimeric partners are targets for the inhibitory effect of NO, and in addition, that NO is able to inhibit not only protein–DNA but also to protein–protein interactions via disruption of zinc finger domains. When using preformed VDR-RXR-VDRE complexes as target for NO, we found that two- to threefold higher SNOC concentrations were required to induce the release of VDR-RXR from their response element as compared with SNOC concentrations necessary to inhibit the DNA-binding activity of VDR-RXR heterodimers that are not DNA-bound. Thus, DNA-bound VDR-RXR heterodimers are more resistant to nitrosative stress than are free VDR-RXR. It is therefore reasonable to speculate that under *in vivo* conditions NO will affect zinc finger transcription factors mostly prior to binding to their respective DNA response elements. Thus, NO-mediated stress will affect zinc finger-dependent regulation of gene transcription mainly during the induction phase.

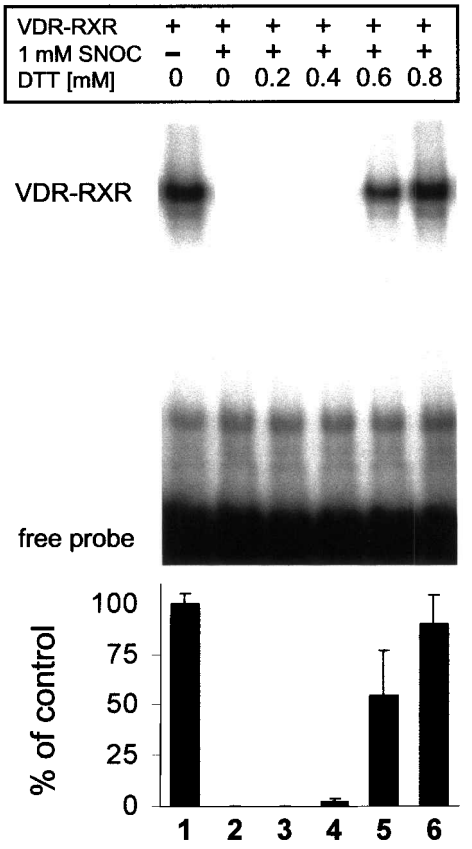


**FIG. 7. The inhibitory effect of NO on the DNA-binding activity of the VDR-RXR heterodimer is reversible.** Gel shift experiments were performed using *in vitro* translated VDR and RXR and the DR3–type VDRE of the rat ANF gene. VDR-RXR heterodimers were incubated without (lane 1) or with 1 m*M* SNOC (lanes 2–6) for 1 h at 30°C. Subsequently, DTT was added in the concentrations indicated, and incubation was continued for 30 min at 30°C. Results show that DTT in a concentration-dependent manner reverses SNOC-mediated inhibition of VDR-RXR DNA-binding activity with nearly complete recovery after treatment with 0.8 m*M* DTT.

To compare nitrosative stress with oxidative stress, we also investigated effects of peroxynitrite and $H_2O_2$, respectively, toward VDR/RXR. Peroxynitrite inhibited VDR/RXR DNA-binding within 10 s with an $IC_{50}$ of ~0.3–0.4 m*M*, which could not be reversed by DTT. In contrast, ~40–50 m*M* $H_2O_2$ was necessary to abrogate the DNA-binding activity of VDR/RXR half-maximally within 90 minutes at 30°C, and about ~50% of the DNA-binding activity could be restored by 20 m*M* DTT (Kröncke, unpublished observations). These results suggest that significant differences do exist with regard

to the effects of oxidative or nitrosative stress toward zinc finger structures.

## NO AFFECTS CELLULAR ZINC FINGER-DEPENDENT TRANSCRIPTION

To investigate whether NO may affect zinc finger-dependent transcription in whole cells, we chose induced expression of the IL-2 gene in lymphocytes. The IL-2 gene is dominantly regulated by the transcription factors Sp1/EGR-1 and/or NFAT (Fig. 8), depending on the cell culture conditions and the activation signals used. Culturing IL-1$\beta$-activated lymphocytes in the presence of nontoxic concentrations of SNOC led to a reversible inhibition of the IL-2 mRNA expression (3). The inhibition of *de novo* transcription correlated with the loss of Sp1 DNA-binding activity in nuclear cell extracts. These results indicate that NO-mediated nitrosative stress inhibits the *de novo* transcription of the IL-2 gene in lymphocytes by reversibly disrupting the zinc fingers of the transcription factor Sp1. Preactivated lymphocytes will not lose their IL-2 mRNA expression upon NO donor addition 30 min after activation with IL-1$\beta$, again indicating that transcription factors having bound to DNA are relatively resistant to NO.

In addition, we investigated whether *in vitro* effects of NO on VDR-RXR activity can also be found in live cells. A luciferase reporter gene construct carrying a VDRE fused to a minimal *tk* promoter was transiently transfected into COS-7 cells together with expression vectors for VDR and RXR. After 4 h, cells were stimulated with 100 n*M* VD and cultured for 20 h in the absence or presence of increasing concentrations of the slow NO donor DETA/NO. Subsequently, $\beta$-galactosidase-normalized luciferase activity was determined from cell extracts, and VD-stimulated gene activity was calculated. Results showed that in live COS-7 cells NO in a concentration-dependent manner indeed inhibited VD signaling with an $IC_{50}$ value of $\sim$1 $\mu M$ NO steady-state concentration (15). However, the opposite also may occur, as has been shown for the TNF-$\alpha$ gene. Here the zinc finger transcription factor Sp1 acts as a repressor, and NO activates the TNF-$\alpha$ gene by abrogating the DNA-binding and thus the repressor activity of Sp1 (43).

These results demonstrate that zinc finger transcription factors are indeed primary targets for NO-derived nitrosative stress in live cells.

## PERSPECTIVES

Evidence is presented that NO-derived nitrosative stress affects zinc finger-dependent *de novo* gene transcription. As this inhibition appears to be reversible, NO at nontoxic concentrations should be regarded as a regulating or modulating molecule. However, numerous zinc finger transcription factors are present within cells, and we currently do not know whether proteins with the various known zinc-sulfur cluster domains show different susceptibilities toward NO. Thus, the question arises whether NO-mediated effects on zinc finger

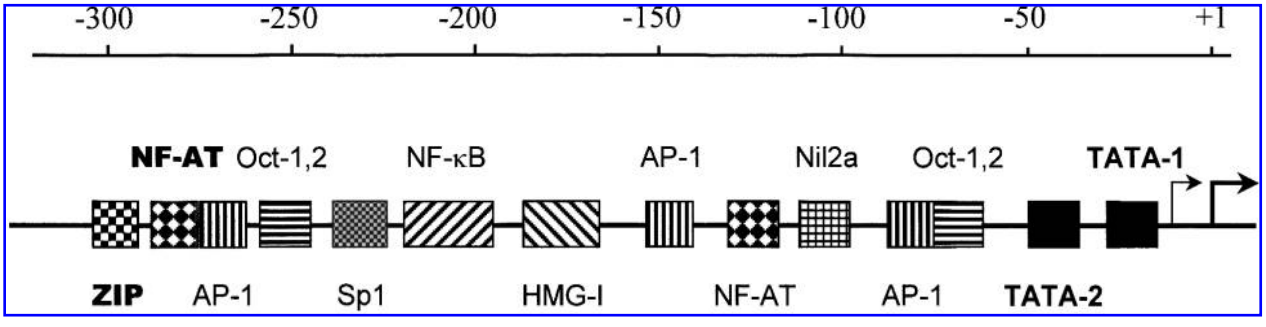


**FIG. 8. Structure of the human IL-2 promoter.** Shown are the protein-binding sites according to Serfling *et al.* (35) and Skerka *et al.* (38) between positions $-50$ and $-300$ upstream of the transcriptional start site. Sp1 and EGR-1, respectively, both binding to the zinc finger protein (ZIP) binding site, as well as NFAT, are the dominant regulating transcription factors, whereas transcription factors binding to other sites play minor modulating roles only.

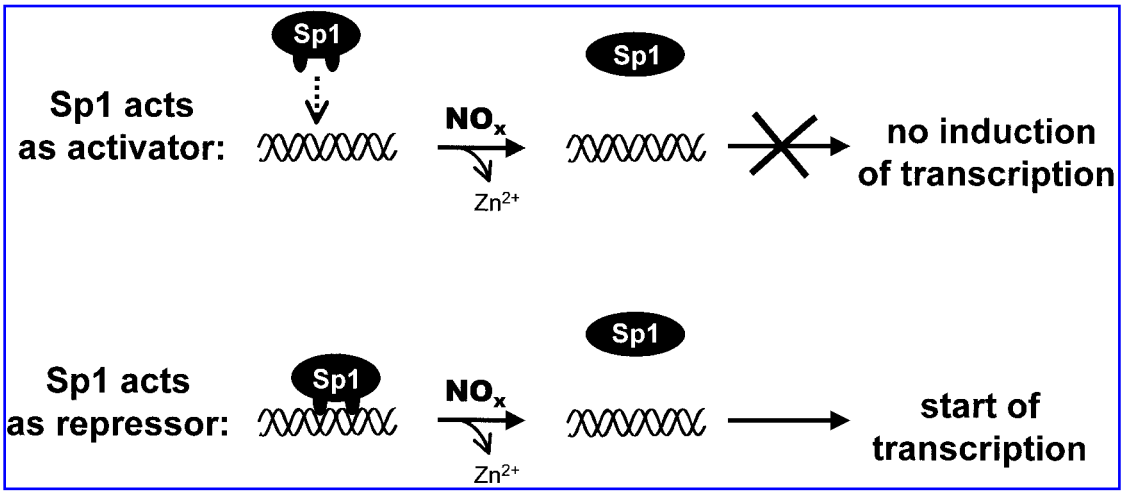


**FIG. 9. Direct effects of NO on zinc finger-dependent gene transcription.** If a zinc finger transcription factor acts as a dominant regulating activator of gene transcription, NO-mediated disruption of the zinc fingers inhibits transcription as has been found with the IL-2 gene (3). In contrast, if a zinc finger transcription factor functions as a repressor, NO-mediated disruption of the zinc fingers may lead to gene transcription as has been found with the TNF-$\alpha$ gene (43), provided that dominant regulating transcription activators of the respective gene are not redox-sensitive. NO may thus inhibit or activate transcription by the same molecular mechanism, depending on the structure of the promoter and the transcription factors involved.

transcription factors can be regarded as specific or not. Prior to answering that question, let us discuss the situation from a different point of view. Considering zinc fingers as sensitive targets of nitrosative or oxidative stress raises the question as to why zinc finger domains have been conserved during evolution as indispensable structures for DNA-binding. However, by using one molecular mechanism, *i.e.*, loss of $Zn^{2+}$ from zinc finger domains, it becomes possible to regulate the transcription of a whole set of genes. Thus, under conditions of nitrosative or oxidative stress, genes that are dominantly regulated by redox-insensitive transcription factors may be selectively transcribed and translated. In contrast, genes that are dominantly regulated by redox-sensitive transcription factors will be turned off. The opposite may occur if a zinc finger transcription factor acts as a dominant repressor. It should be noted, however, that the situation is even more complicated, as different signals may induce different signaling pathways that may affect different transcription factors, some of which may be NO-sensitive, whereas others are not. Thus, although actions of NO on zinc finger transcription factors cannot be regarded as specific, NO-mediated effects on transcription appear to be selective. The same may hold true for reactive oxygen intermediates.

In conclusion, evidence is currently accumulating that high-output NO synthesis serves a regulating role on gene transcription in part mediated by disruption of zinc finger domains. The same molecular mechanism may lead to transcription inhibition of some genes or, depending on the promoter structure and transcription factors involved, activation of other genes (Fig. 9). Thus, depending on the structure of the promoter and on the transcription factors that dominantly regulate the respective gene, NO represents a powerful tool for regulating gene expression within an iNOS-expressing cell, but also within neighboring cells of a tissue.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

AP-1, activator protein-1; DBD, DNA-binding domain; DTT, dithiothreitol; EGR-1, early growth response factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; NFAT, nuclear factor of activated T cell; NO, nitric oxide; RNOI, reactive nitrogen oxide intermediates; RXR, retinoid X receptor; SNOC, *S*-nitrosocysteine; TNF-$\alpha$, tumor necrosis factor-$\alpha$; VD, 1$\alpha$,25-dihydroxyvitamin $D_3$; VDR, VD receptor; VDRE, VD responsive element.

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