Nitric oxide mediates intracytoplasmic and intranuclear zinc release

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Abstract We previously described that NO· leads to destruction of ZnS clusters and release of Zn^{2+} from various proteins including zinc finger transcription factors. To assess the relevance in living cells, we investigated, whether exogenous NO· leads to an increase of cytoplasmic and nuclear free Zn^{2+}. L929 cells, mouse splenocytes, or rat aorta endothelial cells were labeled with Zinquin-E, a Zn^{2+}-specific fluorophore, and were treated with two different spontaneous NO donors, S-nitrosocysteine or DETA/NO. Both NO donors strongly increased the Zn^{2+}-dependent fluorescence in the cellular cytosol and also in nuclei as compared to controls. NO-dependent Zn^{2+} release in splenocytes was quantitated by flow cytometry. These results show for the first time, that nitrosative stress mediates intracellular and intranuclear Zn^{2+} release which may be relevant in altering gene expression patterns.

Key words: Fluorescence microscopy; Flow cytometry; Nitric oxide; Zinc; Zinquin

1. Introduction

Following iron, zinc is the second most widely used transition metal in biology. Nearly 1% of the human genome codes for zinc finger-, ring finger- or the LIM domain-containing proteins (for reviews see [1,2]). The common feature of these structures is that Zn^{2+} is complexed, sometimes together with histidine imidazole nitrogens, by cysteine sulfur ligands, creating tertiary protein structural domains that specifically bind to DNA or RNA sequences. The zinc finger is by far the most prevalent DNA-binding motif. Many of these zinc–sulfur cluster-containing proteins are involved in transcription, replication, recombination or restriction.

The radical nitric oxide (NO·) generated from L-arginine by NO synthases is essential for several functions in physiology and the immune system. Under aerobic conditions, NO· nitrosylates free cysteine SH groups of proteins [3] and inactivates proteins with cysteine residues in or near their active sites [4,5]. NO· has been shown to mediate Zn^{2+} release in vitro from the zinc–sulfur cluster protein metallothionein via S-nitrosylation and subsequent formation of disulfides and to inhibit the DNA-binding activity of the zinc finger transcription factor LAC9 [6]. NO· also inhibits the DNA repair enzyme Fpg containing a zinc finger [7]. However, at present it is unclear whether NO· can possibly mediate changes in gene expression via modification of zinc finger transcription factors. To gain further insight into Zn^{2+} homeostasis in living cells and its modification by NO·, we designed experiments to study whether nitrosative stress in cells mediates increases in detectable free Zn^{2+} concentrations.

2. Materials and methods

2.1. Materials

$ZnCl_2$, L-histidine-HCl, and $NaNO_2$ were purchased from Merck (Darmstadt, Germany), L-cysteine-HCl, propidium iodide and *NNN′N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) from Sigma (Deisenhofen, Germany), Hanks balanced salt solution (HBSS) from Gibco (Heidelberg, Germany), and ethyl(2-methyl-8-*p*-toluenesulphonamido-6-quinoloxy)acetate (Zinquin-E) from Luminis Pty Ltd. (Adelaide, Australia). SNOC and DETA/NO (1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene) were synthesized as described [8,9]. $SNOC_{-NO}$ and $DETA/NO_{-NO}$ were obtained by incubating a 100 mM stock solution of SNOC for 24 h and a 50 mM stock solution of DETA/NO for 72 h at 37°C. The Zn(II)–histidine complex for loading cells with Zn^{2+} was prepared by dissolving 25 mM $ZnCl_2$ and 50 mM L-histidine-HCl in 1 ml of H_2O and neutralizing with 1 M NaOH as described [10].

For all cell cultures RPMI-1640, pH 7.2, was used, supplemented with 6×10^4 U/l penicillin, 60 mg/l streptomycin, 1 mM sodium-pyruvate, 2 mM glutamine, 10 ml/l non-essential amino acids (100-fold concentrated), 10 mM HEPES (all from Gibco), and 10% heat-inactivated fetal calf serum (FCS) purchased from PAA Laboratories (Linz, Austria).

2.2. Target cells

The mouse fibroblast cell line L929 was kindly provided by Dr. B. Urbaschek, University Heidelberg-Mannheim, Germany. Splenocytes were isolated from spleen of anesthesized NMRI-mice. For isolation the spleen was gently pressed through a nylon cell strainer (70 μm, Becton Dickinson, Mountain View, CA). The resulting cell suspension was collected in HBSS, centrifuged ($250\times g$/5 min) and the cell pellet resuspended in HBSS. To remove erythrocytes, the cell suspension was mixed with 2 vol. of lysis buffer (10 mM $KHCO_3$, 155 mM NH_4Cl, 0.1 mM EDTA, pH 7.4) and incubated for 25 min at 4°C. After centrifugation, the resulting lymphocyte cell pellet was dissolved in RPMI and cells were cultured at 37°C. Rat vascular aorta endothelial cells were isolated by outgrowth from rat aortic rings exactly as described [11].

2.3. Labeling of cells and treatment with NO donors

Cells (5×10^5/well) were placed in 12-well flat-bottomed plates (Becton Dickinson) and incubated with 25 μM Zinquin-E for 30 min at 37°C. Subsequently, Zinquin-labeled freshly isolated splenocytes, cultured AEC and L929 cells, respectively, were incubated with SNOC or $SNOC_{-NO}$ for 1 h in serum-free RPMI at the concentrations indicated. Alternatively, cells were cultured in the presence of DETA/NO or $DETA/NO_{-NO}$ for 24 h in RPMI containing 2.5% FCS and were then labeled with Zinquin. Cells were then investigated under the fluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) with the Zeiss filter set number 02 (excitation 365 nm, emission LP 420 nm). As a positive control, cells were loaded with Zn^{2+} as follows: cells were incubated with 100 μM of the Zn(II)–histidine complex [10] for 1 h, washed, labeled with Zinquin-E and investigated as

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described above. As a specificity control, NO-treated cells were incubated with 200 μM of the Zn^{2+} chelator TPEN for 24 h, washed, labeled with Zinquin-E and investigated as described above. To exclude a reaction between NO· and Zinquin or the Zinquin–Zn^{2+} complex, Zinquin-E was incubated with SNOC in the presence or absence of Zn^{2+}. After incubation of Zinquin-E with SNOC for 1 h at 37°C in the absence of Zn^{2+}, Zinquin-E did not become fluorescent. In addition, SNOC treatment did not inhibit or destroy the fluorescence activity of the Zinquin-E–Zn^{2+} complex.

2.4. Flow cytometry

Freshly isolated splenocytes were incubated with Zinquin-E plus SNOC or $SNOC_{-NO}$ for 1 h. For flow cytometric analysis, data of 10^4 stained as well as control cells were collected using a FACStar^{plus} (Becton Dickinson) equipped with a 5-W argon ion laser (Innova 305, Coherent, Palo Alto, CA) operating at 100 mW in a multiline UV (333.6–363.8 nm) mode and analysed using FACStar^{plus} research software (Becton Dickinson). Side scatter was detected with a 330 nm bandpass filter (330WB60, Omega Optical, Brattleboro, VT). Zinquin fluorescence was measured through a 530 nm bandpass filter (530DF30) (Becton Dickinson) and propidium iodide fluorescence was detected at 610 nm (610FS10, Oriel, Stratford, CT).

3. Results

Zinquin-E is a non-fluorescent membrane-permeable fluorophore, which by esterases is intracellularly converted to membrane-non-permeable Zinquin [12]. Upon specific binding of Zn^{2+}, Zinquin becomes strongly fluorescent [13] which was used to detect NO-mediated modulation of intracellular free Zn^{2+} concentrations.

Zinquin-labeled L929 cells were treated with NO donors and subsequently examined by fluorescence microscopy. Zinquin-labeled but otherwise untreated L929 cells showed a weak fluorescence in their cytoplasm with nuclei always unlabeled (Fig. 1A). This demonstrates low concentrations of detectable free Zn^{2+} in cytoplasm and absence of free Zn^{2+} in nuclei. Transillumination confirmed that all cells within the L929 cultures were labeled (Fig. 1B). Treatment of Zinquin-labeled cells with SNOC or with DETA/NO resulted in strong fluorescence detectable over the total cellular area including the nuclei (Fig. 2A) indicative of an increase in detectable free intracellular Zn^{2+} within both, cytoplasm and nuclei. Maximal fluorescence was obtained after treatment with 10 mM SNOC for 1 h or with 2 mM DETA/NO for 24 h. All NO-treated L929 cultures showed some variation of fluorescence intensities between individual cells, but transillumination showed labeling of all cells (Fig. 2B). Increases in labeling are not due to cytotoxicity as treatment of L929 cells with 10 mM SNOC for 1 h or with 2 mM DETA/NO for 24 h resulted in about 7% and 5% dead cells, respectively, as determined by trypan blue exclusion. As negative controls we used the denitrosylated compounds $SNOC_{-NO}$ (10 mM) and $DETA/NO_{-NO}$ (2 mM). Treatment with both control agents under otherwise unchanged conditions resulted in fluorescence intensities exactly as in untreated cells. As a control for Zn^{2+} specificity, L929 cells were treated with SNOC and then with the Zn^{2+} chelator TPEN. Subsequent treatment with Zinquin-E resulted in fluorescence intensities identical to negative controls as an indication of specificity (not shown). As a positive control, L929 cells were preloaded with zinc ions via a Zn(II)–histidine complex prior to Zinquin labeling. Cells thus treated exhibited a strong fluorescence in the cytoplasm as well as in the nuclei comparable to fluorescence of Zinquin-labeled plus NO-treated cells (not shown). This also indicates that the lack of fluorescence in control nuclei is not due to the inability of Zinquin to enter the nuclei.

NO-mediated effects were also found with non-transformed primary cells. Exactly the same experiments as described above were performed with primary cultured aorta endothelial cells (AEC) and with freshly isolated mouse splenocytes. AEC exhibited no fluorescence after Zinquin labeling with no further treatment (Fig. 3A). When AEC were labeled and treated with SNOC (2 mM) for 1 h with DETA/NO (2 mM) for 24 h, each cell within the culture showed homogenous strong fluorescence throughout the total cellular area (Fig. 4A). Identical results were obtained with splenocytes when incubated with 10 mM SNOC for 1 h (not shown).

For further quantification of the effects described above, we analyzed NO-mediated Zn^{2+} release by flow cytometry of freshly isolated splenocytes incubated exactly as described above. Untreated unlabeled (Fig. 5A) and SNOC-treated unlabeled cells (Fig. 5B), respectively, exhibited background fluorescence intensities only. A minor increase in fluorescence intensity was found in labeled but untreated cells (Fig. 5C) as well as labeled plus $SNOC_{-NO}$ treated cells (Fig. 5E). In contrast, a highly significant increase in fluorescence intensity of the total cell population was obtained with labeled and SNOC-treated cells (Fig. 5D). These data exactly match the results obtained with fluorescence microscopy.

4. Discussion

One effect of NO· towards cells is the destruction of Fe-S clusters in proteins and the intracellular release of iron (for review see [14]). We have found in addition that in vitro under aerobic conditions NO· also mediates Zn^{2+} release from the Zn^{2+} storage protein metallothionein via S-nitrosylation and destroys the DNA-binding activity of the yeast zinc finger transcription factor LAC9 [6]. We now show, that exogenous NO· indeed leads to increases of detectable free Zn^{2+} concentrations within living cells as detected with the fluorophore Zinquin. As sources for NO· we used two unrelated chemical NO donors, SNOC and DETA/NO, respectively. The concentrations used, i.e. 2–10 mM SNOC for 1 h or 2 mM DETA/NO for 24 h appear relatively high. However, in cell culture media SNOC has a half-life in the range of several minutes only. Cultures thus are exposed to a burst of NO· most of which probably will be autoxidized or will react with media components before reaching the target cells at the bottom of the culture dish. In contrast, DETA/NO spontaneously releases NO· with predictable first-order kinetics [15] thereby providing a constant NO· discharge over hours. We determined a half-life of 7.7 ± 0.8 h ($n = 3$) for DETA/NO at

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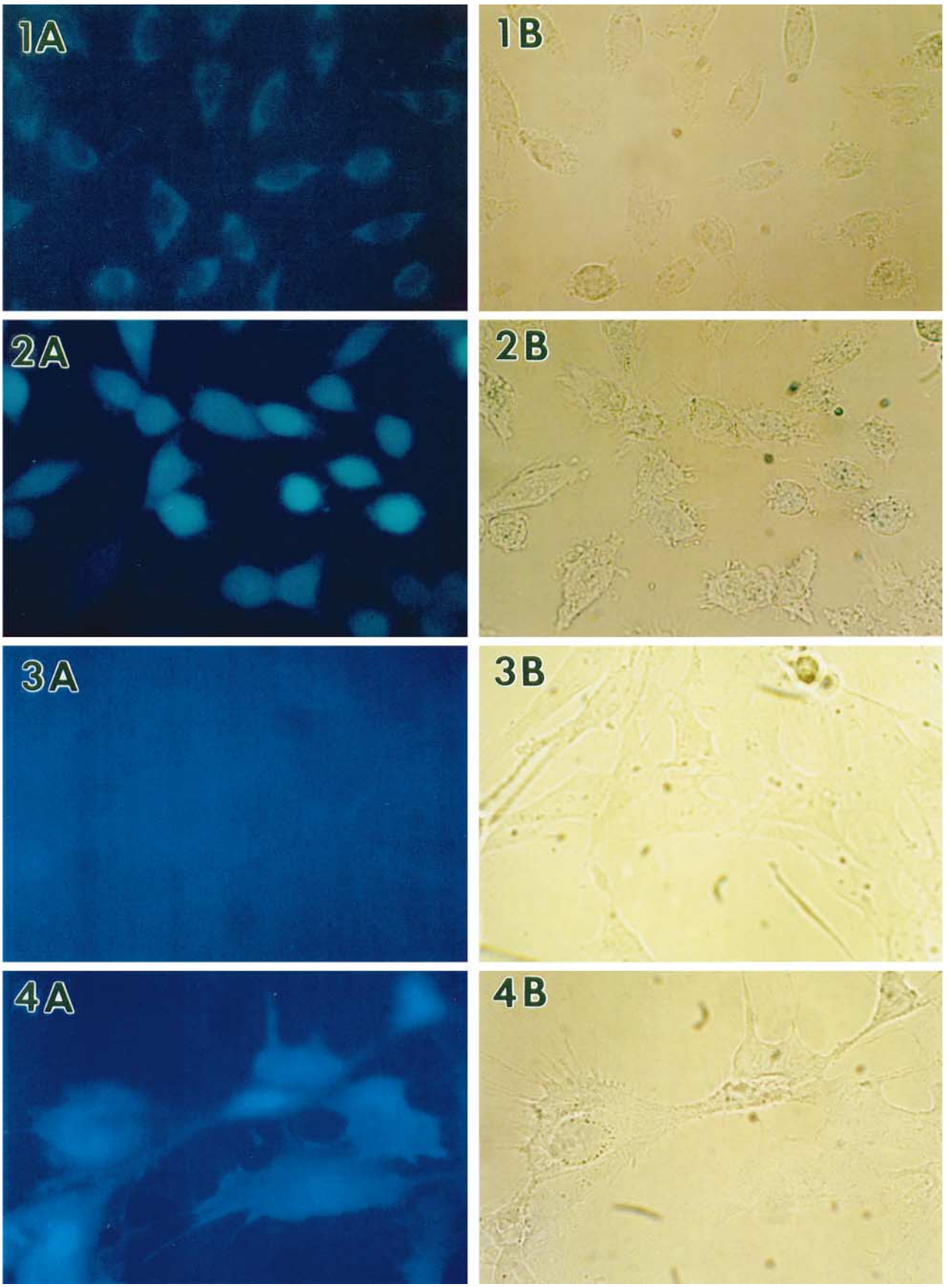
Figs. 1–4. Fluorescence and transillumination micrographs of Zinquin-labeled cells with or without NO-treatment. L929 cells (Figs. 1+2) or rat vascular aorta endothelial cells (Figs. 3+4) were labeled with Zinquin-E and observed for fluorescence (Figs. 1-4A) or with transillumination (Figs. 1-4B). Low or absent fluorescence (Figs. 1A+3A) was found after Zinquin-labeling and no further treatment. Treatment of Zinquin-labeled L929 cells with 10 mM SNOC for 1 h (Fig. 2A) or of endothelial cells with 2 mM DETA/NO for 24 h (Fig. 4A) resulted in strongly enhanced blue fluorescence all over the cytoplasm and the nuclei of every cell. Magnifications: ×300.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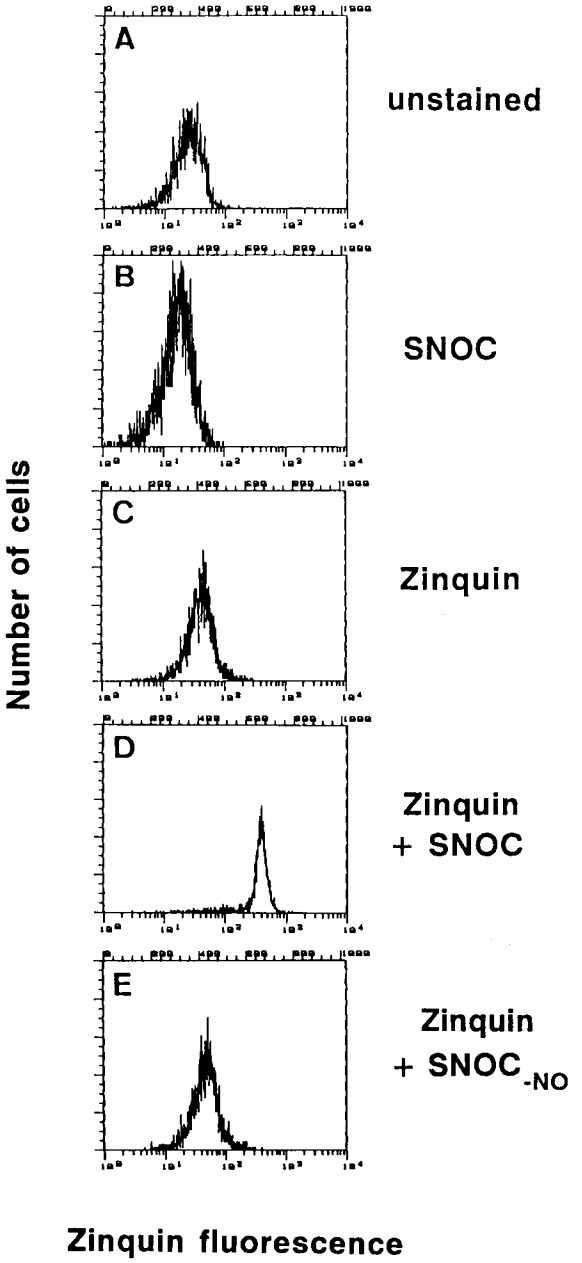

Fig. 5. Histograms of unstained and of SNOC, Zinquin, Zinquin+SNOC and Zinquin+$SNOC_{-NO}$ incubated mouse spleen cells, respectively. Gating of living splenocytes was performed according to light scatter and propidium iodide (0.4 μg/ml) fluorescence. The histograms show: (A) unstained cells, (B) unstained cells treated with 10 mM SNOC (as SNOC treatment control), (C) Zinquin-stained but not otherwise treated cells (as Zinquin-staining control), (D) Zinquin-stained+SNOC (10 mM) incubated cells, and (E) Zinquin-stained+$SNOC_{-NO}$ (10 mM) incubated cells. The Zinquin-+SNOC-treated mouse spleen cells are shifted to 100% into the positive log-scale. Shown are the results of one out of three identical experiments.

37°C, pH 7.4. According to the first-order kinetic laws ($t_{1/2} = \ln 2/k$ and $c = c_0 \cdot e^{-kt}$), 2 mM of DETA/NO generate 6.6 nM NO· per minute. Recently a steady-state concentration of about 4–5 μM of NO· has been calculated to be present in the immediate vicinity of a cell monolayer which enzymatically generates NO· [16]. In consideration of these calculations, the DETA/NO concentrations used appear well within the range of physiological events.

Zinquin fluorescence is highly specific for Zn^{2+}. Otherwise, only Cd^{2+}, which is a rare constituent of biological tissues, weakly enhances Zinquin fluorescence, but no other metal ion, including Fe^{2+} and Fe^{3+}, increase Zinquin fluorescence [13]. The results presented here thus demonstrate that treatment of cells with NO donors results in an increase of the intracellular free Zn^{2+} concentrations throughout the whole cellular area including the nuclei. Identical experiments in the additional presence of a Zn^{2+} chelator resulted in significantly reduced fluorescence intensities showing that we indeed measured a specific Zn^{2+} release within the cells. Preloading of cells with Zn^{2+} prior to Zinquin treatment and no NO-donor addition revealed increased labeling also in the nuclear compartment demonstrating that nuclear fluorescence after NO treatment is not due to NO-mediated translocation or access of Zinquin to the nucleus but indeed reflects a local release of Zn^{2+}. Our experiments suggest that NO· may be able to traverse the cellular cytoplasm and to react with targets in nuclei. Although the effects may well be secondary in nature, we clearly show that exogenously generated NO· can lead to changes even within the nuclear compartment. Local Zn^{2+} release in nuclei appears to be associated with the destruction of zinc finger or zinc finger-related protein structures and would thereby lead to altered gene expression patterns. In view of the recently described activation of a transcription factor via reversible S-nitrosylation [17] an important and new function now emerges for NO· as regulator signal within live cells.

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