Comparing Nitrosative Versus Oxidative Stress toward Zinc Finger-dependent Transcription

UNIQUE ROLE FOR NO*

During inflammatory reactions, cells are under nitrosative and/or oxidative stress. The zinc finger transcription factors vitamin D receptor (VDR) and retinoid X receptor (RXR) were used as a model system to characterize effects of NO and/or reactive oxygen species on zinc finger-dependent gene expression. Nitric oxide (NO) as well as H2O2, singlet oxygen (1O2), peroxyl radicals (ROO), and peroxy-nitrone (ONOO−), respectively, were shown to inhibit VDR/RXR-DNA complex formation in vitro in a dose-dependent manner. While NO-induced inhibition of VDR/RXR-DNA complex formation could be restored nearly completely by subsequent treatment with dithiothreitol, inhibition by H2O2 proved to be only partially reversible, and inhibition by 1O2, ROO or ONOO− was found to be irreversible. In cells transiently transfected with VDR and RXR, subtoxic concentrations of NO or hydroperoxides and intracellular generation of superoxide anion radicals inhibited VDR/RXR-dependent reporter gene activity in a dose-dependent manner. Interestingly, cells can repair the zinc fingers of VDR and RXR after nitrosative stress but not after oxidative stress. The results indicate that, among the reactive species investigated, only NO may act sufficiently gentle to be considered as a regulator and not only as an inhibitor of gene expression via zinc finger transcription factors.

Effects of nitric oxide (NO) on cellular functions are complex and even appear to be contradictory, Janus-headed. NO may act cytotoxically but may also protect cells from toxic insults (1), it may compromise the cellular redox state but may also act as an antioxidant (2, 3), and it may activate or inhibit signal transduction pathways (4, 5) and gene transcription (6–9), respectively. Many of these effects can, at least in part, be explained by different NO concentrations achieved in the respective microenvironment. Nanomolar concentrations of NO, as typically synthesized in a tightly regulated fashion by constitutively expressed nitric oxide synthase (cNOS), serve as a signal molecule activating the soluble guanylate cyclase to produce cGMP, which acts as a second messenger. However, in addition to two cNOS there is also an inducible NOS (iNOS) expressed in a variety of acute or chronic disease states (for reviews see Refs. 10, 11). Originally described as a cytotoxic activated macrophage effector molecule (12) it is now evident that iNOS-derived NO exerts a multitude of biological functions. In an apparently unregulated fashion iNOS synthesizes NO for hours or even days resulting in micromolar concentrations of NO. Under these conditions NO may react with oxygen in a reaction mainly depending on the NO concentration to yield higher nitrogen oxides (NOx such as N2O3, etc.), which display a much broader chemical reaction spectrum than NO itself (13).

A growing body of evidence suggests that NO after reaction to NO helps to orchestrate gene expression, e.g. via posttranslational modifications of transcription factors. A prevalent DNA binding motif of transcription factors is the zinc finger structure with Zn tetrahedrally coordinated between a β-hairpin and a short α-helix, creating a small, functional and independently folded domain (14). In these zinc fingers cysteine thiols and histidine imidazole nitrogens serve as direct ligands for the zinc ion.

We previously found that NO S-nitrosates cysteines in metabolome, mediating the release of Zn from this zinc-storing protein (15), induces Zn release within cells (16) and is able to inhibit zinc finger-dependent transcription (17–19). However, zinc fingers can easily be disrupted by cysteine oxidation, e.g. by reactive oxygen species, or by electrophilic attack, e.g. by alkylating compounds (for reviews see Refs. 20, 21). The question therefore arises, whether the reaction of NO with zinc fingers may have a special role, different from the reaction with other reactive species generated during inflammatory reactions. Such species are superoxide, its dismutation product hydrogen peroxide, the product of its reaction with NO, peroxy-nitrone, or singlet oxygen, as well as peroxyl radicals.

To investigate the impact of these species on the zinc finger integrity, we used the transcription factors VDR and RXR as a model system both containing two Cys-type zinc fingers, which bind to specific promoter sequences as the heterodimeric vitamin D3 receptor; RXR, retinoid X receptor; MAMA/NO, ([Z]-1-[N-methyl-N-[6-(N-methylamino)hexyl]amino]diazen-1-ium-1,2-diolate; DETA/NO (Z)-1-[N-[2-aminoethyl]-N-cyanoethyl]amino]diazen-1-ium-1,2-diolate; NDPO2; 3,30-(1,4-naphthylidene)di(1-propionate 1,4-endoperoxide; t-BHP, tert-butyl hydroperoxide; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; AAPH, 2,2-azobis(2-aminopropane); VD, 1α,25-dihydroxyvitamin D3; VDRE, 1α,25-dihydroxyvitamin D3 response element; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol.

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1 The abbreviations used are: cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; VDR, 1α,25-dihydroxyvitamin D3 receptor; RXR, retinoid X receptor; MAMA/NO, ([Z]-1-[N-methyl-N-[6-(N-methylamino)hexyl]amino]diazen-1-ium-1,2-diolate; DETA/NO (Z)-1-[N-[2-aminoethyl]-N-cyanoethyl]amino]diazen-1-ium-1,2-diolate; NDPO2; 3,30-(1,4-naphthylidene)di(1-propionate 1,4-endoperoxide; t-BHP, tert-butyl hydroperoxide; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; AAPH, 2,2-azobis(2-aminopropane); VD, 1α,25-dihydroxyvitamin D3; VDRE, 1α,25-dihydroxyvitamin D3 response element; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol.
complex VDR/RXR. VDR/RXR-DNA complex formation was quantified after treatment of VDR/RXR with reactive oxygen and nitrogen oxide species in vitro. In addition, VDR/RXR-dependent reporter gene activity was investigated in living cells after treatment with NO or reactive oxygen species. Our results demonstrate that, among the reactive species investigated, NO shows unique characteristics with a potential role in gene regulation.

MATERIALS AND METHODS

Materials—The NO-donors MAMA/NO ([Z]-1-[(N-methylamino)ethyl]amino)diacacen-1-ium-1,2-diolate), and DETANO ([Z]-1-[(2-aminoethyl)-N-(2-ammonioethyl)amino]diacacen-1-ium-1,2-diolate) were synthesized as described (22). Peroxynitrite (ONOO·) was synthesized by reaction of H2O2 with isomyl nitrite (Sigma) on ice at pH 12.5 (23). Contaminating isomyl alcohol was removed by intense washing with chloroform (23), and unreacted H2O2 was removed by reaction with MnO2 (24). The resulting solution was adjusted to pH 14, and the concentration of peroxynitrite was determined spectroscopically at 302 nm (ε = 1670 M−1 cm−1) (25). Stock solutions were stored at −80 °C for several months without loss of absorbance. Degraded ONOO· (ONOO−) was obtained after neutralization of a 6 M HCl stock solution with HCl and incubation for 2 h at 37 °C. The singlet oxygen generator disodium 3,3′-(1,4-naphthylidine) dipropionanitrate (NDPO2) was synthesized as described (26–28). Decomposed endoperoxide (NDPO2(3,5)) verified spectroscopically as NDOX (NDPO2), was obtained by incubating a 50 mM stock solution of NDPO2 for 2 days at 37 °C. Hydrogen peroxide and tert-butyl hydroperoxide (t-BHP) were purchased from Merck, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) was purchased from Alexis (Grünenberg, Germany). 2,2'-azobis-(2-aminopropane) dihydrochloride (AAPH) was purchased from Polyscience (Warrington, PA), and 1,25-dihydroxyvitamin D3 (Vitamin D3) was kindly provided by Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark).

*Gel Shift Assays—Human VDR and human RXRα cDNA were subcloned into the pcDNA3 expression vector (Stratagene, Heidelberg, Germany) (29). Linearized cDNA from VDR and RXR, respectively, were transcribed with T7 RNA polymerase and translated using wheat germ lysate as recommended by the supplier (Promega, Mannheim, Germany). Similar amounts of in vitro translated VDR and RXR proteins were mixed and incubated in the presence or absence of 1 μM VD for 15 min at room temperature in a total volume of 20 μl of binding buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, and 5% glycerol). Indicated amounts of MAMA/NO, H2O2, NDPO2, AAPH or ONOO− were added, and the mixtures were incubated for various times. The DR3-type VD response element (VDRE) of the rat ANF gene (GenBank accession number X98434; sense: 5'-CTCATCCTCTGGCAATCGGA-3'(bases 232–251), antisense: 5'-ATTGAGTCTCCTGCGGACG-3'(bases 771–790). Expression of the housekeeping gene GAPDH was analyzed using primers for GAPDH cDNA (GenBank accession number M17851; sense: 5'-ATGCGCAATGGACATCGACA-3'(bases 153–175), antisense: 5'-TCTCGAGGGGGACATCTTCG-3'(bases 548–568). PCR was carried out following standard procedures (30). To ensure that amplification conditions were within the linear phase, PCR was performed using various numbers of cycles with RNA isolated from transfected and VD-stimulated cells. The following cycle profiles were found to be suitable: 20 cycles at 94 °C/30 s, 56 °C/30 s, and 72 °C/10 s for lAcknowledged mRNA amplification, and 18 cycles at 94 °C/30 s, 58 °C/30 s, and 72 °C/30 s for GAPDH mRNA amplification, respectively. As a control, PCR was performed with all additives but without cDNA or with all additives but only with RNA, respectively, to exclude unspecific amplifications. Equal amounts of DNA were electrophoresed on a 1.8% agarose gel. The luciferase/GAPDH ratio was obtained by densitometric analysis of ethidium bromide-visualized amplification product bands using the Kodak 1D software (Kodak, Stuttgart, Germany).

RESULTS

*In Vitro Studies—To investigate effects of nitrosative and/or oxidative stress toward zinc finger transcription factors, in vitro translated VDR and RXR proteins were used as a model system. VDR-RXR heterodimers (VDR/RXR) were incubated with various concentrations of NO or reactive oxygen species before analyzing VDR/RXR-DNA complex formation to the DR3-type VDRE of the rat ANF gene promoter by gel shift experiments.

*Nitric Oxide—NO was generated by decomposition of the NO-donor MAMA/NO (half-life at 37 °C: 2 min). Fig. 1A shows that NO generated by MAMA/NO inhibits VDR/RXR-DNA complex formation in a concentration-dependent manner with an IC50 value of about 0.5 mM MAMA/NO. Using preformed VDR/RXR-DNA complexes as targets, MAMA/NO with a nearly identical IC50 value (about 0.4 mM) induced the release of VDR/RXR from its response element (Fig. 1B). The DNA complex formation of MAMA/NO-treated VDR/RXR was nearly completely restored by subsequent incubation with 1 or 10 mM diethylthiouretol (DTT) for 30 min at 30 °C (Fig. 1C). To investigate whether NO affects the response element as well, VDRE oligonucleotides preincubated with up to 5 mM MAMA/NO for 30 min at 30 °C before VDR/RXR was added. A significant inhibition was observed only with the highest NO-donor concentration (Fig. 1D).

*Hydrogen Peroxide—Incubating VDR/RXR with increasing concentrations of H2O2 for 90 min at 30 °C provided a significant inhibition of the VDR/RXR-DNA complex formation only at relatively high H2O2 concentrations (>20 mM) with an IC50 value of 40–50 mM H2O2 (Fig. 2A). Similar high H2O2 concentrations were needed to release DNA-bound VDR/RXR from its response element (Fig. 2B). Subsequent incubation with 10–20 mM DTT showed partial restoration of the VDR/RXR-DNA complex formation (Fig. 2C). DTT has been reported to complex zinc ions (34), but even high DTT concentrations (20 mM) apparently did not lead to ejection of Zn2+ from the zinc fingers as the DNA binding activity of VDR/RXR was not affected by treatment with DTT alone (Fig. 2C). Pretreatment of the VDRE
with H$_2$O$_2$ prior to addition of VDR/RXR showed similar IC$_{50}$ values with regard to the formation of VDR/RXR-DNA complexes compared with VDR/RXR (Fig. 2D).

**Singlet Oxygen**—Singlet oxygen ($^1$O$_2$) was generated by decomposition of NDPO$_2$ (half-life at 37°C: 23 min) leading to the formation of NDP and molecular oxygen with a $^1$O$_2$ yield of 50% (28, 35). Incubating VDR/RXR with increasing concentrations of NDPO$_2$ for 90 min at 30°C resulted in a concentration-dependent inhibition of the VDR/RXR-DNA complex formation with an IC$_{50}$ value of about 0.75 mM NDPO$_2$, while the decomposition product NDPO$_{2deg}$ showed no effects (Fig. 3A). Subsequent incubation with up to 20 mM DTT for 30 min at 30°C did not reverse the inhibitory effect of H$_2$O$_2$ on VDR/RXR-DNA complex formation. Representative gels are shown. Relative complex formation is presented in reference to the control in the absence of MAMA/NO. Columns represent means ± standard deviation of at least three independent experiments.

**Peroxyl Radicals**—Peroxyl radicals (ROO$^-$/H$_{2}$$\text{O}_2$) were generated by decomposition of AAPH (half-life at 37°C about 175 h) forming carbon-centered radicals that react swiftly with O$_2$ to yield ROO$^·$. Incubating VDR/RXR with increasing concentrations of AAPH for 2 h at 30°C resulted in a concentration-dependent inhibition of the VDR/RXR-DNA complex formation (Fig. 3C). Pretreatment of the VDRE with NDPO$_2$ before addition of VDR/RXR plus 50 mM DTT to protect VDR/RXR from residual H$_2$O$_2$ resulted in a concentration-dependent inhibition of VDR/RXR-DNA complex formation. Representative gels are shown. Relative complex formation is presented in reference to the control in the absence of H$_2$O$_2$. Columns represent means ± standard deviation of at least three independent experiments.
dependent inhibition of the VDR/RXR-DNA complex formation with an IC_{50} value of about 10 mM AAPH (Fig. 4A). DNA-bound VDR/RXR was found to be slightly more sensitive toward treatment with AAPH (IC_{50} value of about 7 mM) than unbound VDR/RXR (Fig. 4B). Subsequent treatment with up to 20 mM DTT for 30 min at 30 °C did not restore VDR/RXR-DNA complex formation (Fig. 4C). Pretreatment of VDRE with AAPH prior to addition of VDR/RXR plus 50 mM DTT to protect VDR/RXR from residual-generated O_2 resulted in a concentration-dependent inhibition of VDR/RXR-DNA complex formation. Representative gels are shown. Relative complex formation is presented in reference to the control in the absence of NDPO_2. Columns represent means ± standard deviation of at least three independent experiments.

Peroxynitrite—NO and the superoxide anion radical react to form the strong oxidant peroxynitrite anion (ONOO^-), which has a half-life of about 1 s under physiological conditions (24). We used chemically synthesized authentic ONOO^- as a source. Prior to use, the 260 mM ONOO^- stock solution was diluted with ice-cold deionized H_2O to a ONOO^- concentration of 6 mM. To avoid pH changes resulting from the use of up to 2 mM ONOO^- diluted from the 6 mM ONOO^- stock solution, 200 mM phosphate buffer was employed. This buffer had no effect on the VDR/RXR-DNA complex formation (not shown). Incubating VDR/RXR with increasing concentrations of ONOO^- for 10 min at room temperature resulted in a concentration-dependent inhibition of the VDR/RXR-DNA complex formation with an IC_{50} value in the range of about 0.3–0.4 mM ONOO^- (Fig. 5A). Adding VDR/RXR various times after the addition of ONOO^- showed that inhibition of the VDR/RXR-DNA complex formation occurred within 10 s (data not shown). Addition of multiple low ONOO^- doses (5 × 0.2 mM every minute) did not result in
under the control of four copies of the rat ANF DR3-type VDRE (30). To avoid effects of NO or reactive oxygen species on the protein expression values, the cells were allowed to recover after transfection for 8 h and to overexpress inactive VDR and RXR, respectively. Cells were then stimulated with 100 nM VD or solvent (0.1% ethanol) for various time intervals. Subsequent determination of the luciferase activity revealed that VD activated the reporter gene time dependently in a linear fashion: 2 h, 6.1 ± 0.6-fold; 4 h, 25.8 ± 2.7-fold; 6 h, 49.1 ± 4.9-fold; 8 h, 79.6 ± 4.8-fold; 10 h, 98.5 ± 6.6-fold; 24 h, 288.8 ± 22.6-fold (n = 3). To investigate effects of NO’ or reactive oxygen species on VDR/RXR-dependent transcription, the 8 h time period was chosen for further experiments. Only those concentrations of the various compounds were used that proved to be subtoxic (≥95% cell viability investigated by morphological analysis and trypan blue exclusion after 12 h compared with untreated controls).

Nitric Oxide—To investigate effects of NO’ on VDR/RXR-dependent transcription we used DETA/NO, an NO-donor similar to MAMA/NO but with a considerably longer half-life (about 8 h at 37°C). Increasing DETA/NO concentrations inhibited VD-stimulated luciferase activity with an IC_{50} value of about 100 μM DETA/NO (Fig. 6A). In contrast, 1 mM of the control compound DETA had no effect (97.2 ± 4.1% of control) showing that indeed NO’ was the effective compound.

Hydroperoxides—The transiently transfected cells were incubated with increasing concentrations of hydroperoxides. H₂O₂ induced a concentration-dependent inhibition of VD-stimulated luciferase activity with an IC_{50} value of about 300 μM H₂O₂ (Fig. 6B). t-BHP also induced a concentration-dependent inhibition of the reporter gene activity with an IC_{50} value of about 45 μM t-BHP (Fig. 6C).

Intracellular Superoxide—As source for intracellular O₂⁻ generation we used the redox cycler DMNQ. Fig. 6D shows that DMNQ inhibited VDR/RXR-dependent luciferase activity in a concentration-dependent manner with an IC_{50} value of about 5 μM DMNQ.

Reversibility—To ask whether the observed effects on zinc finger-dependent transcription in cells are reversible, the transiently transfected cells were treated with the IC_{50} value concentrations of DETA/NO, H₂O₂, t-BHP, or DMNQ for 8 h. After a recovery period of 5 h in fresh medium, cells were stimulated with 100 nM VD or solvent vehicle for another 8 h. After treatment with 100 μM DETA/NO, 50 μM t-BHP, or 5 μM DMNQ, cells could again be stimulated by VD for reporter gene activity (about 80% restored activity compared with non-pre-treated controls). After treatment with 300 μM H₂O₂, however, cells were refractory to stimulation with VD (Fig. 7).

With this experimental design it was not possible to discriminate whether the disrupted zinc finger transcription factors were repaired by the cells or whether they were synthesized de novo. Therefore, transiently transfected cells were treated for 8 h with the IC_{50} value concentrations of DETA/NO, H₂O₂, t-BHP, or DMNQ, and were then stimulated with 100 nM VD or solvent vehicle in the presence of the translational inhibitor cycloheximide. Treatment with 10 μg/ml cycloheximide inhibited VD-stimulated luciferase activity by 96.3 ± 2.6% (n = 3). RNA was isolated after 15 h, and RT-PCR was performed to quantitate VDR/RXR-dependent luciferase mRNA expression. Fig. 8 shows that VD stimulated luciferase mRNA expression about 7-fold compared with unstimulated cells. Interestingly, even in the presence of cycloheximide a pretreatment of the cells with 100 μM DETA/NO for 8 h still allowed full stimulation of VDR/RXR-dependent luciferase mRNA expression by VD (Fig. 8, lane 4). In contrast, pretreatment with 300 μM H₂O₂, 50 μM t-BHP, or 5 μM DMNQ allowed for subsequent

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**Fig. 5**. Effects of ONOO⁻ on VDR/RXR-DNA complex formation in vitro. Gel shift experiments were performed as described in the legend in Fig. 1. There were free (A) or already VDRE-bound (B) VDR/RXR with authentic ONOO⁻ for 10 min at room temperature resulted in a concentration-dependent inhibition of the VDR/RXR-DNA complex formation, while degraded ONOO⁻ (ONOO⁻_{de}) had no effect. C, subsequent addition of up to 20 mM DTT for 30 min at 30°C did not restore VDR/RXR-DNA complex formation. D, treatment of VDR/RXR with ONOO⁻ in the presence of 20 mM methionine resulted in a significant protection of VDR/RXR-DNA complex formation. E, treatment of VDRE with up to 5 mM ONOO⁻ before addition of VDR/RXR showed no inhibitory effect on the VDR/RXR-DNA complex formation. Representative gels are shown. Relative complex formation is presented in reference to the control in the absence of ONOO⁻. Columns represent means ± standard deviation of at least three independent experiments.

A shift of the IC_{50} value for ONOO⁻ (not shown). DNA-bound VDR/RXR were slightly more sensitive toward treatment with ONOO⁻ (IC_{50} value of ~0.3 mM) than unbound VDR/RXR (Fig. 5B). Subsequent treatment with up to 20 mM DTT for 30 min at 30°C did not restore the VDR/RXR-DNA complex formation (Fig. 5C). Addition of 20 mM methionine nearly completely inhibited the inhibitory effect of 1 mM ONOO⁻ (Fig. 5D). Pretreatment of the VDRE with up to 5 mM ONOO⁻ had no effect on VDR/RXR-DNA complex formation (Fig. 5E). Higher concentrations of ONOO⁻ could not be used due to problems with the pH and the salt concentration essential for optimal VDR/RXR-DNA complex formation.

**Studies with Living Cells**—To investigate whether the results found in vitro are also found with cells, MCF-7 cells were transiently transfected with expression vectors for VDR and RXR, respectively, and a luciferase reporter gene plasmid un-
significant VD-stimulated luciferase mRNA expression (Fig. 8, lanes 5–7). These results demonstrate that cells are able to repair VDR/RXR after nitrosative stress but not after oxidative stress.

DISCUSSION

In Vitro—Nitric oxide is able to nitrosate cysteine thiols in proteins yielding S-nitrosothiols under aerobic conditions, probably via formation of NO\(_2\)-Cys (13, 38). In the case of zinc fingers, which contain up to four cysteines, this reaction leads to the ejection of the zinc ion (15, 39, 40). However, various sulfhydryl-oxidizing compounds such as H\(_2\)O\(_2\) (41–46), O\(_2\)(47), ONOO\(^-\) (48), HOCl (45, 48), or diamide (46, 49), as well as sulphydryl-alkylating compounds, such as iodoacetamide (43, 46) or N-ethylmaleimide (43, 50), have also been shown to disrupt zinc-sulfur complexes in various proteins.

To investigate whether effects of nitrosative and oxidative stress, respectively, differ with regard to zinc finger-dependent transcription factors, we treated VDR/RXR with compounds that are generated under conditions of nitrosative and/or oxidative stress, i.e. NO\(^+\), H\(_2\)O\(_2\), O\(_2\), ROO\(^+\), or ONOO\(^-\). All these compounds inhibited the DNA complex formation and thus the DNA binding activity of VDR/RXR in a concentration-dependent manner (Figs. 1A–5A). The millimolar range of the IC\(_{50}\) value for H\(_2\)O\(_2\) appears to be rather high but fits with concentrations reported for the inhibition of other zinc finger transcription factors, such as the glucocorticoid receptor (41, 42, 51), Sp1 (43, 44), or the estrogen receptor (49). Prebinding to its response element did not protect VDR/RXR from the inhibitory effects (Figs. 1B–5B). In contrast, we found equal or in some cases even slightly enhanced sensitivity of prebound VDR/RXR to our treatment regimens as compared with pretreating VDR/RXR before DNA binding. This suggests that the zinc fingers within the protein-DNA complex are equally or even slightly more accessible for the reactive species investigated. From a functional point of view this means that reactive species are able to inhibit zinc-finger-dependent gene expression during all phases of their action.

NO\(^+\)-induced inhibition of VDR/RXR-DNA complex formation was nearly completely restored by subsequent reduction with DTT (Fig. 1C). After treatment with H\(_2\)O\(_2\), DTT was able to partially (about 50%) restore the DNA complex formation of VDR/RXR (Fig. 2C). In contrast, treatment with 1O\(_2\), ROO\(^+\), or ONOO\(^-\) led to irreversible loss of the DNA binding activity of VDR/RXR since DTT did not restore VDR/RXR-DNA complex formation (Figs. 3C–5C). This suggests that H\(_2\)O\(_2\), 1O\(_2\), ROO\(^+\), or ONOO\(^-\) at least partially oxidize zinc finger cysteine thiols (Cys-SH) beyond the level of disulfides, e.g. to sulfenic (Cys-SO\(_2\)H), sulfinic acid (Cys-SO\(_3\)H) (21), or even thiosulfinate (Cys-SO\(_3\)-S-Cys) (52). After this, the amino acids present in proteins only cysteines are modified by NO\(^+\) (53), while the other reactive species used in this study are able to modify additional amino acids in proteins also, e.g. to oxidize methionine to methionine sulfoxide (49, 54) or, in the case of ONOO\(^-\), to nitrate tyrosine (55, 56). VDR and RXR each contain 17 methionine residues, which in part are located close to the zinc finger
domains, and RXR even contains four tyrosines within or nearby the DNA binding domain. All these amino acid modifications may contribute to inhibition of the VDR/RXR-DNA complex formation by H2O2, $^{3}O_{2}$, ROO$^{-}$, or ONOO$^{-}$.

The reactive species NO$^{*}$, H$_2$O$_2$, $^{1}O_2$, and ROO$^{-}$ were found to affect in a dose-dependent manner the VDRE such that subsequently added VDR/RXR were unable to bind anymore. Interestingly, the ratios of the IC$_{50}$ values found for VDRE and for subsequently added VDR/RXR were 10-fold higher NO$^{*}$ than H$_2$O$_2$. The IC$_{50}$ value found for H$_2$O$_2$ differed considerably. However, differences of two orders of magnitude have also been reported using the zinc finger transcription factor glucocorticoid receptor (51).

In living cells reversibility of the inhibition of VDR/RXR-DNA complex formation was shown after exposure to NO$^{*}$, t-BHP, or DMNQ at concentrations corresponding to the IC$_{50}$ values (Fig. 7). In contrast, after treatment with H$_2$O$_2$ no reversibility was found, although the H$_2$O$_2$ concentration used (300 $\mu$M) also proved to be nontoxic. This suggests that in addition to VDR/RXR-dependent transcription additional cellular functions are affected by H$_2$O$_2$.

To investigate whether cells are able to repair disrupted VDR/RXR, we treated cells with the reactive species and then stimulated them with VD in the presence of the translational inhibitor cycloheximide, which inhibited de novo synthesis of VDR/RXR. Results showed that after exposure to NO$^{*}$ VDR/ RXR-dependent luciferase mRNA expression was unaffected (Fig. 8). In contrast, exposure to the hydroperoxides or DMNQ resulted in significantly impaired expression of luciferase mRNA. These results suggest that cells are able to repair S-nitrosated zinc finger transcription factors, whereas oxidative modifications appear to be irreversible.

Although in this study only transcription factors containing Cys$_3$-type zinc fingers were investigated, experiments using the Cys$_2$/His$_2$-type zinc finger transcription factor Sp1 have shown that the latter is also very susceptible toward H$_2$O$_2$ (43, 44) and NO$^{*}$ (17, 19), respectively. As all known zinc fingers structures contain at least two cysteine residues (14) and as the cysteine thios are the redox-sensitive parts of zinc fingers it is highly likely that our results can be generalized with regard to all zinc-finger containing transcription factors. Whether nitrosative or oxidative stress also affects the translocation of zinc finger transcription factors into the nucleus remains to be determined.

In conclusion, we suggest that the more gentle reactions of NO$^{*}$ permit cells to handle nitrosation of zinc finger transcription factors with minimal collateral damage as hypothesized recently (57) compared with the irreversible modifications induced by oxidative stress. Thus, NO$^{*}$ is not just another compound able to destroy zinc finger domains of proteins but may potentially serve as a gene regulatory molecule during inflammatory reactions.

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