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Nitric Oxide Inhibits c-Jun DNA Binding by Specifically Targeted *S*-Glutathionylation*

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This study addresses potential molecular mechanisms underlying the inhibition of the transcription factor c-Jun by nitric oxide. We show that in the presence of the physiological sulfhydryl glutathione nitric oxide modifies the two cysteine residues contained in the DNA binding module of c-Jun in a selective and distinct way. Although nitric oxide induced the formation of an intermolecular disulfide bridge between cysteine residues in the leucine zipper site of c-Jun monomers, this same radical directed the covalent incorporation of stoichiometric amounts of glutathione to a single conserved cysteine residue in the DNA-binding site of the protein. We found that covalent dimerization of c-Jun apparently did not affect its DNA binding activity, whereas the formation of a mixed disulfide with glutathione correlated well with the inhibition of transcription factor binding to DNA. Furthermore, we provide experimental evidence that nitric oxide-induced *S*-glutathionylation and inhibition of c-Jun involves the formation of *S*-nitrosoglutathione. In conclusion, our results support the reversible formation of a mixed disulfide between glutathione and c-Jun as a potential mechanism by which nitrosative stress may be transduced into a functional response at the level of transcription.

The free radical nitric oxide (NO)[1] has emerged as a major signaling molecule in the immune, cardiovascular, and nervous system (1–5). Accumulating evidence suggests that NO may play a role in the redox control of transcription by modulating the DNA binding activity of transcriptional activators such as OxyR (6), nuclear factor-κB (7), and c-Myb (8) through *S*-nitrosylation of redox-sensitive thiols. Furthermore, NO has been reported to inhibit DNA binding of the transcription factor AP-1 in cerebellar granular cells (9) and to be involved in the post-transcriptional attenuation of AP-1 during NO-induced neuronal cell death (10). Of interest, interferon γ was shown to induce a down-regulation of AP-1 DNA binding activity in human brain-derived cells. This phenomenon is associated with the development of neuroinflammatory diseases and was found to be due to cytokine-mediated induction of NO synthase in these cells (11). However, the molecular mechanisms underlying the inhibition of AP-1 DNA binding by NO remain to be established.

A recent study with purified c-Jun and c-Fos, which constitute the transcriptional activator AP-1, indicates that NO inhibits the DNA binding activity of AP-1 by modifying cysteine residues in the DNA-binding site of these proteins through as yet unknown mechanisms (12). These findings fit well with previous studies on truncated Fos and Jun constructs which mapped redox regulation of AP-1 to a single conserved cysteine residue located in the basic DNA-binding site of c-Fos and c-Jun (13, 14). Reduction of this critical cysteine residue by chemical-reducing agents such as DTT and 2-mercaptoethanol or by the DNA repair enzyme Ref-1 has been shown to convert the inactive and presumably oxidized form of c-Fos and c-Jun into an active state that is permissive for DNA binding (13, 15). *In vitro*, oxidation of c-Jun and concomitant inhibition of its DNA binding activity occur rapidly when the concentration of the reducing agent in the incubation medium (*e.g.* DTT or 2-mercaptoethanol) falls below 0.2 mM (16). The conclusion that NO inhibits AP-1 DNA binding by specifically reacting with cysteine residues in c-Jun and c-Fos, however, was reached from the observation that NO concentrations >0.1 mM inactivate the transcription factor in the presence of low concentrations (0.1 mM) of the dithiol DTT (12). Given the high capacity of NO to decrease thiol levels by *S*-nitrosylation and oxidation (17), this raises the question if the observed effects of NO on Jun/Fos DNA binding are in fact directly related to a protein modification by NO such as *S*-nitrosylation or can be attributed to the oxidation of the transcription factor as described by Curran and co-workers (13, 16) as a consequence of NO-induced thiol depletion.

To address this issue, we analyzed purified recombinant c-Jun DNA binding domains for NO-induced thiol modifications and concomitant changes in DNA binding activity. In our *in vitro* system, special emphasis was given to the role of the reduced sulfhydryl compound GSH, which is present in concentrations of 1–10 mM in mammalian cells (18). GSH not only protects oxidant-sensitive protein thiols against oxidative damage (19, 20) but also critically determines the biological activity of NO (21–23). We show here that in the presence of physiologically relevant concentrations of GSH, NO inhibits c-Jun DNA binding *in vitro* by specifically targeting the formation of a mixed disulfide with GSH to a conserved cysteine residue in the DNA-binding site of the transcription factor. Furthermore,

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[1] The abbreviations used are: NO, nitric oxide; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine sodium salt; GSNO, *S*-nitrosoglutathione; DTT, dithiothreitol, AP-1, activator protein-1; CC-Jun, wild type human c-Jun DNA binding domain; SC-Jun, human c-Jun DNA binding domain with a cysteine 269 to serine mutation; CS-Jun, human c-Jun DNA binding domain with a cysteine 320 to serine mutation; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.

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we provide experimental evidence that GSNO, which is formed by the reaction of NO with GSH, may mediate the NO-dependent *S*-glutathionylation of c-Jun.

EXPERIMENTAL PROCEDURES

Materials—GSH (free acid, SigmaUltra) and GSSG (free acid, SigmaUltra) were purchased from Sigma and Aldrich. DEA/NO and GSNO were from Alexis Biochemicals. Yeast glutathione reductase (120 units/mg) was provided by Roche Molecular Biochemicals. Stock solutions of [^{3}H]GSH were prepared at a final concentration of 20 mM by the addition of 10 volumes of a freshly prepared solution of 22 mM unlabeled GSH (free acid, SigmaUltra) in H_2O to 1 volume of tritium-labeled glutathione ([^{3}H]GSH, 45–50 Ci/mmol, ~0.02 mM, NEN Life Science Products) and stored in small aliquots at −80 °C. Throughout the text, this preparation of the radiolabeled thiol will be referred to as [^{3}H]GSH. As determined by high pressure liquid chromatographic analysis (24) and in agreement with the specifications provided by the manufacturer, the purity of [^{3}H]GSH was ≥98%. The only detectable contamination was GSSG (≤2%).

Preparation of Wild Type and Mutant c-Jun DNA Binding Domains—The insert coding for the DNA binding domain of human c-Jun, corresponding to amino acids 223–327 of the translated sequence with the GenBank™ accession number J04111, was amplified by polymerase chain reaction and cloned into the *Bam*HI-*Hin*dIII site of the expression vector pQE-30 (Qiagen). The obtained hexahistidine fusion protein, which encodes for one cysteine in the basic DNA-binding site (amino acid 269) and a second cysteine in the leucine zipper (amino acid 320) of c-Jun, was designated as CC-Jun. Cysteine 269 to serine (SC-Jun) and cysteine 320 to serine (CS-Jun) mutants were generated by polymerase chain reaction-directed mutagenesis and cloned into the *Bam*HI-*Hin*dIII site of the expression vector pQE-30. The obtained c-Jun plasmids were transformed into competent *Escherichia coli* (M15[pRep4], Qiagen) according to the instructions of the manufacturer. Recombinant clones were verified by restriction analysis and dideoxynucleotide sequencing. The recombinant proteins were expressed and purified by nickel-chelate chromatography as described (16). The obtained protein preparation was dialyzed excessively against a 25 mM phosphate buffer (pH 7.4), containing 1 mM EDTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 0.01% (v/v) Nonidet P-40, and concentrated up to ~0.5 mM using Vivapore 20 concentrators (Vivascience). Protein concentrations were determined by amino acid analysis. Purity of the proteins was estimated to be >95% as judged by Coomassie-stained SDS gels.

Detection of Covalently Linked c-Jun Homodimers—c-Jun DNA binding domains (10 μM) were incubated for 30 min at 37 °C in a 20 mM Tris/HCl buffer (pH 7.5), containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 0.01% (v/v) Nonidet P-40 (buffer A), and 3 mM GSH in the absence and presence of DEA/NO or GSNO. Reactions were stopped by the addition of iodoacetamide (50 mM) and incubation for further 30 min at room temperature. Samples (4 μg of protein) were subjected to non-reducing SDS-PAGE on discontinuous Tris/glycine slab gels (7 × 8 cm), which contained acrylamide and bisacrylamide at final concentrations of 16 and 0.1% (w/v), respectively (25). Gels were stained for protein with Coomassie Blue R-250 and analyzed by densitometry.

Detection of a Mixed Disulfide between c-Jun and Glutathione—c-Jun DNA binding domains (10 μM) were incubated in a final volume of 0.1 ml at 37 °C in buffer A containing 3 mM [^{3}H]GSH (~3 × 10^6 cpm) in the absence and presence of DEA/NO or GSNO. For some experiments, incubations were performed in the presence of [^{3}H]GSNO, which was prepared from [^{3}H]GSH by nitrosation with acidified nitrite (26), or in the presence of 1 mM NADPH and 0.6 units of yeast glutathione reductase. To isolate the glutathionylated protein by trichloroacetic acid precipitation, reactions were stopped by the addition of 0.9 ml of 10% (w/v) ice-cold trichloroacetic acid and incubation on ice for 30 min. Samples were centrifuged for 10 min at 20,000 × *g*, and the supernatant was discarded. Subsequent to washing the precipitated protein three times with 0.9 ml of 10% (w/v) ice-cold trichloroacetic acid, the protein pellet was dissolved by treatment with 0.1 ml of 0.5 N NaOH for 20 min at 70 °C and assayed for incorporation of [^{3}H]GSH by liquid scintillation counting. Results were corrected for protein recovery (68 ± 7%, mean ± S.E., $n = 6$) and blank values (≤0.1 mol of radiolabeled GSH per mol of protein), which were determined as non-DTT-releasable radiolabel by treating the [^{3}H]GSH-protein adduct for 60 min at 37 °C with 10 mM DTT prior to trichloroacetic acid precipitation.

To study the reversibility of GSNO-induced c-Jun glutathionylation, c-Jun DNA binding domains (10 μM CC-Jun) were incubated in a final volume of 0.8 ml for 1 h at 37 °C in buffer A containing 3 mM [^{3}H]GSH (~3 × 10^7 cpm per ml) and 1 mM GSNO prior to isolation of the *S*-glutathionylated protein by chromatography on Sephadex G-25 columns (NAP-10, Amersham Pharmacia Biotech). Protein-containing fractions were pooled, and stoichiometric (0.8–1.1 mol of [^{3}H]GSH per mol of protein) incorporation of [^{3}H]GSH was verified by trichloroacetic acid precipitation as described above. To study dethiolation of c-Jun, the [^{3}H]GSH-labeled protein was incubated in a final volume of 0.1 ml at 37 °C in buffer A in the absence and presence of 3 mM [^{3}H]GSH (~3 × 10^7 cpm per ml) or 10 mM DTT. After 1 h, samples were analyzed for [^{3}H]GSH incorporation as described above. GSH-induced dethiolation was expressed in percent of the radioactivity that was released from the [^{3}H]GSH-labeled protein by 10 mM DTT.

Determination of c-Jun DNA Binding Activity—c-Jun DNA binding domains (10 μM) were preincubated for 30 min at 37 °C in buffer A, which contained 3 mM GSH, in the absence and presence of DEA/NO or GSNO. For the determination of DNA binding activity by EMSA, 2-μl aliquots of the preincubation mixture were diluted into a final volume of 18 μl of buffer A, which additionally contained 0.2 mg/ml bovine serum albumin, 0.1 mg/ml poly(dI-dC), and where indicated 1 mM DTT. Finally, 2 μl of the ^{32}P-radiolabeled double-stranded AP-1 oligonucleotide (5′-GGG CTT GAT GAG TCA GCC GGA CC-3′) were added, and the samples were incubated for further 30 min prior to electrophoresis at 200 V on pre-electrophoresed 6% non-denaturing polyacrylamide gels with 22 mM Tris borate, 0.5 mM EDTA as running buffer. Gels were dried, visualized by autoradiography, and analyzed by densitometry.

To investigate the reversibility of GSNO-induced c-Jun inactivation, c-Jun DNA binding domains (10 μM CC-Jun) were incubated in a final volume of 0.8 ml for 1 h at 37 °C in buffer A containing 3 mM GSH and 1 mM GSNO prior to isolation of the *S*-glutathionylated protein by chromatography on Sephadex G-25 columns. Protein-containing fractions were pooled, and aliquots (2 μl) were incubated for 1 h at 37 °C in the absence or presence of 1 mM GSNO with increasing concentrations of GSH or 1 mM DTT in a final volume of 20 μl of buffer A containing 0.2 mg/ml bovine serum albumin, 0.1 mg/ml poly(dI-dC), and the ^{32}P-labeled oligonucleotide. Samples were cooled to room temperature, subjected to electrophoresis on non-denaturing gels, and the gels analyzed by autoradiography as described above. DNA binding activity of CC-Jun was quantified by densitometry and expressed as percent of maximal DNA binding of the DTT-reactivated reduced protein which was determined in the presence of 1 mM DTT.

Determination of Nitrite, GSNO, and GSSG Concentrations—Nitrite concentrations were determined photometrically by the Griess reaction (27). GSNO concentrations were calculated from the absorbance at 340 nm using an extinction coefficient of 0.75 mM^{-1} cm^{-1} (28). GSSG concentrations were determined by a coupled assay as glutathione reductase-dependent oxidation of NADPH (29). Briefly, samples (0.1–0.7 ml) were assayed for GSSG in a final volume of 1 ml of a 20 mM triethanolamine/HCl buffer (pH 7.6) containing 0.2 mM EDTA and 0.05–0.2 mM NADPH by addition of 0.6 units of yeast glutathione reductase (120 units/ml) and monitoring the absorbance decrease at 340 nm. NADPH consumption was quantified using an extinction coefficient of 6.34 mM^{-1} cm^{-1}. To study the effect of a GSH-regenerating system on NO-induced oxidation of GSH to GSSG, GSH (3 mM) was co-incubated with DEA/NO (1 mM) and CC-Jun (10 μM) in 0.5 ml of buffer A for 1 h at 37 °C in the absence or presence of 6 units/ml glutathione reductase and 1 mM NADPH. Subsequently, the reductase was removed by rapid filtration through microfilters (cut-off, 10 kDa), and aliquots of the filtrate were assayed for GSSG as described above.

Data Evaluation—Data are presented as mean values ± S.E. with the number (n) of experiments in parenthesis. Concentration-response curves were fitted to the experimental data by the Hill equation. Statistical analysis of data was performed by Student's t test and linear regression analysis.

RESULTS

NO-induced Inhibition of c-Jun DNA Binding Activity Involves a Conserved Cysteine Residue in the DNA-binding Site of the Transcription Factor—Incubations of wild type c-Jun DNA binding domains (CC-Jun) in the presence of 3 mM GSH and increasing concentrations of the NO donor DEA/NO resulted in a concentration-dependent inhibition of DNA binding activity of the protein (Fig. 1). Concentrations of 0.01, 0.1, 0.5, and 1 mM of the NO donor inhibited CC-Jun DNA binding to 94 ± 4, 70 ± 8, 44 ± 3, and 13 ± 3% (n = 4–9) of untreated controls, respectively. DNA binding activity was restored by DTT, sug-

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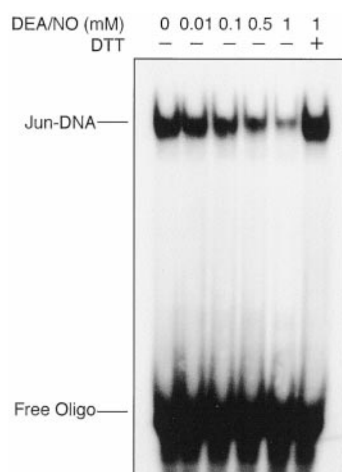

FIG. 1. **Inhibition of CC-Jun DNA binding by NO.** Wild type c-Jun DNA binding domains (CC-Jun) were incubated at final concentrations of 10 μM for 30 min at 37 °C in a 20 mM Tris/HCl buffer (pH 7.5), containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, and 3 mM GSH in the presence of increasing concentrations of DEA/NO. Aliquots (2 μl) were assayed for DNA binding activity by EMSA in the absence and presence of 1 mM DTT as described under "Experimental Procedures." The shown autoradiograph is representative of four experiments.

gesting that the modification of a cysteine residue may be involved in the NO-mediated inhibition of CC-Jun.

The homodimeric c-Jun DNA binding domain contains two pairs of cysteine residues, one located in its basic DNA-binding site (Cys-269) and one located in the leucine zipper-like subunit interface (Cys-320). To assign the inhibitory effect of NO to one of these two pairs of cysteine residues, we compared the effects of NO on the DNA binding activity of wild type and mutant c-Jun constructs. As shown in Fig. 2, NO clearly inhibited DNA binding activity of CC-Jun (87 ± 3% inhibition, $n = 9$), whereas DNA binding of the mutant with a cysteine to serine mutation in the DNA-binding site (SC-Jun) was not significantly affected (8 ± 10% inhibition, $n = 5$). On the other hand, mutation of the cysteine in the leucine zipper domain of the c-Jun DNA binding domain (CS-Jun) did not attenuate the inhibitory effect of DEA/NO (76 ± 11% inhibition, $n = 5$). These data, therefore, render it likely that NO-mediated inhibition of c-Jun DNA binding involves the modification of a single cysteine residue (Cys-269) in the DNA-binding site of the protein.

NO-induced Formation of an Intermolecular Disulfide Bridge between c-Jun Monomers Is Not Involved in the Inhibition of the Transcription Factor by NO—The formation of disulfide bonds between subunits of the AP-1 transcription factor, which is composed by Jun/Jun homodimers or heterodimers between Jun and Fos proteins, was suggested as one potential mechanism by which NO might inhibit AP-1 DNA binding activity (12). As shown in Fig. 3, NO released from 1 mM of the NO donor DEA/NO in the presence of 3 mM GSH in fact induced an SDS-resistant dimerization of wild type c-Jun DNA-binding subunits (CC-Jun). Under these conditions, >70% of CC-Jun were converted into covalently linked dimers as determined by non-reducing SDS-PAGE. The reversibility of this effect by DTT suggests the formation of an intermolecular disulfide bridge between one or both of the two cysteines located in the DNA binding module of c-Jun monomers. A comparison of cysteine to serine mutants shows that SC-Jun but not CS-Jun was converted into disulfide-linked dimers demonstrating that NO specifically targets disulfide formation to cysteine 320 in the leucine zipper domain of c-Jun. Since this cysteine residue is apparently not involved in the NO-mediated loss of c-Jun function (see Fig. 2), these data make intermolecular disulfide formation a highly unlikely mechanism for the inhibition of the transcription factor by NO.

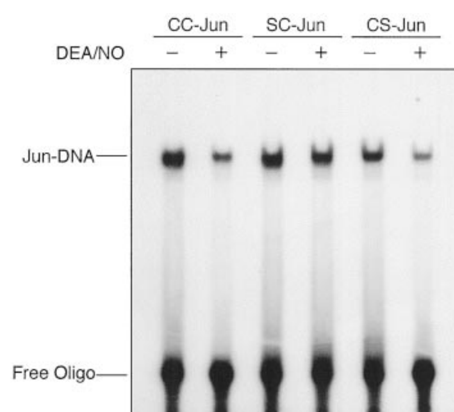

FIG. 2. **Involvement of cysteine residues in the inhibition of CC-Jun DNA binding by NO.** Wild type (CC-Jun) and mutant c-Jun DNA binding domains, in which either the cysteine located in the DNA-binding site (SC-Jun) or adjacent leucine zipper (CS-Jun) were substituted by serine, were incubated with 1 mM DEA/NO in the presence of 3 mM GSH and analyzed for DNA binding activity by EMSA as described under "Experimental Procedures." The shown autoradiograph is representative of five experiments.

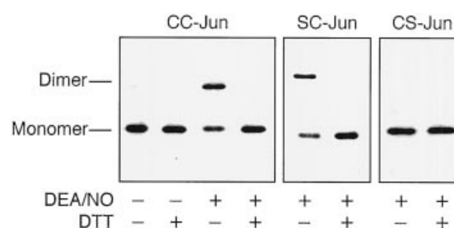

FIG. 3. **NO-mediated formation of an intermolecular disulfide bond between CC-Jun monomers.** Wild type (CC-Jun) and mutant c-Jun DNA binding domains, in which either the cysteine located in the DNA-binding site (SC-Jun) or adjacent leucine zipper (CS-Jun) were substituted by serine, were incubated for 30 min at 37 °C with 1 mM DEA/NO in the presence of 3 mM GSH and analyzed for covalent dimerization by non-reducing SDS-PAGE as described under "Experimental Procedures." The shown gels are representative of at least four similar experiments.

NO Induces the Formation of a Mixed Disulfide between c-Jun and GSH—An alternative mechanism by which protein thiols may transduce oxidative stress into a post-translational modification and functional response is the formation of a mixed disulfide with GSH (30, 31). To investigate if this mechanism may be involved in the NO-induced inhibition of c-Jun, we exposed purified c-Jun DNA binding domains to 1 mM of the NO donor DEA/NO in the presence of 3 mM ^{3}H-labeled GSH, and we isolated the covalent [^{3}H]GSH-protein adduct by trichloroacetic acid precipitation. As shown in Fig. 4*A*, DEA/NO induced a time-dependent incorporation of the radiolabel with an apparent half-time of ~5 min and a maximal incorporation of 0.8–0.9 mol of [^{3}H]GSH per mol of CC-Jun (*closed symbols*). Control incubations in the absence of DEA/NO (*open symbols*) did not yield any significant amounts of protein bound [^{3}H]GSH. DTT-labile incorporation of the radiolabel (see "Experimental Procedures") suggests binding of [^{3}H]GSH to the protein via a disulfide bond.

Fig. 4*B* shows the dependence of [^{3}H]GSH incorporation on the concentration of DEA/NO. At concentrations of 0.01, 0.1, 0.5, and 1 mM, DEA/NO induced binding of 0.05 ± 0.02, 0.28 ± 0.11, 0.62 ± 0.06, and 0.81 ± 0.08 mol of [^{3}H]GSH (n = 3–6) per mol of protein, respectively. Half-maximal mixed disulfide formation was estimated to occur at DEA/NO concentrations of ~300 μM. According to a recently published

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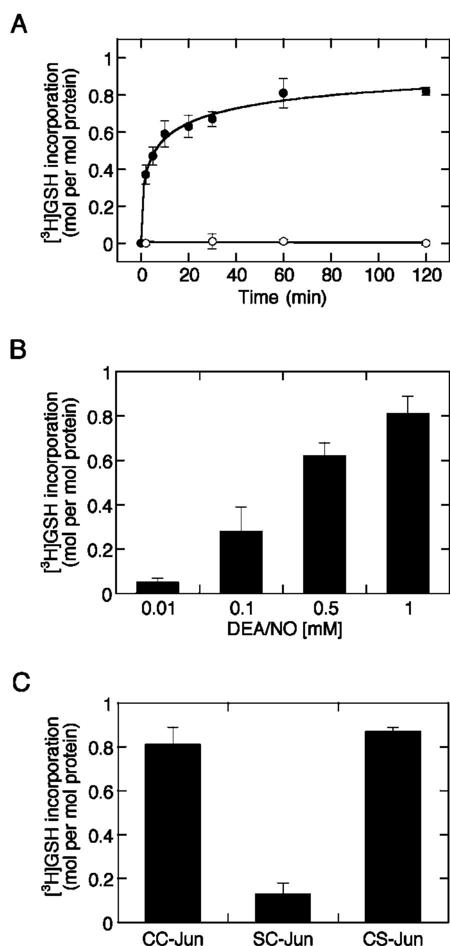

FIG. 4. **NO induces the formation of a mixed disulfide between glutathione and CC-Jun.** Wild type c-Jun DNA binding domains (10 μM CC-Jun) were incubated for the indicated times at 37 °C in the buffer described in the legend to Fig. 1, which contained 3 mM [^{3}H]GSH, in the absence (*open symbols*) or presence (*closed symbols*) of 1 mM DEA/NO (*A*). For DEA/NO concentration-response curves (*B*), incubation times were 60 min. For the comparison of wild type (CC-Jun) and mutant c-Jun proteins, in which either the cysteine located in the DNA-binding site (SC-Jun) or adjacent leucine zipper (CS-Jun) were substituted by serine, the time of incubation and the final concentration of DEA/NO were 60 min and 1 mM, respectively (*C*). Protein *S*-glutathionylation was determined as trichloroacetic acid-precipitable, DTT-labile [^{3}H]GSH incorporation as described under "Experimental Procedures." Data are mean values ± S.E. of three to five experiments.

mathematical model (32), the steady state concentration of NO under these conditions, *i.e.* 300 μM DEA/NO at 37 °C and pH 7.4, reaches a peak level of approximately 20 μM at ~0.6 min and drops exponentially to submicromolar concentrations within 25 min.

As shown in Fig. 4*C*, GSH incorporation into CC-Jun was almost completely abolished by a cysteine 269 to serine mutation (0.13 ± 0.05 mol [^{3}H]GSH/mol SC-Jun, n = 5), whereas a cysteine 320 to serine mutation virtually did not affect the degree of *S*-thiolation (0.87 ± 0.01 mol [^{3}H]GSH/mol CS-Jun, n = 3). These data, therefore, demonstrate that NO specifically targets the formation of a mixed disulfide to a single cysteine residue in the DNA-binding site of c-Jun (Cys-269). The finding that this cysteine residue is involved in the NO-induced inhibition of CC-Jun (see Fig. 2) suggests that NO-induced *S*-glutathionylation mediates the inhibitory effect of NO. This was confirmed by linear regression analysis of data from DNA binding (Fig. 1) and [^{3}H]GSH incorporation (Fig. 4*B*) experiments, which yields a highly significant (p = 0.01) inverse linear correlation between relative DNA binding activity of CC-Jun and DEA/NO-mediated *S*-glutathionylation of the protein (intercept, 1.00; slope, −1.01; r = 0.99).

TABLE I
Nitrite, GSSG, and GSNO formation in incubations of GSH with DEA/NO

GSH (3 mM) was incubated in the absence (control) and presence of increasing concentrations of DEA/NO at 37 °C in a 20 mM Tris/HCl buffer (pH 7.5), containing 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, and 0.01% (v/v) Nonidet P-40. After 60 min, aliquots were assayed for nitrite, GSSG, and GSNO as described under "Experimental Procedures." Data are mean values ± S.E. of 3–6 experiments.

Condition	Nitrite	GSSG	GSNO
		μM	
Control	<1	15 ± 4	<1
DEA/NO 10	9 ± 3	15 ± 5	4 ± 1
100	119 ± 10	17 ± 2	49 ± 1
500	585 ± 33	39 ± 2	257 ± 13
1000	1043 ± 91	241 ± 13	464 ± 24

NO-induced c-Jun S-Glutathionylation Is Not Mediated by a Change in the GSH/GSSG Ratio—Various mechanisms have been suggested to account for the formation of a mixed disulfide between GSH and protein thiols (33). GSSG may directly *S*-thiolate proteins via a thiol/disulfide exchange mechanism. Dependent on the protein examined half-maximal glutathionylation was observed at GSH/GSSG ratios ranging from 27 to 10^{-5} (34). NO was reported to oxidize GSH to GSSG under anaerobic conditions, at low GSH/NO ratios, or via a secondary reaction of GSNO with GSH (22, 35–37). In our experimental system, GSSG concentrations were 15 ± 4 μM (n = 6) under control conditions, *i.e.* in the presence of 3 mM GSH and absence of an NO donor (Table I). During a 60-min incubation at 37 °C, DEA/NO at concentrations of up to 500 μM oxidized less than 3% of the total amount of GSH (3 mM at t = 0) to GSSG. At a concentration of 1 mM, DEA/NO converted ~16% of GSH into GSSG, which results in a decrease of the GSH/GSSG ratio to values <10. These data raise the possibility that an NO-induced shift in the GSH/GSSG ratio may mediate c-Jun mixed disulfide formation. To address this issue, we analyzed CC-Jun for NO-induced [^{3}H]GSH incorporation under conditions where GSSG was recycled continuously to GSH by glutathione reductase (Fig. 5). In the presence of the GSH-regenerating system, NO-dependent GSSG formation was almost completely suppressed (20 ± 11 μM, n = 3) as compared with controls (241 ± 13 μM, n = 3). This ~10-fold decrease in GSSG, which resulted in a >15-fold increase of the GSH/GSSG ratio from <10 to ~130, did not significantly (p > 0.5) affect mixed disulfide formation (0.76 ± 0.04, n = 3 *versus* 0.81 ± 0.08, n = 5). Thus, these data argue against the involvement of GSSG in the NO-induced *S*-glutathionylation of c-Jun.

NO-induced c-Jun S-Glutathionylation May Be Mediated by GSNO—Recently, GSNO has been reported to induce the *S*-glutathionylation of aldose reductase (38). Under aerobic conditions, NO reacts with oxygen to yield the nitrosating species N_2O_3. In the presence of an excess of GSH, hydrolysis of N_2O_3 to nitrite is competitive with the rate of its reaction with GSH to GSNO (35). Accordingly, we found that in the presence of 3 mM GSH 1.08 ± 0.04 mol of nitrite (n = 16) and 0.47 ± 0.01 mol of GSNO (n = 16) were formed per mol of DEA/NO during an incubation period of 60 min at 37 °C (Table I). To investigate if GSNO induces the formation of a mixed disulfide between GSH and c-Jun, we incubated CC-Jun with increasing concentrations of GSNO in the presence of 3 mM ^{3}H-labeled GSH, and we analyzed the protein for *S*-[^{3}H]glutathionylation (Fig. 6, *open symbols*). One mM GSNO induced the incorporation of 0.98 ± 0.07 mol of [^{3}H]GSH/mol of CC-Jun (mean ± S.E., n = 3). From the concentration-response curve shown in Fig. 6, we calculated a half-maximally active GSNO concentration of 160 μM.

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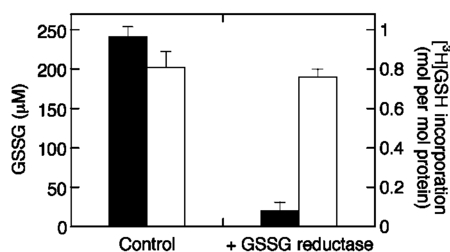

FIG. 5. **Effect of a GSH-regenerating system on NO-mediated GSSG formation and CC-Jun *S*-glutathionylation.** Wild type c-Jun DNA binding domains (10 μM CC-Jun) were incubated with 1 mM DEA/NO in the buffer described in the legend to Fig. 1, which contained 3 mM unlabeled (GSSG determinations) or ^{3}H-labeled ([^{3}H]GSH incorporation) GSH. Incubations were performed for 60 min at 37 °C in the absence (control) or presence (GSSG reductase) of 6 units/ml glutathione reductase and 1 mM NADPH. Samples were assayed for GSSG formation (*closed bars*) and [^{3}H]GSH incorporation into CC-Jun (*open bars*) as described under "Experimental Procedures." Data are mean values ± S.E. of three to five experiments.

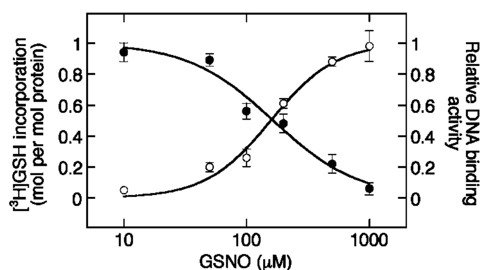

FIG. 6. **Concentration-dependent *S*-glutathionylation and inhibition of CC-Jun by GSNO.** Wild type c-Jun DNA binding domains (10 μM CC-Jun) were incubated with increasing concentrations of GSNO for 60 min at 37 °C in the presence of 3 mM ^{3}H-labeled ([^{3}H]GSH incorporation) or unlabeled (DNA binding assay) GSH and assayed for [^{3}H]GSH incorporation (*open symbols*) and DNA binding activity (*closed symbols*) as described under "Experimental Procedures." Relative DNA binding activity is expressed as the ratio between the DNA binding activity in the presence and absence of GSNO. Data are mean values ± S.E. of three to five experiments.

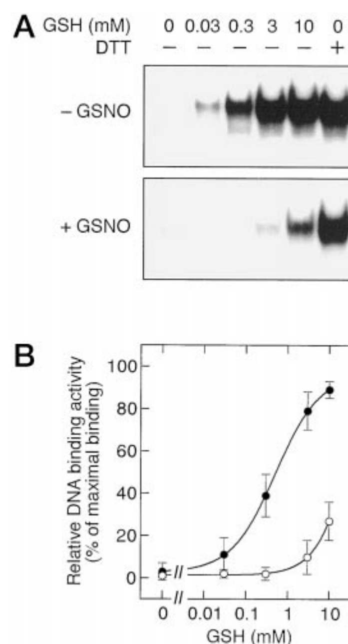

FIG. 7. **Reactivation of GSNO-treated CC-Jun by GSH.** *A,* GSNO-inactivated CC-Jun, which was prepared by incubation with 1 mM GSNO in the presence of 3 mM GSH and isolated as described under "Experimental Procedures," was incubated in the absence (*upper panel*) and presence (*lower panel*) of 1 mM GSNO with increasing concentrations of GSH (30 μM to 10 mM) or DTT (1 mM) for 1 h at 37 °C and analyzed for DNA binding activity by EMSA (see "Experimental Procedures"). The shown autoradiographs are representative of four to six similar experiments. *B,* densitometric analysis of the autoradiographs shown in *A*. Data from experiments performed in the absence (*closed symbols*) or presence (*open symbols*) of GSNO are expressed as the percent of DNA binding activity recovered under each condition relative to the DTT-reactivated transcription factor (mean values ± S.E., n = 4–6).

In agreement with a rapid exchange of NO between GSNO and [^{3}H]GSH, as it can be expected from the high trans-nitrosation rate (~70 $M^{-1} \times s^{-1}$) between equivalent thiol groups (39), essentially the same results were obtained when unlabeled GSNO was replaced by [^{3}H]GSNO (not shown). GSNO-induced *S*-glutathionylation of CC-Jun was paralleled by a loss of DNA binding activity of the transcription factor (Fig. 6, *closed symbols*) and mapped to Cys-269 (not shown). Linear regression analysis confirmed a highly significant (p = 0.002) inverse linear correlation between relative DNA binding activity of CC-Jun and GSNO-mediated *S*-glutathionylation of the protein (intercept, 0.96; slope, −0.88; r = 0.96).

The biological activity of GSNO can be explained in part by the release of NO from the nitrosothiol due to copper-catalyzed homolytic cleavage (40). Alternatively, a direct nucleophilic attack of protein thiols on the nitrosothiol, which does not require cleavage of GSNO, has been suggested as a potential mechanism for GSNO-mediated protein thiolation (38). To address this issue, we measured NO release from GSNO under conditions that elicited quantitative *S*-glutathionylation of CC-Jun by determining the accumulation of nitrite in the presence of 1 mM GSNO and 3 mM GSH during a 1-h incubation at 37 °C. Under these conditions, nitrite accumulation was barely detectable (1.5 ± 0.1 μM, n = 3), indicating that GSNO does not release NO in quantities that could explain the GSNO-induced *S*-glutathionylation of c-Jun. Identical results were obtained when CC-Jun (10–100 μM) was included in the incubations. Furthermore, in accordance with a previous report on the stability of GSNO in the presence of chelating agents such as EDTA and millimolar concentrations of GSH (28), we did not observe any significant decomposition of the nitrosothiol (<2% of total GSNO) as determined by UV spectroscopy, *i.e.* by monitoring the absorbance of the incubation mixture at 340 nm.

Inhibition of c-Jun by GSNO-induced S-Glutathionylation Is Reversible—To investigate if the GSNO-mediated inhibition of c-Jun can be reversed by GSH, we *S*-glutathionylated CC-Jun by incubation with GSNO and separated the protein from GSNO by chromatography on Sephadex G-25 columns. Subsequently, the isolated protein was incubated with increasing concentrations of GSH and analyzed for DNA binding activity by EMSA (Fig. 7*A, upper panel*, and Fig. 7*B, closed symbols*). GSH concentrations of 0.03, 0.3, 3, and 10 mM induced a concentration-dependent recovery of DNA binding activity to 11 ± 8, 39 ± 10, 79 ± 9, and 89 ± 4% (n = 4–6) of maximal DNA binding activity (*i.e.* DNA binding activity of the DTT-reactivated, reduced transcription factor), respectively. In the presence of 1 mM GSNO (Fig. 7*A, lower panel*, and Fig. 7*B, open symbols*) DNA binding activity was only marginally increased by co-incubations with GSH at concentrations up to 3 mM (≤10% of the DTT-reactivated protein) and only partially (27 ± 9%) recovered at the highest GSH concentration (10 mM) investigated. These data, therefore, indicate that inhibition of c-Jun by GSNO-induced mixed disulfide formation may be reverted by the physiological sulfhydryl GSH provided that GSNO, which appears to antagonize the reduction of the mixed disulfide by GSH, is removed from the system. To substantiate that reactivation of GSNO-inactivated c-Jun is associated with dethiolation of the protein, we incubated isolated, *S*-^{3}H-glutathionylated

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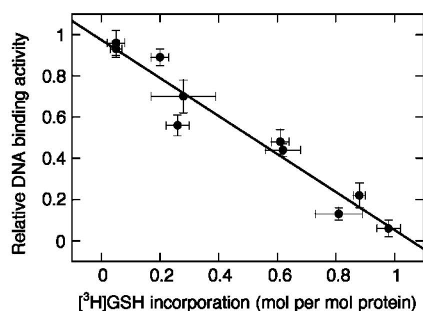

FIG. 8. ***S*-Glutathionylation of CC-Jun correlates with a loss of its DNA binding activity.** To correlate c-Jun inhibition with *S*-glutathionylation, densitometric data from DNA binding assays performed in the absence and presence of DEA/NO and GSNO (Figs. 1 and 6) were re-plotted against the DEA/NO- and GSNO-induced incorporation of [^{3}H]GSH into CC-Jun (Figs. 4*B* and 6). Data, which are mean values ± S.E. of 3–9 experiments, were analyzed by linear regression (intercept, 0.97; slope, −0.92; $r = 0.97$, $p < 0.001$).

CC-Jun (see "Experimental procedures") in the absence and presence of 3 mM [^{3}H]GSH or 10 mM DTT for 1 h at 37 °C and assayed the protein for [^{3}H]GSH incorporation. In the presence of 3 mM [^{3}H]GSH, 77 ± 4% ($n = 3$) of the total DTT-releasable radioactivity were liberated from the protein. When GSNO (1 mM) was additionally present, we did not detect any significant dethiolation (data not shown). The good correlation between recovery of DNA binding activity and dethiolation in the presence of 3 mM GSH suggests that c-Jun *S*-glutathionylation may be reversed by physiologically relevant concentrations of GSH and render dethiolation a likely mechanism of GSH-induced reactivation of the transcription factor.

DISCUSSION

NO and NO donor compounds such as *S*-nitrosothiols and nitrosyl-iron complexes have been implicated in the redox control of protein function in terms of biological signaling as well as nitrosative stress (41–43). Dependent on the reactivity and structural context of the protein thiol on one hand and the reactivity of the NO-derived species on the other hand, various mechanisms may account for the post-translational modification of proteins by NO. It has been suggested that NO may reversibly modify protein-bound cysteines by at least four distinct mechanisms including the following: (i) the covalent attachment of an NO moiety to the thiol, *i.e. S*-nitrosylation; (ii) the reversible oxidation to sulfenic acid; (iii) the formation of intra- or intermolecular disulfide bridges; and (iv) the formation of a mixed disulfide with GSH, *i.e. S*-glutathionylation (44). Here we show that NO and GSNO inhibit DNA binding activity of c-Jun by selectively targeting the formation of a mixed disulfide with GSH to a conserved cysteine residue in the basic DNA-binding site of the transcription factor. A replot of data (Fig. 8) from densitometric analysis of DNA binding assays against [^{3}H]GSH incorporation into CC-Jun, which includes data from assays performed with DEA/NO and GSNO (see Figs. 1, 4*B,* and 6), shows a highly significant ($p < 0.001$) inverse linear relationship between c-Jun *S*-glutathionylation and relative DNA binding activity (intercept, 0.97; slope, −0.92; $r = 0.97$). The excellent correlation between NO/GSNO-mediated GSH incorporation into the Jun protein on one hand and the concomitant loss of DNA binding activity on the other hand as well as the site specificity of both phenomena suggest that *S*-glutathionylation in fact accounts for the inhibition of the transcription factor DNA binding activity by NO and GSNO.

Possible mechanisms of NO/GSNO-induced protein *S*-glutathionylation include the activation of the protein cysteine by *S*-nitrosylation or oxidation to a sulfenate which may subsequently react with GSH to the corresponding mixed disulfide (44, 45). Although we did not observe any *S*-nitrosylation of the relevant c-Jun cysteine as judged by UV/Vis spectroscopy of the DEA/NO-treated protein (not shown), we cannot exclude that *S*-nitrosylated c-Jun may be formed as a short lived intermediate. A c-Jun sulfenate, which has been proposed in a previous study to account for the redox regulation of AP-1 (13), may be another reactive intermediate in the modification of c-Jun by NO donors. In support of this hypothesis, we found that the spectroscopic sulfhydryl/sulfenate probe 7-chloro-4-nitro-2-oxa-1,3-diazole reacts with an intermediate, which was formed during the oxidation of CC-Jun by NO, to an adduct with an absorbance maximum at 350 nm. Although the formation of a compound with these spectral characteristics would be consistent with the intermediary conversion of a protein thiol to a sulfenate (46), quantitative analysis of these data revealed that less than 5% of the protein were scavenged as c-Jun sulfenate.[2] Moreover, we did not detect any c-Jun sulfenate when GSNO was used instead of NO, which argues against a role for this intermediate in the NO/GSNO-mediated thiolation of c-Jun. Definitive conclusions about the formation of presumably short lived intermediates preceding NO/GSNO-dependent *S*-glutathionylation of c-Jun, however, await detailed kinetic analysis by stopped flow techniques.

An alternative explanation for the NO-induced *S*-glutathionylation of c-Jun may be the formation of GSNO due to *S*-nitrosylation of GSH by NO and subsequent reaction of GSNO with the protein. In keeping with a role for GSNO in the modification of c-Jun by NO, we found that the reaction of NO with GSH yields GSNO in concentrations (see Table I), which fitted well with the efficacy and potency of DEA/NO and GSNO to elicit both c-Jun *S*-glutathionylation and inhibition (see Figs. 4*B* and 6). The apparent lack of NO release from GSNO under our experimental conditions further supports the view that GSNO itself and not NO may be the reactive species involved in the transfer of the glutathionyl moiety to the c-Jun protein. This hypothesis fits well with a recent report showing that GSNO directly *S*-glutathionylates human aldose reductase via a nucleophilic attack of the enzyme thiol on the sulfur of GSNO (38). However, although apparently only a small portion of GSNO (<2%) decomposed under our experimental conditions, we cannot exclude the involvement of other as yet unidentified reactive species which might efficiently thiolate the protein at low concentrations. There is evidence that GSH and GSNO may react to the corresponding *N*-hydroxysulfenamide and, depending on the availability of GSH and oxygen, this adduct may undergo a number of reactions yielding GSSG, GSH sulfinic acid, GSH sulfenamide, GSH sulfinamide, GSH sulfenylhydroperoxide, and various presumably short lived radical species on the one hand and nitrite, N_2O, and NH_3 on the other hand (22). Thus, given the complex and as yet not entirely elucidated chemistry of the GSH/GSNO system, further studies are required to establish the molecular mechanism underlying GSNO-induced mixed disulfide formation.

We show that inhibition of c-Jun by NO and GSNO maps to a conserved cysteine residue in the DNA-binding site of c-Jun. This oxidant-sensitive cysteine residue has been identified in previous studies as the amino acid residue that provides redox sensitivity to c-Jun presumably by suffering reversible oxidation to a sulfenic acid (13, 14, 47). In support of a redox-dependent regulation of c-Jun DNA binding, *in vitro* as well as cell culture studies showed that depletion of reducing thiols in the incubation medium (16), treatment of

[2] P. Klatt, E. Pineda Molina, and S. Lamas, unpublished data.

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AP-1 with GSSG or oxidized thioredoxin (48), depletion of intracellular GSH pools (48), or immunodepletion of the nuclear redox protein Ref-1 (15) attenuate AP-1 DNA binding activity. In contrast with the oxidative inactivation of c-Jun and c-Fos seen previously by Curran and co-workers (13) inhibition of c-Jun by NO/GSNO-induced *S*-glutathionylation occurs under reductive conditions, *i.e.* in the presence of millimolar concentrations of GSH and GSH/GSSG ratios >100 (see Table I and Fig. 5). Although the extension of c-Jun *S*-glutathionylation to *in vivo* systems remains to be established, these data raise the possibility that regulation of AP-1 DNA binding activity by oxidative and nitrosative stress may operate independently from each other.

GSNO-induced inhibition of c-Jun by *S*-glutathionylation appears to be a reversible process. In the presence of physiologically relevant concentrations of GSH, removal of the nitrosothiol resulted in dethiolation of c-Jun and recovery of its DNA binding activity. Given that GSNO may accumulate in situations of nitrosative stress on the one hand and may be subjected to decomposition via nonenzymatic copper-dependent mechanisms (28) or recently discovered nitrosothiol-metabolizing enzymes (49) on the other hand, these data suggest that reversible GSNO-dependent thiolation of c-Jun may be a control mechanism linking GSNO formation to regulation of transcription. Of note, the thioredoxin/thioredoxin reductase system, which has been implicated in the redox regulation of the AP-1 transcription factor (15, 50, 51), has been shown recently to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), and to reactivate RNA-binding proteins after exposure to NO (53). It remains to be investigated, however, if this nuclear redox system plays a role in the regulation of AP-1 by GSNO.

It is well established that reactive oxygen and nitrogen species play a key role in the redox regulation of cellular activation, transcription, proliferation, and cell death (44, 54–58). Within this concept of oxidative and nitrosative stress, reversible *S*-glutathionylation of oxidant-sensitive cysteines has been established as one of the post-translational protein modifications that may regulate protein function in response to oxidative stress or protect proteins against irreversible oxidative damage (30, 31). It has been shown that the activation of protein thiols by reactive oxygen species such as superoxide and hydrogen peroxide facilitates the formation of a mixed disulfide between protein thiols and glutathione (59). The results of this study, demonstrating reversible GSNO-dependent *S*-glutathionylation of c-Jun *in vitro*, suggest that *S*-thiolation of a transcription factor triggered by reactive nitrogen species and nitrosothiols may add a novel molecular mechanism to the concept of nitrosative stress. In support of a potential role for mixed disulfide formation as a signal by which nitrosative stress is sensed by cells, *S*-thiolation of endothelial cell proteins in response to NO has been reported recently (60). Given the striking structural similarities between the positively charged DNA-binding site of c-Jun and the DNA binding domain of a number of cysteine-containing transcription factors, including members of the Fos, ATF/CREB, Myb, and Rel/NF-κB families, NO-induced *S*-glutathionylation of a basic DNA binding motif as exemplified by c-Jun may represent a general mechanism by which nitrosative stress is transduced into a functional response at the transcriptional level.

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Nitric Oxide Inhibits c-Jun DNA Binding by Specifically Targeted *S*-Glutathionylation

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