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

(Received for publication, December 3, 1998, and in revised form, February 4, 1999)

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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)† 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-the DNA binding activity of transcriptional activators such as NF-κB (7), and NF-κB (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 occurs 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,
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 GSNG (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 [3H]GSH were prepared at a final concentration of 20 μM by the addition of 10 volumes of a freshly prepared solution of 22 μM unlabeled GSH (free acid, SigmaUltra) in H2O to 1 volume of tritium-labeled glutathione ([3H]GSH, 45–50 Ci/mmol, −0.02 mN, 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 [3H]GSH. As determined by high pressure liquid chromatographic analysis (24) and in agreement with the specifications provided by the manufacturer, the purity of [3H]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 BamH1-HindIII 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 BamH1-HindIII site of the expression vector pQE-30. The obtained c-Jun plasmids were transformed into competent Escherichia coli (M15[Rep4], 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 (25) with 22 mM Tris borate, 0.5 mM EDTA, and 0.02 mM NADPH as running buffer. Gels were dried, visualized by autoradiography, and analyzed by densitometry.

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**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 and a molar extinction coefficient of 13.6 at 340 nm (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
suggesting 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.

**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 MgCl2, 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 as shown in the absence and presence of 1 mM DTT as described under “Experimental Procedures.” The shown autoradiograph is representative of four experiments.

**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 autoradiographs are representative of at least four similar experiments.

**Fig. 3. NO-mediated formation of an intermolecular disulfide bond between c-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.

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 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.

**Fig. 4A.** DEAE/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 [3H]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 [3H]GSH. DTT-labile incorporation of the radiolabel (see “Experimental Procedures”) suggests binding of [3H]GSH to the protein via a disulfide bond.

**Fig. 4B.** Shows the dependence of [3H]GSH incorporation on the concentration of DEAE/NO. At concentrations of 0.01, 0.1, 0.5, and 1 mM, DEAE/NO induced binding of 0.05 ± 0.02, 0.28 ± 0.11, 0.62 ± 0.06, and 0.81 ± 0.08 mol of [3H]GSH (α = 3–6) per mol of protein, respectively. Half-maximal mixed disulfide formation was estimated to occur at DEAE/NO concentrations of ~300 μM. According to a recently published
cysteine 320 to serine mutation virtually did not affect the pH 7.4, reaches a peak level of approximately 20 mM glutathione and CC-Jun.

Wild type c-Jun DNA binding domains (10 that this cysteine residue is involved in the NO-induced inhibition (0.13 ± 0.05 mol [3H]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 [3H]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 [3H]GSH incorporation (Fig. 4B) 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).

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 10–6 (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 mM 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 [3H]GSH incorporation under conditions where GSSG was recycled continuously to GSH by glutathione reduc-tase (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 N2O5. In the presence of an excess of GSH, hydrolysis of N2O5 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 3H-labeled GSH, and we analyzed the protein for S-[3H]glutathionylation (Fig. 6, open symbols). One mM GSNO induced the incorporation of 0.98 ± 0.07 mol of [3H]GSH/mmol 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.

| Condition | Nitrite μM | GSSG μM | GSNO μM |
|-----------|-----------|---------|---------|
| Control   | <1        | 15 ± 4  | <1      |
| DEA/NO 10 | 9 ± 3     | 15 ± 5  | 4 ± 1   |
| 100       | 119 ± 10  | 17 ± 2  | 49 ± 1  |
| 500       | 555 ± 33  | 39 ± 2  | 257 ± 13|
| 1000      | 1043 ± 91 | 241 ± 13| 64 ± 24 |

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 [3H]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 [3H]GSH incorporation as described under “Experimental Procedures.” Data are mean values ± S.E. of three to five experiments.
In agreement with a rapid exchange of NO between GSNO and [3H]GSH, as it can be expected from the high trans-nitrosation rate (~70 s⁻¹ × s⁻¹) between equivalent thiol groups (39), essentially the same results were obtained when unlabeled GSNO was replaced by [3H]GSNO (not shown). GSNO-induced S-glutathionylation of CC-Jun was paralleled by a loss of 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.

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 elicted quantitative S-glutathionylation of CC-Jun by determining the accumulation of nitrite in the presence of 1 mM GSNO and 3 mM GSNO 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 GSNO (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 GSNO, we incubated isolated CC-Jun 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 GSNO (30 μM to 10 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. A, 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).

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CC-Jun (see “Experimental procedures”) in the absence and presence of 3 mM [3H]GSH and GSNO (Figs. 1 and 6), we did not detect any significant dethiolation ([15862]

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 [3H]GSH into CC-Jun (Figs. 4B 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).

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 NO, 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 [3H]GSH incorporation into CC-Jun, which includes data from assays performed with DEA/NO and GSNO (see Figs. 1, 4B, 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 sulphydryl/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. 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. 4B 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 sulfenic acid, GSH sulfenamide, GSH sulfinamide, GSH sulfenylhydroperoxide, and various presumably short lived radical species on the one hand and nitrite, NO2− and NH3 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

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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 deethiolation 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 c-Jun 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), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12), to cleave GSNO (52), to reverse inhibition of AP-1 by NO (12).
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