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Inhibition of AP-1 DNA Binding by Nitric Oxide Involving Conserved Cysteine Residues in Jun and Fos

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Nitric oxide (NO), which has diverse biological effects, can modulate AP-1 activity. Since DNA binding of Jun-Jun and Jun-Fos dimers is regulated *in vitro* by redox control involving conserved cysteines, we hypothesized that the action of NO is mediated via these residues. We performed electrophoretic mobility-shift analyses using Jun and Fos recombinant proteins and NO solutions. Cysteine-to-serine mutants showed that the inhibition of AP-1 activity following NO treatment was dependent on the presence of Cys_{7272} and Cys_{154} in the DNA binding domain of Jun and Fos, respectively. The inhibitory effect of NO was reversed by DTT and the thioredoxin system. Our results demonstrate that NO mediates its inhibitory effect by reacting specifically with the conserved cysteine residues in Jun and Fos.

Nitric oxide (NO) is a short lived free radical gas with many and varied physiological roles. NO has been identified as the primary endogenous vasodilator released from vascular endothelium (1). NO closely mimics macrophage mediated cytotoxicity, while scavengers of NO block its cytotoxic effect, so it has been inferred that NO is the cytotoxic agent of activated macrophages (2). NO has a function in the nervous system as cultured cerebellar granule cells release nitric oxide after exposure to glutamate analogs (3). NO appears to be a neuromodulator or neurotransmitter because nitric oxide synthase (NOS) inhibitors block stimulation of cGMP synthesis in brain slices by glutamate acting at NMDA receptors (4) and block nonadrenergic noncholinergic (NANC) relaxation of intestinal smooth muscle (5). Proteins such as plasminogen (6), glyceraldehyde-3-phosphate dehydrogenase (7) hemoglobin (8) and $p21^{ras}$ (9) have been found to be S-nitrosylated *in vivo.* Furthermore, NO has been suggested to modulate the activity of AP-1 and NF-kB transcription factors (10-12).

The protein products of the proto-oncogenes c-fos and c-jun, Fos and Jun, are components of the AP-1 transcription factor. AP-1 binds to and activates transcription from the TPA-responsive element (TRE) carried by many genes (13). DNA binding of c-Fos and c-Jun apart from phosphorylation, is modulated by the redox status of a single conserved cysteine residue in the DNA-binding domains of the two proteins (Fos-Cys-154 and Jun-Cys-272) (14,15). Reduced cysteine residues cause enhanced DNA-binding whereas oxidation inhibits its binding (14). A specific protein (Ref-1), which enhances binding via reduction of the oxidized cysteine residues has been isolated (15). Oxidized Ref-1 is reduced by thioredoxin. Thioredoxin (12 kDa) has a redox active disulfide located on a protrusion in its three-dimensional structure and is reduced by thioredoxin reductase and NADPH (the thioredoxin system) (16, 17). Thioredoxin is a powerful protein disulfide reductase (18).

The aim of the present study was to investigate whether nitric oxide inhibits Fos/Jun binding by specific posttranslational modification of the conserved redox sensitive cysteine residue. The activity of the AP-1 transcription factor could thus be regulated by a redox mechanism involving NO.

MATERIALS AND METHODS

Materials. NO was a saturated ≈2 mM solution in deoxygenated H_2O, previously energetically flushed with He. The NO concentration was indirectly determined by measuring nitrite utilizing the Griess reaction (19). Only day old solutions were used. Recombinant human thioredoxin and mammalian thioredoxin reductase were prepared as described. (20, 21). Intact c-Jun protein was produced in *E. coli* using a pET8c expression vector. Utilized protein was approximately 80% pure. The truncated polypeptides contained within Fos amino acids 116 to 211 and within Jun amino acids 224 to 334. Mutated Fos and Jun polypeptides contained substitutions in the cysteine residue Cys_{154} and Cys_{272}, respectively (14) and were kindly provided by Dr. Steven Xanthoudakis from Hoffman-La Roche. The double-stranded AP-1 oligonucleotide used as a probe was obtained from Promega

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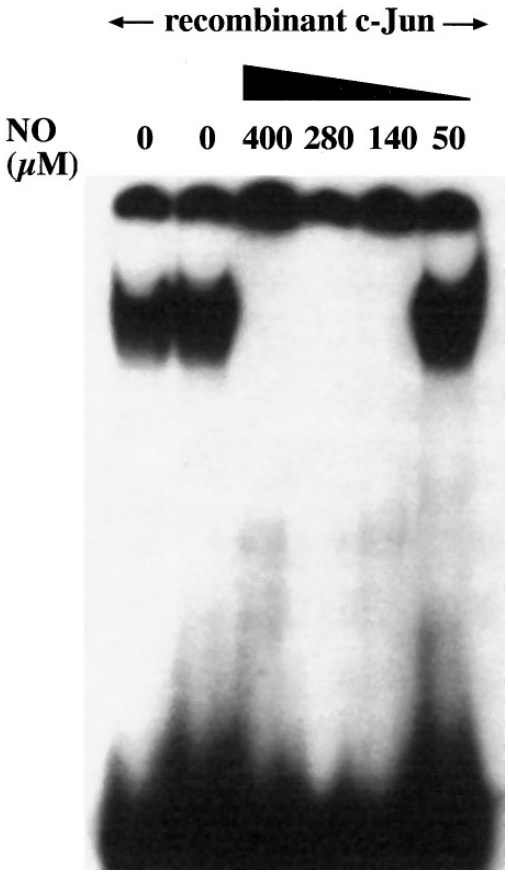

FIG. 1. Inhibitory effect of NO on c-Jun DNA binding. c-Jun (4 μg) was treated with indicated concentrations of NO in a final volume of 10 μl during 15 min at 4°C in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, before the addition of the reaction mixture.

and was labelled with ^{32}P-ATP by T4 polynucleotide kinase from Pharmacia. All other chemicals were from Sigma.

Electrophoretic mobility-shift analysis. Gel shift analysis were performed with 0.4 μg intact c-Jun or with 0.2 μg Fos/Jun polypeptides. All samples contained 0.1 mM DTT. Samples were preincubated with or without NO for 15 min at 4°C. Then a binding buffer, containing 0.2 μg of poly(dI-dC) and 1.5 μg of sonicated salmon sperm DNA in 20 mM HEPES, 0.1 mM EDTA, 12% glycerol, 4 mM $MgCl_2$, 4 mM spermidine, 0.1 mg/ml BSA and 50 mM NaCl, was added to each sample and the samples were incubated on ice for 15 min. The labelled probe was added and the samples were incubated at room temperature for 15 min. The resulting protein-DNA complexes were resolved from unbound oligonucleotides on pre-electrophoresed polyacrylamide gel (29 : 1) with 0.25 x TBE (1 × TBE: 90 mM Tris-borate, 1 mM EDTA) as running buffer. Gels were fixed in 10 % acetic acid, 10 % methanol, dried and visualized by autoradiography.

RESULTS AND DISCUSSION

NO and DNA binding. We examined the binding of intact c-Jun homodimers expressed in *E. coli*, by using a labeled synthetic double stranded AP-1 oligonucleotide probe and EMSA. Treatment of c-Jun with NO during 15 min at 4°C, before addition of binding buffer and ^{32}P-labeled AP-1 oligonucleotide, inhibited AP-1 DNA binding. The effect was concentration dependent and concentrations higher then 140 μM NO resulted in complete inhibition of the binding (Fig. 1).

Mutational analysis of cysteine residues in the DNA binding domains of Jun and Fos. The Fos-Jun heterodimers of truncated polypeptides that contain the leucine zipper and the DNA binding domains, Jun-(225-334) and Fos-(118-211) exhibit efficient DNA binding activity (14) This activity is abolished by oxidizing agents as it is dependent on the redox status of the conserved Cys^{272} and Cys^{154} residues in the DNA binding domains in Jun and Fos, respectively. However, Cys^{272} and Cys^{154} to serine mutations, bind DNA even in the absence of reducing agents (14). We investigated the participation of these cysteine residues, in the inhibition of binding mediated by NO, using the same truncated proteins which are designated J or F for the native sequences and J(C1-S) or F(C1-S) for the cysteine-to serine mutations. Treatment of F and J polypeptides containing the conserved cysteins, resulted in a pronounced inhibition of binding (Fig. 2). In contrast, NO had no effect on the J(C1-S)-F(C1-S). This suggests that the inhibitory effect of NO was elicited by interaction

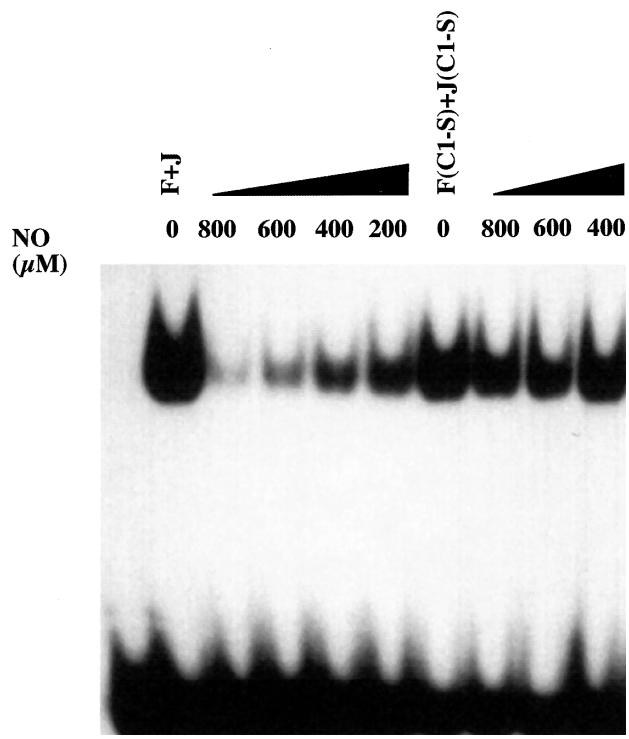

FIG. 2. Effect of NO on the DNA binding activities of F/J truncated polypeptides (lanes 1-5) or the F(C1-S)/C(C1-S) mutant polypeptides (lanes 6-9). The truncated polypeptides (0.2 μg) were treated with indicated concentrations of NO during 15 min at 4°C in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, before the addition of the reaction mixture.

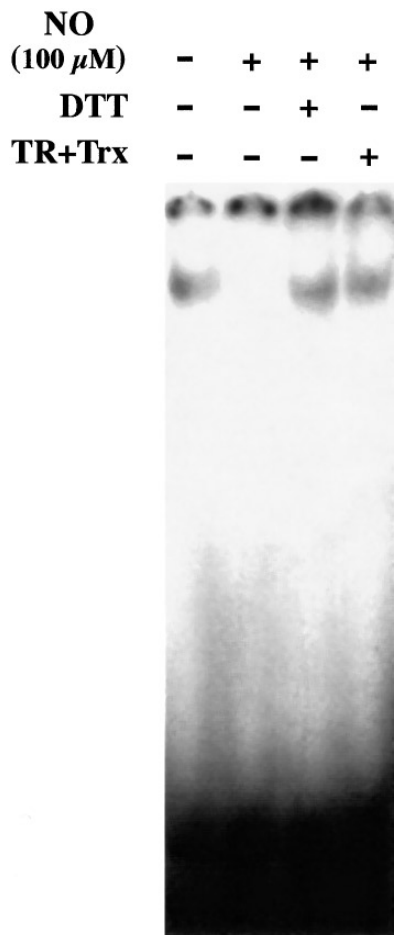

FIG. 3. The inhibitory effect of NO on the c-Jun DNA binding is reversed by DTT or the thioredoxin system. c-Jun (4 μg) was incubated with NO (100 μM) as in Fig. 1 during 15 min at 4°C. Subsequently, DTT (1 mM) or thioredoxin (5 μM), thioredoxin reductase (0.1 μM), and NADPH (100 μM) were added and the samples were further incubated at RT during 15 min.

with specific cysteine residues presumably by nitrosylation or oxidation of the thiol group.

Addition of 1 mM DTT to NO treated c-jun recombinant protein reversed the inhibition (Fig. 3). Likewise the addition of 5 μM thioredoxin, 0.1 μM thioredoxin reductase and NADPH (the thioredoxin system) reversed the inhibitory effect of NO (Fig. 3). Thioredoxin is known to stimulate the AP-1 activity through reduction of the oxidized Ref-1 protein, however the thioredoxin system has no direct effect on this activity. Presumably the thioredoxin system may reverse the inhibitory effect caused by NO, by cleaving the formed S-nitrosothiol. The thioredoxin system has activity towards nitrosothiols, as it cleaves S-nitrosoglutathione, a physiological S-nitrosothiol (22). Alternatively the effect mediated by NO could result in other post-translational modifications such as oxidation and disulfide bridge formation accessible to the actions of the thioredoxin system. Previously it was demonstrated that sodium nitroprusside, an NO donor may directly modulate the AP-1 DNA binding activity in the presence of reducing agents, *in vitro* (10). The participation of superoxide and peroxynitrite was also postulated to be responsible for the inhibiting effect and the NO-mediated neuronal cell death in ischemic brain injury (10, 11). Our results demonstrate that NO modulates AP-1 DNA binding activity by specifically reacting with the conserved Fos-Cys-154 and Jun-Cys-272 cysteines as no effect was observed with their cysteine to serine mutants. These cysteines, which are also conserved in all members of the Jun and Fos families, are flanked by lysine and arginine residues. The pK_a of a cysteine residue in this environment is lowered by the neighboring basic amino acids and the reactivity of the thiolate is greatly enhanced (23). The inhibition of the DNA binding was reversed by DTT or the thioredoxin system suggesting a thiol redox control mechanism. The regulation of AP-1 by NO may therefore be both a direct interaction with the conserved cysteine residue, or a complex interaction involving other reactive species and this would depend largely on the redox status of the cell.

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