[CANCER RESEARCH 57, 3365–3369, August 15, 1997]

Advances in Brief

Nitric Oxide Induces Conformational and Functional Modifications of Wild-Type p53 Tumor Suppressor Protein

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Abstract

Incubation *in vitro* of recombinant wild-type murine p53 protein with *S*-nitroso-*N*-acetyl-DL-penicillamine [a nitric oxide (NO)-releasing compound] has resulted in a change of p53 conformation and also in a significant decrease of its specific DNA binding activity. Similarly, upon treatment with *S*-nitroso-*N*-acetyl-DL-penicillamine (2–5 mM) or *S*-nitrosoglutathione (1–2 mM), human breast cancer cells (MCF-7), which express wild-type p53, rapidly accumulated p53 protein in the nuclei. This p53 protein, however, possessed a significantly decreased activity of specific DNA binding. On the other hand, lower concentrations of NO donors (0.25–0.5 mM) stimulated p53 accumulation as well as its DNA binding activity. These results suggest that excess NO produced in inflamed tissues could play a role in carcinogenesis by impairing the tumor suppressor function of p53.

Introduction

Chronic infection and inflammation are well recognized risk factors for a variety of human cancers (1). It has been proposed that reactive oxygen and nitrogen species, both formed in inflamed tissues, play a role in carcinogenesis. NO,[2] a potentially toxic gas with free radical properties, is generated from L-arginine by constitutive or inducible NOSs (2). NO acts not only as a signal molecule mediating various physiological functions, such as vasodilation and neurotransmission, but also as a mediator of the cytotoxic activity of macrophages, playing an important role in inflammatory processes (3). We and others have recently shown that different isoforms of NOS are expressed in some human precancerous and cancerous tissues (brain, breast, ovarian, stomach, lung, liver, and colon cancers), as well as in a variety of tumor cell lines (4–6).

NO is a highly reactive radical that may react with other radicals to form cytotoxic compounds, such as peroxynitrite, which may cause DNA damage (7). It can also react directly with a variety of enzymes and other proteins to either activate or inhibit their function by oxidizing SH groups, complexing with metal ions, or reacting with tyrosine residues (8). All of these effects could be important for the contribution of NO to the multistage process of carcinogenesis.

We have hypothesized that, in inflamed tissue, excess endogenously formed NO could alter the function of the p53 tumor suppressor protein. The p53 protein is a zinc-dependent transcription factor, which binds specific DNA sequences and transactivates the expression of genes under promoters containing p53 binding sites, therefore playing a critical role in mediating cell cycle arrest in G_1 (9) or apoptosis (10) in response to DNA damage. The sequence-specific DNA binding activity maps to the central core domain of the p53 protein (residues 102–292; Ref. 11). The majority of p53 missense point mutations in human cancers affect residues within this core domain (12), inactivating the protein by impairing its specific DNA binding capacity. Agents that reversibly perturb the metal-dependent folding of this domain, such as chelating and oxidizing agents, also inhibit specific DNA binding (13, 14). Because NO can modify many different proteins, it may modify the structure of the p53 protein, thus affecting its tumor suppressor functions.

In this study, we have investigated whether NO donors affect the conformation and the DNA binding activity of the p53 protein *in vitro*, using a recombinant mwtp53. The effect of NO donors on p53 level and DNA binding activity was also analyzed in cultured human cell lines.

Materials and Methods

Transcription and Translation of p53. RNA for translation of mwtp53 was produced by *in vitro* transcription of the plasmid pSP6p53^{ALA135}, linearized with *Hin*dIII (15). Translation was carried out in rabbit reticulocyte lysate (Promega) for 1 h at 37°C in the presence of 0.75 μM of added [^{35}S]methionine. To eliminate lysate hemoglobin, which reacts with NO rapidly, the translated protein was separated by fractionation on a Bio-select sec-250 (Bio-Rad) column.

Exposure to NO Donors or Peroxynitrite and Immunoprecipitation. SNAP and GSNO were a generous gift from Dr J. C. Decout and Prof. M. Fontecave (Joseph Fourier University, Grenoble, France). NAP and diNONOate were obtained from Fluka (Buchs, Switzerland) and Cayman Chemical Company, respectively. Peroxynitrite and decomposed peroxynitrite were synthesized as described previously (16). The recombinant mwtp53 was incubated for 30 min at 37°C with the various NO donors or with peroxynitrite. The p53 protein was then analyzed by immunoprecipitation, using the monoclonal antibodies Pab248, Pab246, and Pab240. Immunoprecipitates were analyzed on 10% SDS-PAGE. Aliquots of the reaction mixture, in loading buffer with or without DTT, were also applied on 10% polyacrylamide gel. The ^{35}S-labeled protein was detected by autoradiography.

Cell Lines and Extracts. MCF-7 human breast cancer cells and HepG2 human hepatoma cells were grown in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 10% and 5% CO_2, respectively. After treatment with NO donors, cells were lysed in a solution containing 20 mM HEPES, pH 7.6, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP40, 10 mM NaCl supplemented with 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.7 μg/ml pepstatin A. Cytoplasmic extracts were obtained by centrifugation at 2000 × *g* for 4 min. Nuclear extracts were obtained from the pellets treated for 30 min at 4°C with the lysis solution described above, except that the NaCl concentration was increased to 500 mM.[3]

Western Blotting. Nuclear cell extracts containing 10 μg of protein were separated by SDS-PAGE in a 10% gel and electrotransferred onto an Immobilon p15 membrane (Millipore Corp., Bedford, MA). The membranes were blocked with 5% nonfat dry milk in 0.2% Tween 20 in Tris-NaCl for 1 h and incubated overnight at 4°C with DO-7 monoclonal antibody to p53 (Oncogene Science, Uniondale, NY) at a dilution of 1:1000 in blocking solution. Bands were visualized with horseradish peroxidase-conjugated antimouse immunoglobulin (1:5000), enhanced chemiluminescence reagent (Amersham Corp.,

Received 1/30/97; accepted 7/2/97.

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[2] The abbreviations used are: NO, nitric oxide; NOS, NO synthase; diNONOate, diethylamine NONOate; GSNO, S-nitroso-glutathione; mwtp53, wild-type murine p53; NAP, *N*-acetyl-DL-penicillamine; SNAP, *S*-nitroso-NAP.

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Arlington Heights, IL), and subsequent exposure to Hyperfilm-enhanced chemiluminescence (Amersham Corp.).

DNA Binding Assay. Aliquots (50 μl) of recombinant mwtp53 treated with NO donors or peroxynitrite were diluted in 80 μl of DNA binding buffer (100 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.5% NP40, and 50% glycerol) containing 5 ng of ^{32}P end-labeled double-stranded oligonucleotide 5'-GGGCATGTCCGGGCATGTCC-3' (17), 30 ng of herring sperm DNA as a nonspecific competitor, 4 mM DTT, and 100 ng of Pab421. The Pab421 supershifts and stabilizes p53-DNA complexes, and addition of this antibody is required to detect DNA binding of wild-type p53 translated *in vitro* (18). DNA binding with the nuclear cell extracts was performed with 10 μg of protein in 30 μl of the same DNA binding buffer as above containing the oligonucleotide, 1 μg of herring sperm DNA, 4 μg of BSA, 2 mM DTT, and 100 ng of Pab421. Samples were incubated for 30 min at room temperature. Reaction products were analyzed by electrophoresis at 120 V on 4% polyacrylamide gels in 100 mM Tris-borate containing 1 mM EDTA and cooled by water circulation (19).

Levels of p53 protein or p53-DNA complexes were analyzed by Imaging Densitometer Model GS-670, Bio-Rad (Hercules, CA). Statistical analysis was performed using Student's *t* test.

Results and Discussion

As shown in Fig. 1, the ^{35}S-labeled mwtp53 translated *in vitro* reacted more strongly with Pab246 than with Pab240. This immunological phenotype is typical of wild-type p53. The Pab246 antibody reacts with a conformation-dependent epitope exposed in wild-type p53, whereas Pab240 reacts with an epitope that is cryptic in wild-type p53 and exposed in many p53 mutants (20). Pab248, which reacts with both wild-type and mutant forms of p53, is used as a control (19). After exposure to 1 mM SNAP, the reactivity of wild-type p53 with Pab246 was significantly decreased, whereas there was no apparent change in cross-reactivity with Pab240. The ratio of Pab246 to Pab240 for nontreated cells was 4.2 ± 2.3 ($n = 4$), whereas it decreased significantly ($P < 0.05$) to 0.87 ± 0.16 ($n = 4$) for 1 mM SNAP-treated cells (Fig. 1). These results suggest that wild-type p53 changed its conformation upon treatment with SNAP. This change in conformation occurred within 10 min after treatment of the p53 protein with SNAP (data not shown). On the other hand, treatment of mwtp53 with NAP, which has the same chemical structure as SNAP except for the NO group, resulted in an immunological phenotype ($Pab246^+$, $Pab240^-$) identical to that seen with untreated mwtp53; the ratio of Pab246 to Pab240 was 3.4 ± 1.6 ($n = 2$). These results indicate that the effects observed with SNAP are specifically due to NO release (Fig. 1).

As shown in Fig. 2*A*, incubation of the ^{35}S-labeled translated mwtp53 in the presence of a NO-releasing compound (SNAP or diNONOate) or peroxynitrite led to the formation of aggregation products of the p53 protein with very high molecular weight, as detected by SDS-PAGE without DTT (Fig. 2*A*). Such aggregates have also been observed after treatment of p53 with zinc chelators or with oxidizing agents (13, 14). The aggregation products formed in the presence of NO donors accumulated at the top of the stacking gel and/or at the interface between the stacking (5%) and the resolving (10%) gels, whereas the aggregates of p53 formed after treatment with peroxynitrite were observed exclusively at the top of the resolving gel. When SDS-PAGE was carried out after treatment of the sample with DTT (Fig. 2*B*), some of the aggregation product formed in the presence of NO donors was dissociated and migrated with the expected mobility of p53 monomers. In contrast, the aggregation products formed with peroxynitrite were not dissociated by DTT. These results suggest that NO reacted with thiol groups of p53 to mediate the formation of disulfide bonds, possibly through S-NO formation. In contrast, peroxynitrite apparently induces p53 to cross-link in a manner that is not exclusively dependent upon S-S bridging. Peroxynitrite could promote p53 aggregation through the formation of dityrosine, a well-characterized consequence of tyrosine oxidation by peroxynitrite (21).

It is known that the p53 DNA-binding domain contains several cysteine residues, which play an important role in its DNA binding activity (13). As NO can modify cysteine residues leading to the formation of disulfide bonds, it could thus affect the biological functions of p53. Therefore, we have analyzed the DNA binding activity of *in vitro*-translated p53 treated with NO or peroxynitrite. Fig. 2*C* shows that exposure of mwtp53 to NO donors (SNAP and diNONOate) or peroxynitrite before incubation with DNA consensus sequence abolished its ability to complex with DNA. In contrast, exposure to non-NO donors, such as decomposed peroxynitrite or NAP, had no effect or a very limited effect on p53 DNA binding activity.

Taken together, these results indicate that treatment of p53 with NO-releasing compounds or peroxynitrite causes profound structural changes that may be responsible for the conversion from a wild-type to a mutant conformation with loss of DNA binding activity. These changes could result from a direct reaction of NO with the zinc atom in p53 and from oxidation of cysteine residues. However, the DNA binding activity of the SNAP-treated protein was not restored upon reduction with DTT (data not shown). Removal of zinc and alteration of the redox status of p53 have been shown to alter the conformation and activity of the protein both *in vitro* and in intact cells.[3] These results are consistent with the notion that NO can react with many different proteins/enzymes and regulate their activities, as it does with ribonucleotide reductase and aconitase (8).

The effect of NO on the DNA binding activity of p53 was further investigated in cultured cells. Upon treatment of human breast cancer cells (MCF-7), which contain the wild-type *p53* gene, with SNAP, GSNO, or peroxynitrite, rapid accumulation of p53 protein in the nuclei was observed (Fig. 3). We observed similar accumulation in a human hepatoma cell line (HepG2) upon treatment with SNAP or peroxynitrite (data not shown). The accumulation of p53 was dependent upon the concentration of NO donor and was maximum above 2 mM SNAP and 1 mM GSNO (about 8- and 3-fold increases after a 2-h

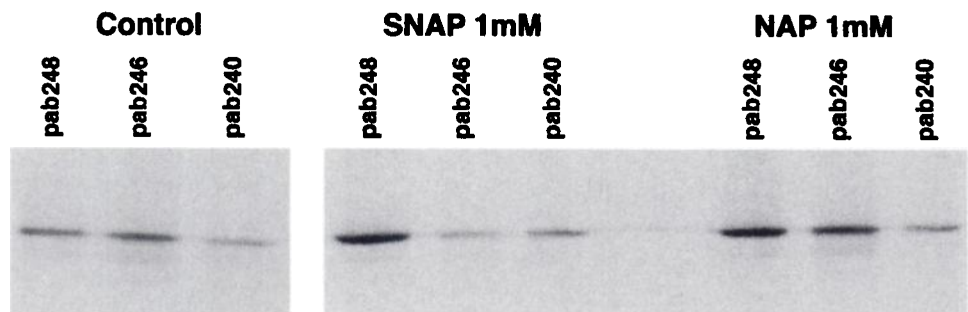

Fig. 1. Effect of SNAP on the conformation of wild-type p53. Wild-type p53 was translated in rabbit reticulocyte lysate at 37°C, and after filtration on a Bio-select sec-250 column, aliquots of protein were exposed to 1 mM SNAP and 1 mM NAP for 30 min at 37°C. The conformation of p53 was analyzed by immunoprecipitation with the conformation-specific monoclonal antibodies Pab248, Pab246, and Pab240; *A*, Pab248 was used as a positive control.

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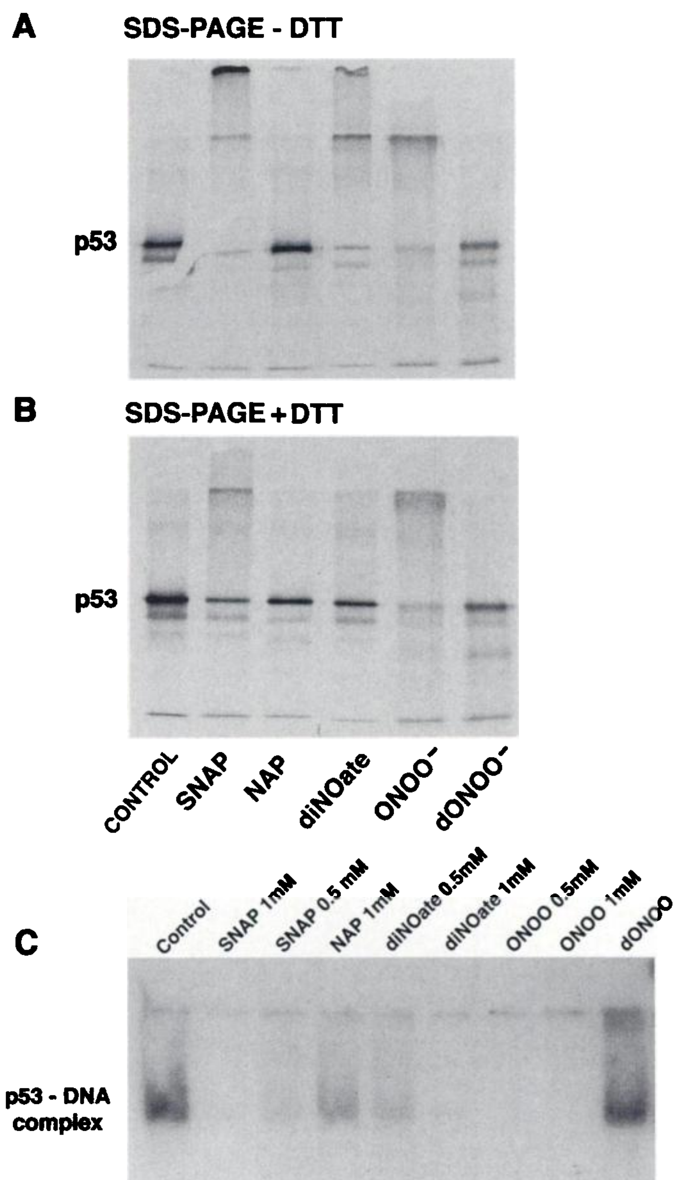

Fig. 2. Effect of various NO donors on the electrophoretic profile and DNA binding activity of wild-type p53. *A* and *B*, aliquots of translated wild-type p53 were exposed to various NO-releasing compounds (SNAP and diNONOate), NAP, peroxynitrite, and decomposed peroxynitrite for 30 min at 37°C. Aliquots were mixed with Laemli buffer containing (*B*) or not containing (*A*) DTT before SDS-PAGE analysis. Full-length p53 is indicated; the minor band corresponds to a putative truncated form of wild-type p53 frequently observed in *in vitro* translation assays (19). *C*, sequence-specific DNA binding activity was assayed using the ^{32}P-labeled double-stranded oligonucleotide 5′-GGGCATGTCCGGGCATGTCC-3′. All reactions were carried out in the presence of Pab 421, which supershifts and stabilizes p53-DNA complexes. Reaction products were analyzed by electrophoresis on 4% PAGE. *diNOate*, diNONOate; *dONOO*, decomposed peroxynitrite; *ONOO*, peroxynitrite.

exposure compared to control, respectively; Fig. 3, *A* and *B*). This accumulation was also transient and time dependent. The level of p53 increased up to 15-fold of control at 6 h after treatment with 2.5 mM SNAP. The p53 level also increased up to 20-fold at 4 h after treatment with 5 mM SNAP, decreased to about ¼ of the maximum at 6 h, and further decreased to the level of controls after longer incubation times (Fig. 3*C*). Accumulation of p53 essentially results from posttranslational modifications that stabilize the protein after cellular stress or DNA damage. The decrease in p53 levels after 4–6 h has already been reported in macrophages exposed to NO donors (22). This decrease may reflect a rapid degradation of accumulated p53 protein through ubiquitin-dependent proteolysis, although these mechanisms are not well understood. In parallel with the accumulation of the p53 protein, we observed a strong reduction in p53 DNA binding activity in these cells with concentrations above 2 mM SNAP and 0.5 mM GSNO (Fig, 4). This inhibition of p53 binding to DNA was dependent upon the concentration of SNAP or GSNO (Fig. 4, *A* and *B*) and upon time (Fig. 4*C*). DNA binding activity increased about 4-fold and 2-fold with SNAP concentrations up to 2 mM and GSNO up to 0.5 mM, respectively. The level of p53-DNA complex then decreased at higher concentrations to become barely detectable at 3 and 1 mM, respectively. This effect on DNA binding does not correlate with the levels of p53 detected by Western blot analysis (Fig. 3). For example, with 2.5 mM SNAP, maximum DNA binding activity was seen after 2 h (Fig. 4*C*), whereas p53 protein accumulated up to 6 h after treatment but in a form that is unable to bind DNA (Fig. 3*C*). These data indicate that NO donors can exert different effects on p53, depending on the concentration. At lower concentrations (up to 2 mM SNAP/0.5 mM GSNO), we observed a parallel increase in p53 protein levels and DNA binding activity, which most probably reflects the formation of NO-induced DNA damage. At higher concentrations, however, we observed that p53 accumulates in a form that is unable to bind to DNA. This is consistent with the notion that NO donors could alter wild-type p53 protein conformation. GSNO was more effective than SNAP in inducing p53 accumulation and modulation, possibly due to the fact that GSNO releases NO more easily than SNAP (23). In inflamed mucosa from patients with ulcerative colitis, increased NOS activities have been reported, ranging from 0.55 to 10 nmol/min/g of tissue (24, 25). This suggests that the amount of NO produced may reach 0.5–10 μmol/liter/min in these tissues. On the other hand, 1 mM GSNO and SNAP have been shown to generate NO at the rate of ~1 to ~4 μmol/liter/min in culture medium containing 10% fetal bovine serum (23). Therefore, the concentrations of NO that are formed locally in intensive inflammatory conditions may be similar to those released by the NO donors in the present study.

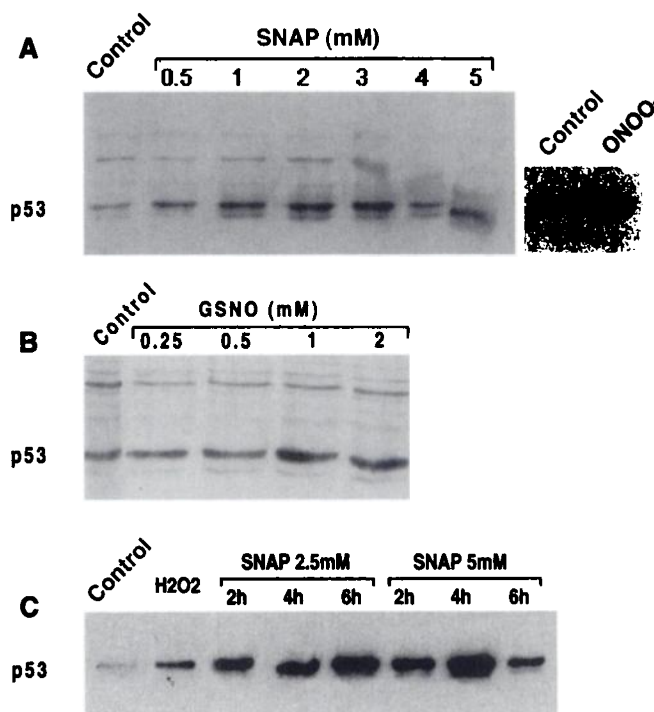

Fig. 3. Effect of various concentrations of SNAP (*A*), GSNO (*B*), and time (*C*) on p53 levels in nuclear extracts of human breast cancer cells (MCF-7). MCF-7 cells were incubated for 2 h at 37°C with different concentrations of SNAP (0–5 mM; *A*) or GSNO (0–1 mM; *B*). p53 levels were analyzed in nuclear extracts at different times (*C*) by Western blot analysis with DO-7 as described in "Materials and Methods."

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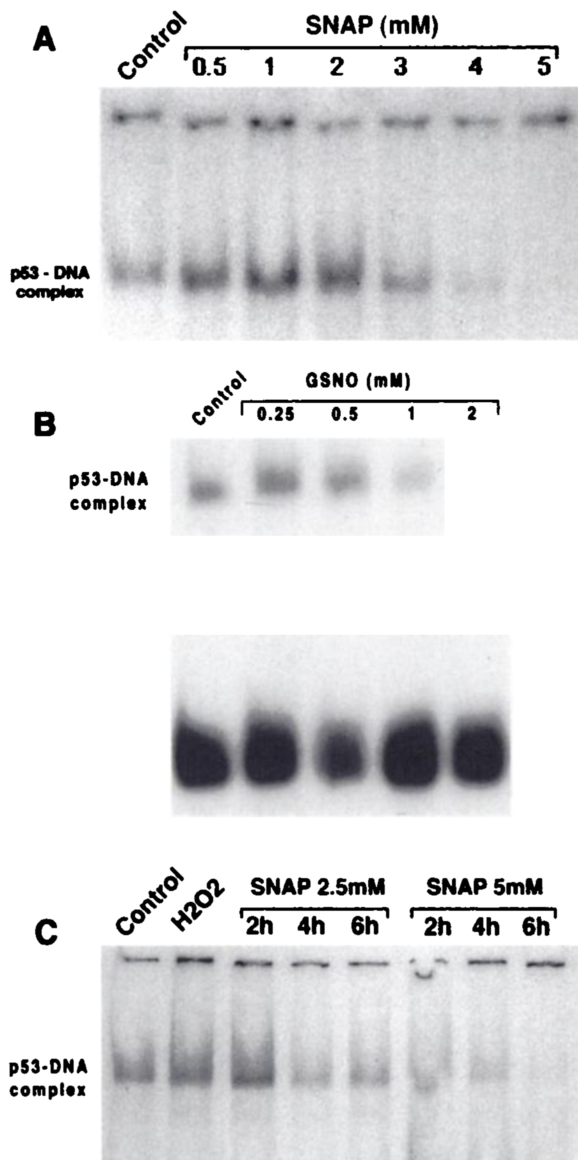

Fig. 4. Effect of various concentrations of 0–5 mM SNAP (*A*), 0–1 mM GSNO (*B*), and time (*C*) on the DNA binding activity of p53 of MCF-7 cells. DNA binding assay was performed as described in "Materials and Methods."

Our data are consistent with recent studies that have reported that NO can stimulate p53 accumulation and apoptosis in rodent macrophages, pancreatic cell lines and murine thymocytes (22, 26, 27). It is now known that NO-induced DNA damage may occur through several mechanisms, including DNA breakage by NO_2 (28), nitrosative deamination (29), DNA alkylation by metabolically activated nitrosamines (30), and nitrosative and oxidative damage by peroxynitrite (31). These DNA alterations can trigger an accumulation of wild-type p53 in these cells (32). On the other hand, although high concentrations of NO can induce p53 accumulation, our results suggest that excess NO can also modify the protein so that its DNA binding activity and subsequent biological activity are lost.

Inactivation of p53 through mutation occurs in one-half of human tumor types and is the most commonly identified molecular alteration detected in human cancer. Recent studies have examined the expression and activity of the inducible NOS in human tumor samples. Increased NOS expression was observed in human gynecological (4), breast (33), and central nervous system (34) tumors. For these tumor types, p53 is mutationally inactivated in 25–40% of cases (35). Interestingly, in the case of breast cancer, the p53 protein is apparently overexpressed in many tumors without *p53* gene mutations, suggesting that alternative mechanisms may contribute to p53 inactivation in these cancers (36, 37). In view of our results, one can hypothesize that in some of the tumors carrying wild-type *p53* alleles, epigenetic events, such as inactivation of p53 protein by overproduction of NO, may play an important role in carcinogenesis.

Recent results have demonstrated that overexpression of wild-type p53 in a variety of tumor cell lines, as well as in murine fibroblasts, resulted in the down-regulation of inducible NOS expression through inhibition of the inducible NOS promoter (38). The control of NO overproduction by p53 supports the hypothesis that close interactions between NO and p53 exist, in which p53 can contribute to the control of intracellular NO production. Our results suggest that p53 and NO could be linked by mutual feedback control mechanisms, with transcriptional control of NOS by p53 and also posttranslational control of p53 activity by NO. As p53 induces expression of a number of growth-regulatory genes, such as *WAF1/CIP1, GADD45,* and *MDM2* (27), we can speculate that NO represents an important regulatory element in the function of p53 as a transcriptional activator *in vivo* in normal and transformed cells.

One physiological consequence of control by NO of p53 conformation would be the inactivation of p53, which may be required to maintain the proliferative potential of cells during wound healing in inflamed tissues. However, this mechanism would also result in a decreased capacity to respond to DNA damage; these cells would therefore be more likely to accumulate genomic alterations and subsequently transform. We are currently investigating whether NO generated by macrophages can affect p53 function in target cells.

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