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Intrastriatal Dopamine Injection Induces Apoptosis Through Oxidation-Involved Activation of Transcription Factors AP-1 and NF-κB in Rats

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ABSTRACT

More and more evidence suggests that increases in dopamine (DA) in striata may participate in neurodegenerative processes during acute ischemia, hypoxia, and excitotoxicity. With a rat model of intrastriatal DA injection, we studied the molecular events involved in DA toxicity. Intrastriatal injections of DA in amounts from 1 to 2 μmol result in apoptotic cell death, as indicated by terminal deoxynucleotidyl transferase labeling of DNA strand breaks and Klenow polymerase-catalyzed [^{32}P]deoxycytidine triphosphate-labeled DNA laddering. Injections of DA produce a strong and prolonged activated protein 1 (AP-1) activity that contains c-fos, c-jun, and phosphorylated c-jun protein. DA injections also stimulate the activity of nuclear factor-κB (NF-κB), an oxidative stress-responsive transcription factor. Injection of curcumin at a dose that selectively inhibits AP-1 activation without affecting NF-κB activity attenuates DNA laddering induced by DA. Preinjection with SN50, a specific permeable recombinant NF-κB translocation inhibitor peptide, reduces DA-induced NF-κB activation and apoptosis. Moreover, preinjection of the antioxidant GSH significantly inhibits both DA-induced activation of transcription factors AP-1 and NF-κB and subsequent apoptosis. Thus, our data suggest that DA-oxidative stress-induced apoptosis in vivo is mediated by activation of transcription factors AP-1 and NF-κB.

Accumulating evidence suggests that a high availability of dopamine (DA) in striata may participate in neurodegenerative processes. These include ischemia, hypoxia (Akiyama et al., 1991; Buisson et al., 1992), local exposure to neurotoxins such as high concentrations of excitatory amino acids (Filloux and Wamsley, 1991), and methamphetamine (Schmidt et al., 1985). For example, the local striatal extracellular DA concentration can reach as high as 0.2 mM in the gerbil ischemic model (Slivka et al., 1988). Depletion of endogenous DA by destruction of the nigrostriatal pathway reduces ischemic damage to the striatum (Globus et al., 1987; Buisson et al., 1992). However, the molecular events for this in vivo DA toxicity are unknown.

The in vitro cell culture studies suggest that DA toxicity is linked to DA-oxidative stress-induced apoptosis. Chemically, DA contains a catechol structure that can spontaneously oxidize in vitro or via an enzyme-catalyzed reaction in vivo to form reactive oxygen species (ROS) and quinones (Graham, 1978). ROS can damage cellular components such as lipids, proteins, and DNA. DA quinones can bind to cysteine or cysteinyl residues on proteins, thereby interfering with protein functions. Both free and protein-bound cysteinyl DA can be detected in the DA-enriched areas in the brain (Fornstedt et al., 1989) or increased by direct intrastriatal DA injections (Hastings et al., 1996). In addition, quinones can further polymerize to form another neurotoxin, neuromelanin, which occurs in the DA-containing neurons of the substantia nigra. Apoptosis is a controlled form of cell death and has been suggested to participate in the cascade of some neurodegenerative diseases, e.g., stroke, Alzheimer's disease, and Parkinson's disease (PD; Mochizuki et al., 1997). The ability of DA to induce apoptosis has been demonstrated both in in vitro cell cultures (Ziv et al., 1994; Luo et al., 1998b) and after in vivo intrastriatal DA injections in rats (Hattori et al., 1998). The apoptotic cells induced by DA are characterized by condensed chromatin, DNA fragmentation, and shrinkage in cell shape. The in vitro studies show that DA (0.01–1.00 mM)-induced apoptosis is associated with ROS because it can be effectively inhibited by application of antioxidants, such as *N*-acetylcysteine, catalase, GSH, and dithiothreitol (DTT; Ziv et al., 1994; Gabbay et al., 1996; Shinkai et al., 1997; Luo et al., 1998b). Moreover, our recent in vitro cell culture studies have demonstrated that the stress-activated protein kinase (SAPK/JNK)-c-jun pathway contributes to DA-oxidation-induced apoptosis (Luo et al., 1998b). However, the molecular events relevant to the processes of DA-oxidative stress-induced apoptosis in vivo are unknown.

ABBREVIATIONS: DA, dopamine; DTT, dithiothreitol; GSH, glutathione; PD, Parkinson's disease; AP-1, activated protein 1; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; EMSA, electrophoretic mobility shift assays.

Recent evidence suggests that prolonged activation of activated protein 1 (AP-1) and nuclear factor-κB (NF-κB) in the CNS may play an important role in determining the cell death in response to oxidative stress, ischemia, and neurotoxins. The AP-1 proteins consist of a homodimer of c-jun or heterodimer of c-fos/c-jun family members (Johnson and McKnight, 1989). AP-1 gene transcription activity is strongly potentiated by phosphorylation of c-jun (Smeal et al., 1991). Recently, the phosphorylation of c-jun has been shown to be tightly associated with induction of apoptosis in several systems. These include the cerebral ischemia-reperfusion model in rats (Herdegen et al., 1998), a kainate excitotoxicity in mice (Yang et al., 1997), survival signal withdrawal in both cerebellar granule and sympathetic neurons (Eilers et al., 1998; Watson et al., 1998), and DA toxicity in cell cultures (Luo et al., 1998b). NF-κB is also an oxidative stress-responsive transcription factor. Unlike c-jun, NF-κB is normally present in the cytosol, where it is bound to an inhibitory protein component IκB (Liou and Baltimore, 1993). On activation, IκB undergoes phosphorylation, ubiquination and degradation, thus releasing active NF-κB and allowing it to translocate into the nucleus. Like the phosphorylation of c-jun, the activation of NF-κB is also linked to the triggering of apoptosis in some systems. These include the apoptotic hippocampal CA1 neurons in the rat global ischemic model (Clemens et al., 1998) and striatal neuronal apoptosis induced by quinolinic acid (Qin et al., 1998). In nonneuronal cells, activation of AP-1 and NF-κB stimulates production of *Fas* ligand (Kasibhatla et al., 1998), a known apoptotic death gene acting through the caspase cascade (reviewed by Nagata, 1997). In this article, we report that intrastriatal DA injection does activate both AP-1 and NF-κB oxidative stress-response transcription factors in rats. DA also induces production of c-fos, c-jun, and the phosphorylated active form of c-jun, which contribute to the AP-1 activity. Both AP-1 activity and NF-κB activation are associated with DA-induced apoptosis. Moreover, the DA-induced transcription activity and the following apoptosis, can be inhibited by administration of the antioxidant GSH.

Experimental Procedures

Materials. Polyclonal anti-c-jun IgG was obtained from Calbiochem (San Diego, CA). Antibody against phospho-specific c-jun (Ser63) II was obtained from New England Biolabs Inc. (Beverly, MA). Monoclonal anti-c-fos was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). DA was obtained from Research Biochemicals International (Natick, MA). Curcumin and GSH were obtained from Sigma Chemical Co. (St. Louis, MO). SN50 and SN50M were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Intrastriatal Injections. The procedures were described previously (Hattori et al., 1998). All surgeries were performed with adult male Wistar rats (6 months old; 360–543 g). The rats were anesthetized with ketamine (100 mg/kg i.p.) and placed into a stereotaxic instrument (David Kopf Instruments, Tajunga, CA). The stereotaxic coordinates were 0.2 mm anterior from bregma, 3 mm from midline, and 7 mm from the skull surface. The chemicals were injected with a Hamilton syringe in a volume of 2 μl. The period for injection was at least 2 min. The animals were sacrificed at the indicated times. Striata were removed and frozen immediately at −80°C.

All solutions were freshly prepared. DA and GSH solutions were prepared with sterile distilled water (pH 7.0–7.5). SN50 was dissolved in 0.9% NaCl solution. Curcumin was dissolved in dimethyl sulfoxide. For control purposes, the same vehicles used for the reagents were prepared and injected on the opposite side of the striatum of the same brain. For inhibitory studies, all inhibitory reagents except GSH were injected 15 min before DA. GSH was injected 1 h before administration of DA.

Genomic DNA Isolation and 3′-OH End Labeling. The genomic DNA was isolated by following a method described elsewhere (Hattori et al., 1998). Briefly, rats were sacrificed at the indicated time. Striata were dissected and homogenized individually in 0.6 ml of lysis buffer containing 10 mM Tris-HCl, 100 mM EDTA, and 0.5% SDS. Samples were first incubated with DNase-free RNase (10 mg/ml) for 3 h and then treated with proteinase K (100 μg/ml) overnight at 55°C. After that, samples were extracted with equal volumes of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) three times. DNA was precipitated with 0.25 volume of 10 M ammonium acetate and 2 volumes of ethanol for 4 h at 4°C. DNA pellets were washed with 70% ethanol, air dried, and dissolved with TE buffer (5 mN Tris-HCl, pH 8.0; 20 mM EDTA). DNA concentration was determined via a UV-visible spectrophotometer (Pharmacia Biotech, Inc., Piscataway, NJ).

The 3′-OH end of DNA was labeled with the procedures described by Rosl (1992) with a minor modification. Briefly, genomic DNA was incubated with 1 U/μg of Klenow polymerase (5000 U/ml; Promega, Madison, WI) and 0.5 μCi/μg [^{32}P]deoxycytidine triphosphate (dCTP; 3000 Ci/mol; Amersham, Arlington Heights, IL) in a buffer containing 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, and 1 mM DTT for 10 min at 30°C. The reaction was terminated with 10 mM EDTA. The labeled DNA was precipitated with 2.5 M ammonium acetate and 2.5 volumes of ethanol in the presence of 50 μM tRNA carrier for 1 h at 4°C. The DNA was collected by centrifugation for 30 min at 4°C. This DNA pellet was then washed twice with ice-cold 70% ethanol and dissolved in 20 μl of TE buffer. The samples were electrophoresed on 1.8% agarose gels. The gels were first stained with ethidium bromide to visualize the molecular-weight standards (Research Genetics, Inc., Huntsville, AL) and photographed for later reference. The gels were then dried and exposed to autoradiography film for visualization of labeled DNA ladders.

Nuclear Extract Preparation. Nuclear extracts were prepared via procedures described elsewhere (Qin et al., 1998). In brief, striatal tissue was homogenized with a Dounce homogenizer in 4 volumes of buffer containing 10 mM HEPES-NaOH, pH 7.9, 0.25 M sucrose, 15 mM KCl, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM DTT, and 1 μg/ml protease inhibitor cocktail (a mixture of phenylmethylsulfonyl fluoride, benzamidine, leupeptin, and antipain, each at 1 μg/ml). The nuclei-containing homogenates were obtained by centrifugation at 1000*g* for 10 min and washed twice in 4 volumes of buffer containing 10 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 μg/ml of the aforementioned protease inhibitor cocktail. These washed nuclear pellets were then resuspended with 4 volumes of buffer containing 10 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 1 M KCl, 1 mM EDTA, 1 mM DTT, and 1 μg/ml protease inhibitor cocktail and incubated on ice for 30 min. The suspension was centrifuged at 14,000*g* for 30 min at 4°C. The supernatant was collected and dialyzed against 100 volumes of buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and the protease inhibitors. The supernatant was then collected, aliquotted, and stored at −80°C. Protein concentrations of the nuclear extracts were determined by a MicroBCA kit from Pierce (Rockford, IL).

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs were performed by use of ^{32}P-labeled double-stranded oligonucleotide containing a specific consensus sequence recognized by each transcription factor. Human metallothionein II_A AP-1 consensus and mutant oligonucleotides were purchased from Santa Cruz Biotechnology. NFκB consensus oligonucleotide was purchased from Promega. Probes were labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP and purified with G-25 spin columns

(Boehringer Mannheim, Indianapolis, IN). The specific activity of the labeled oligonucleotides was about 50,000 to 100,000 cpm/ng. Binding reactions were performed for 30 min at room temperature. The binding reaction contained 5 μg of nuclear extract, 1 μg of poly-(dI.dC), 1 ng of ^{32}P-labeled oligonucleotide, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and protease inhibitors. The reaction volume was 20 μl. For supershift assays, 0.5 to 1.0 μg of antibody was incubated with the nuclear extract in binding buffer for 30 min at room temperature before the binding reaction. For competition studies, a 1- to 25-fold excess of unlabeled oligonucleotides or AP-1 mutant oligonucleotides was added in the binding assay. After the reaction, bound and free probes were separated by electrophoresis on 6% polyacrylamide gels in 0.5 times Tris-Boric acid-EDTA buffer. The gels were dried and exposed to autoradiography film overnight at −80°C.

Lysate Preparation and Western Immunoblotting. Striatal tissue was homogenated with 200 μl of ice-cold lysis buffer containing 25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM DTT, 100 μg/ml phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin, followed by sonication for 10 s on ice. The cellular extract was then centrifuged for 30 min at 14,000 rpm to remove debris. The supernatant was used immediately or aliquotted and stored at −70°C for further use. Protein concentration was determined by a Bio-Rad protein reagent kit (Bio-Rad, Richmond, CA).

For Western immunoblotting, equal amounts of lysate protein (40 μg/lane) were run on 8 to 16% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were first blocked with 10% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 0.01% Tween-20) and then incubated with primary antibodies (phospho-Ser63-specific c-jun, 1:1000; Biolab; polyclonal c-jun, 1:1000; Calbiochem; monoclonal c-fos, 1:500; Santa Cruz) in TBST containing 5% BSA overnight at 4°C. Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and Renaissance substrate (DuPont, Wilmington, DE).

Quantification. All the bands of interest were semiquantified by the National Institutes of Health Image 1.55 program.

Results

Intrastriatal DA Injections and Apoptosis. As we reported previously, intrastriatal DA injections in rats resulted in typical apoptotic cell death (Hattori et al., 1998). The DA-induced dead cells can be labeled easily by the terminal deoxynucleotidyl transferase (tdt)-mediated dUTP-biotin nick-end labeling (TUNEL) technique and show characteristic morphology of apoptosis. The TUNEL-positive cells exhibited condensed granulated and marginated labeling and DNA fragmentation and were shrunken and irregular in shape. Although DNA fragmentation could not be detected by conventional ethidium bromide staining after agarose electrophoresis, it could easily be found with high-sensitivity Klenow polymerase-catalyzed [α-^{32}P]dCTP labeling. The genomic DNA isolated from DA-injected striatum showed a characteristic oligonucleosome-length (about 200-base pairs addition) fragmentation pattern. In contrast to that from DA-injected striatum, the DNA from control striatum (contralateral NaCl injection sites) did not show an obvious [^{32}P]DNA ladder (Fig. 1). Thus, we used the sensitive Klenow polymerase-catalyzed [^{32}P]DNA ladder labeling as a parameter to determine apoptotic processes in our subsequent studies.

DA-induced DNA fragmentation is dependent on time after intrastriatal injection. As shown in Fig. 1A, DNA fragmentation occurred at 24 h and significantly increased at 48 h after injection of 1 μmol of DA. By using the TUNEL technique, we could see apoptotic cells 8 h after intrastriatal injection of 2 μmol of DA (Hattori et al., 1998). With 24 h as a time point, we observed the concentration dependence of DNA laddering induced by DA. The DNA ladder was proportionally increased at DA amounts of 0.5 to 2 μmol (Fig. 1B).

DA-Stimulated AP-1 Activity. By using in vitro neonatal rat striatal cells and nonneuronal cell cultures, we have previously found that DA induces apoptosis through the c-jun-NH_2-terminal kinase, also called SAPK pathway (Luo et al., 1998b). In that study, we observed that a strong and sustained activation of JNK and consequent phosphorylation of c-jun is essential for DA-induced apoptosis. Recently, phosphorylation of c-jun was also shown to be critical for apoptosis in some neuronal systems (Eilers et al., 1998; Herdegen et al., 1998; Watson et al., 1998). Prolonged induction of c-fos is also an important factor to initiate apoptosis in some systems (Smeyne et al., 1993; Haffzi et al., 1997). Thus, we tested whether DA could induce c-jun, phospho-c-jun, and c-fos containing AP-1 transcription activity in vivo.

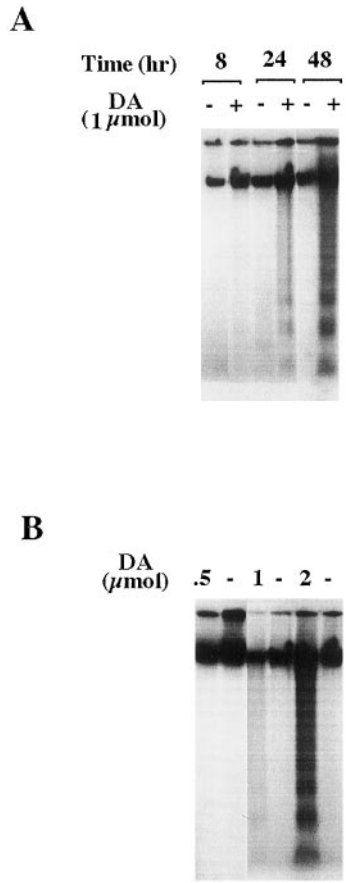

Fig. 1. DA-induced DNA fragmentation in rat striatum. Equal amounts of either DA or NaCl were injected into each side of the striatum of the same rat. Genomic DNA was isolated. The 3′-OH end of the DNA (3 μg/lane) was labeled with [α-^{32}P]dCTP in the presence of Klenow polymerase. A, time course of DNA laddering induced by injections of either 1 μmol of DA (+) or 1 μmol of NaCl (−). B, concentration-dependent studies of DA-induced DNA fragmentation. The rat striatum received injections of DA (+) at the indicated amount for 24 h. (−), injection of NaCl. All experiments were repeated at least three times, and similar results were obtained.

We first examined AP-1 activity with EMSA. As shown in Fig. 2, intrastriatal DA injections in rats significantly increased the binding of nuclear extracts to [^{32}P]-labeled AP-1 consensus sequences. The increase in DA-induced AP-1 binding was dependent on the time after DA injection (Fig. 2A). We used the National Institutes of Health Image 1.55 program to quantify these data. At 8 and 48 h after 2 μmol DA administration, AP-1 activity was increased 2.2-fold ($\pm$0.2; $n = 4$) and 3.0-fold ($\pm$0.2; $n = 4$) compared with 2 μmol NaCl control injection at the 8-h time point (Fig. 2A, bottom). Injection of the same amount of NaCl had little effect on AP-1 binding within 8 to 24 h after injection (Fig. 2A). Within 24 h, we also tested effect of DA dose on AP-1 activation. DA proportionally increased AP-1 binding from 0.5 to 2.0 μmol (Fig. 2B). The [^{32}P]-labeled AP-1 binding of nuclear extract from DA-stimulated striatum could be completely competed by an excess amount of unlabeled AP-1 consensus oligonucleotide but was not affected by same excess of mutated AP-1 oligos (Fig. 2C). This observation indicates a specificity of the nuclear protein to bind to AP-1 consensus oligonucleotide.

Next, we examined whether the DA-induced AP-1 complex contains c-fos, c-jun, and phosphorylated c-jun protein by a supershift assay. The rats were injected with 2 μmol of DA, striata were collected at the indicated times, and nuclear extracts were prepared. Treatment of DA-stimulated extracts with antibodies against the DNA-binding region of either c-fos or c-jun before the binding assay reduced the ability to bind AP-1 consensus oligonucleotide by about 30 to 80% at all time points (Fig. 3). Preincubation of the extracts with anti-phospho-Ser63-specific c-jun increased the broad AP-1-binding band, as shown in Fig. 3. These data suggest that AP-1-binding complexes in DA-stimulated samples contain c-jun, phosphorylated c-jun, and c-fos as a heterodimer or in complex with other AP-1-binding members. To further confirm these results, we also conducted Western immunoblotting assays in whole striatal tissue lysates. As shown in Fig. 4, DA did stimulate phosphorylation of c-jun. The phosphorylated c-jun had a slow migration with a molecular size of about 45 kDa (Fig. 4, top). The molecular weight of c-jun per se was about 39,000. Treatment of the blot with phos-

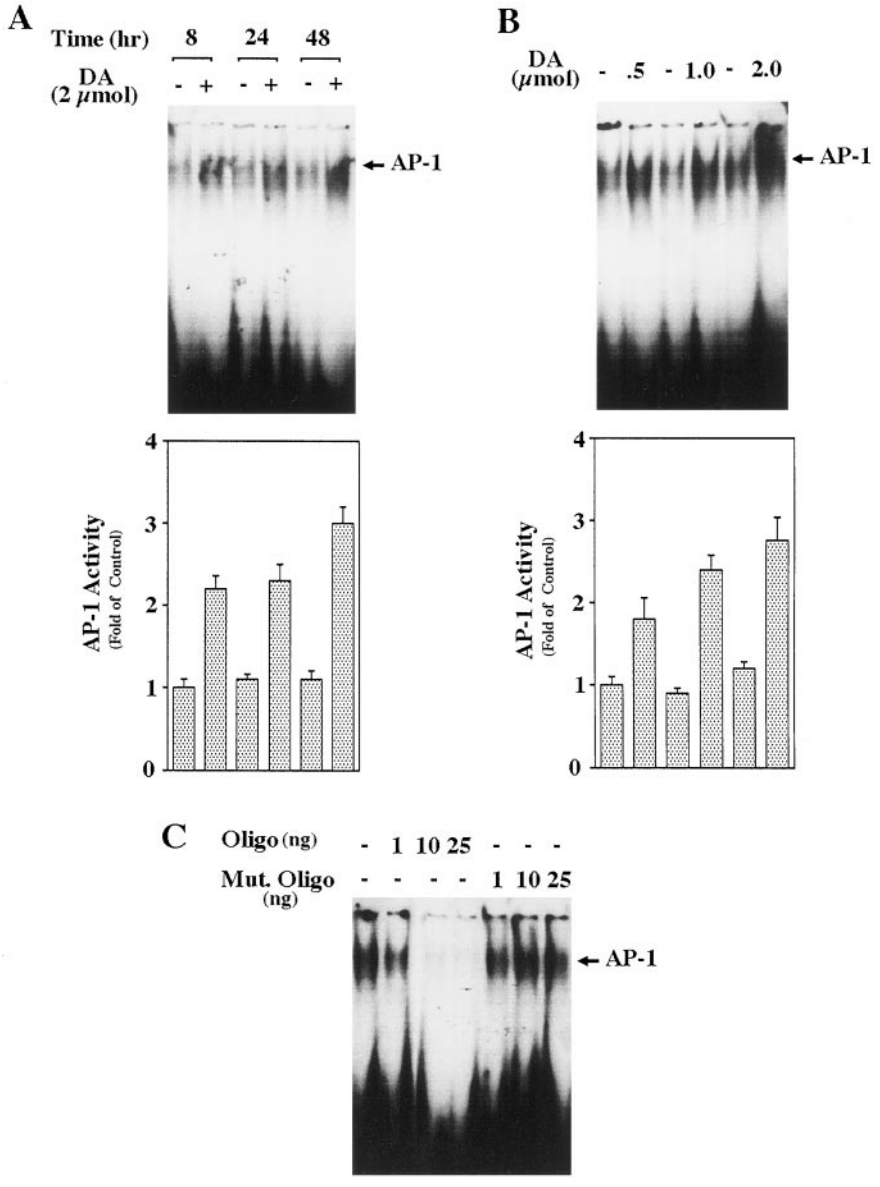

Fig. 2. DA-stimulated AP-1 activity. Striatal nuclear extracts (5 μg/lane) from the DA-injected side (+) and the contralateral NaCl-injected side (−) were used to perform EMSA to bind radiolabeled AP-1 consensus oligonucleotide. A, time course of DA-induced AP-1 activity. B, concentration dependence of DA-stimulated AP-1 binding. In these experiments, the length of DA stimulation in rat striata was 24 h. In both A and B, the top panels are representative autoradiograms for AP-1 activity assays. The bottom shows semiquantification of AP-1 activity from four independent experiment determinations by use of the National Institutes of Health Image 1.55 program. AP-1 activity is expressed as the fold of optical density of [^{32}P]-labeled AP-1-binding band compared with that induced by either 2-μmol NaCl injections for 8 h (Control in A) or 0.5-μmol NaCl injections for 24 h (Control in B). C, specificity of AP-1 activity assay. Nuclear extracts from striata receiving DA injection 24 h prior were prepared. The unlabeled AP-1 consensus oligonucleotide (Oligo) or mutated unlabeled AP-1 oligonucleotide (Mut. Oligo) were first incubated with nuclear extract in binding buffer for 30 min at room temperature. After that, the [^{32}P]-labeled AP-1 was added to perform the normal binding assay. (−), without addition of unlabeled nucleotide. The figure represents at least three independent experiments.

phatase (50 U/ml) could abolish the immunoreactivity of the 45-kDa band to anti-phospho-c-jun IgG without effect on the 39-kDa protein immunoreactivity to anti-c-jun IgG (data not shown), suggesting that the 45-kDa protein was in an active phosphorylated form. The amount of phosphorylation of c-jun in whole lysates was gradually increased from 8 to 48 h after DA injection (Fig. 4, top and middle). At 24 and 48 h, the 45-kDa protein migrated more slowly than proteins at 8 h and their controls, suggesting that multiple phosphorylation of c-jun occurred. This time course of formation of phosphorylation of c-jun was parallel to the time course of DA-induced AP-1 binding activity (Fig. 2A) and apoptosis (Fig. 1A; Hattori et al., 1998). Intrastriatal DA injection also increased the amount of c-jun and c-fos (Fig. 4, top and bottom). Interestingly, this 39-kDa c-jun protein appeared to peak 8 h after DA injection, whereas the 62-kDa c-fos protein peaked 24 h after DA injection. Injections of the same amount of NaCl on the opposite side of the same brain had little effect on the expression of c-jun but slightly increased phosphorylated 45-kDa c-jun (without high phosphorylation; Fig. 4, top) at 24 and 48 h. The amount of c-fos was also slightly increased 48 h after NaCl injection. Taken together, the results suggest that c-fos, c-jun, and phosphorylated c-jun protein participate in DA-induced AP-1 activation, during which apoptosis was observed.

DA-Stimulated NF-κB Activity. NF-κB is an inducible transcription factor and plays an essential role in response to oxidative stress. It has been studied in more detail in tumor necrosis factor-mediated signaling and is believed to have antideath activity in nonneuronal cells (Wu et al., 1998). In the central nervous system, the role of NF-κB is controversial. With in vitro primary hippocampal neuronal cell cultures and neuronal PC12 cells, the activation of NF-κB is associated with neuronal survival (Lezoualc'h et al., 1998). However, in the rat ischemic model and excitotoxicity, the long-term activation of NF-κB is linked with apoptosis (Clemens et al., 1998; Qin et al., 1998). In our study, we examined DA-induced NF-κB activity in vivo.

Intrastriatal injections of DA resulted in stimulation of NF-κB activity. As shown in Fig. 5, NF-κB was activated in a time- and concentration-dependent manner. After administration of 2 μmol of DA, the binding of nuclear extract to NF-κB consensus sequence appeared at 24 h and increased at 48 h (Fig. 5A). Unlike the kinetics of DA-induced AP-1 activation, which was observed 8 h after administration of 2 μmol of DA (Fig. 2A), the onset of NF-κB activation was delayed until 24 h. The NF-κB binding activity was increased when rat striata received 1 to 2 μmol of DA (Fig. 5B). Injection of 0.5 μmol of DA had little effect on NF-κB activation (Fig. 5B). This nuclear NF-κB binding activity is specific

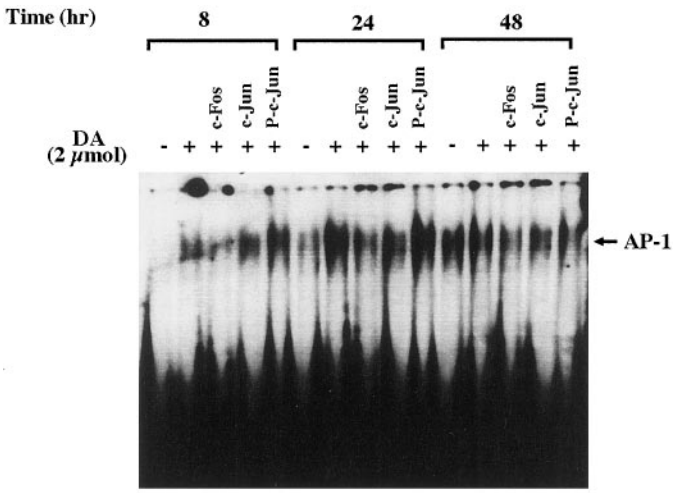

Fig. 3. DA-stimulated AP-1 complex contained both c-jun and c-fos protein. At the indicated times after intrastriatal injection, striatal nuclear extract were prepared. Supershift AP-1 activity assays were performed in the presence of various antibodies (c-fos, 1 μg; c-jun, 0.5 μg; phospho-specific anti-c-jun, 0.5 μg). Autoradiograms representative of striata from three independent injected rats at each time point are shown. A semiquantitative analysis for AP-1 activity in the presence of antibodies is discussed in the text. (+), 2-μmol DA injection; (−), 2-μmol NaCl injection.

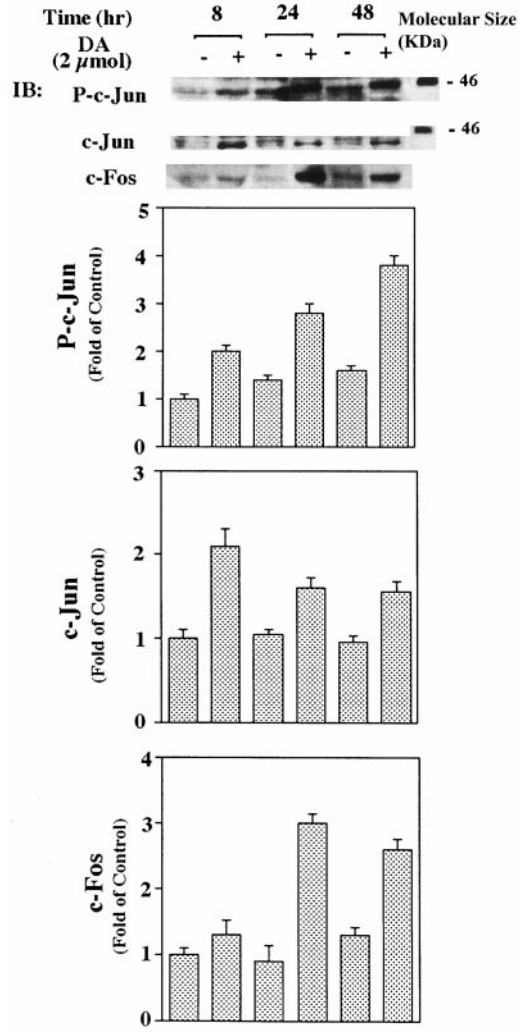

Fig. 4. DA increased productions of c-jun, phosphorylation of c-jun, and c-fos protein. Western immunoblotting of tissue lysates was performed with anti-phospho-specific c-jun (80 μg of lysate protein/lane) or c-jun and c-fos (both having 40 μg of protein/lane). Note that phospho-c-jun has a slower migration than unphosphorylated c-jun (molecular size about 45 versus 39 kDa, respectively; top). At 24 and 48 h after DA injection, the phospho-c-jun protein shows a slower migration than those at 8 h and control sides, suggesting a high phosphorylation of c-jun. The last lane on the top right shows a 46-kDa molecular size standard as a reference. Molecular size for c-fos protein is 62 kDa. The semiquantification of DA-stimulated phosphorylated c-jun, c-jun, and c-fos production was made in comparison with that in the presence of 2 μmol of NaCl for 8 h (Control). These results represent the average ± S.E. of four independent experiments.

because it could be completely competed by an excess amount of unlabeled NF-κB consensus oligonucleotide (Fig. 5C).

DA-Induced Apoptosis and Activation of NF-κB and AP-1. It was important to determine whether activation of AP-1 and NF-κB might contribute to the process for DA-induced apoptosis in vivo. We first studied the effect of curcumin on DA-induced AP-1 activity and subsequent apoptosis. Curcumin is a dietary pigment that has been shown to inhibit c-jun/AP-1 activation (Huang et al., 1991) and NF-κB in nonneuronal cells (Singh and Aggarwal, 1995). Recent evidence suggests that the inhibition of c-jun/AP-1 activation is via inhibition of the JNK pathway by curcumin (Chen and Tan, 1998). As shown in Fig. 6A, preinjections of curcumin did indeed inhibit DA-induced c-jun- and c-fos-associated AP-1 binding activity. At injection amounts of 1 and 10 μmol, curcumin dramatically inhibited AP-1 binding in a concentration-dependent manner (Fig. 6A). Preinjection of 1 μmol of curcumin also inhibited production of c-jun and phosphorylation of c-jun induced by DA (data not shown). Although a small inhibition of DA-induced NF-κB activity was observed by curcumin at 10 μmol, no inhibitory effect was seen at 1 μmol (Fig. 6A), suggesting a specificity to inhibit AP-1 activation by 1 μmol of curcumin in neuronal tissue. Based on these data, we chose 1 μmol of curcumin to examine the effect on DA-induced DNA laddering. As shown in Fig. 6B, the DA-induced DNA ladder was greatly reduced in the presence of 1 μmol of curcumin. However, the injection of same amount of curcumin showed some cytotoxicity, as indicated by the appearance of a DNA ladder (Fig. 6B). This may be caused by the interruption of normal c-jun physiological functions (see *Discussion*). Thus, the AP-1 activation may contribute to DA-induced apoptosis in vivo.

We examined the role of NF-κB activation in DA-induced apoptosis by using SN50. SN50 is a cell-permeable inhibitory peptide and has been shown to block translocation of the NF-κB active complex into the nucleus both in in vitro cell cultures (Lin et al., 1995) and after in vivo brain injections (Qin et al., 1998). SN50 is a specific NF-κB translocation inhibitor. In cell cultures, SN50 specifically inhibits NF-κB activation by various agonists, whereas mutated peptide analog SN50M, which has the same peptide sequence as SN50 except for Lys363 to Asn and Arg364 to Gly in the region of

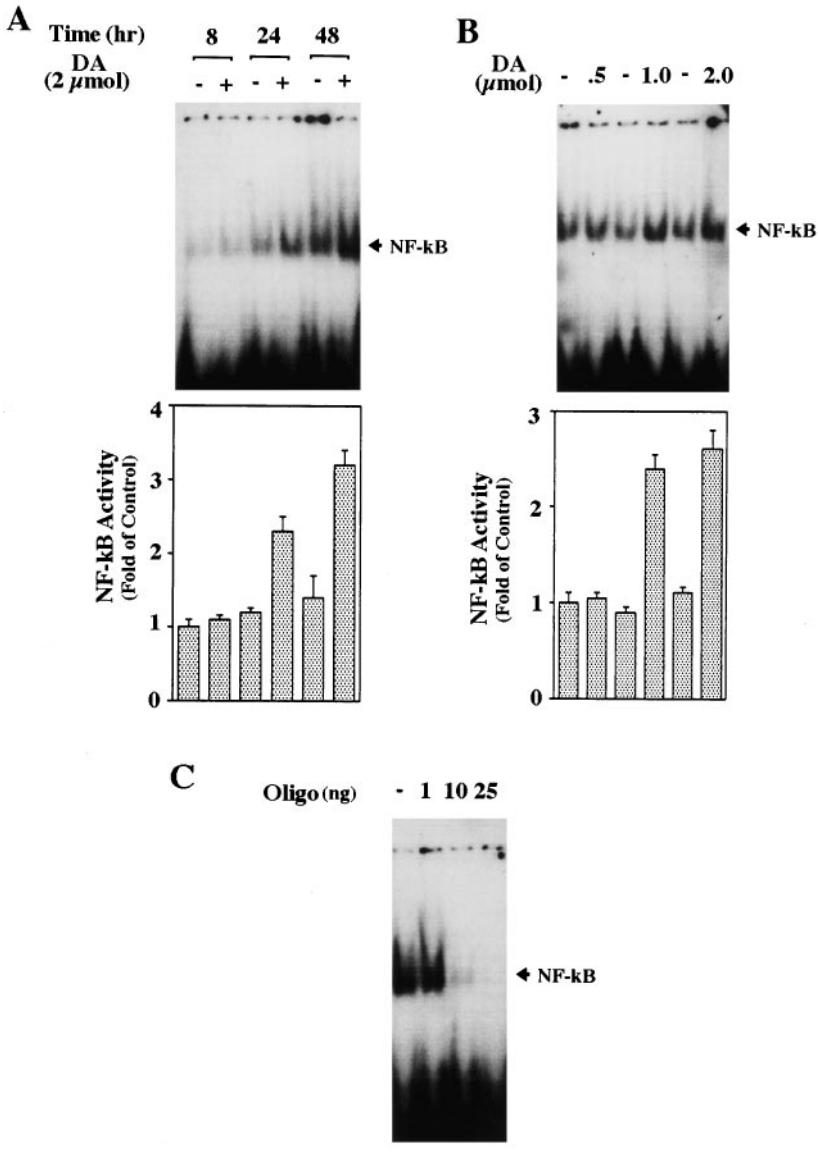

Fig. 5. DA-stimulated NF-κB activity. The procedures for intrastriatal injections, nuclear extract preparation, and EMSAs were identical with those in Fig. 2 except for the use of radiolabeled NF-κB consensus sequence. A, time course of DA-induced NF-κB activity. Note that NF-κB activation occurred 24 h after DA injection. AP-1 activation appeared 8 h after DA administration. Bottom, a quantified result of three independent experiments. The density value of a preparation injected with 2 μmol of NaCl for 8 h was chosen as a control. B, concentration-dependence of DA-stimulated NF-κB binding. In these experiments, the length of DA stimulation in rat striata was 24 h. For quantification, the density of preparation injected with 0.5 μmol of NaCl for 24 h was used as a control. The data are means ± S.E. of three independent experiments. C, specificity of NF-κB activity assay. Nuclear extracts from striata that received a DA injection 24 h before sacrifice were prepared. The unlabeled NF-κB consensus oligonucleotide (Oligo) was preincubated with nuclear extract (5 μg/lane) in binding buffer for 30 min at room temperature. After that, the [^{32}P]-labeled NF-κB consensus oligonucleotide was added to perform gel shift assays. (−), without addition of unlabeled nucleotide. The figure is representative of at least three independent experiments.

nuclear localization signal of NF-κB, have no ability to prevent NF-κB translocation (Lin et al., 1995). In in vivo studies, SN50 has also been shown to specifically inhibit NF-κB activity without affecting activation of AP-1 and a helix-turn-helix transcription factor, OCT-1 induced by intrastriatal injections of quinolinic acid (Qin et al., 1998). Preinjection of SN50 (20 μg) into striata greatly reduced DA-induced NF-κB activity (Fig. 7A). SN50M (20 μg), an inactive peptide serving as a control, has no effect on DA-stimulated NF-κB binding activity (Fig. 7A). We made use of 20 μg of SN50 to examine its role in DA-induced apoptosis. As shown in Fig. 7B, blocking of NF-κB translocation greatly reduced DNA laddering induced by DA (Fig. 7B). However, injections of SN50 alone, like curcumin, showed a cytotoxic effect, as indicated by a weak DNA ladder, suggesting that SN50 interrupts the physiological NF-κB function (see *Discussion*). Taken together, this delayed but sustained NF-κB activation may also contribute to DA-induced apoptosis.

Antioxidant GSH, DA-Induced AP-1 and NF-κB Activation, and Apoptosis. As we mentioned in the Introduction, DA-induced apoptosis is thought to be mediated by oxidative stress (Ziv et al., 1994; Luo et al., 1998b). Because of the inherent instability of the catechol moiety of DA, DA is easily oxidized to form ROS and quinones through either autoxidation or enzyme-catalyzed reactions (Graham, 1978). In striatum, the DA-oxidative products are easily detected by

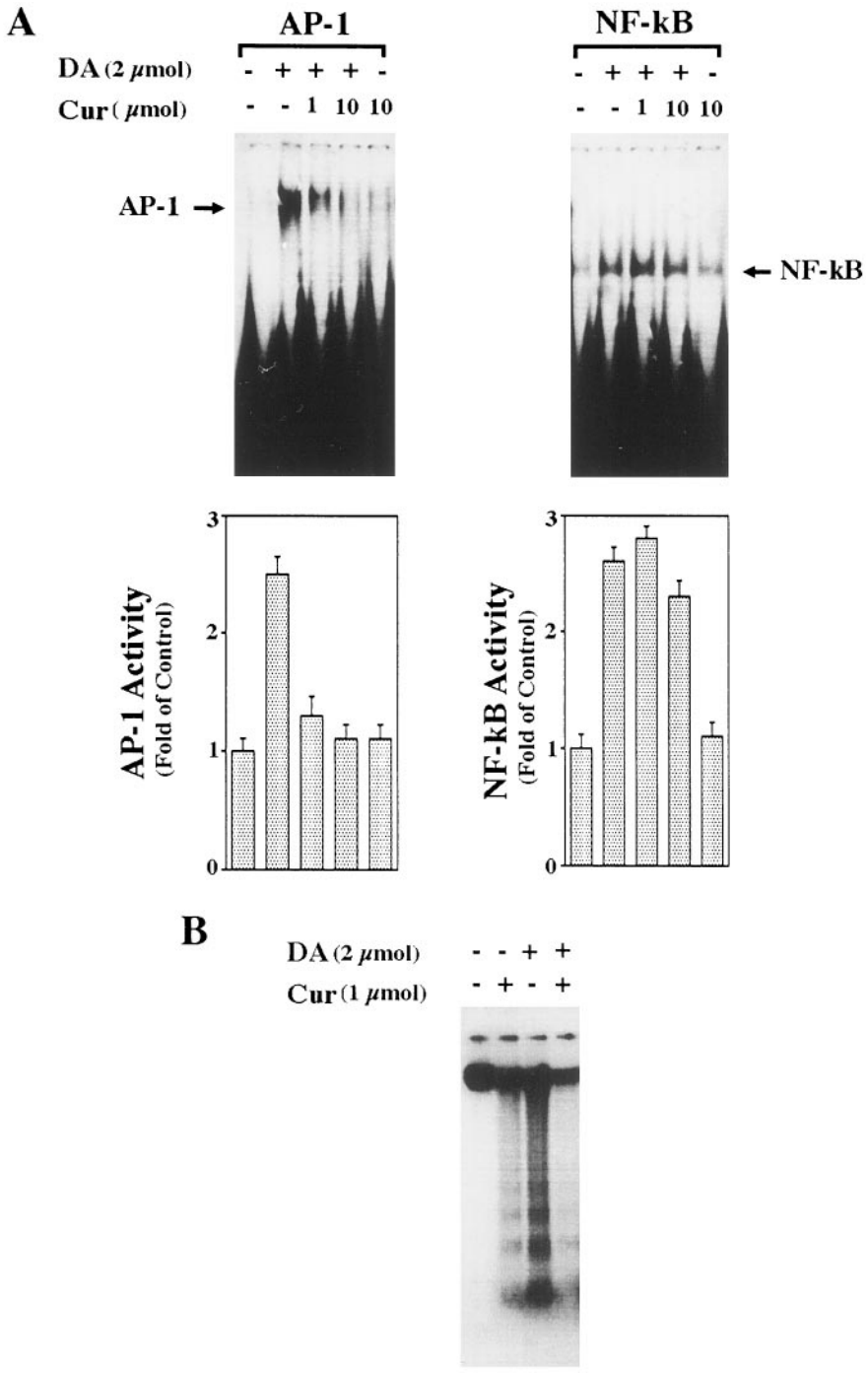

Fig. 6. Curcumin (Cur) reduced both DA-induced AP-1 activity and apoptosis. Curcumin was injected into rat striatum 15 min before DA administration. Twenty-four hours after DA injection, the rats were sacrificed, and striata were removed. A, effects of curcumin on DA-induced AP-1 and NF-κB activity. Assays for AP-1 and NF-κB activity were performed. Top, a representative autoradiograph of assays. Bottom, semiquantification of above assays. Data are averages ± S.E. of three independent experiments. The optical density in the condition of 2-μmol NaCl injection for 24 h was chosen as a control. Note that curcumin at 1 to 10 μmol preferentially inhibited AP-1 activity with little or no effect on NF-κB binding induced by DA. B, DNA ladder analysis. Genomic DNA from each striatum was isolated and labeled with [α-^{32}P]dCTP in the presence of Klenow polymerase. All the above experiments had been independently repeated three times, and similar results were obtained.

formation of free and protein-bound cysteinyl DA, by which the endogenous GSH is greatly exhausted (Hastings et al., 1996). In our study, we examined the effect of exogenous GSH on DA-induced activation of transcription factors AP-1 and NF-κB and subsequent apoptosis in vivo.

As expected, preinjections of GSH (0.2 μmol) prevented both 1-μmol DA-induced AP-1 and NF-κB activation and apoptosis (Fig. 8). Administration of 0.2 μmol of GSH also greatly reduced DNA fragmentation by 2 μmol of DA. Thus, DA-induced apoptosis is mediated by an oxidation-associated activation of AP-1 and NF-κB.

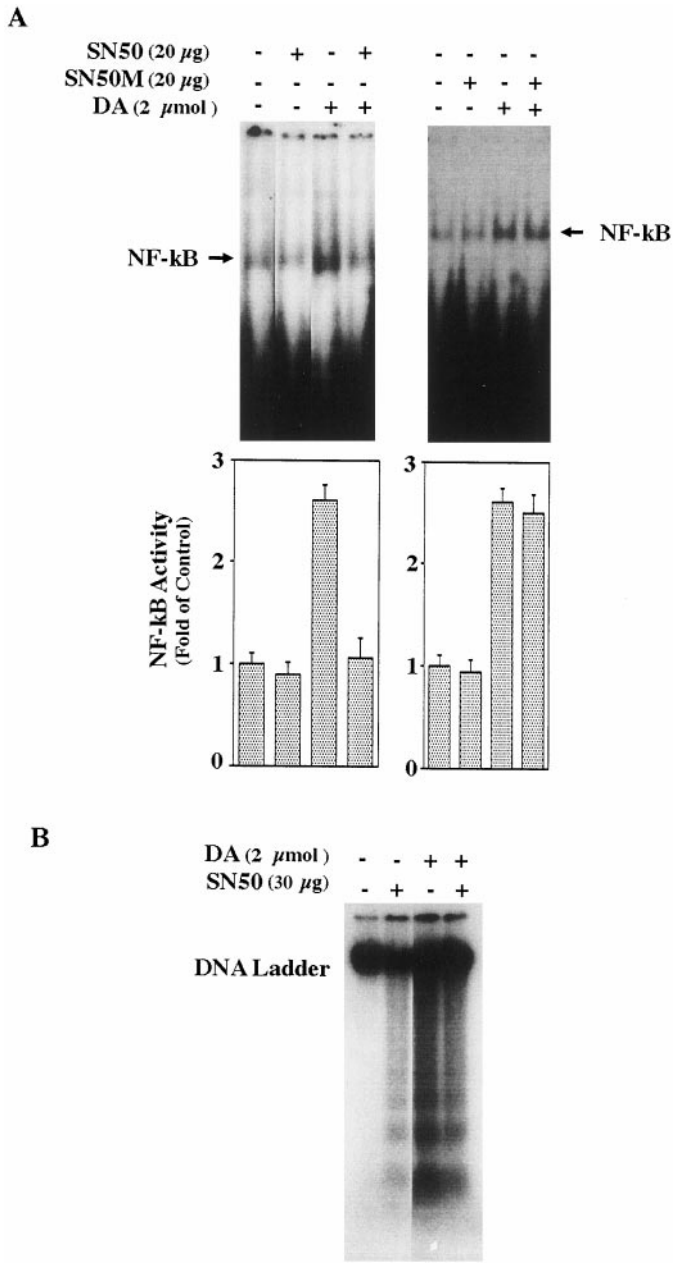

Fig. 7. SN50 reduced both DA-induced NF-kB activation and apoptosis. Identical amounts of either SN50 or SN50M (serving as a peptide control) were injected into rat striata 15 min before DA administration. Forty-eight hours after DA injection, the rats were sacrificed and striata were removed. Striatal nuclear extracts were prepared. The samples (5 μg/lane) were assayed to determine the ability to bind ^{32}P-labeled NF-κB consensus sequence. A, autoradiographs representative of striata (top) or quantified results (bottom) from at least three independent experiments. For studies of the effects of SN50 on DA-induced DNA laddering, the above identical protocol for drug treatments was used. Forty-eight hours after DA injection, genomic DNA was isolated. DNA ladder was detected by the Klenow polymerase-catalyzed [α-^{32}P]dCTP-incorporation technique. B, a representative autoradiograph of three determinations.

Discussion

In this study, we have confirmed that intrastriatal DA injections induce apoptosis in rats (Hattori et al., 1998). As we reported earlier (Hattori et al., 1998), DA-induced apoptosis is restricted to the injected area. Morphologically, the apoptotic cells show typical chromatin condensation, DNA fragmentation, shrinkage, and irregular cell shape. DNA ladders can be detected only by highly sensitive Klenow polymerase-catalyzed [^{32}P]dCTP labeling but not by conventional ethidium bromide staining. This may be because of the small portion of apoptotic cells in vivo. Both TUNEL staining and DNA laddering can readily be detected at 24 h after 2 μmol DA injection.

As in the in vitro cell culture studies (Luo et al., 1998b), DA in vivo stimulates a strong and sustained AP-1 activity. Consistent with this activity, the AP-1 components, including c-fos, c-jun, and phosphorylated c-jun, are also persistently increased (Figs. 3 and 4). Because the AP-1 binding is decreased in the presence of antibody against either c-fos or c-jun, it appears that AP-1 activation may be caused by a c-fos/c-jun heterodimer. Phosphorylation of c-jun enhanced the AP-1 activity. By using immunoblotting, we demonstrated that phosphorylated c-jun occurs, and the extent of phosphorylation of c-jun appears to be higher (Fig. 4A) after 24 h than 8 h after DA injection. Our results appear to differ from those of Schwarzschild et al. (1997), who report that DA at a concentration of 100 μM has no effect on phosphorylation of c-jun in E18 rat striatal cells. Although we used adult rats and injected a different concentration of DA, note that the E18 rat striatal cells in the Schwarzschild et al. article were cultured in a medium with B-27 supplement that contained free-radical scavengers including catalase, superoxide dismutase, DL-α-tocopherol, and GSH. The presence of these antioxidants in the medium may attenuate DA-oxidation-induced JNK activation and subsequent phosphorylation of c-jun. In our in vitro studies, we demonstrated that antioxidants, such as *N*-acetyl-cysteine and catalase, can effectively inhibit the DA-induced JNK-c-jun pathway (Luo et al., 1998b). We also show that preinjection of GSH inhibited phosphorylated c-jun-associated AP-1 activity (Fig. 8). In addition, D1 receptors have been shown to stimulate both JNK and p38 MAPK through a protein kinase A-dependent mechanism in SK-N-MC human neuroblastoma cells (Chen et al., 1998). We have recently shown that D2 receptors can stimulate both the MAPK and the JNK-c-jun pathway through a pertussis toxin-sensitive G protein coupling in C6-D2L cells (Luo et al., 1998a). However, the roles of DA receptors in regulating phosphorylation of c-jun in adult rat striatum have not been examined herein.

Our results suggest that the AP-1 activity is required for DA-induced apoptosis. This is supported by the following evidence. First, the time-course studies show that the AP-1 activation occurs by 8 h (Figs. 2A, 3, and 4), whereas apoptosis is obvious 24 h after DA injection (Fig. 1A; Hattori et al., 1998). The time lag of DNA laddering behind AP-1 activity may be required for expression of a new gene or a set of genes

causing apoptosis. Second, both AP-1 activation and apoptosis can be inhibited by preinjection of the JNK pathway inhibitor curcumin at 1 μmol, at which it did not inhibit DA-induced NF-κB activation (Fig. 6). Third, transfection of Sek1(K→R), a dominant-negative mutant, which prevents phosphorylation of c-jun, inhibits apoptosis induced by DA in 293 cells (Luo et al., 1998b). Fourth, transfection of Flag Δ169, a dominant-negative c-jun in which the NH_2-terminal phosphorylation sites including both Ser63 and Ser73 are deleted, prevents DA-induced apoptosis in both 293 cells and primary neonatal striatal cell cultures (Luo et al., 1998b). Thus, our observations support a positive role of phospho-c-jun and c-fos-contained AP-1 activation in apoptosis that occurs in some neurological models. The phospho-c-jun-involved apoptosis has been demonstrated in the cerebral ischemia-reperfusion model in rats (Herdegen et al., 1998), kainate excitotoxicity in mice (Yang et al., 1997), and survival signal withdrawal in both cerebellar granule and sympathetic neurons (Eilers et al., 1998; Watson et al., 1998). The link of c-fos to apoptosis is also demonstrated in light-induced apoptotic cell death of photoreceptors (Haffzi et al., 1997).

Injection of DA also stimulates NF-κB activity. To the best of our knowledge, this is the first report on DA regulation of nuclear transcription factor NF-κB in vivo. The kinetics of stimulation of NF-κB differ from that of activation of AP-1 by DA. DA-induced NF-κB activation appears at 24 h, whereas

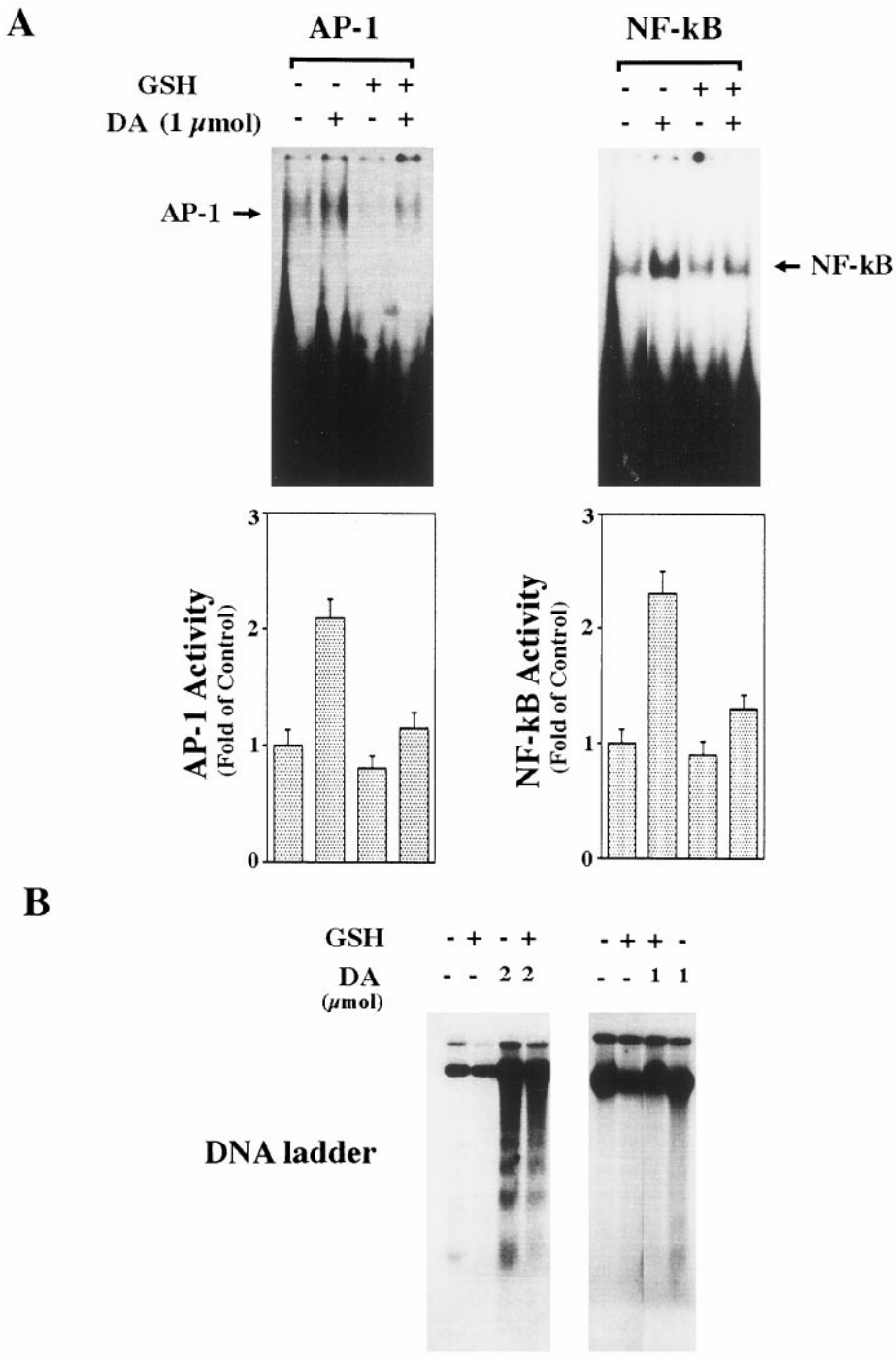

Fig. 8. Antioxidant GSH prevented both DA-induced AP-1 and NF-κB activation and subsequent apoptosis. GSH (0.2 μmol) was injected into rat striatum 1 h before DA administration. Twenty-four hours after DA injection, the rats were sacrificed, and the striata were removed. A, effects of GSH on DA-induced AP-1 and NF-κB activity. Striatal nuclear extracts were prepared, and the AP-1 and NF-κB activities were determined. Top, representative autoradiograph of assays. Bottom, semiquantification (mean ± S.E.) of the above assays from three independent experimental determinations. The samples from 1-μmol NaCl injection sites were used as control. B, DNA ladder analysis. Genomic DNA from each striatum was isolated. The 3′-OH end was labeled with [α-^{32}P]dCTP in the presence of Klenow polymerase. The figure is a representative of at least three determinations.

AP-1 activation is observed at 8 h. This kinetic difference suggests that activation of NF-κB may require a different signaling pathway from that stimulating AP-1. Although the molecular events involved in DA-stimulated AP-1 and NF-κB activation remain elusive, both can be suppressed by preinjection of the antioxidant GSH, suggesting that oxidative stress is involved. Recently, DA oxidation has been reported to inhibit glutamate transport in rat striatal synaptosomes (Berman and Hastings, 1997). If a similar effect occurs in vivo, it would be expected that the glutamate concentration might be significantly increased in striatum, resulting in a delayed activation of NF-κB (Qin et al., 1998).

This delayed NF-κB activation also appears to be involved in DA-induced apoptosis in vivo. Preinjection of SN50, a specific NF-κB translocation inhibitor, greatly reduces NF-kB activity and DNA laddering induced by DA. Preinjection of SN50M, a mutated SN50 peptide serving as a control, has no effect on DA-stimulated NF-κB activation. Our observations support a positive role of NF-κB in apoptosis in some neurodegeneration models. For example, Qin et al. (1998) reported that NF-κB activation contributes to the apoptosis induced by intrastriatal quinolinic acid, a potential model for Huntington's disease. Postmortem studies show that nuclear-active NF-κB is increased about 70-fold in the melanized dopaminergic neurons in PD brains over control groups (Hunot et al., 1997). NF-κB is also observed in degenerating hippocampal neurons after global ischemia. Prevention of activation of NF-κB by pharmacological reagents protects neuron death in this ischemic model (Clemens et al., 1998). Also excitotoxic neuronal death can be blocked by reagents shown to inhibit NF-κB activation (Grilli et al., 1996). In acute traumatic spinal cord injury, nuclear NF-κB is colocalized with inducible nitric oxide synthase (iNOS), a putative NF-κB gene target, in macrophages and neurons (Bethea et al., 1998). In nonneurons, activation of NF-κB is shown to encode the apoptotic death gene *Fas* ligand (Kasibhatla et al., 1998). Although the above evidence favors the promoting role of NF-κB in cell death, the NF-κB activation induced by tumor necrosis factor α has been shown to have cell survival functions (Wu et al., 1998). Thus, NF-κB may play different functions depending on the cell types and environments.

Both AP-1 and NF-κB appear to play important roles in normal physiological neurotransmitter or neuronal survival signal transduction because injections of curcumin and SN50 produces cytotoxic effects. This toxic action is assumed to result from the inhibition of normal AP-1 and NF-κB functions. AP-1 physiologically transduces signals from biological mediators such as cytokines, growth factors, and neurotransmitters. It was also reported that a constitutively active NF-kB occurs in a small population of cortical neurons, suggesting a normal function (Kaltschmide et al., 1994).

Our in vivo studies suggest that GSH plays an important role in protecting against DA-oxidative stress-induced signaling and consequent apoptosis. This is consistent with data from in vitro cell cultures. Application of antioxidants, such as *N*-acetylcysteine and catalase, attenuated DA-induced JNK-c-jun pathway activation and protected against DA toxicity in both neuronal and nonneuronal cells (Luo et al., 1998b). In the human neuronal cell line NMB, the antiapoptotic effect of GSH was selective, because other antioxidants, such as (+)-α-tocopherol (vitamin E) and ascorbic acid (vitamin C) did not show an effect on DA-induced cell death (Gabbay et al., 1996). Inhibition of endogenous GSH synthesis by buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase, enhanced DA toxicity (Gabbay et al., 1996). Moreover, application of DA significantly decreased intracellular GSH levels (Gabbay et al., 1996). The exhaustion of endogenous GSH is caused by DA oxidation, which produces ROS and quinones. This reduction in GSH levels may trigger an apoptotic program.

In summary, we have shown that intrastriatal DA injections can induce apoptosis in rats. The DA-induced apoptosis is dependent on the time and amounts injected and is detectable after 24 h with 1- to 2-μmol DA injections. Corresponding to its apoptotic action, DA strongly activates AP-1 and NF-κB transcription activity. The increased AP-1 activity is accompanied by an increase in c-fos, c-jun, and phospho-c-jun protein. Preinjection of curcumin at a dosage that selectively inhibits AP-1 activation without affecting NF-κB activity attenuates DA-induced apoptosis. Administration of SN50, a specific NF-κB translocation peptide inhibitor, also prevents DA-induced DNA laddering. Exogenous administration of the antioxidant GSH also results in protection against DA-oxidative stress signaling and toxicity. Thus, our results suggest that DA triggers a death program via oxidative stress-mediated activation of nuclear transcription factors AP-1 and NF-κB (Fig. 9). These apoptotic molecular events may explain the DA-related neurodegenerative processes, including chronic PD and acute ischemia and excitotoxicity. Considering the common features of ROS in apoptosis in neuronal cell death, our intrastriatal DA oxidation/apoptosis may serve as an in vivo model for studies of molecular mechanisms in ROS-linked apoptosis.

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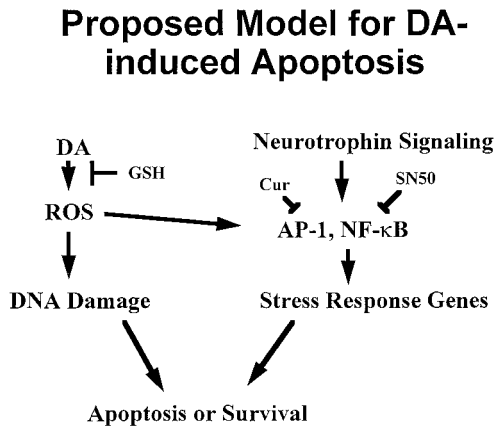

Fig. 9. Proposed model for DA-induced apoptosis in vivo. When DA is injected into rat striatum, it is subject to oxidation, resulting in formation of ROS and semiquinones (Cohen and Heikkila, 1974; Graham, 1978; Hastings et al., 1996). In addition to damage of DNA, these DA-produced ROS strongly activate phospho-c-jun-containing AP-1 and NF-κB transcription factors. This inducible and sustained AP-1 and NF-κB activation seems to be critical for DA-induced apoptosis. Curcumin and SN50, an inhibitor of c-jun/AP-1 activation and a blocker of NF-κB activation, respectively, can effectively prevent DA-induced apoptosis. AP-1 and NF-κB may induce death genes, such as *Fas* ligand, to initiate the caspase activation cascade (Kasibhatla et al., 1998), an executive apoptotic stage. Exogenous antioxidant GSH inhibits both DA-induced AP-1 and NF-κB activation and subsequent apoptosis. Thus, our data suggest that DA-induced apoptosis is mediated by activation of oxidative stress transcription factors AP-1 and NF-κB.

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