JNK-independent Activation of c-Jun during Neuronal Apoptosis Induced by Multiple DNA-damaging Agents*

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Activation of the JNK pathway and induction of the AP-1 transcription factor c-Jun are critical for neuronal apoptosis caused by a variety of insults. Ara-C-induced DNA damage caused rapid sympathetic neuronal death that was associated with an increase of c-jun expression. In addition, c-Jun was phosphorylated in its N-terminal transactivation domain, which is important for c-Jun-mediated gene transcription. Blocking c-Jun activation by JNK pathway inhibition prevented neuronal death after stress. In contrast, neither the JNK inhibitor SP600125 nor the mixed lineage kinase inhibitor CEP-1347 prevented cytosine arabinoside-induced neuronal death, demonstrating that the JNK pathway was not necessary for DNA damage-induced neuronal apoptosis. Surprisingly, SP600125 or CEP-1347 could not block c-Jun induction or phosphorylation after DNA damage. Pharmacological inhibitors of cyclin-dependent kinase (CDK) activity completely prevented c-Jun phosphorylation after DNA damage. These results demonstrate that c-Jun activation during DNA damage-induced neuronal apoptosis was independent of the classical JNK pathway and was mediated by a novel c-Jun kinase. Based on pharmacological criteria, DNA damage-induced neuronal c-Jun kinase may be a member of the CDK family or be activated by a CDK-like kinase. Activation of this novel kinase and subsequent phosphorylation of c-Jun may be important in neuronal death after DNA damage.

Cells experience DNA damage caused by several environmental stresses such as UV irradiation and oxidative stress. When DNA damage is no longer repairable, various cell death pathways are activated before mitosis to prevent the delivery of damaged DNA to offspring. Thus, many chemotherapy regimens are based on DNA-damaging agents. However, DNA damage can also kill postmitotic cells such as neurons. Central nervous system neurotoxicity is a major dose-limiting factor in high dose ara-C treatment for refractory leukemias (1–5). In vitro, ara-C is toxic to postmitotic sympathetic, parasympathetic, and sensory neurons of the peripheral nervous system as well as cerebellar and cortical neurons of the central nervous system (6–10).

In sympathetic neurons, ara-C exposure activates two separate cell death pathways (67). The default pathway is similar to the programmed cell death of neurons upon withdrawal of their trophic factor, nerve growth factor (NGF).1 This pathway kills neurons rapidly and requires the proapoptotic Bcl-2 family member, Bax. Ara-C exposure leads to Bax-mediated mitochondrial cytochrome c release and caspase activation. The second pathway is only observed in the absence of Bax. Activation of cell death in Bax-null neurons is significantly delayed and proceeds with a protracted time course compared with wild type neurons. This slower death is similar to the delayed death observed in camptothecin-treated cortical neurons in the presence of caspase inhibitors or after Bax deletion (11, 12).

AP-1 transcription factor c-Jun is induced in many neuronal cell death paradigms (reviewed in Refs. 13 and 14) and appears to be important for the transcription of proapoptotic genes, such as the BH3-only Bcl-2 family members Bim and Dp5 (15, 16). Inhibition of c-Jun activity by dominant-negative overexpression, neutralizing antibody injection, and genetic deletion prevents sympathetic neuronal apoptosis after NGF deprivation (17–19). Moreover, hippocampal neurons carrying a mutant c-Jun gene (JunAA) that lacks the two critical N-terminal phosphorylation sites show increased resistance to kainate exposure (20).

The N-terminal transactivation domain of c-Jun is phosphorylated by c-Jun N-terminal kinase (JNK) (21). JNK is part of a three-sequential kinase signaling cascade (22, 23). MAP kinase kinase (MKK) activates JNK by dual Tyr and Thr phosphorylation. MKK activation is mediated by MAP kinase kinase kinases, including the mixed lineage kinase (MLK) family in neurons. Selective inhibition of the MLK family in sympathetic neurons by the K252a analog, CEP-1347, demonstrates that MLKs mediate JNK activation after trophic factor deprivation, UV irradiation, and oxidative stress (24–26).

In this study, we examined the changes in c-Jun and JNK pathway activity in sympathetic neurons during DNA damage-induced apoptosis. DNA damage caused c-Jun activation in neurons by two separate mechanisms: induction of c-Jun expression and N-terminal c-Jun phosphorylation. Surprisingly, DNA damage-induced c-Jun expression and phosphorylation were maintained in the absence of neuronal JNK activity. In addition, the JNK pathway was not necessary for DNA damage-induced apoptosis because pharmacological inhibition of the JNK pathway by CEP-1347 or SP600125 did not increase neuronal survival. These results demonstrate that DNA damage-induced neuronal c-Jun activation was independent of the classical JNK pathway. c-Jun activation was blocked by several

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1 The abbreviations used are: NGF, nerve growth factor; CDK, cyclin-dependent kinase; DIV, days in vitro; JNK, Jun-N-terminal kinase; MKK, MAP kinase kinase; MAPK, MAP kinase; MLK, mixed lineage kinase; MEK, MAPK/extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ara-C, cytosine arabinoside.
EXPERIMENTAL PROCEDURES

Animals and Materials—All reagents were purchased from Sigma unless otherwise indicated. Timed-pregnant Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Animal use and treatment complied fully with the Animal Studies Committee of Washington University and the United States Animal Welfare Act (1985). Collagenase and trypsin were purchased from Worthington. Mouse 2.5S NGF was from Harlan Bioproducts (Indianapolis, IN). The goat anti-mouse 2.5 S NGF immunosorbent has been characterized previously (22). CEP-1347 was a kind gift of Cephalon (Westchester, PA). SP600125 was provided by Celgene Inc., San Diego, CA. Etoposide and olomoucine were purchased from Biomol (Plymouth Meeting, PA). Alsterpaullone was obtained from Calbiochem.

Neuronal Culture—Primary sympathetic neuronal cultures were prepared from P0 to P1 rat superior cervical ganglia by using methods described previously (28, 29). Briefly, superior cervical ganglia were dissected from newborn animals and incubated for 30 min each with 1 mg/ml collagenase and 2.5 mg/ml trypsin at 37 °C. The ganglia were dissociated by triturating with a P200 micropipet; cells were plated on collagen-coated plastic tissue culture plates at appropriate densities. Cultures were maintained in AM50 medium (90% minimum essential medium (Invitrogen), 2 mM glutamine, 10% fetal bovine serum (HyClone). Cultures were maintained in AM50 medium (90% minimum essential medium (Invitrogen), 2 mM glutamine, 10% fetal bovine serum (HyClone), 50 ng/ml 2.5 S NGF, 20 μM fluoresceoxydorydine, 20 μM uridine, 100 units/ml penicillin, and 100 units/ml streptomycin), supplemented with 3.3 μM aphidicolin (AG Scientific, Inc., San Diego, CA) for the first 5 days in vitro (DIV) to reduce the number of non-neuronal cells. To deprive neurons of NGF, 5-day-old neuronal cultures were washed three times with AM0 (AM50 medium lacking NGF) and fed with fresh AM0 containing 0.01% anti-NGF antiserum. For the treatment of neurons with various DNA-damaging agents, the culture medium was replaced with fresh AM0 containing the indicated concentrations of the particular DNA toxin. In neuronal cultures used to harvest protein, 25–50 μM of broad spectrum caspase inhibitor, bocaspartyl-(OMe)-fluoromethyl ketone (Enzyme Systems Products, Livermore, CA), was included in all treatment conditions to inhibit neuronal death.

Neuronal Survival—The number of viable cells was assessed after fixing the cells with 4% paraformaldehyde (Fisher) in phosphate-buffered saline and staining with crystal violet. Neurons were scored as viable by a naive observer if the crystal violet-positive cells had well defined cellular outlines. Dead neurons and debris stain faintly or not at all when viewed by a naive observer if the crystal violet-positive cells had large, ill-defined cell outlines. Dead neurons and debris stain faintly or not at all when viewed by a naive observer if the crystal violet-positive cells had large, ill-defined cell outlines.

Immunocytochemistry—Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline, washed with Tris-buffered saline (TBS: 0.1 M Tris-HCl (pH 7.6), 0.9% NaCl), and incubated in blocking solution (5% normal goat serum in TBS, containing 0.3% Triton X-100) for 1 h at room temperature. The cultures were then incubated with primary antibodies in antibody solution (1% normal goat serum in TBS, containing 0.3% Triton X-100) overnight at 4 °C. The following primary antibodies were used: phospho-c-Jun (Ser63) (1:1000, Cell Signaling), phospho-c-Jun (Ser73) (1:1000, Cell Signaling), phospho-SAPK/JNK (Thr183/Tyr185) (1:1000, Cell Signaling), phospho—SEK1/MKK4 (Thr262) (1:1000, Cell Signaling), and tubulin (Clone DM 1A, 1:50,000, Sigma). After washing, blots were incubated for 1 h at room temperature with HRP-linked secondary antibodies (Cell Signaling) diluted 1:2500–1:10,000 in blocking solution. The blots were washed 3 times with TBST and developed with a chemiluminescent substrate (Supersignal, Pierce). To strip and reprobe blots, the membranes were incubated in 100 mM glycine (pH 2.5) twice for 25 min and then washed with TBST. The Western analysis was then repeated. To semiquantitate immunoblots, the films were digitally scanned into Adobe Photoshop, and the selected bands were subjected to pixel analysis by using the UN-SCAN-IT software (Silk Scientific, Orem, UT). All values were normalized using the values obtained for tubulin loading control.

RESULTS

c-Jun Expression Is Induced in Neuronal Apoptosis after DNA Damage—We have reported previously that ara-C kills sympathetic neurons in vitro (8, 10). A recent study (7) has shown that ara-C causes oxidative stress in embryonic cortical neurons and leads to DNA damage and cell death. We determined whether this DNA damage can activate proapoptotic signals in sympathetic neurons. Several lines of evidence suggest that c-Jun has an essential role in neuronal apoptosis. Therefore, we examined the expression of c-Jun during DNA damage-induced neuronal apoptosis. In sympathetic neurons, ara-C exposure caused an increase in the levels of c-Jun protein (Fig. 1). This increase was apparent at 24 h, thus preceding the loss of viability (8). The increased levels of c-Jun protein remained heightened throughout the period of ara-C exposure. After 48 h of ara-C treatment, the migration of neuronal c-Jun became slower in SPADE, suggesting that c-Jun may have been phosphorylated during cell death.

c-Jun Is Phosphorylated in Its N-terminal Transactivation Domain after DNA Damage—One of the most critical steps in c-Jun regulation involves phosphorylation of two specific serine residues, Ser63 and Ser73, located in the N-terminal transactivation domain. Phosphorylation of Ser63 and Ser73 enhances c-Jun-mediated gene transcription (30). To determine whether neuronal c-Jun is subject to posttranslational modification after DNA damage, we examined the potential c-Jun N-terminal phosphorylation using an antibody that specifically detects c-Jun only when Ser63 is phosphorylated. When sympathetic neurons were exposed to increasing concentrations of ara-C, neuronal c-Jun was phosphorylated beginning at 11.1 μM of ara-C, and the levels of phospho-c-Jun were augmented with increasing ara-C concentrations (Fig. 2A). This concentration dependence correlated well with the dose-response curve of ara-C killing in sympathetic neurons (8). Moreover, neurons exposed to ara-C showed increased levels of phospho-c-Jun over time (Fig. 2B). Because ara-C exposure induced c-Jun expression and N-terminal phosphorylation simultaneously, increased phosphorylation after DNA damage might have been increased phosphorylation after DNA damage.
Fig. 2. N-terminal phosphorylation of c-Jun is induced during neuronal apoptosis caused by ara-C exposure. Sympathetic neurons were treated with 100 µM ara-C (NGF+ Ara-C), deprived of NGF (−NGF), or left untreated (−NGF), and neuronal protein was isolated at the indicated times. A, ara-C-induced c-Jun induction and phosphorylation are dose-dependent. Neuronal protein was isolated from sympathetic neurons treated with ara-C for 48 h. Levels of phospho-c-Jun Ser63 and c-Jun increased with greater concentrations of ara-C. B, ara-C-induced c-Jun phosphorylation is time-dependent. Phospho-c-Jun Ser63 levels increased with increasing time of ara-C exposure. C, percentage of N-terminal phosphorylated c-Jun increases in ara-C exposed sympathetic neurons. Levels of c-Jun and phospho-c-Jun Ser63 were semiquantified, and values normalized to tubulin were used to calculate the phospho-c-Jun/c-Jun ratio. Phospho-c-Jun/c-Jun ratio in NGF-maintained, untreated neurons was set to 1. Data are shown as fold increase compared with NGF-maintained, untreated controls. Mean of two separate experiments is shown. D and E, sympathetic neurons exposed to ara-C or left untreated were fixed at the indicated times, and phospho-c-Jun Ser63 was detected by immunocytochemistry. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. Percentage of phospho-c-Jun Ser63-positive neuronal nuclei increased with time after ara-C treatment (E). Untreated neurons did not show nuclear phospho-c-Jun staining. A representative field from neuronal cultures treated for 48 h is shown (D). F, in addition to Ser63, c-Jun is also phosphorylated on the N-terminal Ser73 residue during ara-C-induced neuronal cell death. All Western blots were stripped and reprobed with a tubulin antibody for loading control. Data shown in E represent mean ± S.D. of 3–4 cultures for each treatment. Identical results were obtained in at least two independent trials.

an indirect effect of elevated neuronal c-Jun levels. Therefore, we measured the relative ratio of phosphorylated c-Jun to total c-Jun during ara-C-induced neuronal apoptosis (Fig. 2C). The phospho-c-Jun/c-Jun ratio increased by 3-fold after 48 h of ara-C exposure, thus demonstrating that N-terminal c-Jun phosphorylation was not an indirect effect of increased protein levels. Localization of phospho-c-Jun by immunocytochemistry demonstrated that activated c-Jun was exclusively nuclear during DNA damage-induced neuronal apoptosis (Fig. 2D). About 50% of sympathetic neurons lose their viability after 48 h of ara-C exposure, and most neurons are dead by 96 h (67). Therefore, the time course of N-terminal c-Jun phosphorylation preceded the time course of ara-C-induced sympathetic neuronal death because the number of phospho-c-Jun-positive neurons started to increase at 24 h, at which time all neurons are still alive, and continued until 48 h with 70% of neurons containing nuclear phospho-c-Jun (Fig. 2E). Phosphorylation of Ser73, located in the N-terminal transactivation domain, is also critical for increasing c-Jun-mediated transcription. Similar to Ser63, DNA damage induced by ara-C treatment also led to increased levels of Ser73-phosphorylated c-Jun in sympathetic neurons (Fig. 2F). These results demonstrate that c-Jun was activated by N-terminal phosphorylation after DNA damage. In addition, the time course of c-Jun activation is consistent with increased c-Jun activity being a critical step in neuronal cell death after DNA damage (Fig. 2E).

Neuronal c-Jun Activation Is a Common Stress Response Seen after DNA Damage—Besides causing DNA damage, ara-C can also affect several other metabolic events in neurons, including the synthesis of membrane lipids and glycoproteins, potentially influencing the intracellular levels of lipid second messengers (10). To determine whether c-Jun induction and N-terminal phosphorylation occurred as a specific response to DNA damage after ara-C exposure, we examined the effects of other DNA-damaging agents in sympathetic neurons. Etoposide is a DNA topoisomerase II inhibitor that damages DNA by generating double-stranded DNA breaks. Sympathetic neurons exposed to 10 µM etoposide exhibited a significant increase in c-Jun levels starting at 6 h (Fig. 3A). This increase was associated with rapid neuronal death (data not shown). Etoposide-induced c-Jun protein also migrated more slowly in SDS-PAGE, indicating post-translational modification by increased phosphorylation. Immunoblot analysis of neuronal phospho-c-Jun levels showed that etoposide-induced DNA damage also caused enhanced N-terminal phosphorylation of c-Jun on Ser63 and Ser73 (Fig. 3B). We also exposed sympathetic neurons to another nucleoside analog, ara-A. Similar to ara-C, ara-A interferes with DNA metabolism, but it does not share
other DNA-independent metabolic actions of ara-C. Ara-A-induced sympathetic neuronal death was also associated with c-Jun activation, as shown by increased phospho-c-Jun Ser63 levels in sympathetic neurons after etoposide treatment. Levels of c-Jun, phospho-c-Jun Ser63, and phospho-c-Jun Ser73 were determined by immunoblotting. A, the amount of c-Jun protein increased with time in neurons after etoposide treatment. Both phospho-c-Jun Ser63 (B) and phospho-c-Jun Ser73 (C) levels increased in sympathetic neurons with increasing time of etoposide treatment. D, neuronal death after ara-A exposure is associated with increased c-Jun phosphorylation. Sympathetic neurons exposed to 100 μM ara-A (NGF+Ara-A) or left untreated (NGF) were fixed at 96 h, and phospho-c-Jun Ser63 was detected by immunocytochemistry. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. Percentage of phospho-c-Jun Ser63-positive neuronal nuclei increased after ara-A treatment (NGF+Ara-A). Untreated neurons did not show any nuclear phospho-c-Jun staining (NGF). Mean ± S.D., n = 3–4. E, neuronal death induced by camptothecin (CPT) is not associated with c-Jun phosphorylation or induction. Sympathetic neurons were treated with different concentrations of camptothecin for 48 or 72 h, and levels of phospho-c-Jun Ser63 and total c-Jun were measured by Western blotting. All blots were stripped and reprobed with a tubulin antibody for loading control. Identical results were obtained in at least two independent experiments.

A–C, sympathetic neurons were treated with 10 μM etoposide (NGF+Etoposide) or left untreated (NGF), and neuronal protein was isolated at the indicated times. Levels of c-Jun, phospho-c-Jun Ser63, and phospho-c-Jun Ser73 were determined by immunoblotting. A, the amount of c-Jun protein increased with time in neurons after etoposide treatment. Both phospho-c-Jun Ser63 (B) and phospho-c-Jun Ser73 (C) levels increased in sympathetic neurons with increasing time of etoposide treatment. D, neuronal death after ara-A exposure is associated with increased c-Jun phosphorylation. Sympathetic neurons exposed to 100 μM ara-A (NGF+Ara-A) or left untreated (NGF) were fixed at 96 h, and phospho-c-Jun Ser63 was detected by immunocytochemistry. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. Percentage of phospho-c-Jun Ser63-positive neuronal nuclei increased after ara-A treatment (NGF+Ara-A). Untreated neurons did not show any nuclear phospho-c-Jun staining (NGF). Mean ± S.D., n = 3–4. E, neuronal death induced by camptothecin (CPT) is not associated with c-Jun phosphorylation or induction. Sympathetic neurons were treated with different concentrations of camptothecin for 48 or 72 h, and levels of phospho-c-Jun Ser63 and total c-Jun were measured by Western blotting. All blots were stripped and reprobed with a tubulin antibody for loading control. Identical results were obtained in at least two independent experiments.

DNA Damage-induced c-Jun Induction and Phosphorylation Are Primarily Mediated by a JNK-independent Signaling Pathway—Phosphorylation of c-Jun N-terminal transactivation domain is believed to be performed exclusively by JNKs after cellular stress (13, 22, 23). This JNK-mediated N-terminal phosphorylation is critical for the transcriptional activity of c-Jun (30). Neuronal c-Jun phosphorylation induced by ara-C and other DNA-damaging agents suggests that the JNK pathway was activated during DNA-damage-induced neuronal apoptosis. During neuronal cell death caused by trophic factor deprivation, UV irradiation, or oxidative stress, MLKs mediate phosphorylation-coupled JNK activation by stimulating JNK-kinase MKK4/7 (24, 32). Phosphorylation of MKKs and JNKs is

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*a* C. G. Besirli and E. M. Johnson, Jr., unpublished observations.
required for catalytic activity of these kinases. Therefore, activation of the JNK pathway can be indirectly examined by analyzing the levels of phosphorylated kinases, as increased phosphorylation is coupled to increased catalytic activity. We determined the levels of phospho-MKK4 in sympathetic neurons by using an antibody that specifically recognizes Thr261-phosphorylated MKK4, which is important for kinase activity. Although we did not detect any significant increase above basal levels after DNA damage at 12 h (Fig. 4A), our data indicated that there might be a slight increase in phospho-MKK4 levels in ara-C-treated neurons at 24 and 36 h. However, quantitative data showed that there was no significant difference in phospho-MKK4 levels between NGF-maintained and ara-C-treated neurons at these time points (Fig. 4B). In contrast, NGF-deprived neurons, in which the JNK pathway is activated, showed significantly higher phospho-MKK4 levels compared with NGF-maintained, untreated neurons (Fig. 4A). Similar to phospho-MKK4, we did not detect any increase in the amount of phospho-JNKs after ara-C treatment (Fig. 4C). Indeed, the amount of phospho-JNK declined during DNA damage-induced neuronal death, because sympathetic neurons showed high basal levels of JNK phosphorylation when maintained in NGF. These results suggest that activation of the JNK pathway was not increased by DNA damage and, in fact, was decreased and that JNKs did not mediate c-Jun phosphorylation. However, MKK7 might have been the JNK kinase activated after DNA damage, and phospho-MKK4 antibody might not have detected phosphorylated MKK7 in neurons. In addition, even though the total phospho-JNK levels were decreased in ara-C-exposed sympathetic neurons, a subset of JNKs might have been activated by DNA damage, as the JNK family consists of 3 isoforms and 10 splice variants. The detection of this potential isoform-specific JNK activation with a pan-phospho-JNK antibody may not be possible. Therefore, we determined whether the JNK pathway activation was responsible for c-Jun phosphorylation in DNA-damaged neurons by assessing the effect of pharmacological JNK pathway inhibition on c-Jun activation. CEP-1347 is a selective MLK inhibitor that prevents JNK activity and neuronal apoptosis (15, 24–26). When we treated sympathetic neurons with ara-C in the presence of 1.3 μM CEP-1347, the majority of N-terminal c-Jun phosphorylation was maintained (Fig. 5, A and B). In contrast, N-terminal c-Jun phosphorylation induced by NGF deprivation was completely prevented in sibling neuronal cultures (Fig. 5, A and B), demonstrating that CEP-1347 fully prevented MLK-dependent activation of JNKs in neurons as reported previously (26).

The inability of CEP-1347 to prevent ara-C-induced N-terminal c-Jun phosphorylation indicates that MLKs did not activate the JNK pathway in sympathetic neurons after DNA damage. However, the JNK pathway might be activated by another MKK kinase such as a member of the MEK kinase (MEKK) family. Therefore, we proceeded to inhibit the JNK pathway at the last catalytic step, JNK phosphorylation of substrates such as c-Jun. JNK activity was blocked selectively by the anthrapyrazolone JNK inhibitor SP600125 (33, 34), and neuronal c-Jun phosphorylation was analyzed after ara-C treatment. When DNA damage was induced with ara-C in sympathetic neurons in the presence of 14 μM SP600125, N-terminal c-Jun phosphorylation on Ser63 was unaltered (Fig. 5C). The number of sympathetic neuronal nuclei positive for phospho-c-Jun staining after ara-C treatment was the same as that seen in sister cultures containing SP600125 (Fig. 5D). In contrast, SP600125 completely prevented N-terminal c-Jun phosphorylation after NGF deprivation, demonstrating its full inhibitory effect on JNK catalytic activity (Fig. 5, C and D). Therefore, similar to CEP-1347, SP600125 was unable to prevent c-Jun phosphorylation associated with DNA damage-induced neuronal apoptosis. Immunocytochemical results were confirmed by evaluating phospho-c-Jun Ser63 levels by immunoblotting after ara-C or etoposide treatment in the presence of JNK-pathway inhibitors (Fig. 6). Unlike NGF deprivation, CEP-1347 or SP600125 could not prevent N-terminal c-Jun phosphorylation or induction during ara-C and etoposide-in-

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**Fig. 4.** JNK pathway is not activated at detectable levels in sympathetic neurons after DNA damage. 5 DIV rat sympathetic neurons were treated with 100 μM ara-C with or without 1.3 μM CEP-1347 (CEP), deprived of NGF, or left untreated, and neuronal protein was isolated at the indicated times. Levels of phosphorylated MKK4 and JNKs were determined by immunoblotting. A, ara-C treated neurons (NGF+Ara-C) did not show any increase in MKK4 phosphorylation above the basal levels seen in untreated controls (NGF). In contrast, MKK4 activity was increased as indicated by the elevated levels of phospho-MKK4 in NGF-deprived neurons (−NGF). Phospho-MKK4 levels in NGF-maintained cells at 12 h was set to 1. C, JNK phosphorylation was decreased in ara-C-exposed sympathetic neurons (1st to 3rd lanes) compared with high basal levels seen in untreated controls (NGF). In contrast, MKK4 activity was Increased JNK activity was indicated by the elevated levels of phospho-JNKs in neurons after NGF deprivation (right panel). The MLK-inhibitor CEP-1347 completely inhibited JNK phosphorylation in neurons (4th lane). Blots were stripped and reprobed with a tubulin antibody for loading control. Identical results were obtained in three independent experiments.
duced death of sympathetic neurons (Fig. 6, A and B). However, both drugs showed a partial inhibition of phospho-c-Jun Ser63 levels in Western blots (45% (CEP-1347) and 35% (SP600125) decrease when normalized to c-Jun, Fig. 6A). Similar results were obtained when phospho-c-Jun Ser73 antibody was used to detect N-terminal c-Jun phosphorylation in Western analysis (data not shown). Continued N-terminal c-Jun phosphorylation in sympathetic neurons in the absence of JNK activity indicated that DNA damage activated a novel kinase that phosphorylated c-Jun transactivation domain during neuronal death.

JNK Pathway Is Not Necessary for Neuronal Death after DNA Damage—Inhibition of the JNK pathway varies by method, including pharmacological intervention with the MLK inhibitor CEP-1347, overexpression of dominant-negative constructs, and genetic deletion prevents neuronal apoptosis induced by trophic factor deprivation and other stress stimuli (24, 26, 35–39). Although JNK inhibition did not prevent the majority of c-Jun phosphorylation after DNA damage, JNK activity may still be important for the activation of the cell death machinery in DNA-damaged neurons. Therefore, we examined whether blocking the JNK pathway can attenuate neuronal apoptosis induced by DNA damage. Mean ± S.D., n = 3–4. Similar results were obtained in at least two independent experiments.

![Fig. 5.](image)

**Fig. 5.** c-Jun induction and phosphorylation during DNA damage-induced neuronal death is independent of the classical JNK pathway. 5-DIV sympathetic neurons were exposed to 100 μM ara-C, deprived of NGF, or left untreated and were fixed after 48 h. In some neuronal cultures, ara-C treatment or NGF deprivation was performed in the presence of 1.3 μM CEP-1347 (CEP) or 14 μM SP600125 (SP). N-terminal c-Jun phosphorylation was examined by the detection of neuronal phospho-c-Jun Ser63 immunocytochemically. Concurrent staining with bisbenzimide was performed to detect the nuclei of neurons. Neither MLK-inhibitor CEP-1347 (A and B) nor JNK-inhibitor SP600125 (C and D) blocked the majority of c-Jun phosphorylation after ara-C-induced DNA damage. Mean ± S.D., n = 3–4. Similar results were obtained in at least two independent experiments.

![Fig. 6.](image)

**Fig. 6.** MLK or JNK inhibition does not prevent DNA damage-induced c-Jun expression and N-terminal phosphorylation. Sympathetic neurons were treated with 100 μM ara-C, 10 μM etoposide, deprived of NGF, or left untreated, and neuronal protein was isolated at the indicated times. Some neuronal cultures contained 1.3 μM CEP-1347 or 14 μM SP600125 throughout the treatment period. A, phospho- or total c-Jun was detected by immunoblotting. CEP-1347 or SP600125 only partially blocked ara-C-induced N-terminal c-Jun phosphorylation or c-Jun induction (6th to 8th lanes). B, neither CEP-1347 nor SP600125 inhibited the majority of c-Jun induction or N-terminal phosphorylation induced by etoposide. Blots were stripped and reprobed with a tubulin antibody for loading control. Similar results were obtained in at least two independent experiments.
JNK-independent Neuronal c-Jun Activation

FIG. 7. The JNK pathway is dispensable for neuronal apoptosis after DNA damage. Sympathetic neuronal cultures maintained in the presence of NGF for 5 days were exposed to 100 μM ara-C or deprived of NGF for 48 h, and the fraction of surviving neurons was determined by measuring viability as described. 1.3 μM CEP-1347 (CEP) or 14 μM SP600125 (SP) was present in some neuronal cultures throughout the treatment period. A, CEP-1347 had no significant effect on neuronal survival after ara-C-induced DNA damage (Ara-C versus Ara-C+CEP). Neuronal apoptosis was completely blocked by CEP-1347 after NGF deprivation (~NGF versus ~NGF+CEP). B, specific JNK inhibition by SP600125 did not prevent neuronal death induced by ara-C. Sympathetic neurons died normally after ara-C treatment in the presence of SP600125 (Ara-C versus Ara-C+SP). NGF deprivation-induced neuronal apoptosis was mostly prevented by SP600125 (~NGF versus ~NGF+SP). C, JNK pathway was not necessary for caspase activation after DNA damage in neurons. Sympathetic neurons were exposed to 100 μM ara-C (Ara-C) for 48 h or left untreated (NGF). In some cultures, ara-C treatment was performed in the presence of 25 μM c-Jun phosphorylation in the absence of JNK activity suggests the presence of another kinase that was activated in response to DNA damage. p44/42 MAPK has been reported to phosphorylate c-Jun in its N terminus during PC12 cell differentiation (40). To determine whether p44/42 MAPK is responsible for c-Jun phosphorylation, we induced DNA damage in sympathetic neurons in the presence of 25 μM U0126, which selectively prevents the activation of MAPK kinase MEK and inhibits the phosphorylation-dependent activation of p44/42 MAPK. U0126 completely prevented neuronal p44/42 MAPK phosphorylation, but c-Jun phosphorylation was actually slightly enhanced (data not shown). p38, another stress-activated protein kinase, has also been reported to regulate c-Jun activity (41, 42). However, inhibiting p38 catalytic activity with 50 μM SB203580 did not affect N-terminal c-Jun phosphorylation during ara-C-induced neuronal apoptosis (data not shown). DNA damage-induced c-Jun N-terminal kinase was also not a phosphatidylinositol 3-kinase-like enzyme because c-Jun phosphorylation was only enhanced when sympathetic neurons were exposed to ara-C in the presence of 50 μM LY294002 (data not shown).

CDKs are implicated in neuronal cell death induced by trophic factor deprivation and DNA damage (12, 43–45). cyclin D1 expression is significantly increased in sympathetic neurons undergoing NGF deprivation-induced apoptosis (46). Moreover, overexpression of dominant-negative CDKs and pharmacological CDK inhibitors have been reported to prevent neuronal cell death after DNA damage and trophic factor deprivation (31, 47). To determine whether CDK activation mediates c-Jun induction and phosphorylation after DNA damage, we examined the effects of several pharmacological CDK inhibitors during ara-C-induced sympathetic neuronal death. Sympathetic neurons were exposed to ara-C in the presence of three structurally different CDK inhibitors. The purine derivative olomoucine, a semi-selective CDK inhibitor (48), was able to block both c-Jun induction and N-terminal phosphorylation completely at 200 μM (Fig. 8A). In addition, olomoucine also prevented NGF deprivation-induced N-terminal c-Jun phosphorylation in sympathetic neurons (Fig. 8A). Roscovitine, a CDK inhibitor structurally similar to olomoucine, had the same inhibitory effect on c-Jun phosphorylation at 50 μM (data not shown). Inhibition of c-Jun expression and N-terminal phosphorylation simultaneously is expected, because c-Jun regulates its own expression, and phosphorylation is tightly coupled
to c-jun transcription. Furthermore, the kinases (e.g. JNK) that phosphorylate c-jun can also activate other transcription factors that regulate c-jun expression (e.g. ATF-2). Thus, inhibition of the kinase activity can reduce both gene expression and protein phosphorylation at the same time (see Ref. 49 and references therein). We also tested the potential effect of flavone-compound flavopiridol, which is a pan-CDK inhibitor (50). Flavopiridol was also very effective in preventing ara-C-induced c-Jun phosphorylation on Ser^{63} (Fig. 8B). Finally, 3 μM of semi-selective CDK-inhibitor alsterpaullone, a paullone derivative (51), significantly reduced N-terminal c-Jun phosphorylation in sympathetic neurons after ara-C exposure (Fig. 8C). Consistent with previous reports on the protective effects of these compounds on DNA damage-induced neuronal death (31, 47), we confirmed that all CDK inhibitors tested were able to prevent neuronal death induced by ara-C only at the concentrations that prevented N-terminal c-Jun phosphorylation (data not shown). Simultaneous inhibition of N-terminal c-Jun phosphorylation and DNA damage-induced neuronal death by CDK inhibitors suggests that c-jun activity is critical for neuronal apoptosis induced by DNA damage. Moreover, JNK-independent c-Jun activation appears to be mediated by a CDK-like activity during DNA damage-induced neuronal death.

**DISCUSSION**

In this study, we investigated the role of JNK pathway and c-Jun activation in neuronal death induced by DNA damage. Our data demonstrate that neurons undergoing DNA damage-induced apoptosis activated the AP-1 transcription factor c-Jun. This activation was regulated by both transcriptional mechanisms via increased c-jun expression and post-translational mechanisms via N-terminal c-Jun phosphorylation. Induction of c-Jun activity in degenerating neurons preceded caspase activation and cell death. Remarkably, in contrast to other neuronal death paradigms in which c-Jun activity is exclusively regulated by the JNK pathway, DNA damage-induced c-Jun activation was primarily mediated by a signaling pathway other than the classical JNK pathway. We also show that unlike neuronal apoptosis induced by trophic factor deprivation or oxidative stress, DNA damage-induced apoptosis did not require JNK pathway activity. The novel c-Jun phosphorylation/activation pathway was inhibited by the semi-selective CDK inhibitors known to block DNA damage-induced neuronal death. Thus, activation of this JNK-independent signaling pathway most likely has a critical role in neuronal death induced by DNA-damaging agents.

C-Jun is implicated in many different neuronal death paradigms (reviewed in Refs. 13 and 49). In particular, NGF deprivation-induced apoptosis of sympathetic neurons requires c-Jun activity, because microinjection of a c-Jun-blocking antisemur or dominant-negative c-Jun construct into neurons prevents neuronal death (17, 18). Confirming these previous results, Palmada and colleagues (19) recently reported that c-Jun-deficient sympathetic neurons are resistant to NGF deprivation in vitro. Our data demonstrate that c-Jun expression is significantly induced in sympathetic neurons after DNA damage (Figs. 1 and 3). In addition, we observed increased phosphorylation of Ser^{63} and Ser^{73} in the N-terminal transactivation domain of c-jun (Figs. 2 and 3). This is in contrast to a previous study reporting that there was no c-Jun phosphorylation after ara-C treatment in sympathetic neurons (52). This inconsistency may be because of the different culture paradigm used by that study. c-Jun expression and phosphorylation preceded cell death, suggesting that c-Jun-mediated gene transcription may be necessary for the activation of the cell death pathway after DNA damage (8). Neuronal genes regulated by c-Jun during apoptosis are still unknown. A pair of likely targets are the proapoptotic BH3-only Bcl-2 family members Bim and DP5 (15, 16). Similar to NGF deprivation (16, 53), Bim expression is up-regulated in sympathetic neurons after ara-C exposure (67). c-Jun activity has also been reported to regulate FasL expression (54, 55). Ara-C-treated neurons show increased levels of Fas mRNA, but FasL expression does not change. In addition, deletion of FasL or Fas in sympathetic neurons does not affect neuronal survival after ara-C exposure, suggesting that these genes are not the critical c-Jun targets during DNA damage-induced apoptosis. Although our data shows that DNA damage clearly activated c-Jun in sympathetic neurons, the importance of c-Jun-mediated gene transcription during neuronal death remains to be tested. It is possible that proapoptotic signaling events leading to c-Jun

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3 R. M. Easton and E. M. Johnson, Jr., unpublished observations.
activation, but not c-Jun alone, may be required for DNA damage-induced neuronal death, and c-Jun activation may be an important, but not a necessary, marker of these signaling pathways.

Whereas we observed c-Jun activation with multiple DNA toxins, this activation appeared to depend on the type of DNA damage and the specific neuronal population. Interestingly, different DNA-damaging agents, i.e. camptothecin, UV irradiation, ara-C, or etoposide, exerted distinct biological effects on sympathetic neurons. For example, we did not observe c-Jun phosphorylation in sympathetic neurons exposed to various concentrations of camptothecin (Fig. 3E). This is in contrast to the findings of Gahremani et al. (56) who reported JNK-dependent phosphorylation of c-Jun in embryonic cortical neurons after camptothecin-induced DNA damage. UV irradiation also kills sympathetic and cortical neurons in vitro, but unlike ara-C-induced death, UV-induced death does not require new protein synthesis (8, 57), although the mechanism of death may be dependent on the dose. In addition to causing DNA damage by creating pyrimidine dimers, UV irradiation also induces the photo-oxidation of proteins and membrane lipids and generation of reactive oxygen species (58, 59). Therefore, neuronal death after UV damage is potentially more complicated than a simple DNA-damage response and may not require DNA damage to proceed. Interestingly, unlike ara-C or etoposide, camptothecin (56) and UV irradiation-induced (24) neuronal c-Jun phosphorylation is MLK-dependent, and MLK inhibition protects neurons from both types of DNA damage.

JNKs are the only mammalian kinases reported to phosphorylate c-Jun in its N-terminal transactivation domain during cellular stress. In particular, activation of JNK family members is required for neuronal death caused by various stress stimuli including trophic factor deprivation and oxidative stress (reviewed in Refs. 13 and 22). However, we did not observe any ara-C-induced increase in kinase-active forms of MKK4 or JNKs in neurons (Fig. 4). Furthermore, neither the MLK inhibitor CEP-1347 nor the JNK inhibitor SP600125 prevented N-terminal c-Jun phosphorylation or neuronal death after DNA damage (Figs. 5–7), although both drugs could partially reduce both c-Jun expression and N-terminal phosphorylation (Fig. 6). There are at least two potential explanations for this partial effect. First, classical JNK pathway may contribute to c-Jun activation after DNA damage. Alternatively, pharmacological inhibitors of the JNK pathway may also cause incomplete inhibition of the novel DNA damage-induced c-Jun kinase pathway as these kinases may be similar to JNK pathway kinases, thus making them sensitive to similar pharmacological inhibitors. Regardless, most of the c-Jun activation was maintained in the absence of JNK activity (Figs. 5 and 6), and complete inhibition of the classical JNK pathway did not prevent (or even delay) DNA damage-induced neuronal death (Fig. 7). Therefore, the JNK pathway was not necessary for DNA damage-induced c-Jun activity or neuronal apoptosis. Activation of c-Jun by multiple stress stimuli such as NGF deprivation and DNA damage suggests that c-Jun may be the critical converging point for intracellular signaling pathways for the initiation of neuronal apoptosis. Whereas NGF-deprived neurons used the classical JNK pathway to activate c-Jun, neurons with DNA damage stimulated a separate JNK-independent pathway to increase c-Jun activity.

Inhibition of N-terminal c-Jun phosphorylation and neuronal apoptosis by CDK inhibitors suggested that a CDK family member (or a downstream kinase) might be the DNA damage-induced c-Jun kinase. Previous studies suggest that CDK4 and -6 are activated during neuronal apoptosis (31, 47). However, these studies use dominant-negative CDK overexpression or pharmacological CDK inhibitors. Many potential problems exist with these techniques, complicating and limiting the interpretation of these data. Although relatively selective for the CDK family, CDK inhibitors can also inhibit other structurally related kinases such as GSK-3β (60). In addition, both olomoucine and roscovitine inhibit JNK1 activity (61), which probably explains the observation that these drugs also inhibited c-Jun phosphorylation mediated by NGF deprivation (Fig. 8 and data not shown). Another complicating factor is that none of the pharmacological CDK inhibitors currently available is specific for any CDK isoform (see reviews in Refs. 62 and 63). Flavopiridol is a pan-CDK inhibitor that inhibits all CDKs tested. The newer generation of CDK inhibitors, olomoucine, roscovitine, and alsterpaullone, are semi-selective for CDKs 1, 2, and 5. Although these inhibitors prevented neuronal cell death after NGF deprivation and DNA damage, in vitro kinase assays demonstrated that the same drug concentrations do not significantly block the catalytic activities of two CDK isoforms most commonly reported to be required for cell death in these paradigms, CDK4 and -6.

A more significant problem with using pharmacological CDK inhibitors is that these drugs inhibit other CDK family members that are not involved in cell cycle regulation. One of these is CDK9, a critical regulator of the RNA transcription machinery (64, 65). In fact, we found that CDK inhibitors significantly reduced protein synthesis in sympathetic neurons. For instance, flavopiridol decreased neuronal protein synthesis by 50% at the concentration that prevented death (data not shown). This result is consistent with a recent study (66) showing global repression of gene transcription by flavopiridol. Therefore, part of the cell survival-promoting effects of CDK inhibitors may be an indirect consequence of protein synthesis inhibition (8). Overexpressing dominant-negative CDK proteins may also have a similar inhibitory effect on protein synthesis by sequestering regulatory molecules required for the function of other CDK family members. Similar to CDK9, there are other CDK family members, including CDK5, -7, and -8 and probably more CDKs remaining to be identified, which are essential for non-cell cycle-related functions. An indirect effect such as the inhibition of CDK-mediated RNA transcription, or direct inhibition of another neuronal CDK-like protein not involved in cell cycle, may contribute to the prosurvival effects observed with molecular or pharmacological CDK inhibition. Therefore, the results reported here and previous results based on these techniques need to be interpreted cautiously.

Considering the potential disadvantages of using pharmacological CDK inhibitors, we cannot conclude from our data that DNA damage-induced N-terminal c-Jun phosphorylation was mediated by a known CDK or was related to cell cycle regulation in sympathetic neurons. Instead, we suggest that DNA damage-induced c-Jun N-terminal phosphorylation is diminished by pharmacological CDK inhibitors. This activity may control c-Jun activation directly by phosphorylating c-Jun N-terminal transactivation domain or indirectly by activating a novel Jun N-terminal kinase other than the JNK family members. c-Jun expression and N-terminal phosphorylation by DNA toxins strongly suggest a proapoptotic role of c-Jun in DNA-damaged neurons. The inability to prevent c-Jun activation or neuronal death by JNK pathway inhibition after DNA damage demonstrates that JNK-independent signaling pathways must exist in neurons that can stimulate c-Jun-mediated gene transcription and apoptosis. Therefore, depending on the type of neuronal stress, prevention of pathological neuronal loss by blocking c-Jun-mediated gene transcription may require inhibiting the classical JNK pathway and/or other
JNK-independent signaling events that regulate neuronal c-Jun activity.

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REFERENCES

1. Winkelman, M. D., and Hines, J. D. (1983) Ann. Neurol. 14, 520–527
2. Sylvester, R. K., Fisher, A. J., and Lobell, M. (1987) Drug Intell. Clin. Pharm. 21, 177–180
3. Lazarus, H. M., Herzig, R. H., Herzig, G. P., Phillipa, G. L., Roessman, U., and Fishman, D. J. (1981) Cancer (Philad.) 48, 2577–2582
4. Vogel, H., and Horoupian, D. S. (1995) Cancer (Philad.) 71, 1303–1308
5. Rosar, L. M., Phillips, P. C., Kastan, M. B., Leventhal, B. G., Bowman, P. W., and Civen, C. I. (1995) Cancer (Philad.) 71, 117–123
6. Dossi, F., Pollard, H., Moreau, J., Ben-Ari, Y., and Charriaut-Marlangue, C. (1995) J. Neurochem. 64, 1890–1897
7. Geller, H. M., Cheng, R. Y., Goldsmith, N. K., Romero, A. A., Zhang, A. L., Morris, E. J., and Grandison, L. (2001) J. Neurochem. 77, 299–303
8. Bliss, V. P., and Collingridge, G. L. (1993) Nature 361, 31–39
9. Tomkins, C. E., Edwards, S. N., and Tolkovsky, A. M. (1994) J. Neurosci. 14, 11542–11551
10. Geller, H. M., Cheng, K. Y., Goldsmith, N. K., Romero, A. A., Zhang, A. L., Morris, E. J., and Grandison, L. (2001) J. Neurochem. 77, 299–303
11. Stefanis, L., Park, D. S., Friedman, W. J., and Greene, L. A. (1999) J. Neurosci. 19, 8890–8897
12. Morris, E. J., Keramaris, E., Rideout, H. J., Slack, R. S., Dyson, N. J., Stefanis, L., and Park, D. S. (2001) J. Neurosci. 21, 5017–5026
13. Ham, J., Eilers, A., Whiffled, J., Neame, S. J., and Shah, B. (2000) Biochem. Pharmacol. 60, 1015–1021
14. Herdegen, T., Skene, P., and Bahr, M. (1997) Trends Neurosci. 20, 227–231
15. Harris, C. A., and Johnson, E. M., Jr. (2001) J. Biol. Chem. 276, 37774–37760
16. Whitfield, J., Neame, S. J., Paquet, L., Bernard, O., and Ham, J. (2001) Neuron 29, 629–643
17. Ham, J., Bahij, C., Whiffled, J., Pfarr, C. M., Lallemend, D., Yaniv, M., and Rubin, L. L. (1990–1991) Neuron 4, 827–838
18. Eilers, A., Xu, Z., Maroney, A. C., Kukekov, N. V., and Greene, L. A. (2001) Neuron 29, 809–820
