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c-Jun N-Terminal Kinase Mediates Apoptotic Signaling Induced by *N*-(4-Hydroxyphenyl)retinamide

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ABSTRACT

N-(4-Hydroxyphenyl)retinamide (4-HPR), a retinoic acid analog, induces apoptosis in several cell types. The mechanism by which 4-HPR initiates apoptosis remains poorly understood. We examined the effects of 4-HPR on two prostate carcinoma cell lines, LNCaP (an androgen-sensitive, $p53^{+/+}$ cell line) and PC-3 (an androgen-insensitive, $p53^{-/-}$ cell line). 4-HPR caused sustained c-Jun N-terminal kinase (JNK) activation and apoptosis in LNCaP cells but not in PC-3 cells at the dosages tested. Activation of JNK by 4-HPR was independent of caspases because a pan-caspase inhibitor failed to suppress JNK activation. Ultraviolet-C and γ-radiation induced JNK activation in both LNCaP and PC-3 cells, suggesting that the failure of PC-3 cells to respond to 4-HPR was due to defects upstream of the JNK pathway. Furthermore, γ-radiation-induced JNK activation was suppressed by an antioxidant, but 4-HPR-induced JNK activation was not, indicating that these two stimuli induced JNK activation through different mechanisms. Forced expression of JNK1, but not a JNK1 mutant, caused apoptosis in both LNCaP and PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. 4-HPR-induced apoptosis in LNCaP cells was suppressed by curcumin, which inhibits JNK activation. Expression of dominant-negative mutants in the JNK pathway also inhibited 4-HPR-induced apoptosis in human embryonic kidney 293 cells. Collectively, these results suggest that the JNK pathway mediates 4-HPR-induced apoptotic signaling.

Retinoic acid and its synthetic analogs have diverse effects on development, morphogenesis, and homeostasis, and have been tested in the prevention and treatment of cancers (Means and Gudas, 1995; Lotan, 1996). Natural retinoids show limited effects on many cancer cells; however, several synthetic retinoids exhibit potent biological activities. For example, *N*-(4-hydoxylphenyl)retinamide (4-HPR) shows promise in the treatment and prevention of several cancers (Pienta et al., 1993; Kelloff et al., 1994). 4-HPR suppresses cell proliferation in cancer cells as does retinoic acid. However, 4-HPR induces apoptosis, whereas retinoic acid usually does not (Delia et al., 1993; Oridate et al., 1995; Ponzoni et al., 1995; Fanjul et al., 1996). The ability to arrest growth and to induce apoptosis gives 4-HPR great potential as an effective anticancer agent. To date, the mechanism by which 4-HPR induces apoptosis is poorly understood.

c-Jun N-terminal kinase (JNK; also named stress-activated protein kinase) is a member of the mitogen-activated protein kinase (MAPK) family, which also includes extracellular signal-regulated kinase and p38-MAPK. JNK was first identified by its ability to respond to environmental stresses, proinflammatory cytokines, and mitogens (for review, see Kyriakis and Avruch, 1996; Ip and Davis, 1998). The JNK pathway was later found to be important in apoptosis signaling (for review, see Ip and Davis, 1998). Interference with the JNK pathway suppresses the induction of apoptosis by various agents (Xia et al., 1995; Chen et al., 1996b, 1998; Verheij et al., 1996; Zanke et al., 1996). JNK phosphorylates the Ser63/Ser73 residues in the N-terminal *trans*-activating domain of c-Jun, strongly augmenting its transcriptional activity (Whitmarsh and Davis, 1996). In addition, the JNK pathway activates activating transcription factor-2 (Gupta et al., 1995), Elk-1 (Whitmarsh et al., 1995), and Sap-1a transcription factors (Janknecht and Hunter, 1997), and interacts with the nuclear factor-κB pathway (Meyer et al., 1996; Lee et al., 1997). The mechanisms by which the JNK pathway participates in these diverse cellular functions are unclear. Our previous data suggest that the duration of JNK activation determines cell fate (Chen et al., 1996a,b). In this study, we examined the apoptotic effect of 4-HPR on two prostate carcinoma cell lines, LNCaP and PC-3. We found that 4-HPR induced sustained JNK activation and apoptosis in LNCaP

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ABBREVIATIONS: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; z-VAD-FK, z-Val-Ala-Asp-fluoromethyl ketone; UV-C, ultraviolet C; X-gal, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside; NAC, *N*-acetyl-L-cysteine; TGF, transforming growth factor; HEK293 cells, human embryonic kidney 293 cells.

but not in PC-3 cells. Interference with the JNK signaling suppressed apoptosis induced by 4-HPR. Our results suggest that the JNK pathway mediates apoptotic signaling induced by 4-HPR.

Materials and Methods

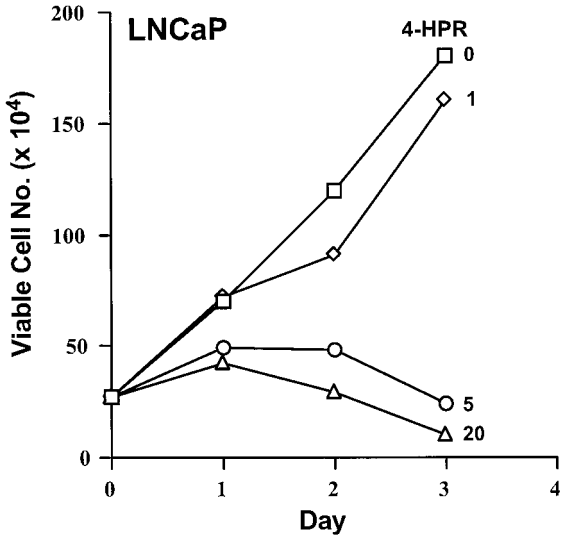
Cells and Antibodies. The culture of human embryonic kidney 293 cells (HEK293 cells) was described previously (Chen et al., 1998). The prostate carcinoma cell lines LNCaP and PC-3 were kindly provided by K.-M. Tchou-Wong (New York University Medical School, New York, NY), and were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and streptomycin/penicillin. The rabbit anti-JNK1 antibody Ab101 was described previously (Chen et al., 1996a). The rabbit anti-p38-MAPK antibody Ab221 was generated against the peptide containing the carboxy-terminal 18 amino acids (DEVISFVPPPLDQEEMES) of p38 kinase. Anti-caspase 3 (anti-p11; no. K-19), anti-Bcl-2 (no. 100), and anti-Bcl-X_L (no. S-18) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-HA (12CA5) antibody was purchased from Boehringer Mannhein Corp. (Indianapolis, IN). Anti-Flag (M2), anti-glutathione *S*-transferase (GST), and horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Reagents and Radiation Treatments. 4-HPR was purchased from Sigma Chemical Co. and prepared as concentrated stock solutions in ethanol. SB202190 and curcumin were obtained from Calbiochem Corp. (La Jolla, CA) and Sigma Chemical Co., respectively, and dissolved in dimethyl sulfoxide. *N*-Acetyl-L-cysteine was obtained from Sigma Chemical Co. The caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-FK) was obtained from Kamiya Biomed. Co. (Thousand Oaks, CA). Ultraviolet-C (UV-C) irradiation was performed with a UV Stratalinker 1800 from Stratagene, Inc. (La Jolla, CA). Gamma irradiation was performed with a Gammacell 1000 ^{137}Cs source (Atomic Energy of Canada Ltd., Commercial Products, Ottawa, Ontario, Canada).

Plasmids. The GST-Jun(1–79) plasmid was described previously (Chen et al., 1996b). The pHA-JNK1 plasmid was provided by Dr. J. R. Woodgett (Ontario Cancer Institute, Toronto, Canada) (Pombo et al., 1995; Yao et al., 1997). The pEBG-GST-SEK1(AL) was provided by L. I. Zon (Children's Hospital, Boston, MA; Pombo et al., 1995), pcDNA3-Flag-JNK1(APF) was provided by R. J. Davis (University of Massachusetts, Worcester, MA; Gupta et al., 1995).

Apoptosis Assays. For nuclear morphology staining, the harvested cells were fixed with 1% paraformaldehyde (in 1× PBS) for 10 min, washed once with 1× PBS, then stained with Hoechst 33258 (2.5 ng/ml in PBS). The nuclear morphology was examined with a fluorescence microscope, and cells with condensed or fragmented nuclei were identified as apoptotic cells. For flow cytometry analyses of DNA staining profile, 5×10^5 or 10^6 cells were collected and washed with PBS once, then fixed with 70% ethanol. Fixed cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing propidium iodide (10 μg/ml; Sigma Chemical Co.). The stained cells were analyzed by flow cytometry (model XL; Coulter Corp., Hialeah, FL). Forward light scatter characteristics were used to exclude the cell debris from the analysis. Apoptotic cells were determined by their hypochromic, subG1 staining profiles. DNA fragmentation assays were performed as described in Herrmann et al. (1994).

Cell Extract Preparation and Immunocomplex Kinase Assays. Whole cell extract was prepared by suspending 2×10^6 cells in 200 μl of lysis buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4]. The cell lysate was kept on ice for 20 min and vigorously mixed in a Vortex mixer every 5 min. The lysate was cleared by centrifugation at 15,000*g* for 3 min, and the supernatant was stored at −80°C. JNK assays were carried out as described in Chen et al. (1996b). For p38-MAPK assays, endogenous p38-MAPK was precipitated by incubation with an anti-p38 antibody (Ab221) and protein A-agarose beads (Bio-Rad Laboratories, Inc., Richmond, CA) in the lysis buffer at 4°C for 3 h. The precipitates were washed twice with the lysis buffer and twice with kinase buffer [25 mM HEPES (pH 7.6), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM Na_3VO_4], then mixed with 5 μg of myelin basic protein, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer. The kinase reaction was per-



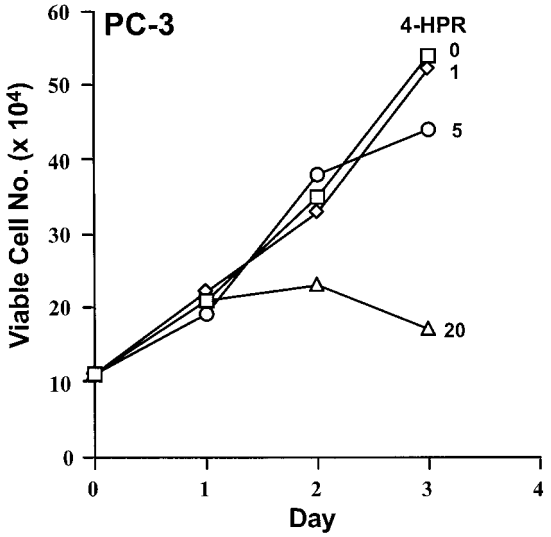


Fig. 1. 4-HPR decreases cell viability in LNCaP and PC-3 cells. LNCaP (A) and PC-3 cells (B) were plated in six-well plates 24 h before treatment. The cells were treated with 4-HPR at the indicated concentrations on day 0, and harvested on days 1, 2, and 3. Viable cells per well were determined by the trypan blue exclusion assay.

formed at 30°C for 30 min, then terminated by adding SDS-sample buffer. The reaction mixtures were boiled and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Transient Transfection-Protection/Apoptosis Assays. Transient transfection-protection assays were performed as described with modifications (Chen et al., 1996b); HEK293 cells were plated in a 35-mm-well plate (1.5×10^5 cells/well) the day before transfection. Cells were transfected with plasmids encoding β-galactosidase (1 μg) in combination with empty vector or the indicated plasmids encoding dominant-negative kinase mutants (2 μg for each). The transfections were performed by a calcium phosphate precipitation method (Specialty Media, Phillipsburg, NJ). Transfected cells were cultured in complete medium for 6 h after removing the transfection mixture, and then treated with 4-HPR (20 μM) or left untreated for 12 h. Cells were fixed in 1% paraformaldehyde for 10 min, washed twice with PBS, and stained with the staining solution [PBS (pH 7.4), 1 mM $MgCl_2$, 10 mM $K_4[Fe(CN)_6]$, 10 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal)]. Transfected cells (blue color) with rounding up, shrinkage, or membrane-blebbing morphology were identified as apoptotic cells. Apoptosis induction was represented as percentage of apoptotic cells per 300 blue cells. For transient transfection-apoptosis assays, LNCaP or PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with the indicated kinase plasmids as

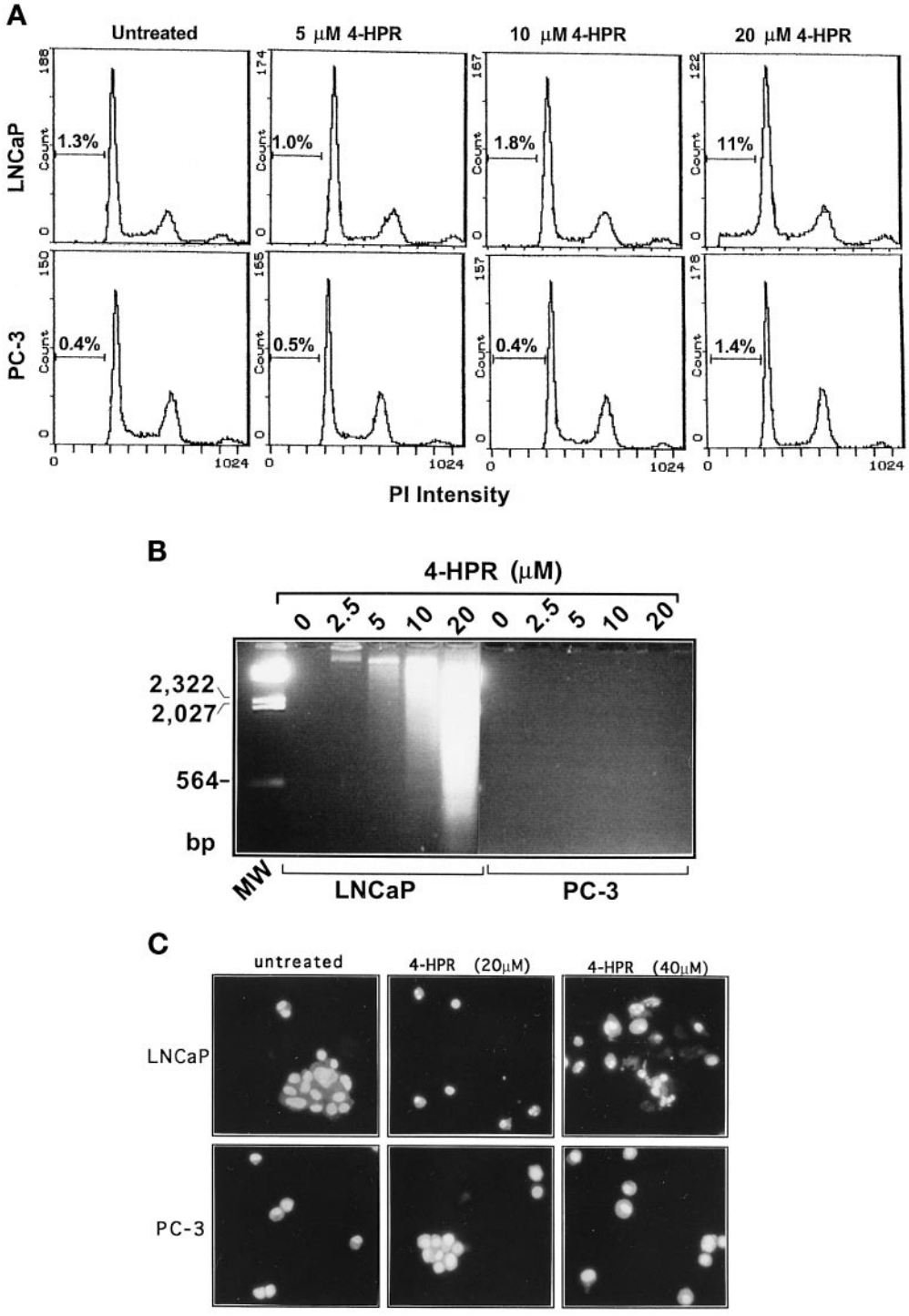


Fig. 2. 4-HPR induces apoptosis in LNCaP but not in PC-3 cells. LNCaP and PC-3 cells were treated with 4-HPR at the indicated concentrations. A, cells were harvested 36 h post-treatment, fixed, stained with propidium iodide (PI), and analyzed for DNA staining profiles as described in *Materials and Methods*. B, cells were harvested 36 h post-treatment and analyzed for DNA fragmentation as described in *Materials and Methods*. MW, λ-DNA/*Hin*dIII markers. C, LNCaP and PC-3 cells were harvested 24 and 48 h, respectively, after treatment. Harvested cells were fixed, stained, and examined for nuclear morphology.

described in figure legends. Cells were stained with X-gal 24 or 48 h after the transfections, and apoptosis induction was measured as described above. Two hundred transfected cells (blue color) were examined in every transfection.

Western Blot Assays. The cells were lysed as described above. The lysate was resolved by SDS-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with a primary antibody (anti-caspase 3, 1:200 dilution; anti-Bcl-2, anti-Bcl-X_L, and anti-GST, 1:1000; anti-HA, 1 μg/ml; anti-Flag, 5 μg/ml), washed, and blotted with a secondary antibody conjugated with horseradish peroxidase (1:1000 dilution). The membrane was then developed in the enhanced chemiluminescence reagent from Amersham (Piscataway, NJ) and exposed to an X-ray film.

Results

4-HPR Induces Apoptosis in LNCaP Cells But Not in PC-3 Cells. Two prostate carcinoma cell lines, LNCaP and PC-3, were treated with various concentrations of 4-HPR and were examined by trypan blue exclusion assays at different time points after treatment. 4-HPR had a strong suppressive effect on LNCaP cell growth. At concentrations >5 μM, 4-HPR effectively decreased the growth of LNCaP cells in 1-day cultures (Fig. 1A), and this inhibitory effect was more evident at higher concentrations of 4-HPR or at later time points (Fig. 1A). In contrast, PC-3 cells were more resistant to 4-HPR treatment because low concentrations of 4-HPR (1 and 5 μM) had no apparent effect on cell viability (Fig. 1B). 4-HPR (20 μM) effectively suppressed PC-3 cell growth after a 2-day incubation (Fig. 1B).

4-HPR induces apoptosis in several cancer cell lines, including breast cancer cells (Fanjul et al., 1996), leukemia cells (Delia et al., 1993), cervical carcinoma cells (Oridate et al., 1995), and neuroblastoma cells (Ponzoni et al., 1995). Decreases of cell viability in LNCaP and PC-3 cells after 4-HPR treatment (Fig. 1) suggest that 4-HPR may induce apoptosis in these two prostate carcinoma cells. Various assays were performed to examine the ability of 4-HPR to induce apoptosis in LNCaP and PC-3 cells. With flow cytometry, we detected apoptosis induction, as defined by the appearance of cells with a sub-G1 staining pattern, in LNCaP cells treated with 20 μM 4-HPR (Fig. 2A). No significant apoptosis was detected in PC-3 treated with various doses of 4-HPR (Fig. 2A). DNA fragmentation also was observed in 4-HPR-treated LNCaP cells in a dose-dependent manner, but not in 4-HPR-treated PC-3 cells (Fig. 2B). In addition, 4-HPR (20 μM) caused nuclear condensation and fragmentation in LNCaP cells (Fig. 2C), but it failed to do so in PC-3 cells (Fig. 2C).

Caspase 3 is an important mediator in the apoptotic pathway, and the cleavage of caspase 3 is a hallmark of apoptosis in various cell types. By Western blot analyses, we also observed the processing of caspase 3 in 4-HPR-treated LNCaP cells but not in PC-3 cells (Fig. 3, A and B). Collectively, our results indicate that LNCaP cells were more sensitive to 4-HPR-induced apoptosis than were PC-3 cells. Although 4-HPR was capable of suppressing PC-3 cell growth (Fig. 1B), it did not cause apparent apoptosis in PC-3 cells at concentrations tested.

4-HPR Induces JNK Activation in LNCaP Cells But Not in PC-3 Cells. Although 4-HPR effectively induces apoptosis in various cell types, the biochemical mechanism is unclear. Recently, the JNK kinase pathway was shown to play an important role in apoptosis signaling (Xia et al., 1995; Chen et al., 1996b, 1998; Verheij et al., 1996; Zanke et al., 1996). We decided to examine whether the JNK pathway participates in 4-HPR-induced apoptosis. In LNCaP cells treated with 4-HPR (20 μM), JNK activity was detected after the 6-h time point. The kinase activity plateaued at 14 h and remained elevated up to 36 h after treatment (Fig. 4, A and B). 4-HPR failed to induce JNK in PC-3 cells (Fig. 4A), as would be expected from the absence of apoptosis (Figs. 2 and 3). We did not observe significant p38-MAPK activation in either LNCaP (Fig. 4C) or PC-3 cells (data not shown) treated with 4-HPR. These results show an association between JNK activation and apoptosis induction in these two prostate carcinoma cells.

Caspases are important effector molecules in apoptotic process. To establish the molecular order between the JNK pathway and caspases, we examined JNK induction by 4-HPR in the presence or absence of a pan-caspase inhibitor, z-VAD-FK, which suppresses the activation of multiple caspases. The caspase inhibitor failed to suppress 4-HPR-induced JNK activation, although it blocked the processing of caspase 3 (Fig. 4B). In addition, JNK activation preceded the cleavage of caspase 3 in 4-HPR-treated LNCaP cells (Figs. 3A and 4A). These data suggest that JNK activation by 4-HPR is independent of caspase activation and, therefore, should be an initiating signal rather than an end effect of 4-HPR-induced apoptosis.

Radiation and 4-HPR Induce JNK Activation through Different Pathways. The differential induction of JNK and apoptosis by 4-HPR in LNCaP and PC-3 cells sug-

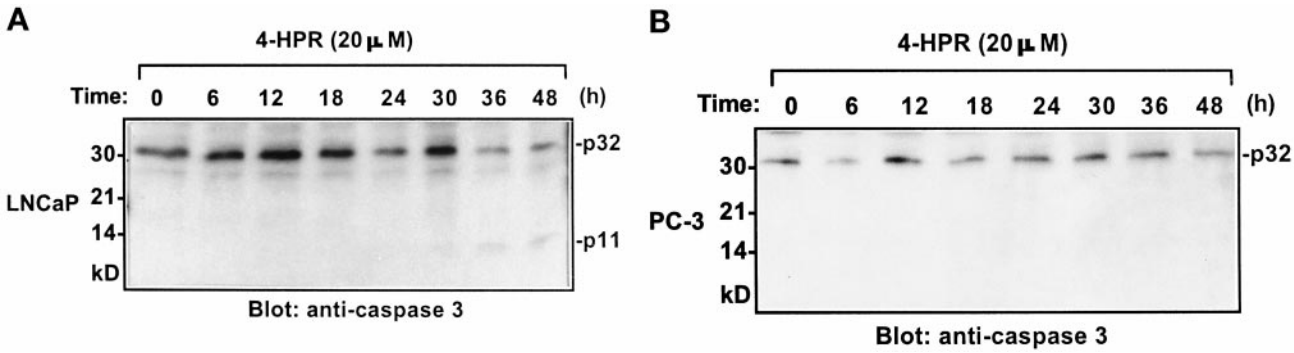


Fig. 3. 4-HPR induces cleavage of caspase 3 in LNCaP but not in PC-3 cells. LNCaP (A) and PC-3 cells (B) were treated with 4-HPR (20 μM) and harvested at the indicated time points; endogenous caspase 3 was examined by Western blotting with a specific antibody.

gests that the failure of 4-HPR to induce JNK activation and apoptosis in PC-3 cells may result from defects in apoptotic signaling. To determine whether the JNK pathway is functional in PC-3 cells, we used γ-radiation and UV-C, strong JNK and apoptosis inducers, to activate the JNK pathway. Both γ-radiation and UV-C induced JNK activation in PC-3 cells, although the activation was slower than that in LNCaP cells (Fig. 5, A and B). The radiation-induced apoptosis was detected by examination of morphological changes 24 to 48 h after irradiation (data not shown). This result shows that the JNK pathway in PC-3 cells is responsive to the radiation treatments. Therefore, the JNK pathway is functional in PC-3 cells, and the failure of these cells to respond to 4-HPR may be due to other defects in cellular signaling upstream of JNK.

We and others have shown that oxidative stress induces JNK activation in apoptosis induced by γ-radiation, anticancer agents, and growth factor withdrawal (Park et al., 1996; Chen et al., 1998). Therefore, we examined whether 4-HPR-induced JNK activation is mediated through oxidative stress. Cotreatment of LNCaP cells with 4-HPR and an antioxidant, *N*-acetyl-L-cysteine (NAC), did not block JNK activation by 4-HPR (Fig. 5C). In contrast, NAC completely suppressed γ-radiation-induced JNK activation. This result indicates that, unlike γ-radiation-induced JNK activation, the 4-HPR-induced JNK activation is not mediated through oxidative stress. Collectively, these data suggest that γ-radiation and 4-HPR use different pathways to induce JNK activation.

Expression of Bcl-2 Was Decreased in 4-HPR-Induced Apoptosis in LNCaP Cells. Expression of antiapoptotic molecules, such as Bcl-2 and Bcl-X_L, is associated with resistance to apoptotic stimuli (for review, see Adams and Cory, 1998). We examined the correlation between the expression of antiapoptotic molecules Bcl-2 and Bcl-X_L and the susceptibility to 4-HPR-induced apoptosis in LNCaP and PC-3 cells. These two cell lines expressed comparable levels of Bcl-X_L, however, Bcl-2 expression was slightly higher in

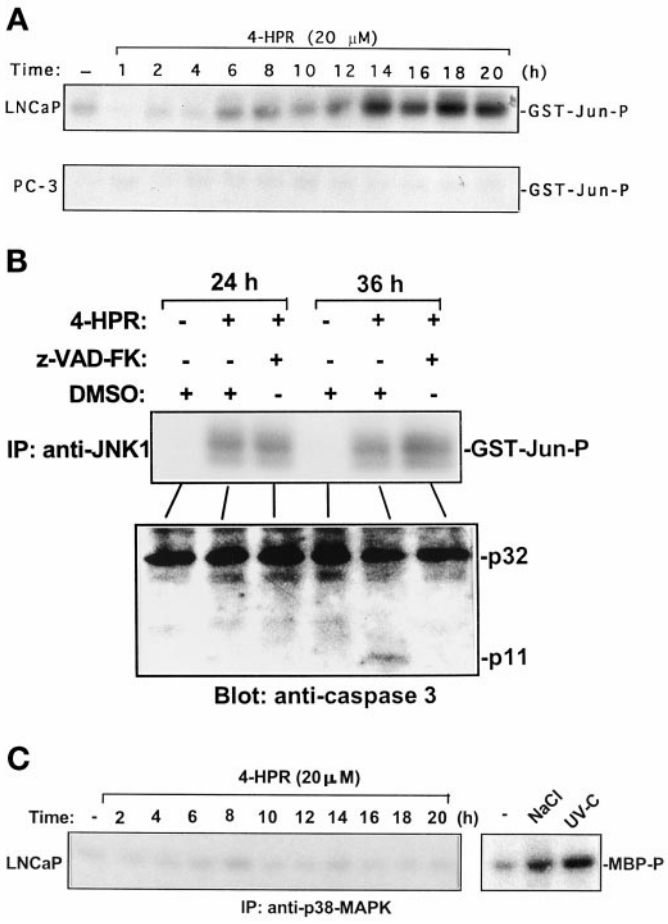


Fig. 4. 4-HPR induces persistent JNK activation in LNCaP but not in PC-3 cells. A, LNCaP and PC-3 cells were treated with 20 μM 4-HPR. The cells were collected at the indicated time points, and the endogenous JNK activity was determined by immunocomplex kinase assays. B, LNCaP cells treated with 20 μM 4-HPR in the presence or absence of the caspase inhibitor, z-VAD-FK, for 24 or 36 h. Endogenous JNK activity was examined by immunocomplex kinase assays, and endogenous caspase 3 was examined by Western blotting with a specific antibody. C, LNCaP cells were treated with 20 μM 4-HPR, osmotic shock (250 mM NaCl; 30 min), or UV-C (200 J/m^2). The cells were collected at the time points indicated and endogenous p38-MAPK activity was determined by immunocomplex kinase assays with myelin basic protein as a substrate. Activation of p38-MAPK by osmotic shock and UV-C served as controls for p38-MAPK assays.

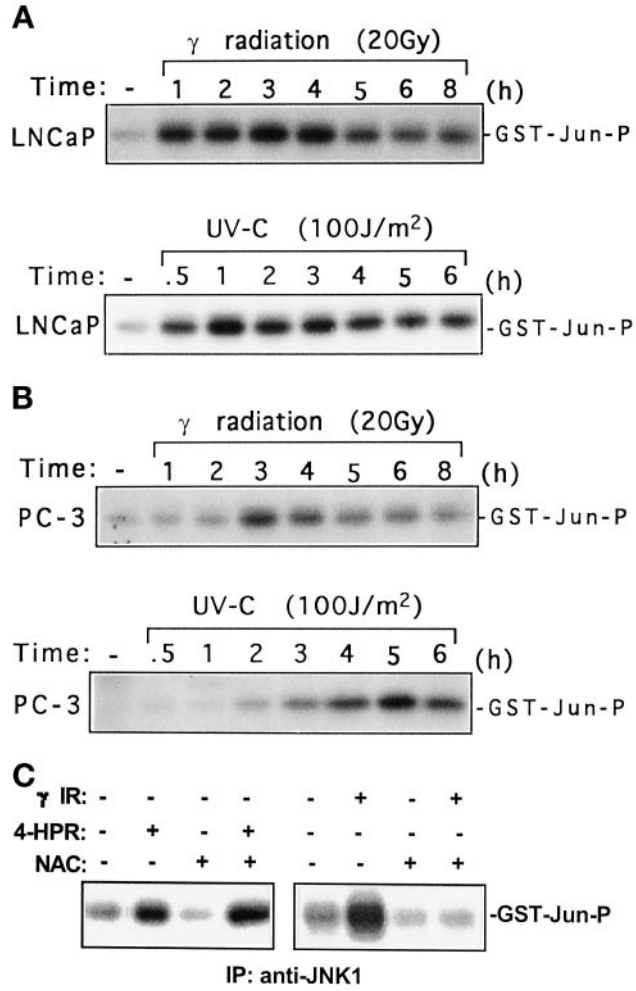


Fig. 5. 4-HPR and γ-radiation induce JNK activation through different mechanisms. LNCaP (A) and PC-3 cells (B) were treated with γ-radiation (20 Gy) or UV-C (100 J/m^2). The cells were harvested at the time points indicated and the endogenous JNK activity was determined by immunocomplex kinase assays as described in *Materials and Methods*. C, LNCaP cells were treated with 4-HPR (20 μM) or γ-radiation (20 Gy) in the presence or absence of NAC (20 mM). Cells were collected 1 h (γ-radiation) or 12 h (4-HPR) after treatment, and endogenous JNK activity was examined by immunocomplex kinase assays.

LNCaP cells than in PC-3 cells (Fig. 6A). Therefore, the difference in susceptibility to 4-HPR in LNCaP and PC-3 cells cannot simply be explained by the expression levels of Bcl-2 and Bcl-X_L. The expression of Bcl-2, but not Bcl-X_L, gradually decreased in LNCaP cells after 4-HPR treatment and became undetectable at the 24-h time point (Fig. 6B). This decrease in Bcl-2 expression occurred after the JNK activation and, therefore, is unlikely to be the cause of JNK induction. The expression levels of Bcl-2 and Bcl-X_L did not change significantly by 4-HPR treatment in PC-3 cells (Fig. 6B).

Activation of JNK Pathway Induces Apoptosis in Both LNCaP and PC-3 Cells. We also tested whether the apoptotic signaling downstream of JNK is intact in PC-3 cells. We transfected an empty vector, HA-tagged JNK1, or Flag-tagged kinase-dead JNK1 mutant (JNK1[APF]) plasmid into LNCaP and PC-3 cells, and studied the induction of apoptosis in the transfected cells. HA-JNK1 has been shown to be activated by forced expression in transfected cells (Yao et al., 1997). The liposome used in the tansfection caused a background of apoptosis (Fig. 7, A and B); however, we observed an increase in apoptosis in the wild-type JNK1-transfected cells compared with cells transfected with the control plasmid or JNK1[APF] (Fig. 7, A and B). These data indicate that both LNCaP and PC-3 cells can undergo apoptosis following induction of the JNK pathway, and that the apoptotic signaling downstream of JNK is functional in PC-3 cells. Because PC-3 is a $p53^{-/-}$ cell line, these results also suggest that p53 is not required for JNK-induced apoptosis.

Interference with JNK Pathway Suppresses 4-HPR-Induced Apoptosis. If JNK activation is required for 4-HPR-induced apoptosis, interference with the JNK pathway should suppress apoptosis induction by 4-HPR. LNCaP cells were treated with 4-HPR in the presence or absence of a chemical that inhibits p38-MAPK (SB202190; Lee et al., 1994) or JNK activation (curcumin; Chen and Tan, 1998). Cotreatment with curcumin suppressed 4-HPR-induced JNK activation and also decreased apoptosis induction (Fig. 8). As expected, because 4-HPR did not induce p38-MAPK activation in LNCaP cells (Fig. 4C), SB202190 failed to affect 4-HPR-induced apoptosis (Fig. 8). SB202190 also did not affect JNK activity at the concentrations tested.

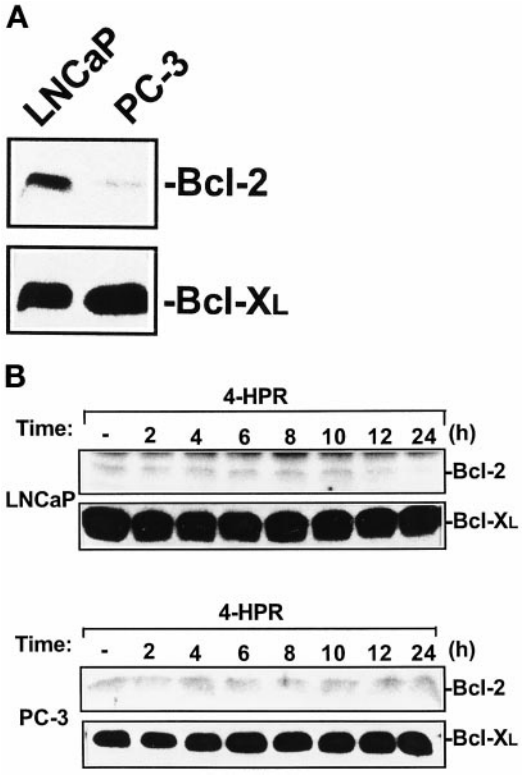


Fig. 6. Bcl-2 expression decreases after 4-HPR treatment in LNCaP cells. A, equal amounts of cell lysate (50 μg) from untreated LNCaP and PC-3 cells were subjected to Western blot analyses for expression of Bcl-2 (exposure time after enhanced chemiluminescence reaction, 5 min) and Bcl-X_L (exposure time, 1 min). B, LNCaP and PC-3 cells were treated with or without 4-HPR (20 μM). At the time points indicated, the cells were harvested and examined by Western blot assays for the expression levels of Bcl-2 (exposure times, 1 min in LNCaP and 3 min in PC-3 cells) and Bcl-X_L (exposure time, 1 min in both LNCaP and PC-3 cells).

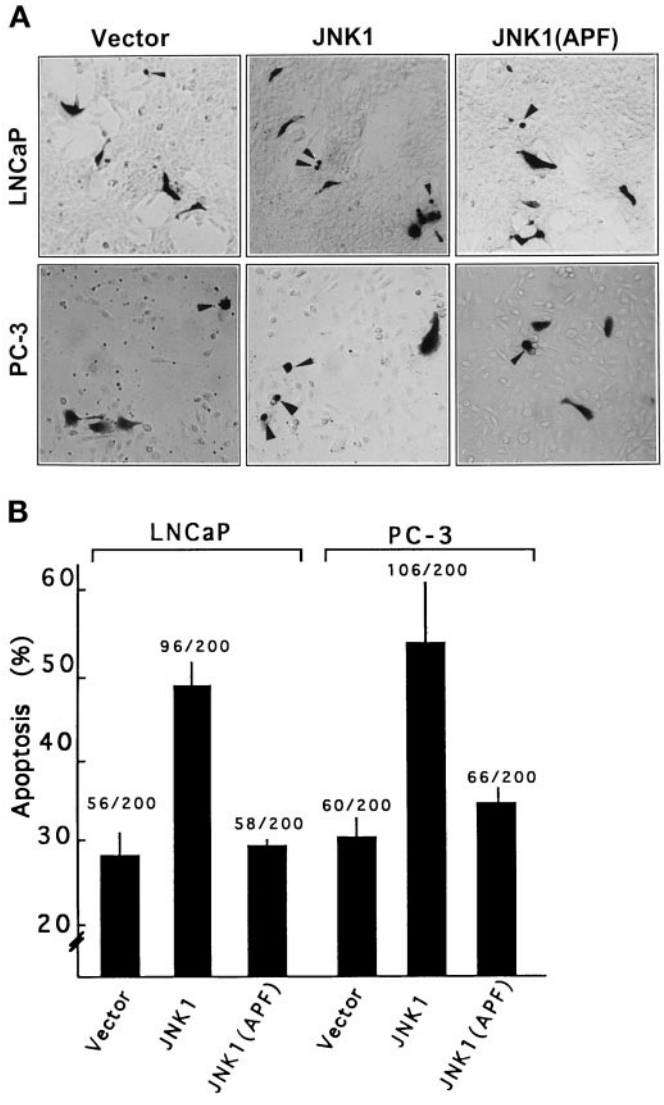


Fig. 7. Forced expression of JNK1 induces apoptosis in both LNCaP and PC-3 cells. LNCaP and PC-3 cells were transfected with plasmids encoding β-galactosidase (3 μg) in combination with the indicated plasmids [HA-JNK1, 2 μg; Flag-JNK1(APF), 2 μg]. The total amount of transfected DNA was normalized with empty vectors. A, cells were stained with X-gal either 24 (PC-3) or 48 h (LNCaP) after transfection. Transfected cells (dark color) with rounding up, shrinking, or membrane-blebbing morphology were identified as apoptotic cells (indicated by arrowheads). B, apoptosis induction was represented as percentage of apoptotic cells per 200 blue cells. Data represent the means ± S.D. of four experiments.

We have not been able to block 4-HPR-induced apoptosis in LNCaP cells by dominant-negative mutants of the JNK pathway in transient transfection-protection assays (data not shown). This was probably because the expression of the mutated kinases was not sufficient to suppress the endogenous kinases in LNCaP cells. HEK293 cells, which allow efficient expression of transfected genes, were used to examine the requirement of the JNK pathway in 4-HPR-induced apoptosis. HEK293 cells were sensitive to 4-HPR-induced JNK activation and apoptosis (Fig. 9, A–C). Interference with the JNK pathway by forced expression of a dominant-negative mutant of JNK1 [JNK(APF)] or SEK1 [SEK1(AL)] suppressed 4-HPR-induced apoptosis (Fig. 9, B and C). The mutated kinase SEK1(AL), which acts immediately upstream of JNK, blocked the activation of cotransfected HA-JNK1 by 4-HPR (Fig. 9D). Collectively, these results suggest that the JNK signaling pathway is important in 4-HPR-induced apoptosis.

Discussion

Our results suggest that the JNK pathway participates in 4-HPR-induced apoptosis. Apoptosis induced by 4-HPR was associated with sustained JNK activation. By forced expression of JNK1, we induced apoptosis in transfected LNCaP and PC-3 cells. Interference with the JNK pathway by dominant-negative kinase mutants suppressed 4-HPR-induced apoptosis in HEK293 cells, suggesting the requirement of the JNK pathway in 4-HPR-induced apoptosis in these cells. Curcumin, an inhibitor of JNK activation, blocked 4-HPR-induced apoptosis in LNCaP cells, suggesting the importance of the JNK signaling. However, the requirement of JNK in 4-HPR-induced apoptosis in LNCaP was not conclusively proved in this study.

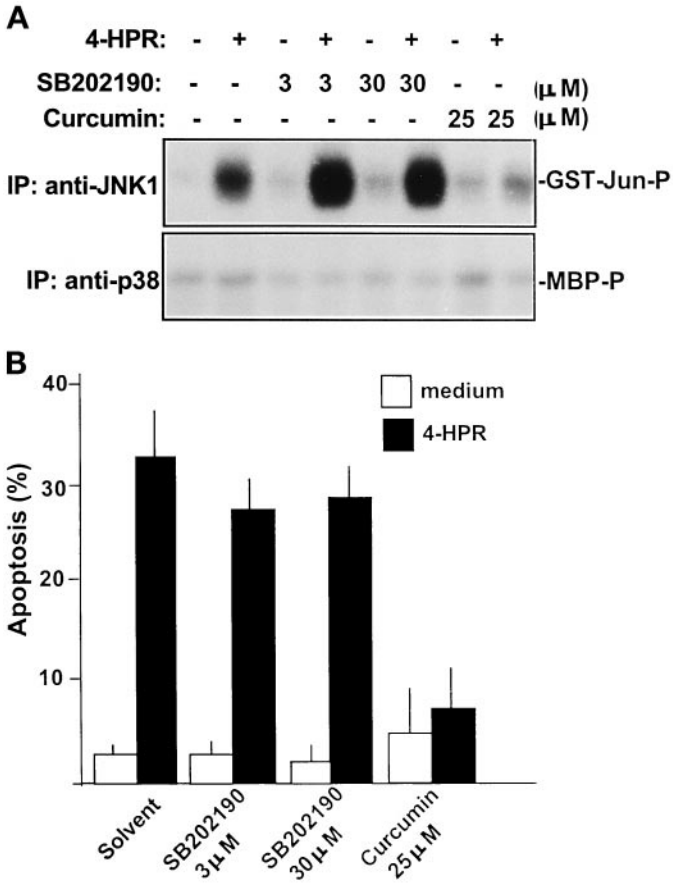


Fig. 8. Interference with the JNK pathway suppresses 4-HPR-induced apoptosis. LNCaP cells were treated with 4-HPR (20 μM) in the presence or absence of SB202190 (30 μM) or curcumin (25 μM). A, cells were collected at the 12-h time point, and endogenous JNK and p38-MAPK activities were examined by immunocomplex kinase assays. B, treated cells were collected at the 30-h time point for nuclear staining. Cells with condensed and fragmented nuclei were identified as apoptotic cells. The results presented are means ± S.D. of three independent experiments.

We found that LNCaP cells were more sensitive to 4-HPR-induced JNK activation and apoptosis than were PC-3 cells. Roberson et al. (1997) reported that 4-HPR is capable of inducing apoptosis in PC-3 cells through a transforming growth factor-β (TGF-β)-dependent pathway. We did observe a growth-inhibitory effect of 4-HPR on PC-3 cells (Fig. 1); however, no significant apoptosis was detected in 4-HPR-treated PC-3 cells by four criteria (flow cytometric analysis, DNA fragmentation, nuclear morphology, and cleavage of caspase 3; Figs. 2 and 3). It is noted that Roberson et al. (1997) used two assays (flow cytometric and DNA fragmentation assays) to detect apoptosis, and they did not compare PC-3 to LNCaP cells. Furthermore, this discrepancy may be due to variations between the different PC-3 lines used in these two studies. One possible variation is in the production of TGF-β or in various components of the TGF-β receptor-signaling pathway. TGF-β has been shown to cause sustained JNK activation (Atfi et al., 1997; Zhou et al., 1999). Whether TGF-β is required for 4-HPR-induced JNK activation is unknown. If that is the case, and TGF-β is required for 4-HPR-induced apoptosis in PC-3 cells as reported (Roberson et al., 1997), defects in TGF-β production or TGF-β receptor signaling may significantly suppress 4-HPR-induced JNK activation and apoptosis. The involvement of TGF-β and the JNK pathway in 4-HPR-induced apoptosis needs to be elucidated by further examination of different cell types. Nevertheless, our data clearly showed the difference in 4-HPR responsiveness between PC-3 and LNCaP cells.

Other genetic factors may result in the differential regulation of JNK and apoptosis by 4-HPR in LNCaP and PC-3 cells. PC-3, an androgen-insensitive, bone marrow-derived, metastasized tumor cell line, is a more progressive prostate carcinoma cell line than the lymph node-derived, androgen-sensitive LNCaP cells (Kaighn et al., 1979; Horoszewicz et al., 1983). Therefore, the failure of PC-3 cells to respond to 4-HPR-induced apoptosis may be due to some defects in the apoptotic-signaling pathway that are not present in LNCaP cells. In addition to androgen unresponsiveness, PC-3 cells have no p53 protein products due to deletions at both p53 alleles (Rubin et al., 1991; Planchon et al., 1995). In contrast, LNCaP cells have wild-type p53 genes. It has been shown that p53 is important for apoptosis induced by γ-radiation and by the adenovirus E1A protein (Debbas and White, 1993; Lowe et al., 1993). It is possible that the lack of p53 protein may contribute to the resistance of PC-3 to apoptosis induction. However, JNK can be activated by radiation in PC-3 cells, although with a slower activation kinetics, in the absence of functional p53 protein. This suggests that p53 is not required for JNK activation. Because JNK phosphorylates both murine and human p53 proteins in vitro (Milne et al., 1995; Alder et al., 1997), it has been suggested that p53 is a downstream effector of the JNK pathway. However, in this

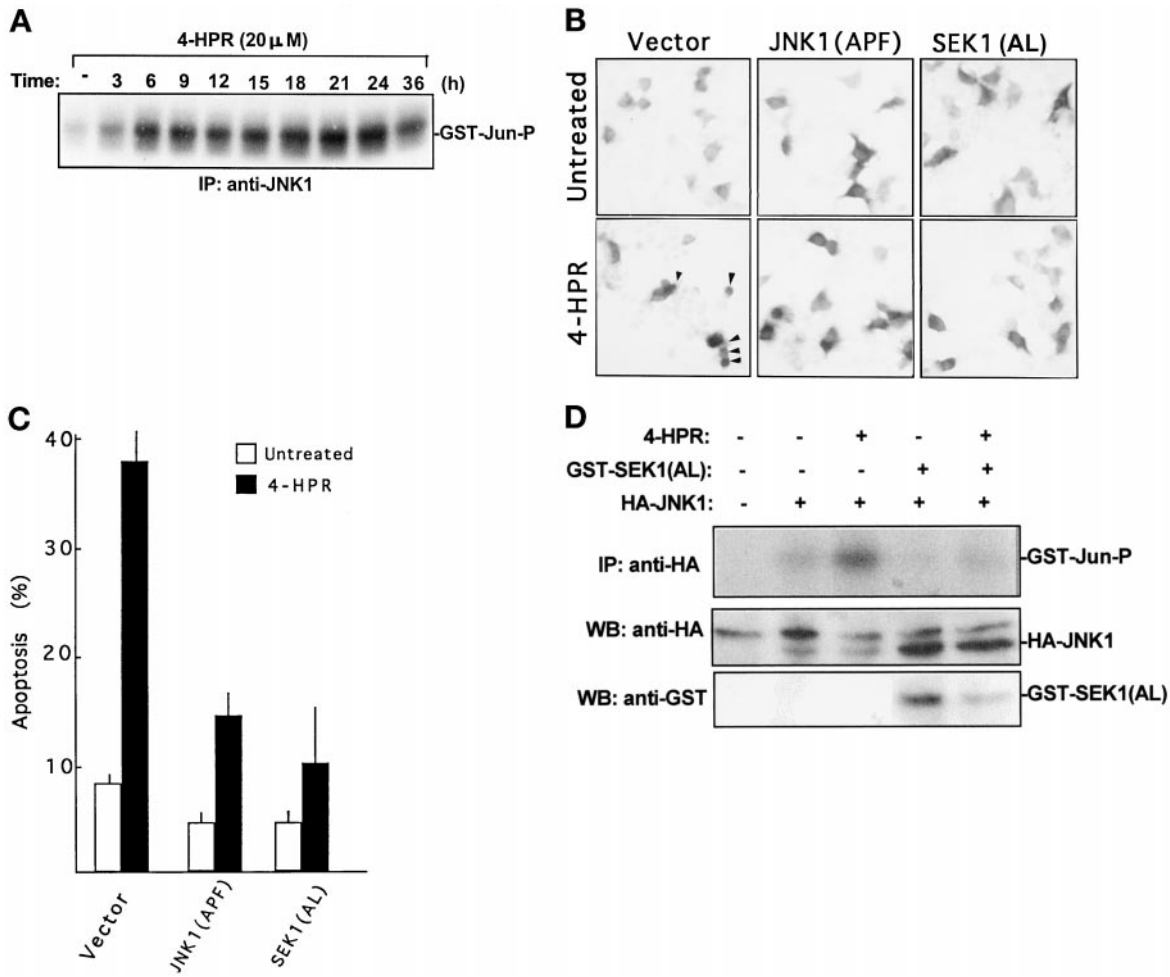


Fig. 9. Expression of dominant-negative mutants in the JNK pathway suppresses 4-HPR-induced apoptosis. A, HEK293 cells were treated with 20 μM 4-HPR; cells were collected at the time points indicated and examined for endogenous JNK activity. B, HEK293 cells were transfected with plasmids encoding β-galactosidase (1 μg) in combination with empty vector or the indicated plasmids encoding dominant-negative kinase mutants (2 μg of each). Transfected cells were cultured in complete medium for 6 h after removing the transfection mixture, treated with or without 4-HPR (20 μM) for 12 h, and then stained with X-gal. Transfected cells (dark color) with rounding up, shrinking, or membrane-blebbing morphology were identified as apoptotic cells (indicated by arrowheads). C, apoptosis induction was represented as percentage of apoptotic cells per 300 blue cells. D, HEK293 cells were transfected with HA-JNK1 plasmid (0.5 μg) plus empty vector (2 μg), or HA-JNK1 (0.5 μg) plus GST-SEK1(AL) (2 μg) plasmids. Cells were treated with or without 4-HPR (20 μM) before harvest. HA-JNK1 activity in the transfected cells was determined by immunocomplex kinase assays. The expressions of the transfected genes were determined by Western blot analyses.

article, we show that forced expression of JNK1 induced apoptosis in the $p53^{-/-}$ PC-3 cells, suggesting that p53 is not required for JNK-mediated apoptosis. Collectively, the data suggest that p53 is not essential for the activation of JNK, and that the p53 protein is not required for the JNK-induced apoptosis. However, this study does not exclude the possibility that p53 may synergize with the JNK pathway to induce apoptosis.

The ability of an antioxidant (NAC) to block γ-radiation-induced JNK activation, but not 4-HPR-induced JNK activation, indicates that these two agents induce JNK through distinct mechanisms. The activation of the JNK pathway by radiation but not by 4-HPR in PC-3 cells shows that genetic alterations in tumor cells may affect one but not other signaling pathways involved in the induction of JNK and apoptosis. Induction of apoptosis in PC-3 cells by forced expression of JNK1 suggests that we may be able to bypass the genetic defects in tumor cells that prevent apoptosis induction by activating JNK directly. Further examination of JNK-mediated apoptotic signaling will be important in the design of more effective cancer therapeutic agents.

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