The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival

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The mammalian response to stress is complex, often involving multiple signalling pathways that act in concert to influence cell fate. To examine potential interactions between the signalling cascades, we have focused on the effects of a model oxidant stress in a single cell type through an examination of the relative influences of mitogen-activated protein kinases (MAPKs) as well as two proposed apoptosis regulators, nuclear factor κB (NF-κB) and Bcl-2, in determining cell survival. Treatment of HeLa cells with H_2O_2 resulted in a time- and dose-dependent induction of apoptosis accompanied by sustained activation of all three MAPK subfamilies: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38. This H_2O_2-induced apoptosis was markedly enhanced when ERK2 activation was selectively inhibited by PD098059. Apoptosis decreased when JNK/SAPK activation was inhibited by expression of a dominant negative mutant form of SAPK/ERK kinase 1. Inhibition of the p38 kinase activity with p38-specific inhibitors SB202190 and SB203580 had no effect on cell survival. Because NF-κB activation by H_2O_2 is potentially related to both the ERK and JNK/SAPK signalling pathways, we examined the effects of inhibiting the activation of NF-κB; this interference had no effect on the cellular response to H_2O_2. Overexpression of the anti-apoptotic protein Bcl-2 significantly decreased the apoptosis seen after treatment with H_2O_2 without altering ERK or JNK/SAPK activities. Our results suggest that ERK and JNK/SAPK act in opposition to influence cell survival in response to oxidative stress, whereas neither p38 nor NF-κB affects the outcome. Bcl-2 acts independently and downstream of ERK and JNK/SAPK to enhance the survival of H_2O_2-treated cells.

INTRODUCTION

Oxidative stress constitutes a major threat to organisms living in an aerobic environment, and for humans it might have a causative role in many disease processes. Oxidants can trigger the activation of multiple signalling pathways that influence the cytotoxicity observed in affected cells, including the phosphorylation cascades leading to the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB).

MAPKs, which include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 subfamilies, are important regulatory proteins through which various extracellular signals are transduced into intracellular events [1–3]. They are responsible for the phosphorylation of a variety of proteins including downstream kinases and transcription factors. ERK, JNK/SAPK and p38 can all be activated by a variety of stimuli including growth factors, cytokines and different cellular stresses. However, the subfamilies are differentially affected by particular stimuli such that, in general, ERK shows greater activation than either JNK/SAPK or p38 in response to mitogenic stimulation, whereas JNK/SAPK and p38 are activated to a much greater extent by stressful stimuli [2,3]. High levels of JNK/SAPK activity have been correlated in many instances with the induction of apoptosis [4–10]. However, there are reports of situations in which JNK/SAPK activation occurs without influencing cell death [11,12], and still others in which JNK/SAPK activation actually promotes proliferation and/or cellular transformation [13–16]. In many conditions of stress leading to apoptosis, ERK activity is suppressed. This has led to the suggestion that the balance between JNK/SAPK and ERK activities is a key factor in regulating apoptosis [4]. Although p38 has been less well studied than JNK/SAPK or ERK, a limited number of reports have provided evidence indicating that p38 might have an active role in the induction of apoptosis [4,17–20]. Oxidative stress constitutes an interesting exception to the generalities described above, in that ERK is highly activated by treatment with oxidants such as H_2O_2 and butylated hydroxytoluene hydroperoxide [21,22] and, at least in NIH 3T3 mouse fibroblasts and rat PC12 phaeochromocytoma cells, this ERK activation seems to be important for long-term survival after treatment.

NF-κB is also presumed to have an important role in determining cell fate during stress. Although most published reports have provided evidence suggesting that it is anti-apoptotic, NF-κB has also been linked to the onset of apoptosis in certain cell types and conditions of stress [23,24]. A heterodimeric transcription factor, NF-κB leads to the transcriptional activation of numerous stress response genes [24]. Interestingly, both the ERK and the JNK/SAPK signalling pathways have been implicated in NF-κB activation through phosphorylation of its inhibitor IκB [25–28]. Although NF-κB is known to be activated after treatment with H_2O_2 [29], its role in influencing cell survival under such conditions has not been addressed.

Although MAPK signalling pathways and NF-κB activation have both been implicated in regulating the cellular response to stress, there remains conflicting evidence regarding the import-

Abbreviations used: DAPI, 4′,6′-diamidino-2-phenylindol; DTT, dithiothreitol; ERK, extracellular signal-regulated protein kinase; GST, glutathione S-transferase; HA, haemagglutinin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2; MBP, myelin basic protein; MEK, MAPK/ERK kinase; MEKK1, MAPK kinase kinase 1; NF-κB, nuclear factor κB; SAPK, stress-activated protein kinase; SEK1, SAPK/ERK kinase 1.

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ance of certain mediators from one study to another as highlighted above. This confusion could arise from a number of variables, including differences in the cell type or stress agent. Furthermore, because most studies have looked at a given pathway in isolation, the potential interactions between pathways have often not been addressed. In the present study we sought to investigate systematically the relative contributions of ERK, JNK/SAPK and p38 MAPK as well as NF-κB in influencing cell survival during treatment with a model oxidant (H_2O_2) in a single cell type (HeLa). We provide evidence that H_2O_2-induced apoptosis of HeLa cells is potentiated by inhibition of ERK and decreased by inhibition of JNK/SAPK. Neither p38 activation nor NF-κB activation seems to influence the response. Bcl-2 overexpression protects cells against apoptosis without altering the activity of either ERK or JNK/SAPK, suggesting that it acts independently and downstream of these kinases to regulate H_2O_2-induced cell death.

MATERIALS AND METHODS

Reagents and plasmids

PD098059, SB203580 and SB202190 were purchased from Calbiochem (La Jolla, CA, U.S.A.). They were dissolved in DMSO and stored as 10 mM stock solutions. H_2O_2 and myelin basic protein (MBP) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant human Hsp27 protein was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). The anti-ERK2, JNK, NF-κB, IκBα and Bcl-2 polyclonal antibodies, anti-(glutathione S-transferase) (anti-GST) monoclonal antibody and NF-κB consensus oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-haemagglutinin (anti-HA) monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The rabbit anti-p38 serum was a gift from Dr. John Lee. The GST–c-Jun(1–135) construct was provided by Dr. James Woodgett. Vectors expressing wild-type and mutant SAPK/ERK kinase 1 (SEK1) [SEK1wt and SEK1(K-R) respectively] were provided by Dr. John Kyriakis [30]. The rabbit polyclonal anti-(MAPK-activated protein kinase 2) (anti-MAPKAPK2) antibody was a gift from Dr. Jacques Huot. The pCMX vector was obtained from Dr. Tony Hunter, the pCMX-IkBαM from Dr. Inder Verma, and the pSFFV-neo and Bcl-2 expression vectors from Dr. Gabriel Nunez. The purified GST and GST–Bcl-2 proteins were provided by Dr. Lawrence Fritz.

Cell culture and transfections

HeLa (human cervical carcinoma) cells were maintained in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Life Technologies, Gaithersburg, MD, U.S.A.) and were cultured at 37 °C in an air/CO_2 (19:1) atmosphere. Cells (4×10^5) were plated in 60 mm dishes 14 h before H_2O_2 treatment. All H_2O_2 treatments were performed in medium containing serum. Although this necessitates the use of higher H_2O_2 concentrations to induce apoptosis and activate MAPK than under serum-free conditions, it avoids the introduction of additional complexities into the model system due to the stress of serum starvation.

Transfections were performed by precipitation with calcium phosphate [31]. For the generation of stable lines, transfected cells were selected under 1 mg/ml G418 (Life Technologies) for 3 weeks. Expression of the foreign proteins was verified by Western blot analysis.

Staining with 4′,6′-diamidino-2-phenylindol (DAPI) and analysis by FACS for apoptosis

Nuclear staining with DAPI (Sigma Chemical Co.) was performed as described previously [32]. In brief, cells were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature and permeated with ice-cold ethanol. The fixed cells were stained with DAPI (1 μg/ml) for 30 min and washed, then examined by fluorescence microscopy. Apoptotic cells were scored on the basis of the presence of highly condensed or fragmented nuclei. Although a small fraction of the total cells did become detached after treatment with H_2O_2, only cells that remained attached to the plates were analysed. For FACS analysis, 10^6 cells were fixed in 70% (v/v) ethanol at 4 °C for 24 h, after which they were washed twice with PBS, resuspended in 0.9 ml of PBS and incubated with RNase A for 30 min at 37 °C. Propidium iodide was added to the cells; they were then subjected to FACScan analysis. The presence of a sub-G_1 compartment of cells was indicative of apoptosis [33].

Western blot analysis

Total cell proteins were extracted from cell monolayers, and 25 μg of protein lysate was size-separated by SDS/PAGE [12% (w/v) gel]. After electrophoresis, proteins were transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.). After incubation of the membranes with appropriate antibodies, specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, U.S.A.).

Immunoprecipitations and kinase assays

After treatment with H_2O_2 for the indicated times, cells were ruptured in 0.5 ml of lysis buffer [20 mM Hepes (pH 7.4)/2 mM EGTA/50 mM β-glycerophosphate/1 mM Na_3VO_4/5 mM NaF/1% (v/v) Triton X-100/10% (v/v) glycerol/1 mM dithiothreitol (DTT)/1 mM PMSF/10 μg/ml leupeptin/10 μg/ml aprotinin] for 30 min on ice. Cell debris was removed by centrifugation at 10000 ***g*** for 10 min at 4 °C. JNK/SAPK, ERK and p38 were then immunoprecipitated by the addition of 1 μg of the appropriate antibody to the cell lysates for 3 h, with the addition of 40 μl of 50% slurry Protein A–Sepharose during the final hour. The beads were pelleted by centrifugation and then washed three times each in lysis buffer and wash buffer [500 mM LiCl/100 mM Tris/HCl (pH 7.6)/0.1% (v/v) Triton X-100/1 mM DTT]. The beads were left as a 1:1 suspension in assay buffer and 20 μl (0.3 mg/ml) of either GST–c-Jun (for the JNK/SAPK assay) or MBP (for the ERK2 and p38 assays) was added. Kinase reactions were initiated by the addition of 15 μl of γ-^{32}P-labelled Mg^{2+}/ATP solution {50 mM $MgCl_2$/500 μM ATP/10 μCi of [γ-^{32}P]ATP} and performed at 30 °C for 20 min. Reactions were stopped by the addition of Laemmli sample buffer and boiling for 5 min. Samples were separated by SDS/PAGE [12% (w/v) gel], and after drying were subjected to autoradiography. Quantification was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). For the MAPKAPK2 kinase assay, the enzyme was immunoprecipitated from lysates with anti-MAPKAPK2 antibody. The

immunoprecipitates were washed as for the p38 and JNK/SAPK assays, and kinase activity was assayed with recombinant Hsp27 as substrate [34].

Nuclear extract preparation and gel shift analysis

Nuclear extracts were prepared essentially as described [35] with the addition of 20% (v/v) glycerol to buffer C [20 mM Hepes (pH 7.9)/0.4 M NaCl/1 mM EDTA/1 mM DTT/1 mM PMSF]. In brief, HeLa cells were treated with H_2O_2 at the concentrations and for the times indicated in the Figure legends. At the time of harvesting, cells were washed twice with ice-cold PBS, scraped from the plates and suspended in 400 μl of cold buffer A [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/1 mM DTT/1 mM PMSF]. The cells were left to swell on ice for 15 min, then 25 μl of 10% (v/v) Nonidet P40 was added. The mixture was vortex-mixed for 10 s and incubated for 15 min on ice. Nuclei were collected with a 30 s spin, washed with cold buffer A and collected again. The nuclear pellet was resuspended in ice-cold buffer C, then rocked vigorously at 4 °C for 15 min followed by three cycles of freezing (−80 °C) and thawing (on ice). The nuclear extract was clarified by centrifugation at 12000 ***g*** for 5 min at 4 °C; the resulting supernatant was frozen at −80 °C.

For gel shift assays, nuclear extracts (4 μg of protein) were incubated in a 25 μl reaction mixture containing 10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 1 μg of BSA and 2 μg of poly(dI-dC). The double-stranded ^{32}P-labelled oligonucleotide corresponding to an NF-κB consensus binding site (AGTTGAGGGGACTTTCCCAGGC, positive strand) was added to the reaction mixture and incubated at 25 °C for 30 min. Then 2 μl of loading dye [50% (v/v) glycerol/0.25% Bromophenol Blue] was added and the samples were subjected to non-denaturing PAGE in 4% gels. The gels were dried and analysed with a PhosphorImager.

Statistical analyses

A paired Student's *t* test was used to assess differences between H_2O_2-treated and control groups.

A P value of < 0.05 was considered significant.

RESULTS

Treatment with H_2O_2 induces apoptosis and activates MAPK pathways in HeLa cells

The cytotoxic effects of H_2O_2 were evaluated 24 h after the treatment of HeLa cells with concentrations ranging from 200 to 1000 μM. Although little cytotoxicity was evident at concentrations below 300 μM, there was a dose-dependent decrease in cell number with higher concentrations, such that 600 μM H_2O_2 resulted in more than 50% loss in cell viability as assessed by Trypan Blue dye exclusion (results not shown). H_2O_2-treated cells exhibited morphological alterations consistent with apoptosis including shrinkage and membrane blebbing, and DAPI-stained cells showed evidence of nuclear condensation and fragmentation (Figure 1A). These hallmarks of apoptosis were not evident in control (untreated) cells, which showed homogeneous nuclear staining. That H_2O_2-treated cells died via apoptosis was further confirmed by FACS analysis, where a sub-G_1 peak was evident in treated cells but absent from control populations (Figure 1B).

The numbers of apoptotic cells present in control and 600 μM H_2O_2-treated cultures at various times after addition of the oxidant were quantified by nuclear staining with DAPI. Whereas less than 1% of untreated cells showed signs of apoptosis at any of the time points examined (results not shown), H_2O_2 treatment resulted in a time-dependent increase in numbers of apoptotic cells (Figure 1C). Some degree of apoptosis was evident as early as 6 h after treatment, and a significant percentage of apoptotic cells was observed by 12 h. At 24 h after treatment, more than 50% of remaining cells showed signs of apoptosis.

Because various members of the MAPK family have been implicated in influencing cell survival in response to stress, we examined the activation of ERK2, JNK1/SAPK1 and p38α isoforms of the respective subfamilies in HeLa cells treated with 600 μM H_2O_2. Immunocomplex kinase assays were used to assess the activities of each of these kinases. The anti-ERK2 and anti-p38α antibodies display cross-reactivity with ERK 1 and other p38 isoforms respectively, and thus probably reflect levels of total ERK and p38 complexes. The JNK1 antibody is highly selective for JNK1 and therefore our analysis is restricted to this isoform. As shown in Figure 2, activities of all three kinases increased after treatment. Maximum activation of both ERK2 and p38α occurred within 1 h of the addition of H_2O_2, but maximum activation of JNK1/SAPK1 occurred after 3 h of treatment. Although ERK2 activity decreased at later time points, it remained above levels seen in the absence of treatment. In contrast, JNK1/SAPK1 and p38α kinase activity remained at maximal levels throughout the entire 12 h period.

ERK activation promotes the survival of H_2O_2-treated cells

Previous studies from our laboratory suggested that activation of ERK was an important factor in determining the survival of PC12 and NIH3T3 after treatment with H_2O_2 [21]. Therefore we investigated the influence of ERK activation on H_2O_2-induced apoptosis in HeLa cells employing the inhibitor of MAPK/ERK kinase (MEK) activation, PD098059 [36]. MEK lies immediately upstream of ERK in the signalling cascade and is responsible for its phosphorylation and activation. As shown in Figure 3(A), pretreatment of HeLa cells with 20 μM PD098059 for 1 h before the addition of 600 μM H_2O_2 significantly inhibited ERK activation. An assessment of apoptosis in similarly treated cells revealed that the addition of the MEK inhibitor before treatment of cells with H_2O_2 led to a significant increase in apoptosis (Figure 3B). These findings are consistent with our previous studies implicating early ERK activation as a factor for surviving oxidant injury [21,22].

Sustained JNK/SAPK activation is required for the induction of apoptosis after treatment with H_2O_2

Because JNK/SAPK has been implicated in mediating apoptosis in response to stressful stimuli, we sought to examine whether JNK/SAPK activation was linked to apoptosis of H_2O_2-treated HeLa cells. For this purpose we transfected HeLa cells with plasmids expressing either wild-type SEK1 or a dominant-negative SEK1 mutant [SEK1(K-R)], the upstream kinase responsible for the activation of JNK/SAPK [30]. Control cells received the empty pCDNA3 host vector. Stable transfectants expressing either GST-tagged wild-type or mutant SEK1 were identified by Western blot analysis with an anti-GST antibody (Figure 4A) and then analysed for their response to treatment with H_2O_2. Overexpression of wild-type SEK1 had no significant effect on the magnitude of JNK/SAPK activation after treatment with H_2O_2, indicating that the endogenous SEK1 was sufficient to activate JNK/SAPK maximally in a dose-dependent fashion (Figure 4B). However, the SEK1 wild-type transfectants did exhibit a delay in the attenuation of JNK/SAPK activity

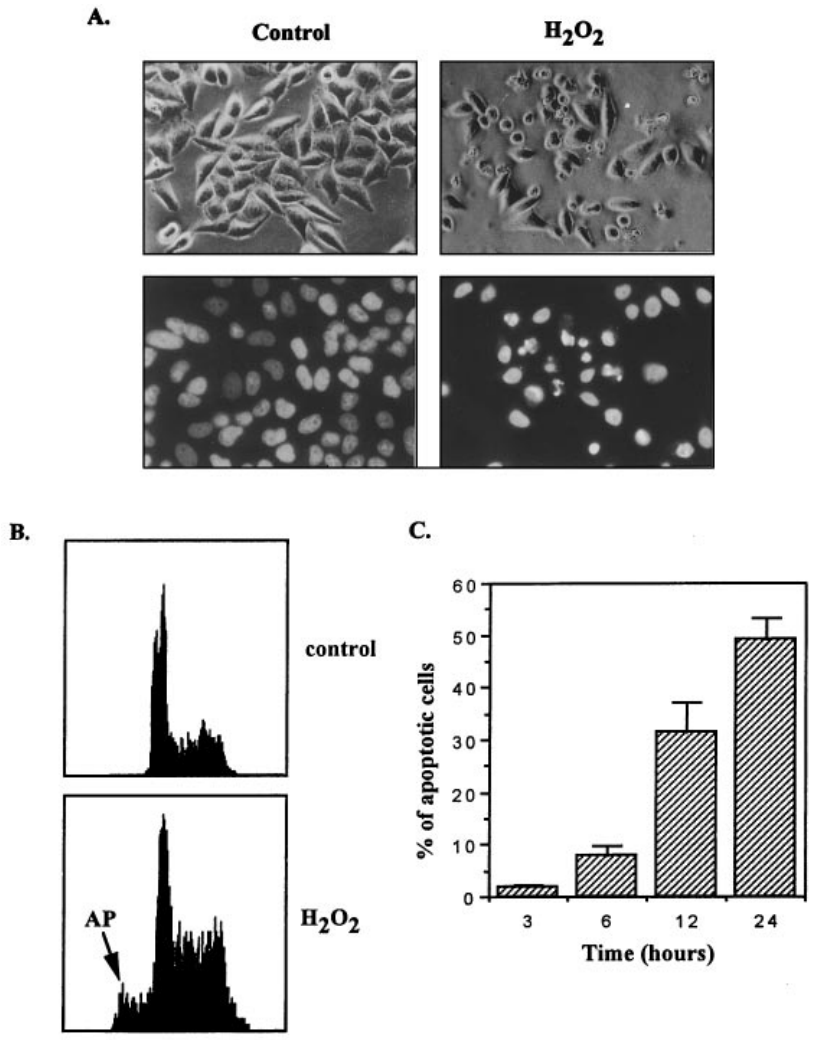

Figure 1 H_2O_2 induces apoptosis of HeLa cells

(**A**) Upper panels: phase-contrast micrographs showing the morphology of control and H_2O_2-treated HeLa cells. Cells were examined 24 h after treatment with 600 μM H_2O_2. Lower panels: DAPI staining showing nuclear condensation and fragmentation in H_2O_2-treated cells. Cells were fixed and stained 24 h after exposure to H_2O_2. (**B**) FACS analysis of cells 12 h after treatment with H_2O_2. AP denotes the presence of a sub-G_1 peak, indicative of apoptotic cells in H_2O_2-treated, but not in control, populations. (**C**) Quantification of apoptotic cells at various times after treatment with H_2O_2. Cells treated with 600 μM H_2O_2 were stained with DAPI to identify apoptotic cells. Cells exhibiting condensed and fragmented nuclei were scored. Values are means $\pm$ S.D. for three separate determinations.

compared with the vector control cells treated similarly with 600 μM H_2O_2 (Figure 4C). Overexpression of the dominant-negative mutant SEK1(K-R), in contrast, resulted in a marked shift in the dose–response relationship for JNK/SAPK activation by H_2O_2, almost completely abolishing JNK/SAPK activation at the 3 h time point at concentrations below 500 μM (Figure 4B). In addition, the SEK1(K-R) expression significantly decreased the duration of JNK/SAPK activation in response to treatment with 600 μM H_2O_2 (Figure 4C). To determine whether this decreased JNK/SAPK activity was correlated with altered cell survival, the percentage of apoptotic cells was assessed by DAPI staining after treatment of the wild-type and mutant SEK1-expressing cells with 600 μM H_2O_2. As shown in Table 1, the SEK1(K-R)-expressing cells displayed a marked decrease in number of apoptotic cells, whereas the wild-type SEK1-overexpressing cells were slightly more sensitive than the control cells to the H_2O_2. These findings are consistent with the view that JNK/SAPK activation is an important event in the induction of apoptosis by H_2O_2.

H_2O_2-induced apoptosis is not dependent on p38

To ascertain whether activation of p38 MAPK influenced survival of HeLa cells after treatment with H_2O_2, the pharmacological inhibitors SB202190 and SB203580 were utilized. At low concentrations (below 10 μM), these agents have been shown to act as specific inhibitors of the activity of p38 without affecting the activities of either ERK or JNK/SAPK [37]. HeLa cells were pretreated for 1 h with various concentrations of the inhibitors before treatment with 600 μM H_2O_2. Cells were harvested 1 h later and assessed for inhibition of p38 by measuring the activity of a downstream target of p38, MAPKAPK2 [38]. The MAPKAPK2 activity present in cell lysates was determined with an

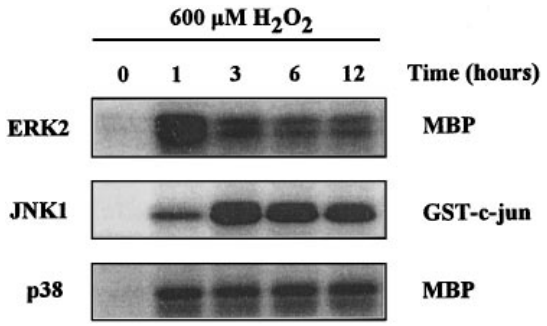

Figure 2 MAP kinase activation after treatment with H_2O_2

Time course of ERK2, JNK1 and p38 MAPK activation in HeLa cells treated with 600 μM H_2O_2 for the indicated times. Polyclonal anti-ERK2, anti-JNK1 and anti-p38 antibodies were used for immunoprecipitation of the respective kinases from cell lysates. Kinase activity was assessed by immunocomplex kinase assays with MBP (for ERK2 and p38) or GST–c-Jun (for JNK1) as substrates.

immunocomplex kinase assay employing recombinant Hsp27 protein as a substrate. Both p38 inhibitors acted in a dose-dependent manner to decrease MAPKAPK2 activity in H_2O_2-treated cells to levels below those of control populations (Figure 5A): 1 μM was sufficient to abolish MAPKAPK2 activation completely. However, as shown in Figure 5(B), these agents did not alter the number of apoptotic cells seen after treatment with the oxidant. Thus p38 activation does not seem to influence cell survival in response to treatment with H_2O_2.

Activation of NF-κB and its influence on survival of H_2O_2-treated cells

Recent studies have demonstrated that activation of the transcription factor NF-κB has an essential role in protecting cells from apoptosis mediated by a variety of stressful stimuli, including tumour necrosis factor α, γ-irradiation and certain chemotherapeutic agents [23,24]. In such instances, NF-κB activation occurs through phosphorylation and subsequent degradation of the NF-κB inhibitor IκB. This results in the accumulation of free NF-κB, which can then translocate to the nucleus and interact with DNA. Interestingly, recent studies have implicated both the ERK-regulated kinase $p90^{RSK}$ and a kinase in the JNK/SAPK phosphorylation cascade as mediators of IκB phosphorylation [25–27,39]. Because NF-κB is known to be activated after H_2O_2 treatment of HeLa cells [29], we examined whether such activation influenced HeLa cell survival. NF-κB activation was assessed by its ability to bind to DNA by using gel mobility-shift assays with an oligonucleotide containing a consensus NF-κB-binding element. As shown in Figure 6, NF-κB protein levels did not change but NF-κB DNA binding activity in crude nuclear extracts increased after treatment with H_2O_2. Competition assays utilizing excess unlabelled oligonucleotides confirmed that the binding activity was specific (results not shown). This NF-κB activation was associated with a loss of the inhibitor IκBα protein (presumably owing to its degradation). To determine the importance of NF-κB activation in the regulation of H_2O_2-induced apoptosis, we transfected HeLa cells with a plasmid expressing a dominant-negative form of IκBα protein (IκBαM) that is resistant to phosphorylation and degradation and therefore acts to inhibit NF-κB activation [40]. Protein immunoblotting demonstrated that the mutated protein IκBαM, which migrates differently from the endogenous IκBα, was indeed expressed in the transfectants (Figure 7A). Although expression

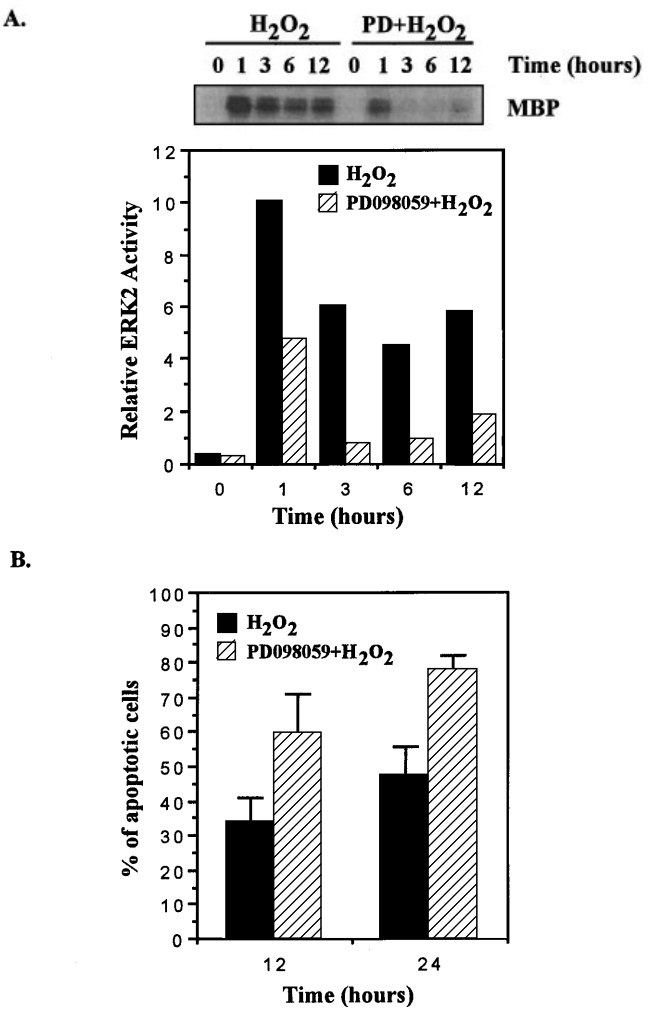

Figure 3 Pretreatment of HeLa cells with the MEK1 inhibitor PD098059 enhances H_2O_2-induced apoptosis

(**A**) PD098059 inhibits ERK activation in H_2O_2-treated cells. HeLa cells were pretreated with 20 μM PD098059 for 1 h before the addition of 600 μM H_2O_2. Cells were lysed at different times after treatment, and ERK2 activity in cell lysates was determined by an immunocomplex kinase assay with 6 μg of MBP as substrate. (**B**) PD098059 pretreatment enhances H_2O_2-induced apoptosis. Apoptosis was assessed as described for Figure 1 after staining with DAPI. Values are means $\pm$ S.D. for three independently performed experiments. $P < 0.05$ comparing the H_2O_2-treated and H_2O_2/PD098059-treated groups at both time points.

of the mutant IκBα diminished the NF-κB DNA-binding activity in response to H_2O_2 (Figure 7B), this inhibition of NF-κB activation was not associated with altered survival of H_2O_2-treated cells (Figure 7C).

Bcl-2 protects against apoptosis without altering JNK/SAPK activity

Numerous studies have provided evidence that Bcl-2 and the related protein Bcl-x_L can prevent apoptosis induced by a variety of stimuli [41,42]. Western blot analysis of HeLa cell lysates exhibited no change in the endogenous levels of either Bcl-2 or Bcl-x_L expression after treatment with H_2O_2 (results not shown). Expression of Bax, a third member of the Bcl-2 family, which is pro-apoptotic and acts to antagonize the protective influence of Bcl-2, was also unchanged after treatment with H_2O_2 (results not

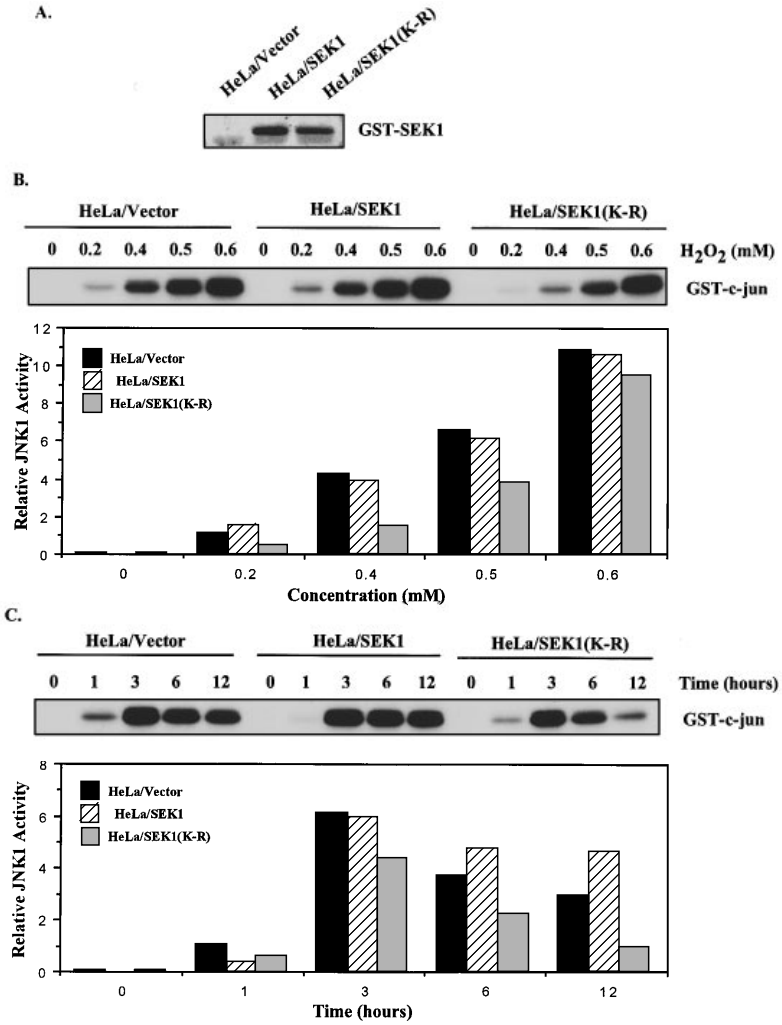

Figure 4 Expression of dominant-negative mutant SEK1(K-R) inhibits JNK/SAPK activation by H_2O_2

(**A**) Western blot analysis of wild-type SEK1 and SEK1(K-R) protein expression in stably transfected HeLa cells. An anti-GST antibody was used to detect the expression of the GST–SEK1 and GST–SEK1(K-R) fusion proteins. (**B**) Dose–response relations for the activation of JNK/SAPK in control cells and in SEK1- and SEK1(K-R)-expressing cells. Cells were assayed for JNK/SAPK activity 3 h after treatment. (**C**) Kinetics of JNK/SAPK activation and attenuation in control cells and in SEK1- and SEK1(K-R)-expressing cells treated with 600 μM H_2O_2.

Table 1 SEK1(K-R)-expressing HeLa cells show decreased apoptosis in response to treatment with H_2O_2

Control cells (vector) and cells expressing wild-type SEK1 and mutant SEK1(K-R) were treated with 600 μM H_2O_2, then stained with DAPI 12 or 24 h later for quantification of apoptosis. Values are means $\pm$ S.D. for three separate determinations. $^*P < 0.05$ compared with HeLa/vector controls.

	Apoptotic cells (% of total)	
Cells	12 h	24 h
HeLa/vector	34.4 $\pm$ 4.6	53.2 $\pm$ 4.8
HeLa/SEK1	41.3 $\pm$ 2.8	66.4 $\pm$ 1.7*
HeLa/SEK1(K-R)	11.2 $\pm$ 2.5*	18.7 $\pm$ 2.9*

shown). To address whether overexpression of exogenous Bcl-2 could influence H_2O_2-mediated cell death in HeLa cells, cells were stably transfected with a plasmid that constitutively produces high levels of Bcl-2. The stable transfectants strongly expressed Bcl-2 protein (Figure 8A); this Bcl-2 overexpression greatly suppressed H_2O_2-induced apoptosis (Figure 8B), consistent with its known anti-apoptotic functions. Although the mechanism by which Bcl-2 exerts this protective effect has not been elucidated, several studies have suggested that it might act through inhibition of the JNK/SAPK signalling pathway [9,43,44]. Given this fact, and our observation that alterations in JNK/SAPK activity affect the level of apoptosis seen in H_2O_2-treated cells, we examined whether Bcl-2 overexpression could modulate the level of JNK/SAPK activation seen after treatment with H_2O_2. Despite the significant inhibition of apoptosis seen in the Bcl-2-overexpressing cells, JNK/SAPK activity did not differ significantly from that seen in cells stably transfected with the empty expression vector pSSFV-neo (Figure 8C). The level of ERK activation seen after treatment with H_2O_2 was also not affected by Bcl-2 overexpression (results not shown).

These findings seem to contradict recent reports suggesting that Bcl-2 might act to inhibit JNK/SAPK signalling; we therefore undertook two additional approaches to investigate this issue further. In the first approach, we transiently transfected HeLa cells with a plasmid expressing HA-tagged JNK1 with a

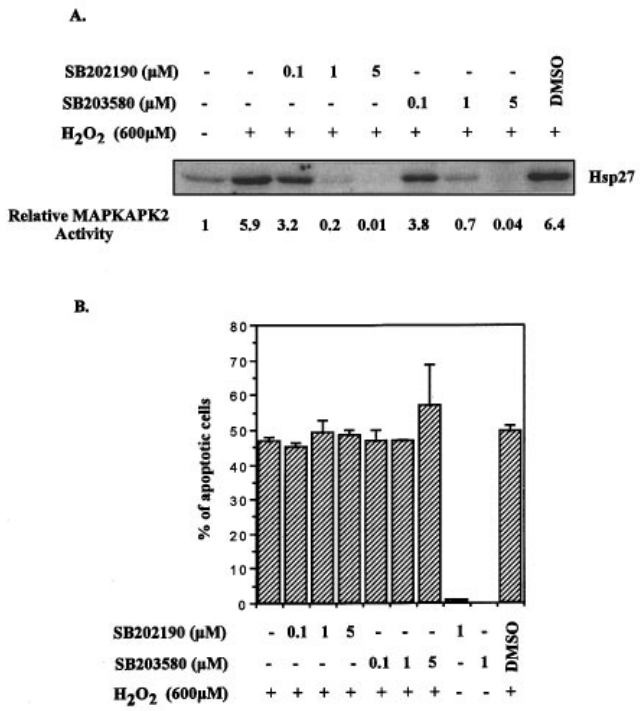

Figure 5 p38 activation does not influence H_2O_2-induced apoptosis

(**A**) The p38-specific MAPK inhibitors SB202190 and SB203580 block H_2O_2-induced activation of MAPKAPK2 kinase. HeLa cells were pretreated with the inhibitors for 1 h before the addition of 600 μM H_2O_2. After 1 h, treated cells were harvested and assayed for MAPKAPK2 kinase activity with Hsp27 protein as a substrate. (**B**) H_2O_2-induced apoptosis in the presence and in the absence of the p38 inhibitors. HeLa cells were pretreated with the inhibitors for 1 h before exposure to 600 μM H_2O_2. After 24 h the cells were fixed and stained with DAPI for quantification of apoptosis as described in the legend to Figure 1. Values are means $\pm$ S.D. for three independently performed experiments.

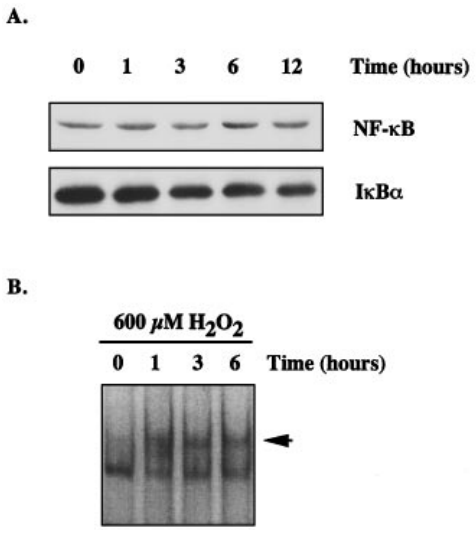

Figure 6 IκBα and NF-κB expression and NF-κB DNA-binding activity in H_2O_2-treated HeLa cells

(**A**) Western blot analysis of IκBα and NF-κB protein levels in HeLa cells at various times after treatment with 600 μM H_2O_2. (**B**) Treatment with H_2O_2 enhances the DNA binding activity of NF-κB in HeLa cells. Nuclear extracts were prepared from cells at various times after treatment with 600 μM H_2O_2 and analysed for binding activity to an oligonucleotide containing a consensus binding site for NF-κB. The arrow denotes specific NF-κB–DNA complexes.

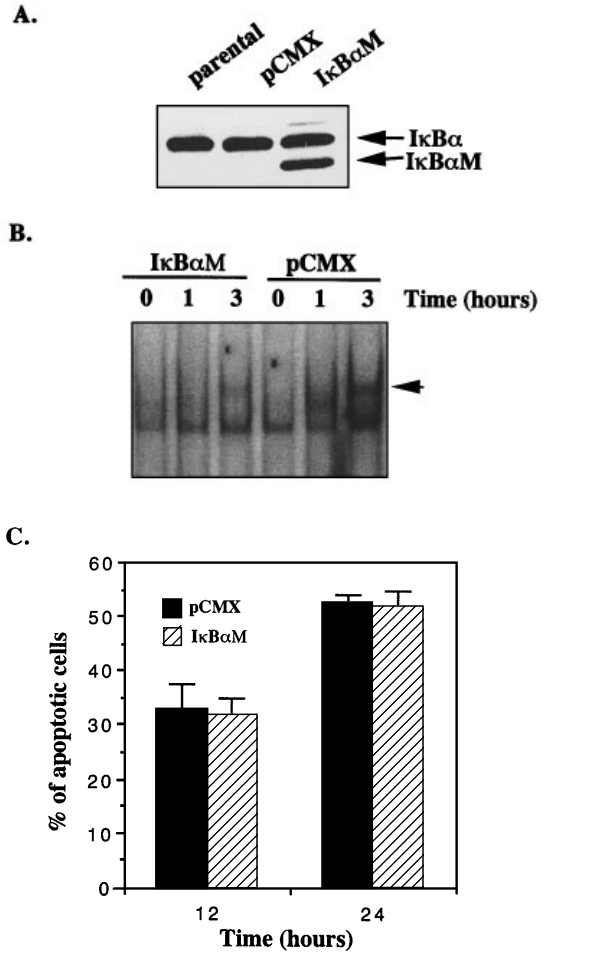

Figure 7 NF-κB activation has no effect on H_2O_2-induced apoptosis

(**A**) Western blot analysis verifying the expression of mutant IκBα protein (IκBαM) in cells stably transfected with a vector driving its expression [32]. The mutant protein migrates differently from the endogenous IκBα protein. (**B**) Overexpression of IκBαM prevents the activation of NF-κB. Nuclear extracts were prepared from control (cells transfected with empty pCMX vector) and IκBαM-expressing cell lines at 1 or 3 h after treatment with 600 μM H_2O_2. DNA-binding activity of NF-κB in these extracts was examined with an oligonucleotide containing a consensus NF-κB-binding site. (**C**) IκBαM expression does not alter the sensitivity to H_2O_2. Control and IκBαM-expressing cells were assayed for apoptosis by staining with DAPI 12 or 24 h after their treatment with 600 μM H_2O_2. Apoptotic cells were scored and plotted as described in the legend to Figure 1. Values are means $\pm$ S.D. for three independently performed experiments.

Bcl-2 overexpression vector. Transfected cells were either left untreated or were treated with H_2O_2. HA–JNK1 protein was then immunoprecipitated from cell extracts with anti-HA monoclonal antibody; the immunocomplex was then assayed for its ability to phosphorylate the GST–c-Jun substrate (Figure 9A). Although Western blot analysis showed equal expression of HA–JNK1 protein, the HA–JNK1 activity was markedly elevated after treatment with H_2O_2, but this activation was not affected by co-expression of Bcl-2. The second approach examined the effect of adding purified Bcl-2 protein directly to the kinase reaction *in vitro*. As shown in Figure 9(B), recombinant Bcl-2 had no effect on the endogenous JNK1 kinase activity, which was greatly stimulated by H_2O_2 treatment. Taken together, the findings obtained with three different experimental approaches suggest that, whereas treatment with H_2O_2 greatly enhances

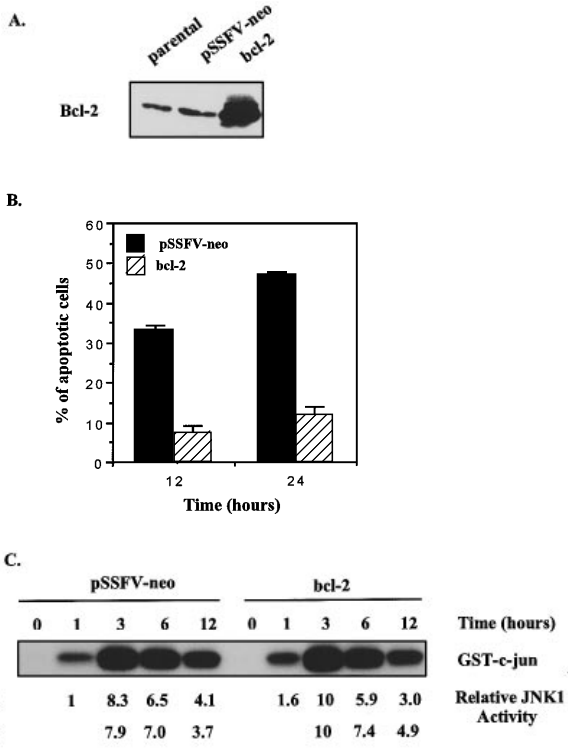

Figure 8 Bcl-2 overexpression protects cells against H_2O_2-induced apoptosis without affecting JNK/SAPK activity in stably transfected cells

(**A**) Western blot verifying high Bcl-2 expression in cells stably transfected with pSSFV-Bcl-2, but not in those transfected with the vector (pSSFV-neo) alone. (**B**) Apoptosis in control (empty pSSFV-neo vector) and Bcl-2-overexpressing cells after treatment with 600 μM H_2O_2. Apoptosis was assessed by staining with DAPI as described in the legend to Figure 1. (**C**) Bcl-2 overexpression does not prevent H_2O_2-induced JNK/SAPK activation. Control and Bcl-2-overexpressing cells were treated with 600 μM H_2O_2 and examined at various times thereafter for JNK/SAPK activity with an immunocomplex kinase assay and GST–c-Jun as a substrate. JNK/SAPK activity was quantified with a PhosphorImager. Results of two independent experiments are shown. Note that the JNK/SAPK activity is expressed relative to that seen in control cells 1 h after treatment with H_2O_2. JNK/SAPK was not readily detectable in untreated cells.

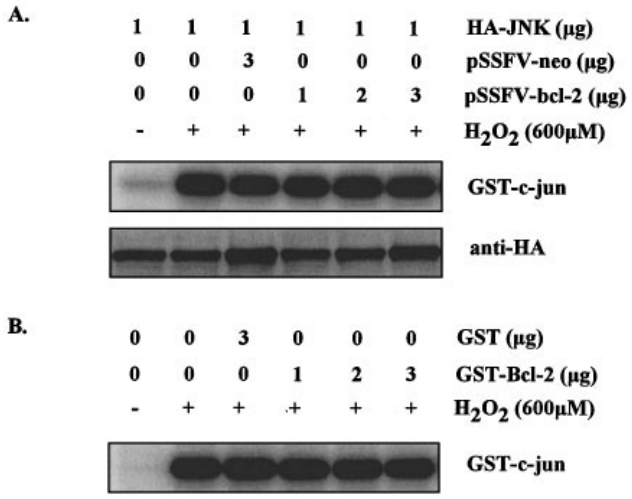

Figure 9 Bcl-2 does not inhibit JNK/SAPK activity *in vivo* or *in vitro*

(**A**) Activation of HA-tagged JNK1 by H_2O_2 is not affected by Bcl-2 overexpression in transiently transfected cells. Various amounts of the Bcl-2 expression construct were co-transfected with an HA-tagged JNK1 expression plasmid into HeLa cells. Transfected cells were treated with 600 μM H_2O_2 for 3 h, then JNK1 activity was determined in cell extracts by using the immunocomplex kinase assay. (**B**) Bcl-2 protein does not inhibit JNK/SAPK activity *in vitro*. JNK/SAPK activity was stimulated by exposure of HeLa cells to 600 μM H_2O_2; cells were lysed after 3 h. The kinase activity of JNK/SAPK was assayed by determining its ability to phosphorylate GST–c-Jun in the presence of various amounts of purified GST or GST–Bcl-2 protein.

JNK/SAPK activity associated with decreased cell survival, Bcl-2 acts independently of JNK/SAPK to protect cells from H_2O_2-induced apoptosis.

DISCUSSION

Through the use of specific inhibitors of ERK, JNK/SAPK and p38, we have addressed the relative contributions of these kinases in influencing the survival of H_2O_2-treated HeLa cells. Inhibition of either ERK or JNK/SAPK significantly altered the survival of H_2O_2-treated HeLa cells, and the effects seen were in opposition: inhibition of ERK led to enhanced apoptosis, whereas inhibition of JNK/SAPK decreased the number of apoptotic cells. These findings support and extend the general hypothesis, put forth by Xia et al. [4], that the dynamic balance between ERK and JNK/SAPK pathways is important in determining whether a cell survives or undergoes apoptosis. However, our findings differ in several respects. In the earlier studies of Xia et al., the model was one of growth-factor withdrawal in which ERK activity was suppressed at the same time as JNK/SAPK was activated. This resulted in a major shift in the ratio of JNK/SAPK activity to ERK activity. Indeed, it was suggested that the concurrent inhibition of ERK and sustained activation of JNK/SAPK was necessary for the induction of apoptosis in that model system. With H_2O_2 treatment, both ERK and JNK/SAPK activities are elevated, and thus a smaller change in the actual ERK-to-JNK/SAPK ratio occurs relative to that seen with growth factor withdrawal. However, even small shifts in this balance achieved through manipulation of either the ERK or JNK/SAPK pathways produces marked changes in the survival of H_2O_2-treated cells. It is therefore likely that the differences in sensitivity of various cell types to the cytotoxic effects of H_2O_2 reflect the relative activation of ERK and JNK/SAPK. In this regard it is worth noting that we have observed considerable variation in ERK and JNK/SAPK activation in different cell types in response to H_2O_2 treatment [21]. High ERK activation has also been observed with other agents that result in oxidant injury such as asbestos and butylated hydroxytoluene hydroperoxide [22,45]. Certainly the relationship between ERK activation and survival after oxidant injury is not restricted to HeLa cells, as we have previously reported that inhibition of ERK also decreases the survival of NIH 3T3 and rat PC12 cells treated with H_2O_2 [21]. However, in our earlier studies we did not address the mechanism (i.e. apoptosis compared with necrosis) of cell death and did not examine the contribution of JNK/SAPK or p38 to the response. In agreement with our current findings, Aikawa et al. [46] have likewise recently provided evidence that ERK activation protects cardiac myocytes from apoptotic death after oxidative stress.

Small changes in the level or duration of JNK/SAPK activation can produce major changes in survival, as is evident in the time-course studies evaluating the effects of overexpressing wild-type and dominant-negative SEK1 on both JNK/SAPK activation and cell survival after treatment with 600 μM H_2O_2. The magnitude of JNK/SAPK activation by such treatment at the 3 h time point did not differ significantly between control

cells, cells overexpressing wild-type SEK1 and cells expressing the mutant SEK1(K-R). However, SEK1(K-R)-expressing cells failed to maintain elevated levels of JNK/SAPK at later time points; SEK1 cells maintained higher JNK/SAPK activity relative to control cells (Figure 4B). Survival of the SEK1- and SEK1(K-R)-overexpressing cells was correlated precisely with the relative JNK/SAPK activities seen at the later time points. Hence, in predicting the apoptotic response to treatment with H_2O_2, the duration of the JNK/SAPK activation seems more important than the level of activation attained. This relationship is consistent with that seen in other stress conditions that result in apoptosis, including growth factor withdrawal, and radiation treatment [4,5,8].

Among the potential MAPK-regulated transcription factors known to be activated in response to oxidative stress is NF-κB. Under normal growth conditions, NF-κB is sequestered in the cytoplasm in an inactive state through its interaction with one or more members of the IκB family of inhibitory proteins. Conversion of NF-κB to an active form requires the release of IκB. This is accomplished through the phosphorylation of IκB leading to its ubiquitination and degradation. Two upstream regulators of JNK/SAPK, MAPK kinase kinase 1 (MEKK1), and a novel MEKK1-related protein, NIK (NF-κB-inducing kinase), have been implicated in the pathway leading to the phosphorylation of IκB [25,26,28,39]. The ERK signalling pathway has also been linked to NF-κB activation through the finding that the ERK-regulated kinase $p90^{RSK}$ can phosphorylate IκB, leading to its inactivation in response to mitogenic stimulation [27]. There are conflicting reports on the role of NF-κB as a regulator of apoptosis in different model systems and different cell types [23,24]. It was therefore of interest to determine the influence of NF-κB on the cellular response to H_2O_2 in our model system. In keeping with previous studies in HeLa cells, treatment with H_2O_2 resulted in the activation of NF-κB, although the increase in DNA-binding activity was relatively modest in our system. Inhibition of this NF-κB activation did not alter the survival of H_2O_2-treated cells. These findings are similar to those recently reported in another model of oxidative stress, that of hyperoxia treatment of cardiac myocytes [47]. Although hyperoxia led to a significant increase in NF-κB DNA-binding activity, it did not influence cell survival. However, in that model system the myocytes seemed to die via a necrotic, rather than an apoptotic, mechanism. Treatment of the same cells with H_2O_2 did lead to apoptosis, but no activation of NF-κB was observed. Hence although NF-κB has an important role in regulating apoptosis in response to some stresses, it seems not to be important in influencing survival after treatment with H_2O_2.

Several recent studies have provided evidence suggesting that Bcl-2 exerts its anti-apoptotic function through suppression of the JNK/SAPK signalling pathway. For example, overexpression of Bcl-2 was found to promote survival and to block JNK/SAPK activation caused by withdrawal of nerve growth factor in PC12 cells [43]. More recently, Bcl-2 overexpression was found to prevent JNK/SAPK activation and to suppress apoptosis of N18TG neuroglioma cells caused by a variety of agents [44]. Although JNK/SAPK overexpression was able to antagonize this effect of Bcl-2, it did not seem to act directly on JNK/SAPK to inhibit its activity because the activation of MEKK1, an upstream intermediate of the JNK/SAPK cascade, was also prevented by Bcl-2 overexpression [44].

The observation that Bcl-2 does not directly alter JNK/SAPK activity is supported by our finding that Bcl-2 did not affect JNK/SAPK activity when added *in vitro* to cell extracts from H_2O_2-treated cells. However, our studies differ from those mentioned above in that we did not observe any suppression of H_2O_2-induced JNK/SAPK activation *in vivo* when Bcl-2 was overexpressed. This was true both in cell lines stably overexpressing Bcl-2 and in experiments in which transiently transfected HA-tagged JNK was assessed for activity in cells co-transfected with the Bcl-2-expressing plasmid. Hence, at least for this model system, Bcl-2 seems to act downstream of JNK/SAPK to regulate apoptosis. These observations are particularly important given the possibility that Bcl-2 itself might act as an antioxidant [41]. If Bcl-2 were acting in such a fashion in our model system, it would be expected to decrease the level of oxidative stress and therefore dampen the activation of the MAPK signalling pathways.

In summary, our findings provide new insight into the relative contributions of ERK, JNK/SAPK and p38 MAPK (i.e. anti-apoptotic, pro-apoptotic and no effect respectively) in regulating apoptosis in response to oxidant injury. In addition they provide novel information about the relative contribution of NF-κB (no effect) and the stage in the apoptotic pathway (downstream of JNK/SAPK) at which Bcl-2 acts to inhibit cell death. Future studies will be directed towards identifying the downstream effectors of the ERK and JNK/SAPK activities responsible for mediating their effects on cell survival after stress.

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REFERENCES

1 Davis, R. J. (1994) Trends Biochem. Sci. **19**, 470–473
2 Robinson, M. J. and Cobb, M. H. (1997) Curr. Opin. Cell Biol. **9**, 180–186
3 Waskiewicz, A. J. and Cooper, J. A. (1995) Curr. Opin. Cell Biol. **7**, 798–805
4 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Science **270**, 1326–1331
5 Chen, Y. R., Meyer, C. F. and Tan, T. H. (1996) J. Biol. Chem. **271**, 631–634
6 Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M. et al. (1996) Nature (London) **380**, 75–79
7 Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zon, L., Kyriakis, J., Liu, F. F. and Woodgett, J. R. (1996) Curr. Biol. **6**, 606–613
8 Chen, Y. R., Wang, X., Templeton, D., Davis, R. J. and Tan, T. H. (1996) J. Biol. Chem. **271**, 31929–31936
9 Goillot, E., Raingeaud, J., Ranger, A., Tepper, R. I., Davis, R. J., Harlow, E. and Sanchez, I. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 3302–3307
10 Yang, X., Khosravi-Far, R., Chang, H. Y. and Baltimore, D. (1997) Cell **89**, 1067–1076
11 Liu, Z. G., Hsu, H., Goeddel, D. V. and Karin, M. (1996) Cell **87**, 565–576
12 Lenczowski, J. M., Dominguez, L., Eder, A. M., King, L. B., Zacharchuk, C. M. and Ashwell, J. D. (1997) Mol. Cell. Biol. **17**, 170–181
13 Nishina, H., Fischer, K. D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E. A., Bernstein, A., Mak, T. W., Woodgett, J. R. and Penninger, J. M. (1997) Nature (London) **385**, 350–353
14 Raitano, A. B., Halpern, J. R., Hambuch, T. M. and Sawyers, C. L. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 11746–11750
15 Xu, X., Heidenreich, O., Kitajima, I., McGuire, K., Li, Q., Su, B. and Nerenberg, M. (1996) Oncogene **13**, 135–142
16 Rodrigues, G. A., Park, M. and Schlessinger, J. (1997) EMBO J. **16**, 2634–2645
17 Graves, J. D., Draves, K. E., Craxton, A., Saklatvala, J., Krebs, E. G. and Clark, E. A. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 13814–13818
18 Kawasaki, H., Morooka, T., Shimohama, S., Kimura, J., Hirano, T., Gotoh, Y. and Nishida, E. (1997) J. Biol. Chem. **272**, 18518–18521
19 Kummer, J. L., Rao, P. K. and Heidenreich, K. A. (1997) J. Biol. Chem. **272**, 20490–20494
20 Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F. and Gulbins, E. (1997) J. Biol. Chem. **272**, 22173–22181
21 Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N. J. (1996) J. Biol. Chem. **271**, 4138–4142
22 Guyton, K. Z., Gorospe, M., Kensler, T. W. and Holbrook, N. J. (1996) Cancer Res. **56**, 3480–3485

23 Baichwal, V. R. and Baeuerle, P. A. (1997) Curr. Biol. **7**, R94–R96
24 Baeuerle, P. A. and Henkel, T. (1994) Annu. Rev. Immunol. **12**, 141–179
25 Meyer, C. F., Wang, X., Chang, C., Templeton, D. and Tan, T. H. (1996) J. Biol. Chem. **271**, 8971–8976
26 Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoue, J. and Ohno, S. (1996) J. Biol. Chem. **271**, 13234–13238
27 Schouten, G. J., Vertegaal, A. C., Whiteside, S. T., Israel, A., Toebes, M., Dorsman, J. C., van der Eb, A. J. and Zantema, A. (1997) EMBO J. **16**, 3133–3144
28 Malinin, N. L., Boldin, M. P., Kovalenko, A. V. and Wallach, D. (1997) Nature (London) **385**, 540–544
29 Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) EMBO J. **12**, 2005–2015
30 Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M. and Zon, L. I. (1994) Nature (London) **372**, 794–798
31 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051
32 Wang, X., Gorospe, M., Huang, Y. and Holbrook, N. J. (1997) Oncogene **15**, 2991–2998
33 Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P. and Traganos, F. (1992) Cytometry **13**, 795–808
34 Huot, J., Houle, F., Marceau, F. and Landry, J. (1997) Circ. Res. **80**, 383–392
35 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Nucleic Acids. Res. **17**, 6419
36 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7686–7689
37 Gallagher, T. F., Seibel, G. L., Kassis, S., Laydon, J. T., Blumenthal, M. J., Lee, J. C., Lee, D., Boehm, J. C., Fier-Thompson, S. M., Abt, J. W. et al. (1997) Bioorg. Med. Chem. **5**, 49–64
38 Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T. and Nebreda, A. R. (1994) Cell **78**, 1027–1037
39 Lee, F. S., Hagler, J., Chen, Z. J. and Maniatis, T. (1997) Cell **88**, 213–222
40 Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. and Verma, I. M. (1996) Science **274**, 787–789
41 Reed, J. C. (1995) Curr. Opin. Oncol. **7**, 541–546
42 Yang, E. and Korsmeyer, S. J. (1996) Blood **88**, 386–401
43 Park, D. S., Stefanis, L., Yan, C. Y. I., Farinelli, S. E. and Greene, L. A. (1996) J. Biol. Chem. **271**, 21898–21905
44 Park, J., Kim, I., Oh, Y. J., Lee, K., Han, P. L. and Choi, E. J. (1997) J. Biol. Chem. **272**, 16725–16728
45 Zanella, C. L., Posada, J., Tritton, T. R. and Mossman, B. T. (1996) Cancer Res. **56**, 5334–5338
46 Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y. and Yazaki, Y. (1997) J. Clin. Invest. **100**, 1813–1821
47 Li, Y., Zhang, W., Mantell, L. L., Kazzaz, J. A., Fein, A. M. and Horowitz, S. (1997) J. Biol. Chem. **272**, 20646–20649

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