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Activation of Mitogen-activated Protein Kinase by H_2O_2

ROLE IN CELL SURVIVAL FOLLOWING OXIDANT INJURY*

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The mitogen-activated protein kinase (MAPK) family is comprised of key regulatory proteins that control the cellular response to both proliferation and stress signals. In this study we investigated the factors controlling MAPK activation by H_2O_2 and explored the impact of altering the pathways to kinase activation on cell survival following H_2O_2 exposure. Potent activation (10–20-fold) of extracellular signal-regulated protein kinase (ERK2) occurred within 10 min of H_2O_2 treatment, whereupon rapid inactivation ensued. H_2O_2 activated ERK2 in several cell types and also moderately activated (3–5-fold) both c-Jun N-terminal kinase and p38/RK/CSBP. Additionally, H_2O_2 increased the mRNA expression of MAPK-dependent genes c-*jun*, c-*fos*, and MAPK phosphatase-1. Suramin pretreatment completely inhibited H_2O_2 stimulation of ERK2, highlighting a role for growth factor receptors in this activation. Further, ERK2 activation by H_2O_2 was blocked by pretreatment with either *N*-acetyl-cysteine, *o*-phenanthroline, or mannitol, indicating that metal-catalyzed free radical formation mediates the initiation of signal transduction by H_2O_2. H_2O_2-stimulated activation of ERK2 was abolished in PC12 cells by inducible or constitutive expression of the dominant negative Ras-N-17 allele. Interestingly, PC12/Ras-N-17 cells were more sensitive than wild-type PC12 cells to H_2O_2 toxicity. Moreover, NIH 3T3 cells expressing constitutively active MAPK kinase (MEK, the immediate upstream regulator of ERK) were more resistant to H_2O_2 toxicity, while those expressing kinase-defective MEK were more sensitive, than cells expressing wild-type MEK. Taken together, these studies provide insight into mechanisms of MAPK regulation by H_2O_2 and suggest that ERK plays a critical role in cell survival following oxidant injury.

The cellular response to diverse external stimuli is controlled via a complex array of phosphorylation cascades. The extracellular signal-regulated protein kinase (ERK)[1] cascade is a prominent component of the mitogen-activated protein kinase (MAPK) family that in particular plays an integral role in both growth factor and stress signaling (reviewed in Ref. 1). The majority of ERK activity in most cell types arises from ERK1 (p42) and ERK2 (p44) isoforms (1), which are believed to have functional redundancy. Interestingly, at least some stress signals (*e.g.* UVC irradiation (2)) utilize the same signaling pathways for ERK activation as do mitogens. This well characterized cascade (reviewed in Ref. 3) is initiated by growth factor binding, which stimulates receptor tyrosine kinases. The sequential activation of the GTP-binding protein Ras and the serine kinase Raf then ensues (3–5). Raf then activates MAPK kinase (MEK), a threonine/tyrosine dual specificity kinase that directly activates ERK (6). ERK activation culminates in the phosphorylation of downstream cytosolic and nuclear factors that control a variety of cellular processes (7).

Oxidant injury is thought to play a critical role in the degenerative alterations that occur with aging and in the etiology of many disease processes including cancer and atherosclerosis (8–10). Many of the basic molecular aspects regulating the cellular response to oxidative stress in bacteria are well established (11). However, the pathways mediating the control of gene expression by oxidants and sensitivity to oxidant injury in mammalian systems are less well defined. Herein we examine the activation of MAPK pathways by the oxidative agent H_2O_2, with particular focus on the cellular consequences of modulating the ERK signaling cascade. Our findings support a pivotal role for the ERK pathway in determining cell survival following oxidant injury.

MATERIALS AND METHODS

Cell Culture and Treatment—Primary cultures and cell lines were cultured in a 37 °C humidified environment containing 10% CO_2 in air. Cell lines were cultured in Dulbecco's modified Eagle's medium with gentamicin (50 ng/ml), supplemented with 10% calf serum (NIH 3T3), 10% fetal bovine serum (HeLa and Rat1), or 10% fetal bovine serum and 5% horse serum (PC12). Primary smooth muscle cells were isolated by enzymatic digestion of rat aorta as described (12) and cultured in medium 199 supplemented with 20% calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Serum starvation was achieved by incubation in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum for at least 16 h prior to the direct addition of H_2O_2 into this culture medium (with 200 μM H_2O_2 equivalent to 1 μmol of H_2O_2/10^6 cells). Suramin (0.3 mM), *N*-acetyl-cysteine (20 mM), *o*-phenanthroline (0.1 mM), mannitol (100 mM), or cycloheximide (40 μg/ml) were added to the culture medium 45 min before the direct addition of H_2O_2.

Immunoprecipitation and Kinase Activity Assays—Cell cultures (60–80% confluent) were treated, washed twice with ice-cold phosphate-buffered saline, and lysed in buffer containing 20 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin, 2 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μM okadaic acid. Soluble extracts were prepared by centrifugation at 10,000 × *g* for 10 min at 4 °C. Following normalization of protein content, endogenous ERK2, c-Jun N-terminal kinase-1 (JNK1/SAPK), or p38/RK/CSBP were immunoprecipitated from the cell extracts using rabbit polyclonal antibodies against $p42^{ERK2}$ or $p46^{JNK1}$ (Santa Cruz Biotechnology, Santa Cruz, CA), or

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[1] The abbreviations used are: ERK, extracellular signal-regulated kinase; JNK1/SAPK, c-Jun N-terminal kinase-1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; MEK_{wt}, wild-type MEK; MEK_{ca}, constitutively active MEK; MEK_{kd}, kinase-defective MEK; MKP-1, MAPK phosphatase-1; rMKP-1, rat MKP-1; SMC, aortic smooth muscle cell primary cultures; MOPS, 4-morpholinepropanesulfonic acid; p38/RK/CSBP, 38-kDa MAPK-related protein/reactivating kinase/cytokine-suppressive anti-inflammatory drug binding protein; UVC, short wavelength UV radiation.

$p38^{CSBP2}$ (provided by J.C. Lee, Smithkline Beacham Pharmaceuticals, King of Prussia, PA), respectively. Kinase activity was assayed for 20 min at 37 °C in the presence of 6 μg of substrate, 30 μM ATP, and 20 μCi of [γ-^{32}P]ATP in 55 μl of assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 20 mM $MgCl_2$). GST-c-Jun-(1–135) (13) was used as a substrate for JNK1/SAPK, and myelin basic protein (MBP) was used for assaying ERK2 and p38/RK/CSBP activity. After completion of kinase assays, the proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the gels were dried and subjected to autoradiography. The incorporation of ^{32}P was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis—Protein extracts were prepared and subjected to Western analysis as described previously (14) using a monoclonal antibody specific for ERK2 (Transduction Laboratories, Lexington, KY). Phosphotyrosine was detected using the monoclonal anti-phosphotyrosine antibody RC20H (Transduction Laboratories). Immune complexes were visualized using an enhanced chemiluminescence detection kit (Amersham Corp.) following the manufacturer's specifications.

RNA Isolation and Northern Analysis—Total RNA was extracted from treated cells using RNA Stat-60™ (Tel-Test "B", Friendswood, TX). RNA (10–20 μg/lane) was size-separated in agarose/formaldehyde gels and transferred onto GeneScreen Plus nylon membranes (DuPont NEN). cDNA probes for c-Fos, c-Jun, and MKP-1 were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Boehringer Mannheim). Hybridization and washes were performed according to the method of Church and Gilbert (15), and the hybridization signal was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Hybridization to a 24-base pair oligonucleotide complementary to 18 S RNA (5′-ACGGTATCTGATCGTCTCGAACC-3′) that had been 3′-end-labeled with [α-^{32}P]ATP by terminal deoxynucleotidyltransferase (Life Technologies, Inc.) was used to control for variation in loading and transfer among samples.

Transfections and Luciferase Assay—HeLa cells were transiently transfected by $CaPO_4$ precipitation with 1 μg *fos*-luciferase (16) together with either 10 μg of carrier DNA (pSG5), 6 μg of carrier DNA and 4 μg of pSG5-rMKP-1, or 10 μg of pSG5-MKP-1as (14). Cells were treated with 200 μM H_2O_2 the following day, and luciferase activity was measured in cell extracts prepared 24 h after treatment using a luciferase assay system kit (Promega, Madison, WI).

Cell Viability Assays—Cells were plated at a density of 50×10^3 cells/well (PC12) or 5×10^3 cells/well (NIH 3T3) into 96-well microtiter plates. The plates were treated with H_2O_2 the following day and stained 48 h after treatment with crystal violet for assessment of cell viability using a microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA)

RESULTS

H_2O_2 Stimulates Protein Tyrosine Phosphorylation and Potently Activates ERK2—As shown in Fig. 1*A*, H_2O_2 stimulated the rapid and transient phosphorylation of several proteins of 33–44 kDa in NIH 3T3 cells. Kinases of the MAPK family have mobilities consistent with the proteins that became recognized by the anti-phosphotyrosine antibody within 5–10 min of H_2O_2 treatment. Indeed, direct measurement of kinase activity by immune complex kinase assay confirmed that H_2O_2 activated multiple members of the MAPK family (Fig. 1*B*). While JNK1/SAPK and p38/RK/CSBP were only activated 3–5-fold over control levels, H_2O_2 stimulated a striking increase in ERK2 activity of 25-fold. Enhancement of ERK2 activity was evident with as little as 10 μM H_2O_2, with dose-dependent increases up to 200 μM H_2O_2. To determine if ERK2 activation constitutes a widespread cellular response to oxidants such as H_2O_2, we examined the effect of H_2O_2 on ERK2 in several cell types including HeLa, Rat1, NIH 3T3, and PC12 cell lines as well as in primary aortic smooth muscle cell cultures (SMC). While minimal ERK2 activation by H_2O_2 has been reported in SMC (17), we found significant ERK2 stimulation (10–20-fold) in all cell types tested, including these primary cultures (Fig. 2).

The kinetics of ERK2 activation in NIH 3T3 cells following H_2O_2 exposure are shown in Fig. 3. H_2O_2 stimulation of ERK2 activity occurred within 5 min of treatment and was maximal by 10 min after H_2O_2 exposure (Fig. 3*A*). A rapid inactivation of

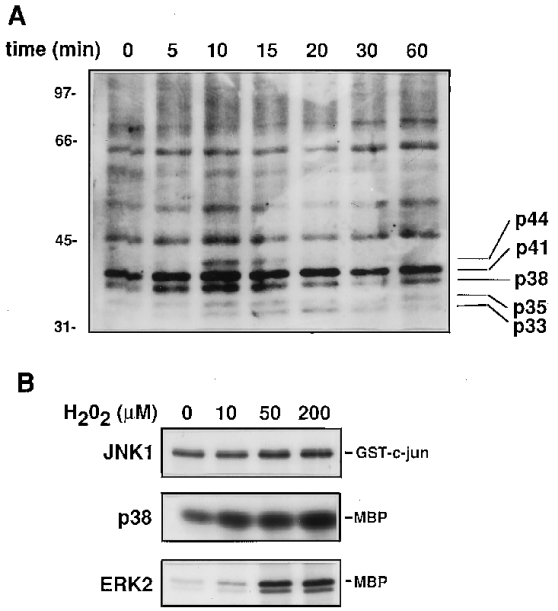

FIG. 1. **Protein tyrosine phosphorylation and kinase activation by H_2O_2.** *A*, Western blot analysis using the antiphosphotyrosine antibody RC20H. NIH 3T3 cell lysates were prepared following H_2O_2 treatment for the indicated times. Tyrosine-phosphorylated proteins of 33, 35, 38, 41, and 44 kDa are indicated. *B*, dose-response analysis of ERK2, JNK1/SAPK, and p38/RK/CSBP kinase activation by H_2O_2. NIH 3T3 cells were treated with the indicated doses of H_2O_2 at the time of maximal activation (10 min for ERK2; 15 min for JNK1/SAPK and p38/RK/CSBP), and polyclonal anti-ERK2, anti-JNK1/SAPK, or anti-p38/RK/CSBP antibodies were used for kinase immunoprecipitation from the soluble fraction of cell lysates. Kinase activity was then assessed by immune complex kinase assay using bovine brain MBP (for ERK2 and p38/RK/CSBP) or GST-c-Jun (for JNK1/SAPK) as a substrate.

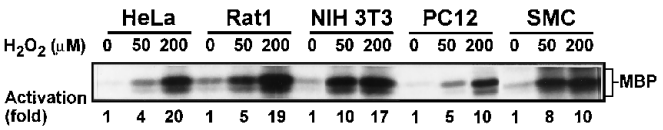

FIG. 2. **Activation of ERK2 by H_2O_2 in HeLa, Rat1, NIH 3T3, PC12, and SMC.** The indicated cell types were treated with 50 or 200 μM H_2O_2 for 10 min, and ERK2 was analyzed in the soluble fraction of lysates by immune complex kinase assay.

ERK2 then ensued, with a return to basal ERK2 levels occurring within 30 min of H_2O_2 exposure. The increase in ERK2 kinase activity following H_2O_2 treatment was paralleled by a shift in the electrophoretic mobility of ERK2 protein seen on Western blots, indicating phosphorylation of ERK2 protein (Fig. 3*B*); however, the abundance of ERK2 protein expressed remained unchanged.

MAPK-dependent Gene Expression following H_2O_2 Exposure—An important cellular consequence of MAPK activation is the modulation of the expression of transcription factors and other key regulatory proteins. Therefore, we investigated the effect of H_2O_2 on the expression of several such early response genes. As shown in Fig. 4*A*, H_2O_2 treatment stimulated a transient but dramatic increase in the mRNA expression of MKP-1, c-*jun*, and c-*fos*. H_2O_2 also activated a *fos* promoter-luciferase reporter gene construct in transiently transfected HeLa cells (Fig. 4*B*). The dependence of *fos* promoter activation by H_2O_2 on the ERK pathway was further demonstrated by cotransfection experiments, in which coexpression of MKP-1 (which is known to abrogate MAPK activity (18, 19)), attenuated *fos*-luciferase activity by H_2O_2, while expression of antisense MKP-1 was without effect.

While MKP-1 has been implicated in regulating ERK2 activ-

ity in response to growth factor stimulation (18), the physiological role of MKP-1 in mitigating the rapid activation of ERK2 activity by H_2O_2 is less well defined. The reversion of ERK2 protein to the unphosphorylated state that accompanies the rapid loss of kinase activity following ERK2 stimulation by H_2O_2 in Fig. 3*A* suggests that protein phosphatases function in ERK2 regulation following H_2O_2. However, inhibition of protein synthesis by cycloheximide pretreatment did not affect the kinetics of ERK2 activation by H_2O_2 (Fig. 4*C*), indicating that newly synthesized phosphatases such as MKP-1 do not participate in inactivating ERK2. These results are consistent with the rapid kinetics of ERK2 inactivation and suggest that ERK2 activity is instead regulated by preexisting phosphatases.

Initiation of ERK2 Signaling by H_2O_2: Role of Growth Factors and Free Radicals—Mitogen-stimulated signal transduction leading to ERK2 activation is initiated through the interaction of peptide growth factors with their receptors. Receptor activation can also be mediated by sulfhydryl oxidation, and through such mechanisms H_2O_2 has been postulated to mimic the actions of insulin and other receptor-binding proteins (20). We therefore explored the role of growth factor receptors in the initiation of ERK2 activation by H_2O_2. Suramin is known to block ligand-receptor interactions and can inhibit ERK2 activation by epidermal growth factor as well as by UVC irradiation (2). As shown in Fig. 5, suramin pretreatment also blocked H_2O_2-stimulated ERK2 activation.

The nature of the chemical signal generated from H_2O_2 that initiates the ERK2 cascade was also investigated. Enhancing the cellular antioxidant potential by pretreatment with the glutathione precursor *N*-acetyl-cysteine abolished the ability of H_2O_2 to stimulate ERK2 (Fig. 6). These results confirm that oxidant stress initiates ERK2 activation by H_2O_2. The iron chelator *o*-phenanthroline also effectively inhibited ERK2 activation by H_2O_2 (Fig. 6), suggesting that metal-dependent reactions are required for kinase activation by H_2O_2. In the presence of metal ions, H_2O_2 can undergo conversion via dismutation reactions to other oxygen-derived free radical species including hydroxyl radical (21). Indeed, mannitol, a free radical scavenger with specificity for hydroxyl radical, also blocked H_2O_2-mediated ERK2 activation. Taken together, these results suggest that H_2O_2 undergoes metal-catalyzed conversion to a hydroxyl radical-like species and that oxidation by this free radical initiates signal transduction leading to ERK2 activation by H_2O_2.

Role of Ras in ERK2 Activation by H_2O_2—Although Ras plays a prominent role in the activation of multiple MAPK family members in response to both mitogen stimulation and UVC irradiation (5), MAPK activation can also occur through Ras-independent pathways (22). To determine if Ras is a component of the signaling cascade leading to ERK2 activation by H_2O_2, we examined the inhibitory effect of the dominant negative Ras-N-17 allele on ERK2 activation in PC12 cells (23, 24). Dexamethasone pretreatment of PC12 cells carrying a murine mammary tumor virus-driven Ras-N-17 expression vector resulted in a near complete abolition of ERK2 activation by H_2O_2 (Fig. 7, *left panel*), suggesting that Ras is required for ERK2 activation by H_2O_2. Similar results were obtained in PC12 cells constitutively expressing the dominant negative Ras-N-17 allele (Fig. 7, *right panel*). By comparison with that in wild-type PC12 cells, ERK2 activation by H_2O_2 in these Ras-N-17-expressing cells was markedly reduced. Taken together, the results from these two model systems of mutant Ras expression indicate a requirement for Ras in mediating ERK2 activation by H_2O_2.

Effect of Modulating ERK Activation on Survival following H_2O_2 Exposure—To investigate the potential physiological role of ERK in mediating the cellular response to oxidative stress, we undertook a comparative analysis of cell survival following H_2O_2 exposure in PC12/Ras-N-17 cells and wild-type PC12 cells. H_2O_2 stimulated a dose-dependent decrease in cell survival in both cell lines as assessed by crystal violet staining. However, PC12/Ras-N-17 cells exhibited significantly greater sensitivity to H_2O_2 than their wild-type counterparts, as evidenced by reduced survival at identical doses of H_2O_2 (Fig. 8). Indeed, the LD_{50} for H_2O_2 was 150 μM in PC12/Ras-N-17 cells, as compared with 380 μM in wild-type PC12 cells. Similar results were found by colony formation assay (data not shown).

In addition to mediating signal transduction to ERK, Ras is

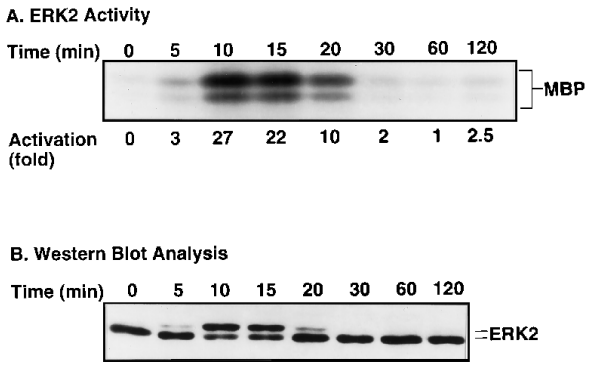

FIG. 3. **Time course for activation of ERK2 by H_2O_2.** NIH 3T3 cells were treated with 200 μM H_2O_2 for the indicated times, after which cells were harvested and the soluble fraction was analyzed for ERK2. *A*, kinetics of ERK2 activation in H_2O_2-treated cells. ERK2 was immunoprecipitated using a polyclonal anti-ERK2 antibody, and kinase activity was assayed using bovine brain MBP as a substrate. *B*, Western blot analysis of the expression and phosphorylation of ERK2.

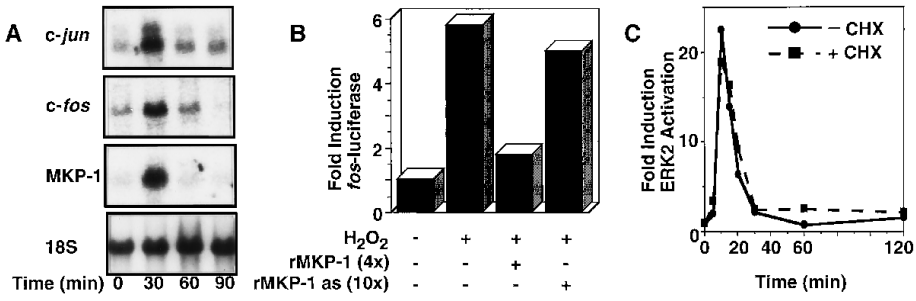

FIG. 4. **H_2O_2 stimulates MAPK-dependent gene expression.** *A*, Northern blot analysis for the induction of c-*jun*, c-*fos*, and MKP-1 mRNA expression by H_2O_2. Following treatment with 200 μM H_2O_2 for the indicated times, RNA was isolated and Northern blots were probed with the indicated cDNAs. The 18 S signal is shown as a control for variations in loading and transfer. *B*, effect of cotransfection with rMKP-1 or rMKP-1as on *fos*-luciferase expression stimulated by H_2O_2 (200 μM). *C*, effect of cycloheximide pretreatment on ERK2 activation by H_2O_2. Cycloheximide (40 μg/ml) was added 45 min prior to treatment with 200 μM H_2O_2 for the indicated times, and ERK2 activity was assessed in a soluble fraction of cell extracts by immune complex kinase assay.

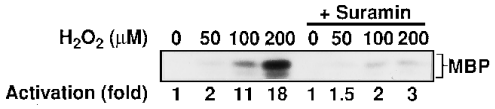

FIG. 5. **Suramin inhibits ERK2 activation by H_2O_2.** Suramin (0.3 mM) was added 45 min before the direct addition of H_2O_2, and cells were harvested 10 min later. ERK2 activity was analyzed in the soluble fraction of cell lysates by immune complex kinase assay.

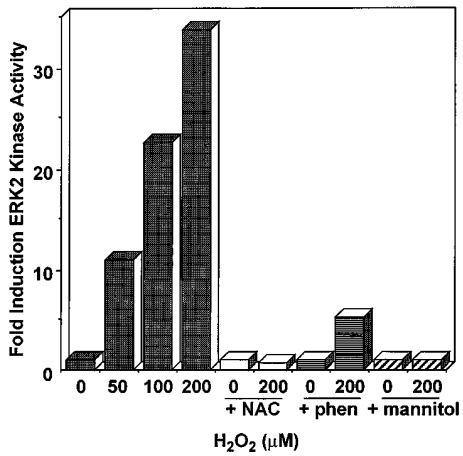

FIG. 6. **Role of free radicals in H_2O_2-mediated ERK2 activation.** *N*-acetyl-cysteine (20 mM), *o*-phenanthroline (100 μM), or mannitol (100 mM) was added 45 min before the direct addition of H_2O_2, and cells were harvested 10 min later for analysis of ERK2 activity by immune complex kinase assay. Data are expressed as the -fold induction in ERK2 activity over inhibitor alone controls. The inhibitors alone did not activate ERK2.

known to participate in the activation of other MAPK family members, including JNK1/SAPK (13, 25). Thus, the dramatic effect of Ras-N-17 on cell survival following H_2O_2 may not solely arise as a consequence to modulation of the ERK signaling pathway. In order to investigate the possible contribution of the JNK1/SAPK pathway to the enhanced sensitivity of PC12/Ras-N-17 cells to H_2O_2, we compared H_2O_2-stimulated JNK1/SAPK activity in Ras-N-17 and wild-type PC12 cells. However, we found that the modest activation of JNK1/SAPK (4-fold with 200 μM H_2O_2) by H_2O_2 was not affected by cellular Ras status (data not shown). By contrast, the reduced potential for cell survival following oxidant injury of PC12/Ras-N-17 cells correlates with, and may indeed arise as a consequence of, the decreased capacity for ERK activation in these cells (Fig. 7).

In order to further address the function of the ERK pathway in the cellular response to H_2O_2, we compared cell survival in NIH 3T3 cell lines in which the activity of MEK, the immediate upstream regulator of ERK, had been altered (26). With increasing dosage of H_2O_2, cell survival diminished accordingly in cell lines expressing wild-type MEK (MEK_{wt}), constitutively active MEK (MEK_{ca}), or MEK lacking a kinase domain (MEK_{kd}) (Fig. 9). However, the sensitivity to H_2O_2 was correlated with MEK activity; MEK_{ca} cells exhibited enhanced resistance, while MEK_{kd} cells showed diminished resistance, as compared with MEK_{wt} cells. This effect of MEK was evidenced by a separation in the dose-response curves for cell survival following H_2O_2 exposure in the three cell lines and in marked differences in the LD_{50} for H_2O_2. While 120 μM resulted in a 50% decrease in survival in MEK_{kd} cells, 200 μM and 320 μM were required for the same effect in MEK_{wt} and MEK_{ca} cells, respectively. Likewise, the same dose of H_2O_2 could differentially affect cell survival in the three cell lines: 180 μM H_2O_2 mediated a 10, 45, or 65% loss of survival in MEK_{ca}, MEK_{wt}, and MEK_{kd} cells, respectively. Comparable findings resulted from colony formation assays (data not shown). These results

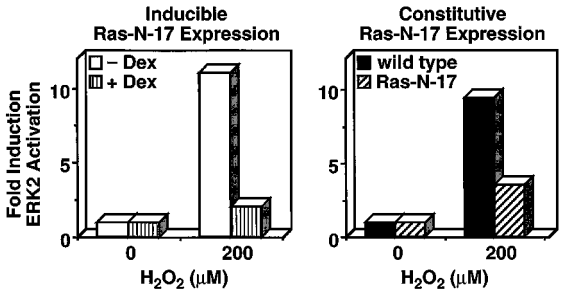

FIG. 7. **Effect of inducible and constitutive dominant-negative Ras-N-17 on H_2O_2-mediated ERK2 activation.** PC12 cells were treated with H_2O_2 for 10 min, whereupon cells were harvested and ERK2 activity in the soluble fraction of cell lysates was assessed by immune complex kinase assay. *Left*, PC12 cells expressing murine mammary tumor virus-Ras-N-17 were cultured overnight in the presence or absence of dexamethasone (1 μM) prior to treatment with H_2O_2. *Right*, comparison of the -fold activation of ERK2 by H_2O_2 in parental PC12 cells and those constitutively expressing Ras-N-17.

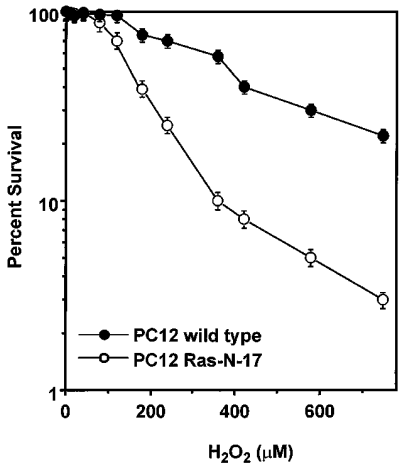

FIG. 8. **Comparative effect of H_2O_2 on cell viability in parental and constitutive Ras-N-17-expressing PC12 cells.** PC12 cells were cultured overnight in 96-well plates, treated with H_2O_2 in complete medium, and stained 48 h later with crystal violet for assessment of cell viability. Values represent mean ± S.E. for seven wells.

are consistent with, and extend our findings in, PC12 cells expressing wild-type Ras or Ras-N-17 to suggest that cell survival following exposure to H_2O_2 can be accordingly modulated by either enhancing or suppressing the pathway to ERK activation.

DISCUSSION

In this report, we demonstrate that multiple members of the MAPK family are stimulated by H_2O_2 and that ERK2 in particular is highly activated in a variety of cell types (Figs. 1 and 2). The rapid and transient nature of ERK2 activation by H_2O_2 (Fig. 3) highlights the reversible and direct nature of alterations stimulated by H_2O_2 and underscores a role for phosphatases in regulating this response. However, the involvement of newly synthesized proteins in regulating ERK2 activation following H_2O_2 exposure is precluded by both the rapidity of inactivation and the cycloheximide insensitivity of ERK2 activation (Fig. 4*C*). Dephosphorylation of ERK2 protein may instead be reliant on preexisting phosphatases (19, 27, 28). The early temporal control of ERK2 activation by both kinase and phosphatase activity is also reflected in the transient stimulation of MAPK-dependent gene expression by H_2O_2 (Fig. 4*A*).

The function of numerous cellular proteins, including transcription factors, calcium-regulatory proteins, and other cell and organelle surface molecules, is subject to redox regulation

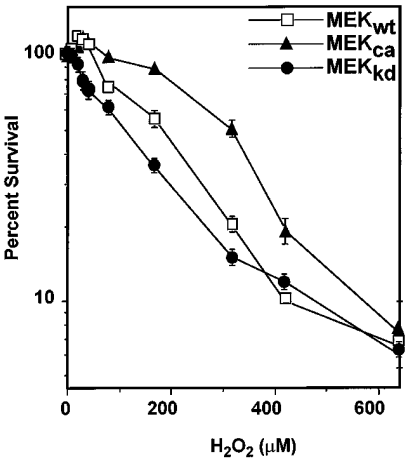

FIG. 9. **Effect of cellular MEK status on H_2O_2-stimulated loss of cell viability.** NIH 3T3 cells expressing MEK_{wt}, MEK_{ca}, or MEK_{kd} were cultured overnight in 96-well plates, treated with H_2O_2 in complete medium, and stained 48 h later with crystal violet for assessment of cell viability. Values represent mean ± S.E. for seven wells.

(10, 29–34). Oxidation-reduction mechanisms are in fact a likely physiological means for reversible regulation of protein function and provide a likely target through which exogenous oxidants can usurp normal signal transduction pathways. For example, many growth factor and cytokine receptors have cysteine-rich motifs, the oxidation of which can simulate ligand binding (35, 36). That suramin can block H_2O_2-stimulated ERK2 activation (Fig. 5) suggests that oxidation of such cell surface receptors may mediate signal initiation by H_2O_2. Indeed, the sulfhydryl reactivity of the oxidant signal generated from H_2O_2 was confirmed by the inhibitory actions of *N*-acetylcysteine (Fig. 6). Free radical species generated from H_2O_2 may directly oxidize and thereby activate cell surface receptors, although the oxidative modification of other molecules, including those involved in phosphatase regulation, may also function in the regulation of ERK2 by H_2O_2. These findings further suggest that free radicals or other redox mechanisms may constitute a critical component of the signaling pathways to ERK activation normally utilized by growth factors and other stimuli. Our demonstration that ERK2 can be activated by exposure to low doses of H_2O_2 (10 μM), such as may typically occur in cells (37), supports this assertion. That H_2O_2-stimulated ERK2 is regulated through Ras (Fig. 7), as has been reported for serum and growth factors (5), further emphasizes the significant overlap between the pathways for oxidative stress and normal physiological signals.

While MAPK activation has been reported in response to both proliferation and stress stimuli (1), an understanding of the function of the ERK phosphorylation cascade in regulating the downstream cellular effects that occur pursuant to stimulation is only beginning to emerge. Recent reports have provided evidence that constitutive MAPK activation is associated with the transformed phenotype (38) and that likewise unregulated activation of MEK, the immediate upstream activator of MAPK, can alone cause cellular transformation (26, 39). These findings are in keeping with the known oncogenic potential of other molecules (*i.e.* Ras and Raf) that play a critical role in signal transduction pathways (reviewed in Ref. 40). In the present study we demonstrate in two model systems that modulation of the pathway to ERK activation by H_2O_2 affects cellular survival following H_2O_2. Expression of dominant negative Ras in PC12 cells and kinase-defective MEK in NIH 3T3 cells results in enhanced sensitivity to H_2O_2, while a constitutively active MEK variant engendered greater resistance. Thus, we provide evidence that altered responsiveness to extracellular stress is an important consequence of these potentially oncogenic alterations. This type of "response modification" has been proposed to contribute to carcinogenic development by oxidant and other stimuli (41, 42). While the precise MAPK-dependent cellular alterations engendering a modified response to oxidants remain to be defined, the present study provides strong support of a crucial role for the MAPK pathway in regulating cellular protection and proliferation in response to oxidative stress.

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