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$_2$O$_2$, and explored the impact of altering the pathways to kinase activation on cell survival following H$_2$O$_2$ exposure. Potent activation (10–20-fold) of extracellular signal-regulated protein kinase (ERK2) occurred within 10 min of H$_2$O$_2$ treatment, whereupon rapid inactivation ensued. H$_2$O$_2$ 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$_2$O$_2$ increased the mRNA expression of MAPK-dependent genes c-jun, c-fos, and MAPK phosphatase-1. Suramin pretreatment completely inhibited H$_2$O$_2$ stimulation of ERK2, highlighting a role for growth factor receptors in this activation. Further, ERK2 activation by H$_2$O$_2$ was blocked by pretreatment with either N-acetyl-cysteine, o-phenanthroline, or manitol, indicating that metal-catalyzed free radical formation mediates the initiation of signal transduction by H$_2$O$_2$. H$_2$O$_2$-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$_2$O$_2$ toxicity. Moreover, NIH 3T3 cells expressing constitutively active MAPK kinase (MEK, the immediate upstream regulator of ERK) were more resistant to H$_2$O$_2$ 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$_2$O$_2$ 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) 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) utilize the same signaling pathways for ERK activation as do mitogens. This well-characterized cascade (reviewed in Ref. 3) is initiated by growth factor signals which stimulate mitogen-activated protein 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$_2$O$_2$, 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 μg/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$_2$O$_2$ into this culture medium (with 200 μM H$_2$O$_2$ equivalent to 1 μM of H$_2$O$_2$/10$^6$ cells). Suramin (0.3 mM), N-acetyl-cysteine (20 mM), o-phenanthroline (0.1 mM), manitol (100 mM), or cycloheximide (40 μg/ml) were added to the culture medium 45 min before the direct addition of H$_2$O$_2$.

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 mM Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM diethiothreitol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM Leupetin, 2 μM aprotinin, 2 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM okadac 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, and Ras-N-17 were immunoprecipitated from the cell extracts using rabbit polyclonal antibodies against p42$^{N\text{term}}$ or p44$^{N\text{term}}$ (Santa Cruz Biotechnology, Santa Cruz, CA), or p38$^{N\text{term}}$ (Santa Cruz Biotechnology, Santa Cruz, CA).
p38CSBP2 (provided by J. C. Lee, Smithkline Beecham 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-32P]ATP in 55 μl of assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 20 mM MgCl2). GST-cjun un-1(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 32P 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 [γ-32P]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′-ACGGTATCTGATCGTCTCGACC-3′) that had been 3′-end-labeled with [α-32P]dATP 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 CaPO4 precipitation with 1 μg of substrate, 30 μg of carrier DNA, and pSG5-MKP-1 as (14). Cells were treated with 200 μM H2O2 for 24 h after treatment using 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 × 10^5 cells/well (NIH 3T3) into 96-well microtiter plates. The plates were treated with H2O2, the following day, and luciferase activity was measured in cell extracts prepared 24 h after treatment using a microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA).

RESULTS

H2O2 Stimulates Protein Tyrosine Phosphorylation and Potently Activates ERK2—As shown in Fig. 1A, H2O2 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 H2O2 treatment. Indeed, direct measurement of kinase activity by immune complex kinase assay confirmed that H2O2 activated multiple members of the MAPK family (Fig. 1B). While JNK1/SAPK and p38/RK/CSBP were only activated 3–5-fold over control levels, H2O2 stimulated a striking increase in ERK2 activity of 25-fold. Enhancement of ERK2 activity was evident with as little as 10 μM H2O2, with dose-dependent increases up to 200 μM H2O2. To determine if ERK2 activation constitutes a widespread cellular response to oxidants such as H2O2, we examined the effect of H2O2 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 H2O2 has been reported in SMC (17), we found significant ERK2 stimulation (20–50-fold) in all cell types tested, including these primary cultures (Fig. 2). The kinetics of ERK2 activation in NIH 3T3 cells following H2O2 exposure are shown in Fig. 3. H2O2 stimulation of ERK2 activity occurred within 5 min of treatment and was maximal by 10 min after H2O2 exposure (Fig. 3A). A rapid inactivation of ERK2 then ensued, with a return to basal ERK2 levels occurring within 30 min of H2O2 exposure. The increase in ERK2 kinase activity following H2O2 treatment was paralleled by a shift in the electrophoretic mobility of ERK2 protein seen on Western blots, indicating phosphorylation of ERK2 protein (Fig. 3B); however, the abundance of ERK2 protein expressed remained unchanged.

MAPK-dependent Gene Expression following H2O2 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 H2O2 on the expression of several early response genes. As shown in Fig. 4A, H2O2 treatment stimulated a transient but dramatic increase in the mRNA expression of MKP-1, c-jun, and c-fos. H2O2 also activated a fos promoter-luciferase reporter gene construct in transiently transfected HeLa cells (Fig. 4B). The dependence of fos promoter activation by H2O2 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 H2O2, 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$_2$O$_2$ 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$_2$O$_2$ in Fig. 3A suggests that protein phosphatases function in ERK2 regulation following H$_2$O$_2$. However, inhibition of protein synthesis by cycloheximide pretreatment did not affect the kinetics of ERK2 activation by H$_2$O$_2$ (Fig. 4C), 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$_2$O$_2$: 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$_2$O$_2$ 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$_2$O$_2$. 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$_2$O$_2$-stimulated ERK2 activation.

The nature of the chemical signal generated from H$_2$O$_2$ that initiates the ERK2 cascade was also investigated. Enhancing cellular antioxidant potential by pretreatment with the glutathione precursor N-acetyl-cysteine abolished the ability of H$_2$O$_2$ to stimulate ERK2 (Fig. 6). These results confirm that oxidant stress initiates ERK2 activation by H$_2$O$_2$. The iron chelator ω-phenanthroline also effectively inhibited ERK2 activation by H$_2$O$_2$ (Fig. 6), suggesting that metal-dependent reactions are required for kinase activation by H$_2$O$_2$. In the presence of metal ions, H$_2$O$_2$ 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$_2$O$_2$-mediated ERK2 activation. Taken together, these results suggest that H$_2$O$_2$ 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$_2$O$_2$.

Role of Ras in ERK2 Activation by H$_2$O$_2$—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$_2$O$_2$, 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$_2$O$_2$ (Fig. 7, left panel), suggesting that Ras is required for ERK2 activation by H$_2$O$_2$. 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$_2$O$_2$ 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$_2$O$_2$.

Effect of Modulating ERK Activation on Survival following H$_2$O$_2$ 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$_2$O$_2$ exposure in PC12/Ras-N-17 cells and wild-type PC12 cells. H$_2$O$_2$ 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$_2$O$_2$ than their wild-type counterparts as evidenced by reduced survival at identical doses of H$_2$O$_2$ (Fig. 8). Indeed, the LD$_{50}$ for H$_2$O$_2$ 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
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 H2O2 may not solely arise as a consequence to modulation of the ERK signaling pathway. In order to investigate the possible contribution of the J NK1/SAPK pathway to the enhanced sensitivity of PC12/Ras-N-17 cells to H2O2, we compared H2O2-stimulated J NK1/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 H2O2) by H2O2 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 H2O2, 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 H2O2, cell survival diminished accordingly in cell lines expressing wild-type MEK (MEKwt), constitutively active MEK (MEKca), or MEK lacking a kinase domain (MEKkd) (Fig. 9). However, the sensitivity to H2O2 was correlated with MEK activity; MEKca cells exhibited enhanced resistance, while MEKkd cells showed diminished resistance, as compared with MEKwt cells. This effect of MEK was evidenced by a separation in the dose-response curves for cell survival following H2O2 exposure in the three cell lines and in marked differences in the LD50 for H2O2. While 120 µM resulted in a 50% decrease in survival in MEKkd cells, 200 µM and 320 µM were required for the same effect in MEKwt and MEKca cells, respectively. Likewise, the same dose of H2O2 could differentially affect cell survival in the three cell lines: 180 µM H2O2 mediated a 10, 45, or 65% loss of survival in MEKca, MEKwt, and MEKkd cells, respectively. Comparable findings resulted from colony formation assays (data not shown). These results 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 H2O2 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 H2O2 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 H2O2 (Fig. 3) highlights the reversible and direct nature of alterations stimulated by H2O2 and underscores a role for phosphatases in regulating this response. However, the involvement of newly synthesized proteins in regulating ERK2 activation following H2O2 exposure is precluded by both the rapidity of inactivation and the cycloheximide insensitivity of ERK2 activation (Fig. 4C). 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 H2O2 (Fig. 4A).

The function of numerous cellular proteins, including transcription factors, calcium-regulatory proteins, and other cell and organelle surface molecules, is subject to redox regulation.
tracellular 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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Fig. 9. Effect of cellular MEK status on H2O2-stimulated loss of cell viability. NIH 3T3 cells expressing MEKwt, MEKca, or MEKkd were cultured overnight in 96-well plates, treated with H2O2 in complete medium, and stained 48 h later with crystal violet for assessment of cell viability. Values represent mean ± S.E. for seven wells.