Epidermal Growth Factor Receptor-dependent Akt Activation by Oxidative Stress Enhances Cell Survival*

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The serine/threonine kinase Akt (also known as protein kinase B) is activated in response to various stimuli by a mechanism involving phosphoinositide 3-kinase (PI3-K). Akt provides a survival signal that protects cells from apoptosis induced by growth factor withdrawal, but its function in other forms of stress is less clear. Here we investigated the role of PI3-K/Akt during the cellular response to oxidant injury. H2O2 treatment elevated Akt activity in multiple cell types in a time- (5–30 min) and dose (400 μM-2 mM)-dependent manner. Expression of a dominant negative mutant of p85 (regulatory component of PI3-K) and treatment with inhibitors of PI3-K (wortmannin and LY294002) prevented H2O2-induced Akt activation. Akt activation by H2O2 also depended on epidermal growth factor receptor (EGFR) signaling; H2O2 treatment led to EGFR phosphorylation, and inhibition of EGFR activation prevented Akt activation by H2O2. As H2O2 causes apoptosis of HeLa cells, we investigated whether alterations of PI3-K/Akt signaling would affect this response. Wortmannin and LY294002 treatment significantly enhanced H2O2-induced apoptosis, whereas expression of exogenous myristoylated Akt (an activated form) inhibited cell death. Constitutive expression of v-Akt likewise enhanced survival of H2O2-treated NIH3T3 cells. These results suggest that H2O2 activates Akt via an EGFR/PI3-K-dependent pathway and that elevated Akt activity confers protection against oxidative stress-induced apoptosis.

Oxidative stress poses a major threat to organisms living in an aerobic environment and is believed to play a causative role in many disease processes. Cells respond to oxidant injury with the activation of multiple signal transduction pathways that serve to coordinate the cellular response and ultimately determine the outcome. Depending on the particular stimulus encountered or the cell type involved, the response can range from proliferation and transformation, to growth arrest or cell death (1–3). Among the major signaling pathways and/or key mediators known to influence survival of cells subjected to oxidant injury are the phosphorylation cascades leading to activation of mitogen-activated protein kinases (MAPK)1 including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (4–6), NF-κB (7), and the tumor suppressor protein p53 (8).

The serine/threonine kinase Akt, also known as protein kinase B, was originally identified as the cellular homolog of the v-akt oncogene (9). It is activated via a phosphoinositide 3-kinase (PI3-K)-dependent signaling pathway when cells or tissues are exposed to growth factors, insulin, certain cytokines, and integrin-linked extracellular stimuli (10–16). Akt has received widespread attention as an important anti-apoptotic protein through which various survival signals suppress cell death induced by growth factor withdrawal, cell cycle discordance, and detachment of cells from their extracellular matrix (17–23). However, its potential role in influencing cell fate during other conditions of stress is less clear.

Several reports have shown that Akt can be activated by some stresses such as heat, hyperosmotic stress, H2O2, cadmium chloride (CdCl2), and sodium arsenite (24–27). However, other studies have provided evidence that apoptosis-inducing stresses including ceramide, hyperosmotic stress, UVC, and ionizing radiation result in down-regulation of the PI3-K/Akt pathway (28–30). Little is known regarding the mechanisms involved in altering Akt activity during stress or the functional significance of such changes in Akt activity. Indeed, the high concentrations of certain of the agents utilized to modulate Akt activity in many of the studies noted above raise concerns about the biologic relevance of the observations.

In the present study we sought to explore the mechanisms involved in activation of Akt during the cellular response to oxidant injury and to determine its influence on cell survival. By using H2O2 as a model oxidant, we show that sub-millimolar concentrations lead to activation of Akt in a variety of cell types, and we further provide genetic, enzymatic, and pharmacological evidence indicating that this occurs through an epidermal growth factor receptor (EGFR)/PI3-K-dependent signaling pathway. Most importantly, using various strategies to inhibit or enhance Akt activity, we present findings suggesting a pivotal role for the PI3-K/Akt pathway in promoting cell survival following oxidant injury.

EXPERIMENTAL PROCEDURES

Materials—Hydrogen peroxide (H2O2), phosphatidylinositol (PI), and phosphatidylinositol 3-phosphate (PIP) were purchased from Sigma. Histone H2B was from Roche Molecular Biochemicals. Wortmannin and LY294002 were from Calbiochem. Anti-hemagglutinin (HA) monoclonal antibody, 12CA5, was from Roche Molecular Biochemicals. The anti-Akt1, anti-ERK2, anti-JNK1, and anti-EGFR polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-active MAPK and anti-active JNK polyclonal antibodies were from Xcelligence Systems, Inc. (Männedorf, Switzerland).

The abbreviations used are: MAPK, mitogen-activated protein kinase; H2O2, hydrogen peroxide; PI3-K, phosphatidylinositol 3-kinase; H2B, histone H2B; MBP, myelin basic protein; PI, phosphatidylinositol; PIP, phosphatidylinositol 3-phosphate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; UVC, ultraviolet C; NAC, N-acetylcycteine; EGFR, epidermal growth factor receptor; HA, hemagglutinin; DAPI, 4′,6′-diamidino-2-phenylindole; Bis-Tris, 2-[bis(2-hydroxyethyl)aminoo]-2-(hydroxymethyl)propane-1,3-diol.
Promega (Madison, WI). The anti-rat PI3-K and anti-phosphotyrosine antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). The plasmid expressing hemagglutinin epitope (HA)-tagged Akt has been described (11). The plasmid expressing a dominant-negative mutant form of the p85 subunit of PI3-K (p85), which lacks a binding site for the Akt insert (a null) was a gift from Dr. Julian Downward (32).

**Cell Culture**—HeLa, A549, NIH3T3, NIH3T3/p-akt and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD). Jurkat cells were grown in RPMI 1640 (Biological Industries, Inc.). All media were supplemented with 10% fetal bovine serum. We have demonstrated that these conditions reflect true proliferation (6). 32P-Labeled protein was separated by 15% SDS-PAGE and detected by autoradiography, and the relative intensity of labeling was determined using a PhosphorImager.

**Antibodies**—The following antibodies were used: mouse anti-Akt, rabbit anti-phospho-Akt (Ser473), rabbit anti-phospho-ERK (Thr202/Tyr204), rabbit anti-phospho-JNK (Thr183/Tyr185), goat polyclonal anti-Akt or mouse monoclonal anti-HA (12CA5) (Biolabs Inc., Beverly, MA). ERK and JNK kinase activities were measured as described previously (6). 32P-labeled protein was separated by 15% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Immunoblot analysis was carried out using the appropriate antibodies. Specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

**Apoptosis and Colony Formation Assays**—HeLa cells were assessed for apoptosis by staining with 4'6'-diamidino-2-phenylindole (DAPI) as described previously (6). Apoptotic cells were scored based on the presence of highly condensed or fragmented nuclei. That the DAPI staining reflects true cell death was confirmed by FACS analysis (6).

**Western Blot Analysis**—Cells were washed in ice-cold PBS containing 100 μM Na3VO4, and then lysed in buffer containing 20 mM Heps, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM Na3VO4, 5 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin. Five hundred μg protein was incubated with 160 μl of 50% slurry of protein A-Sepharose for 4 h at 4 °C. Immune complexes were washed four times with the same lysis buffer and resuspended in 2× sample buffer. For Western analysis, samples were electrophoresed through 4–12% NuPAGE Bis-Tris gels (NOVEX, San Diego, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). Immunoblot analysis was carried out using the appropriate antibodies. Specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

**RESULTS**

**Characterization of Akt Activation by H2O2 in Different Cell Lines**—To examine the effect of H2O2 treatment on Akt activity, different cell types were exposed to various doses of the oxidant for 30 min, after which an immunocomplex kinase assay was used to assess Akt activity. The particular doses of H2O2 used were based on the relative sensitivity of the cells to toxic effects of the oxidant. As shown in Fig. 1A, endogenous Akt activity was stimulated by H2O2 treatment in all cell types, with the increase in activity ranging from 2- to 3-fold over untreated controls. UVC irradiation and several chemotherapeutic agents that are known to induce apoptosis were also examined for their abilities to activate Akt. Representative results shown in Fig. 1B indicate that, of the agents tested, only H2O2 resulted in significant activation of Akt, and none of the other agents lowered Akt activity.

To examine the kinetics and dose-response relationships for Akt activation by H2O2 treatment, NIH3T3 cells were transiently transfected with a hemagglutinin-tagged Akt (HA-Akt) expression vector, and 24 h later cells were treated either with 800 μM H2O2 for the indicated times or with various doses of H2O2 for 30 min. HA-Akt was then immunoprecipitated from cells using an anti-HA antibody, and kinase activity was determined. Activation of the HA-Akt was evident within 5 min exposure to 800 μM H2O2 (Fig. 2A). Maximum levels of Akt activity were seen 15–30 min after addition of H2O2, followed by a return to basal levels by 90 min. Akt activation was also dependent; activity increased approximately 2-fold with a dose of 400 μM and further increased to levels ~20-fold higher than those seen in control cells with a dose of 2 mM H2O2 (Fig. 2B). Western blot analysis with an anti-HA antibody was used to verify that equal amounts of HA-Akt protein were present in the immunoprecipitates regardless of the treatment conditions.

That the activation of Akt by H2O2 was the result of oxidant
injury was supported by experiments in which the ability of N-acetylcysteine (NAC) to block activation was assessed. NAC is a glutathione precursor and can enhance the antioxidant capacity of the cell both by acting directly as a scavenger of free radicals and by increasing glutathione levels (35). As shown in Fig. 2C, NAC treatment acted in a dose-dependent manner to inhibit Akt activation by H2O2 in both HeLa and NIH3T3 cells. This effect is most likely attributed to the free radical scavenging effect of NAC, rather than its modulation of glutathione levels, as the effects of NAC were rapid and other agents such as diethyl maleate and buthionine sulfoximine which deplete cellular glutathione did not alone lead to Akt activation (results not shown).

**Dependence of H2O2-induced Akt Activation on PI3-K**—Several strategies were employed to evaluate the role of PI3-K in the activation of Akt by H2O2. PI3-K consists of a regulatory subunit, p85, and a catalytic subunit, p110. In response to growth factor and cytokine treatment, phosphorylation of p85 leads to activation of p110. A dominant negative mutant form of p85 (Δp85), which lacks the binding site for p110, has previously been shown to inhibit growth factor-induced PI3-K activity (10, 31). Therefore, if PI3-K were involved in the activation of Akt by H2O2, the dominant negative p85 mutant should block this activation. As shown in Fig. 3A, transfection of cells with Δp85 along with an HA-Akt expression plasmid completely inhibited HA-Akt activation by H2O2 in NIH3T3 cells and decreased H2O2-induced HA-Akt activation by ~85% in HeLa cells. Further evidence for the involvement of PI3-K in H2O2-induced Akt activation was provided by treatment of cells with two potent inhibitors of PI3-K, wortmannin and LY294002. As shown, these agents abolished activation of Akt by H2O2 in both NIH3T3 and HeLa cells (Fig. 3A). Fig. 3B shows the dose-dependent effect of wortmannin in NIH3T3 cells where even 5 nM of the inhibitor led to greater than 40% reduction in H2O2-induced Akt activation.

To assess directly whether PI3-K was activated in response to H2O2 treatment, PI3-K was immunoprecipitated from H2O2-treated HeLa cells using an anti-p85 antibody and examined for kinase activity. As a positive control, insulin-treated HeLa cells were also examined. The results of a representative experiment are shown in Fig. 4. Although the effect of the oxidant was not as great as that seen with insulin treatment, a significant increase in PI3-K activity was observed with two different doses of H2O2 and at two different time points.

**Role of EGFR in Mediating H2O2-induced Akt Activation**—We and others (36–38) have previously provided evidence that growth factor receptors, and EGFR in particular, play an important role in mediating the activation of ERK MAPK in response to oxidant injury. Since PI3-K/Akt is also strongly activated by EGF stimulation, we investigated the possibility that EGFR plays a role in mediating Akt activation following H2O2 treatment. To demonstrate phosphorylation of EGFR by H2O2 treatment, EGFR was immunoprecipitated using an anti-EGFR antibody, and the precipitated proteins were then analyzed on Western blots using an anti-phosphotyrosine antibody. As shown in Fig. 5A, EGFR underwent rapid and
transient phosphorylation in response to H$_2$O$_2$ treatment. The kinetics of EGFR phosphorylation and its attenuation were consistent with those for H$_2$O$_2$-induced Akt activation. Evidence for the contribution of this EGFR phosphorylation in mediating Akt activation by H$_2$O$_2$ was provided using two different strategies. First, we examined the influence of several inhibitors of EGFR signaling on Akt activation by H$_2$O$_2$. The three agents used, PD153035, compound 56, and AG1478, all of which inhibit EGFR tyrosine kinase activity, were effective in inhibiting Akt activation in response to H$_2$O$_2$ treatment (Fig. 5B). The second strategy relied on the fact that continued presence of growth factors in the medium often results in down-regulation of receptor levels on the cell surface (36, 39). Such receptor down-regulation is associated with transient refractoriness to subsequent stimulation. Therefore, we examined the influence of EGF pretreatment on the ability of H$_2$O$_2$ to activate Akt. As shown in Fig. 5C, EGF pretreatment greatly inhibited activation of Akt in response to H$_2$O$_2$ treatment. Taken together, these findings indicate that the EGFR plays an important role in mediating Akt activation by oxidants.

PI3-K/Akt Activation Inhibits H$_2$O$_2$-induced Apoptosis—We have previously shown (6) that H$_2$O$_2$ treatment leads to death in a variety of other cell types and, in particular, apoptosis of HeLa cells. The importance of growth factor signaling pathways in contributing to survival is supported by the fact that suramin, a broad inhibitor of growth factor receptor, reduced survival of H$_2$O$_2$-treated cells (5). In keeping with our previous findings, treatment of HeLa cells with the EGF receptor tyrosine kinase inhibitor AG1478 likewise resulted in enhanced cell death; the percentage of apoptotic cells seen 24 h following treatment with 600 $\mu$M H$_2$O$_2$ increased from 47 $\pm$ 1.8% to 69.8 $\pm$ 6.6% ($n = 3$) in the presence of AG1478. Since the PI3-K/Akt pathway serves an anti-apoptotic function in some circumstances, and is activated downstream of the EGFR in the response to H$_2$O$_2$ treatment, we investigated the influence of PI3-K/Akt activation on H$_2$O$_2$-induced cell death. First, we examined whether inhibiting activation of the pathway would alter survival of H$_2$O$_2$-treated cells. Pretreatment of HeLa cells with either 100 nM wortmannin or 25 $\mu$M LY294002 (shown above to completely block Akt activation in response to H$_2$O$_2$ treatment) prior to the addition of 600 $\mu$M H$_2$O$_2$ led to a significant increase in apoptosis (Fig. 6). Importantly, these agents were not cytotoxic for cells when given alone. These findings suggest that PI3-K activity is important for survival during the cellular response to H$_2$O$_2$.

Several groups have reported the involvement of PI3-kinase in the activation of ERK and JNK (40–43). On the other hand, other studies have suggested that PI3-K may mediate its anti-apoptotic effects via inhibiting JNK activation (44). We have previously demonstrated that both ERK and JNK are activated in response to H$_2$O$_2$ treatment of HeLa cells and act in opposing directions to influence cell survival (5, 6); ERK was shown to inhibit apoptosis, whereas JNK promoted cell death. To rule out the possibility that the pro-survival effect of PI3-K observed in the present study might be attributed to effects on either of these pathways, we examined whether perturbation of PI3-K activity would affect ERK and JNK activities in HeLa cells. As shown in the top panel of Fig. 7A, neither wortmannin nor LY294002 treatment affected the magnitude of ERK and JNK activation seen in response to H$_2$O$_2$.

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negative mutant p85 (Δp85) or constitutively active mutant p110 (p110-CAAX) in HeLa cells also failed to alter JNK activation by H2O2 (Fig. 7A, lower panel). Similar results were obtained with H2O2-treated NIH3T3 cells when ERK and JNK activities were assessed using phospho-specific antibodies to recognize the activated forms of these proteins (Fig. 7B). Thus, the influence of PI3-K activity on cell survival is independent of the MAPK signaling pathways.

If Akt is a pro-survival signal during H2O2 treatment, then constitutive elevation of Akt activity would be expected to inhibit apoptosis of cells following treatment with the oxidant. To test this possibility, HeLa cells were infected with an adenovirus directing expression of myristoylated Akt (Ad.Akt), a constitutively active form of the kinase, prior to treatment with H2O2. Control cells received a similar adenoviral construct lacking the Akt insert (Ad.null). That the Ad.Akt-infected cells contain elevated amounts of Akt protein is shown at the top of Fig. 8A. The results of several experiments examining the influence of this elevated Akt expression on survival of cells subsequently treated with various doses of H2O2 are summarized in the graph. The Ad.Akt expressing cells showed approximately 40% fewer apoptotic cells compared with cells receiving the vector alone, indicating that elevated Akt activity protects cells against H2O2-induced apoptosis.
and LY294002, enhances H2O2-induced apoptosis in HeLa cells. HeLa cells were pretreated with either 100 nM wortmannin or 25 μM LY294002 for 30 min prior to addition of 600 μM H2O2. Apoptosis was assessed by DAPI staining 24 h later as described previously (33). Values are the means ± S.D. obtained from three independent experiments. p < 0.05 comparing values from H2O2 alone-treated and H2O2 plus wortmannin- or LY294002-treated cells.

We also performed experiments to compare survival of NIH3T3 cells with NIH3T3 cells stably expressing v-Akt, a constitutively active form of the kinase. Detachment of these cells from the plates precluded the assessment of survival via DAPI staining as we had done for HeLa cells. Therefore, we employed a clonogenic assay to examine the long term survival of control and v-Akt-expressing cells following treatment with various doses of H2O2. The results, presented in Fig. 8A, demonstrate greater survival in cells expressing the v-Akt. These findings further support the view that Akt provides a pro-survival signal during the cellular response to oxidative injury.

**DISCUSSION**

The major findings of this study are that Akt is activated by biologically relevant doses of H2O2, that this activation occurs via an EGFR/PI3-K-dependent mechanism, and most importantly, that Akt provides a pro-survival signal that can protect cells against oxidative stress.

Akt is an established downstream target of PI3-K following stimulation of cells with growth factors, and a role for Akt in promoting cell growth and protecting against growth factor withdrawal-induced apoptosis is well established (17, 18, 20, 22, 45, 46). Whether Akt activation occurs in response to other stressful stimuli is more controversial (24–26, 47). Several studies have reported activation of Akt by H2O2 as well as by other stresses that are known to exert their toxic effects, at least in part, through an oxidative stress mechanism (24–26).

In contrast, three recent studies have reported that ceramide and other stresses known to elevate cellular ceramide levels and induce apoptosis down-regulate Akt activity (28–30). In one such study, Zundel and Giaccia (29) provided evidence to indicate that UVC, ionizing radiation, and sorbitol all inhibit Akt, through stress-induced increases in ceramide that lead to inhibition of PI3-K and Akt. However, Zhou et al. (28) suggested that ceramide inhibits Akt independently of PI3-K. Although H2O2 was not examined in either of these studies, like the other stresses examined, it is known to induce sphingomyelin hydrolysis to generate ceramide (48) and, accordingly, would be expected to inhibit PI3-K and/or Akt. Clearly we did not observe such an effect, but rather we saw a significant activation of both PI3-K and Akt in H2O2-treated cells. It is also worth noting that, unlike the above-mentioned studies, we did not observe a down-regulation of Akt activity in response to UVC irradiation.

Several studies have inferred a role for PI3-K in the activation of Akt by H2O2 based on the sensitivity of Akt activation to inhibition by wortmannin. Our studies have confirmed these observations with pharmacological inhibitors and have provided additional evidence for the dependence of H2O2-induced Akt activation on PI3-K using genetic mutant forms of p85 that prevent PI3-K activation. Second, inhibition of PI3-K signaling has no effect on either ERK or JNK activation by H2O2, in NIH3T3 cells. NIH3T3 cells were either transfected with Δp85 or treated with 100 nM wortmannin. Thirty min after treatment with 600 μM H2O2 and 3 h later assayed for JNK activity. Anti-HA antibody was used to verify similar levels of HA-JNK expression in the different transfectants. Inhibition of PI3-K signaling has no effect on either ERK or JNK activation by H2O2. Inhibition of PI3-K signaling has no effect on either ERK or JNK activation by H2O2. Inhibition of PI3-K signaling has no effect on either ERK or JNK activation by H2O2.

**FIG. 7. Activation of ERK and JNK in response to H2O2 treatment occurs independently of the PI3-K/Akt signaling pathway.** A, upper panel, H2O2-induced ERK and JNK activations were assessed 30 min (ERK) or 3 h (JNK) later by immunocomplex kinase assays as described previously using myelin basic protein (MBP) and GST-c-Jun fusion protein as substrates for ERK and JNK, respectively. Lower panel, the level of JNK activation following H2O2 treatment is not affected by enhancement or inhibition of PI3-K activity. HA-tagged JNK was cotransfected into HeLa cells with either a plasmid expressing a dominant negative mutant form of p85 (Δp85), constitutively active mutant p110-CAAX, or empty control vector. Twenty-four h after transfaction, cells were treated with 600 μM H2O2 and 3 h later assayed for JNK activity. Anti-HA antibody was used to verify similar levels of HA-JNK expression in the different transfectants. B, inhibition of PI3-K signaling has no effect on either ERK or JNK activation by H2O2.

Several studies have suggested a role for PI3-K in the activation of Akt by H2O2 based on the sensitivity of Akt activation to inhibition by wortmannin. Our studies have confirmed these observations with pharmacological inhibitors and have provided additional evidence for the dependence of H2O2-induced Akt activation on PI3-K using genetic mutant forms of p85 that prevent PI3-K activation. However, our findings further support the view that Akt provides a pro-survival signal that can protect cells against oxidative stress.

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EGFR-dependent Akt Activation and H$_2$O$_2$-induced Apoptosis

Fig. 8. Akt overexpression confers a survival advantage to H$_2$O$_2$-treated cells. A, adenovirus-directed myristoylated-Akt expression inhibits apoptosis of HeLa cells in response to H$_2$O$_2$ treatment. HeLa cells were infected with 100 plaque-forming units/cell adenovirus expressing myristoylated Akt (Ad.Akt) or an empty adenovirus vector (Ad.null). Twenty four h after infection, cells were treated with the indicated concentrations of H$_2$O$_2$ and 24 h later were assessed for apoptosis by DAPI staining as described under “Experimental Procedures.” Western blot analysis verified increased Akt expression in Ad.Akt-infected cells. Quantitation of apoptosis in Ad.null (control) and Ad.Akt-infected cells is displayed graphically. Values shown are the means ± S.D. obtained from four independent experiments, $p < 0.05$ comparing number of apoptotic cells in myristoylated Akt-expressing clones relative to control cells with all H$_2$O$_2$ concentrations. B, colony formation assays demonstrating enhanced survival of v-Akt-expressing NIH3T3 cells in response to H$_2$O$_2$ treatment. Cells were treated with the indicated concentrations of H$_2$O$_2$ for 30 min. Twenty four h later, the cells were trypsinized and plated into 100-mm dishes. Colonies were counted 10–14 days after plating.

studies from our laboratory and others (36–38), showing that the EGFR plays an important role in mediating activation of ERK MAPK in response to oxidative stress. However, and importantly, the Akt and ERK activations rely on independent signaling pathways, as treatment of cells with wortmannin (which completely prevents Akt activation) did not alter ERK activation (Fig. 7). Likewise, treatment of cells with PD98059, a specific MEK1/2 inhibitor, that prevents ERK activation did not interfere with Akt activation by H$_2$O$_2$ (data not shown). A recent study has implicated focal adhesion kinase (FAK), a non-receptor tyrosine kinase, as the upstream mediator of PI3-K activation in T98 glioblastoma cells (27). It was shown that FAK underwent tyrosine phosphorylation in response to H$_2$O$_2$ treatment and associated with PI3-K in a time frame consistent with Akt activation. In that study, however, the kinetics of FAK phosphorylation and Akt activation (earliest activation occurring at 1 h and peaking at 4 h) were significantly delayed relative to what we have observed in our studies for both NIH3T3 and HeLa cells (activation seen as early as 5 min, peaking at 15–30 min, and returning to basal levels within 1 h of treatment). It is possible that different receptor and membrane-associated nonreceptor tyrosine kinases can contribute to the initiation of the response, dependent on the cell type and/or circumstances. In this regard, it is worth noting that we have obtained preliminary evidence that the platelet-derived growth factor receptor also undergoes phosphorylation in response to H$_2$O$_2$ treatment, and inhibitors capable of preventing this phosphorylation also partially inhibited Akt activation. Additional experiments will be required to understand better the role of growth factor receptors in mediating the response.

Finally, and most importantly, our study has provided the first direct evidence that Akt activation contributes to the survival of H$_2$O$_2$-treated cells. Prior to our study, all reports of Akt activation in response to H$_2$O$_2$ treatment involved H$_2$O$_2$ concentrations in excess of 1 mM, with most experiments performed with 5–10 mM H$_2$O$_2$. The use of such high concentrations raises concerns regarding the biologic relevance of the response as such concentrations can be markedly toxic for cells, leading to rapid necrosis. We have demonstrated that concentrations as low as 400 μM can result in activation of Akt. We had previously shown that H$_2$O$_2$ treatment leads to apoptosis of HeLa cells in a dose-dependent manner over a range of concentrations from 400 μM to 1.2 mM (6). That Akt activation occurs over a similar dose-response range supports its biologic relevance, and a role for Akt activation in protecting cells against H$_2$O$_2$-induced apoptosis was suggested by the finding that inhibition of Akt activation with PI3-kinase inhibitors (wortmannin and LY294002) and AG1478 (the EGFR tyrosine kinase inhibitor) led to enhanced apoptosis in response to H$_2$O$_2$ (Fig. 6). Direct evidence for the ability of Akt to confer protection against H$_2$O$_2$-induced apoptosis was obtained in two different cell types using two different strategies to constitutively elevate Akt activity as follows: infection of HeLa cells with adenovirus expressing myristoylated Akt, and constitutive expression of v-Akt in NIH3T3 cells. In both model systems, elevated Akt activity was associated with enhanced resistance of cells to apoptosis following H$_2$O$_2$ treatment.

An important question remaining is what are the downstream targets responsible for the protective influence of Akt? A number of different Akt targets have been identified that are believed to contribute to its anti-apoptotic function as follows: BAD, a proapoptotic member of the Bel-2 family; caspase-9, a protease that functions as an initiator and effector of apoptosis; forkhead, a transcription factor believed to regulate the activity of other apoptosis-related genes; and IKKα, a kinase involved in activation of NF-κB (49–53). The possible contribution of these downstream targets is currently under investigation. However, a role for IKKα appears to be ruled out by earlier studies from our laboratory (6) indicating that activation of NF-κB does not play a role in influencing survival of H$_2$O$_2$-treated HeLa cells.

In conclusion, although the precise mechanisms through which the PI3-K/Akt signaling pathway acts to modulate the
response to oxidants remains to be determined, our current findings provide strong support for a crucial role of the PI3-K/Akt pathway in regulating cellular protection during the response to oxidative stress. It is important to remember, however, that this is only one of many signaling pathways activated in response to oxidant injury. Some of these are generally pro-survival (e.g. ERK and NF-κB), whereas others appear to be pro-apoptotic (e.g. JNK and p53). Thus, the ultimate cellular outcome in a given cell type will reflect the relative balance between these various activities.

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