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Molecular Pathways Leading to Oxidative Stress-Induced Phosphorylation of Akt

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ABSTRACT

Oxidative stress can activate a variety of intracellular signaling pathways. The authors previously reported the CaM-K inhibitor KN-93 inhibited hydrogen peroxide-induced phosphorylation of Akt on threonine 308 (T308). In this report they demonstrate that phosphorylation of T308 in response to hydrogen peroxide treatment is not inhibited by LY294002, suggesting that phosphorylation of this residue in response to oxidative stress is largely PI3K independent. In contrast, hydrogen peroxide-induced phosphorylation of Akt on serine 473 (S473) was downregulated by both PI3K and CaM-K inhibition, indicating that hydrogen peroxide-induced phosphorylation of Akt on S473 was largely dependent on both PI3K and a CaM-K activity. Further, it is reported that $p56^{Lck}$ had a substantial role in hydrogen peroxide-induced phosphorylation of S473, but only a minimal role in hydrogen peroxide-induced phosphorylation of T308. These data suggest that in response to hydrogen peroxide, two pathways are activated in Jurkat T lymphocytes that converge to result in the phosphorylation of Akt on S473 and T308. One pathway involves the CaM-Ks that may directly phosphorylate Akt on T308. In this pathway, neither the Src kinases nor PI3K are required. The other pathway mediated by hydrogen peroxide results in the phosphorylation of Akt on S473 and requires CaM-K, PI3K, and Src activity. *Antioxid. Redox Signal.* 8, 1749–1756.

INTRODUCTION

THE PROTEIN KINASE AKT, an important intermediate in the phosphatidylinositol-3-kinase (PI3K) signaling pathway, is proposed to have a role in anti-apoptotic responses mediated via growth factor receptors (33, 41, 45). Full activation of Akt is achieved by phosphorylation of this serine/threonine kinase on two critical residues, threonine 308 (T308) and serine 473 (S473). However, partial activation of this kinase can be achieved by phosphorylation of T308 alone (2).

Akt is phosphorylated in response to many growth factors. Growth factor-induced receptor ligation results in the activation of PI3K, which phosphorylates the 3′ position of the phosphatidylinositol (PI) ring (14, 22, 33, 34, 39). Akt is able to bind to one of the products of this reaction [PI (3,4,5) P3] via its pleckstrin homology domains. Formation of PI (3,4,5) P3 also results in the membrane recruitment and activation of two kinases known as Phosphatidylinositol Dependent Kinase (PDK) 1 and 2 via their pleckstrin homology domains (1–4, 6, 42). PDK1 and PDK2 are reported to phosphorylate Akt on T308 (PDK1) and S473 (PDK2), resulting in Akt activation (1, 2, 4, 6, 42). Once activated, Akt can mediate anti-apoptotic activity via the phosphorylation of a number of downstream substrates such as the forkhead transcription factor and GSK-3β (20). Although growth factor-induced Akt phosphorylation appears to be due to the PI3K pathway, it is possible that other stimuli may induce phosphorylation of Akt by alternative pathways.

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Oxidative stress has been shown to induce a number of intracellular signaling pathways, including the PI3K pathway (7, 10, 26, 30, 32, 35, 37, 38). Cells can be exposed to oxygen radicals intrinsically through the leakage of electrons from the mitochondrial electron transport chain. These electrons can then pass directly to oxygen, forming O_2^-. Triggering of surface receptors on certain cells can also lead to the generation of oxygen radicals (29). Oxygen radicals also play a role in insulin (32), epidermal growth factor (EGF) (9), and vascular endothelial growth factor (VEGF) signaling (13), as well as in response to cytokines such as tumor necrosis factor alpha (TNF-α) (23) and interleukin 1β (IL-1β) (37). Extrinsic exposure to oxygen radicals can occur in inflammatory environments.

Our results, and those of others, demonstrated that Akt is phosphorylated in response to oxidative stress on T308 (25, 27). Qin *et al*. (38) demonstrated that PI3K is activated by hydrogen peroxide, and Wang *et al*. (47) demonstrated that inhibition of the PI3K pathway prevented hydrogen peroxide-induced Akt kinase activity. Although it is reported that PI3K can be activated in response to hydrogen peroxide and result in the phosphorylation of Akt on both S473 and T308, PI3K inhibitors have only been reported to prevent the phosphorylation of S473 to this stimuli.

Our previous results demonstrated that calcium/calmodulin-dependent kinases (CaM-K) were involved in hydrogen peroxide-induced phosphorylation of T308 on Akt (25). Others have demonstrated that CaM-KII and CaM-KK could directly phosphorylate T308 on Akt (36, 48). Together, these results suggest that the CaM-Ks can substitute for PDK1 in hydrogen peroxide-induced Akt phosphorylation. They also suggest that the phosphorylation of Akt on T308 may be independent of PI3K activation (*i.e.*, CaM-K-dependent), although maximal Akt kinase activity appears to require PI3K activation.

To gain a better understanding of hydrogen peroxide-induced Akt phosphorylation and activation, we examined the role of PI3K, CaM-K, and the Src family kinases in the phosphorylation and activation of Akt on both S473 and T308. We report that hydrogen peroxide-induced Akt phosphorylation on S473 required the functionality of all three of these kinases. Hydrogen peroxide-induced phosphorylation of Akt on T308 was CaM-K-dependent and did not require PI3K and $p56^{Lck}$ activity. These results suggest a role for the CaM-Ks in hydrogen peroxide-induced PI3K activation, as well as a direct role of CaM-Ks in the phosphorylation of Akt.

MATERIALS AND METHODS

Cells and reagents

The Jurkat and HuT 78 human T lymphocyte cell lines were obtained from ATCC (Rockville, MD). J.CaM1/Rep3 and J.CaM1/Lck human T lymphocyte cell lines were kindly provided to us by Dr. Art Weiss (UCSF, San Francisco, CA). The J.CaM1/Rep3 cell line represents $p56^{Lck}$-deficient Jurkat cells that are transfected with an empty Rep3 vector (44). These cell lines were cultured in RPMI 1640 with 5% fetal bovine serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. KN-92, KN-93, K252a, LY294002, PD98059, and PP2 were purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. Hydrogen peroxide (H_2O_2) and catalase were purchased from Sigma (St. Louis, MO). Rabbit anti-Akt, rabbit anti-phospho-T308-Akt, rabbit anti-phospho-S473-Akt, rabbit anti-I-κB, rabbit anti-glycogen synthase kinase-3 (GSK3) β, and rabbit anti-phospho-S21/9-GSK3α/β were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-$p56^{Lck}$ and monoclonal mouse anti-ERK2 antibodies were purchased from Zymed Laboratories (South San Francisco, CA). Rabbit anti-phospho-ERK1 and 2 antibodies were purchased from Promega (Madison, WI). Alkaline phosphatase (AP)-conjugated-goat anti-rabbit IgG (Fc), AP-conjugated-goat anti-mouse IgG (H+L), and NBT/BCIP Color Development Substrate (ProtoBlot® II AP System) were purchased from Promega.

Sample preparation for immunoblot analysis

Sample preparation was carried out essentially as previously described (8, 16, 17). Cells were washed and resuspended in RPMI 1640 with 5% FBS. One ml, containing 1.25 $\times$ 10^6 cells, was added to microfuge tubes and placed at 37°C for at least 30 min prior to the start of the experiment. Cells were stimulated with various treatments by adding 10 μl of the stimulant to the tube and then vortexing. Following stimulation, tubes were microfuged for 30 sec at 14,000 rpm, supernatants were aspirated, cell pellets were resuspended in 110 μl of cold lysis buffer (25 m*M* Tris-hydrochloric acid, pH 7.4; 50 m*M* sodium chloride; 2% IGEPAL® CA-630; 0.2% sodium dodecyl sulfate; 0.5% sodium deoxycholate; 1 m*M* phenylmethylsulfonyl fluoride; 50 μg/ml aprotinin; 50 μ*M* leupeptin; 0.5 m*M* sodium orthovanadate), and then placed on ice for 15 min. Lysates were microfuged for 10 min at 14,000 rpm, supernatants (98 μl) were removed and mixed with 42 μl of 3.3X sample buffer (200 m*M* Tris-hydrochloric acid, pH 6.8; 33% glycerol; 6.6% sodium dodecyl sulfate; 16.6% 2-mercaptoethanol; 0.04% bromophenol blue). Samples were boiled for 5 min and frozen.

Immunoblot analysis

Immunoblot analysis was carried out essentially as previously described (8, 16, 17). Fifteen microliters of prepared lysates were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel, and proteins were electrophoretically transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C in blocking buffer (25 m*M* Tris-hydrochloric acid, pH 8.0; 125 m*M* sodium chloride; 0.1% Tween® 20; 1% bovine serum albumin; 0.1% sodium azide). Membranes were then incubated for 2 h with the primary antibody diluted in blocking buffer [anti-Akt, anti-phospho-T308-Akt, anti-phospho-S473-Akt, rabbit anti-GSK3β, anti-phospho-S21/9-GSK3α/β, anti-ERK2 (all at 1:1000), anti-$p56^{Lck}$, anti-I-κB (at 1:2000), anti-phospho-ERK1 and 2 (1:5,000)]. The blots were washed twice in Tris buffered saline with Tween® 20 (TBST) [25 m*M* Tris-HCl, pH 8.0; 125 m*M* NaCl; 0.025% Tween 20] and incubated with AP-conjugated goat anti-rabbit (anti-Akt, anti-phospho-T308-Akt, anti-phospho-S473-Akt, anti-phospho-ERK1 and 2, and anti-I-κB) or goat

anti-mouse (anti-p56^{Lck} and anti-ERK2) immunoglobulin (Promega) (1:10,000 in TBST) for 1 h at room temperature. The blots were washed twice in TBST and developed with the colorogenic substrates BCIP and NBT (Promega Proto-Blot® II AP System).

RESULTS

Hydrogen peroxide stimulation induces phosphorylation of Akt on residues T308 and S473 in Jurkat and HuT 78 cells

To determine if Akt was phosphorylated on T308 and S473 in Jurkat T lymphocytes, we treated cells with hydrogen peroxide for 1 to 60 min. Lysates were prepared from the cells and then subjected to immunoblotting using total-Akt, phospho-T308-Akt, and phospho-S473-Akt. As shown by the immunoblots in Fig. 1A, hydrogen peroxide induced the phosphorylation of both T308 and S473. The phosphorylation of both of these residues occurred very rapidly in response to hydrogen peroxide and could be noted within a minute. The phosphorylation of S473 appeared to be more transient peaking at 1–5 min and declining to background values after 15 min. Phosphorylation of T308 also peaked at 1–5 min, but was more prolonged with increased phosphorylation still evident at 60 min poststimulation. Since the Jurkat cell line lacks PTEN, we wanted to determine if the expression of PTEN modified our response. To do this, we carried out similar experiments on the PTEN-positive T cell line, HuT 78. The overall response of the HuT 78 cells was similar to the Jurkat cells (Fig. 1B). The kinetics of the phosphorylation in HuT 78 cells, however, was more transient than Jurkat cells in regards to phosphorylation of residue T308. The addition of catalase to cultures prior to stimulation with hydrogen peroxide blocked the increase in phosphorylation of both of these residues, indicating that these effects were mediated via hydrogen peroxide (data not shown).

Hydrogen peroxide induces the phosphorylation of GSK-3β

We next wanted to determine if hydrogen peroxide-induced phosphorylation of these two residues on Akt correlated with increased Akt activity. GSK-3β is a downstream target of Akt. To determine if Akt was activated in response to oxidative stress, we measured GSK-3β phosphorylation in these cells using phospho-specific antibodies. It would appear that hydrogen peroxide treatment of Jurkat cells results in Akt activity as indicated by the phosphorylation of GSK-3β (Fig. 2). Phosphorylation of the GSK-3β protein was rapid, occurring within a minute, and transient. It should be noted that we have observed a reproducible slight increase in the amount of total GSK-3β recognized in these samples following treatment with hydrogen peroxide. We do not know the cause for this, as other proteins do not exhibit increases. Similar to the phosphorylation of GSK-3β, a related protein, GSK-3α, which differs in its cellular localization, was also phosphorylated (Fig. 2).

KN-93 decreases hydrogen peroxide-induced phosphorylation of Akt at both the T308 and S473 phosphorylation sites in Jurkat cells

We had previously demonstrated that hydrogen peroxide-induced phosphorylation of Akt on residue T308 was sensitive to inhibition by the CaM-K inhibitors KN-93 and K252a [(23) and Fig. 3]. When we examined the ability of KN-93 and K252a to inhibit phosphorylation of S473, using immunoblot analysis, a strong inhibition of S473 with KN-93 and a partial inhibition following treatment with K252a was observed (Fig. 3). The inactive analog of KN-93, KN-92, did not affect hydrogen peroxide-induced Akt phosphorylation nor did any of the treatments influence the level of Akt expressed. These data suggests that a CaM-K-dependent pathway is mediating Akt phosphorylation in response to oxidative stress.

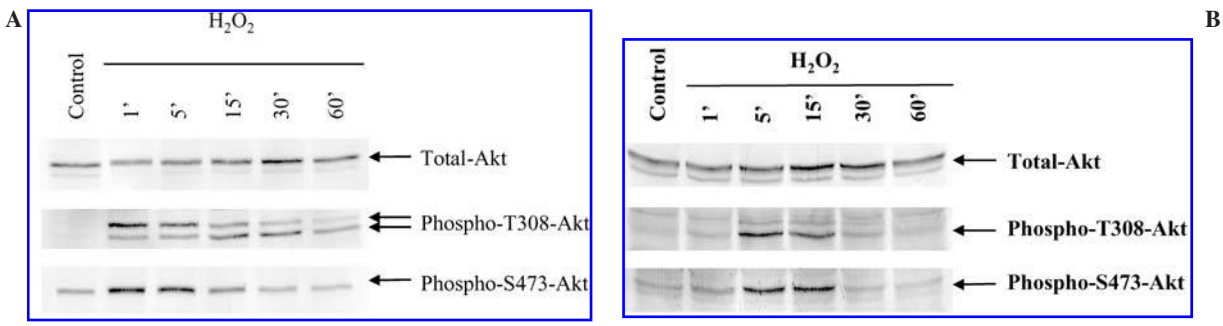

FIG. 1. Hydrogen peroxide phosphorylation of Akt on both T308 and S473. Jurkat (**A**) or HuT 78 (**B**) cells were washed and resuspended in serum-free RPMI 1640 at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with 5 m*M* hydrogen peroxide (H_2O_2) for the indicated periods of time. Cellular lysates were prepared and subjected to immunoblot analysis using an antibody against total-Akt, phospho-T308-Akt, and phospho-S473-Akt. Immunoblotting was performed as previously described (18, 19, 21).

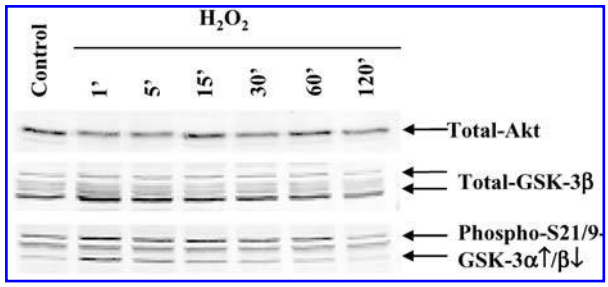

FIG. 2. Hydrogen peroxide phosphorylation of GSK-3β. Jurkat cells were washed and resuspended in serum-free RPMI 1640 at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with 5 m*M* hydrogen peroxide (H_2O_2) for the indicated periods of time. Cellular lysates were prepared and subjected to immunoblot analysis using an antibody against total-Akt, total-GSK-3β, and phospho-S21/9-GSK-3α/β. Immunoblotting was performed as previously described (18, 19, 21).

$p56^{Lck}$ is a crucial intermediate in the phosphorylation of Akt on S473 but not T308

We first determined if hydrogen peroxide induced the phosphorylation of $p56^{Lck}$. $p56^{Lck}$ phosphorylation can be detected by decreased mobility of $p56^{Lck}$ on immunoblots. Jurkat T lymphocytes were stimulated with hydrogen peroxide in the presence of DMSO, KN-93, or the inactive analog of KN-93, KN-92. Lysates were then prepared and subjected to immunoblot analysis using an antibody that recognizes all forms (both phosphorylated and nonphosphorylated) of $p56^{Lck}$. Shifts in mobility of $p56^{Lck}$ were evident following treatment with hydrogen peroxide, indicating that $p56^{Lck}$ was phosphorylated in response to oxidative stress (Fig. 4). Hydrogen peroxide-induced shifts in the mobility of $p56^{Lck}$ were blocked by KN-93, indicating a role for a CaM-K in this response (Fig. 4). A slight decrease in hydrogen peroxide-induced shifts in $p56^{Lck}$ mobility could be noted with the inactive analog; however, this decrease was not as great as the decrease seen with the active CaM-K inhibitor, KN-93 (Fig. 4).

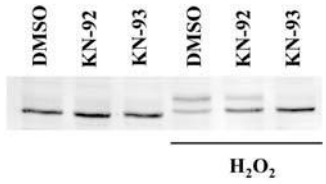

FIG. 4. KN-93 decreases hydrogen peroxide-induced shifts in mobility of $p56^{Lck}$ on immunoblots. Jurkat cells were washed and resuspended in RPMI 1640 at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with KN-92 (5 μ*M*) or KN-93 (5 μ*M*) for 30 min. The cells were then stimulated with 5 m*M* hydrogen peroxide (H_2O_2) for 5 min. Cellular lysates were prepared and subjected to immunoblot analysis using antibodies against $p56^{Lck}$. Immunoblotting was performed as previously described (18, 19, 21).

J.CaM1/Rep3 cells are a Jurkat cell derivative that lacks $p56^{Lck}$. When we compared hydrogen peroxide-induced phosphorylation in $p56^{Lck}$-negative cells with Jurkat cells that express $p56^{Lck}$, we found that Akt is phosphorylated at residue T308 in response to hydrogen peroxide in both Jurkat and J.CaM1/Rep3 cells, but to a slightly lesser extent in the latter (Fig. 5). The hydrogen peroxide-induced increase in the phosphorylation of Akt at residue S473 occurred in the Jurkat cells, but was completely absent in the $p56^{Lck}$-deficient cell line J.CaM1/Rep3 (Fig. 5). Transfection of $p56^{Lck}$ into the J.CaM1/Rep3 cells restored hydrogen peroxide-induced increases in Akt S473 phosphorylation.

We also measured the phosphorylation of both T308 and S473 in response to hydrogen peroxide in the presence of Src kinase (PP2) and PI3K (LY294002) inhibitors. Both PP2 and LY294002 completely blocked hydrogen peroxide-induced

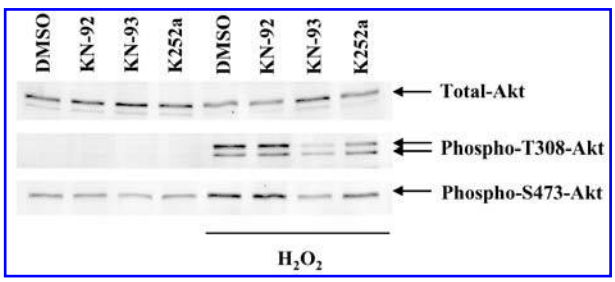

FIG. 3. KN-93 decreases hydrogen peroxide-induced phosphorylation of Akt at both T308 and S473 phosphorylation sites in Jurkat cells. Jurkat cells were washed and resuspended in RPMI 1640 at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with KN-92 (5 μ*M*), KN-93 (5 μ*M*), or K252a (25 n*M*) for 30 min. The cells were then stimulated with 5 m*M* hydrogen peroxide (H_2O_2) for 5 min. Cellular lysates were prepared and subjected to immunoblot analysis using antibodies against total-Akt, phospho-T308-Akt, and phospho-S473-Akt. Immunoblotting was performed as previously described (18, 19, 21).

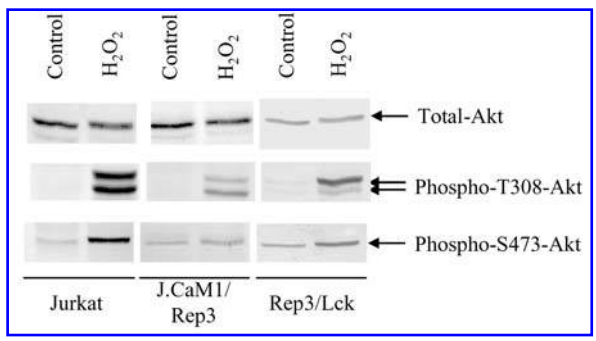

FIG. 5. The $p56^{Lck}$-negative Jurkat cell derivative J.CaM1 fails to phosphorylate Akt on S473 in response to hydrogen peroxide. Jurkat, Rep3/Lck, and J.CaM1/Rep3 cells were washed and resuspended in RPMI 1640 at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated for 5 min with 5 m*M* hydrogen peroxide (H_2O_2). Cellular lysates were prepared and subjected to immunoblot analysis using antibodies against total-Akt, phospho-T308-Akt, and phospho-S473-Akt. Immunoblotting was performed as previously described (18, 19, 21).

phosphorylation of Akt at S473, but not at T308 in Jurkat cells (Fig. 6). These data suggest that PI3K and p56^{Lck} are largely required for S473 phosphorylation of Akt but not for phosphorylation of T308. They also suggest that there is a PI3K-independent CaM-K-dependent pathway leading to the phosphorylation of Akt on T308.

PD98059 does not inhibit phosphorylation of Akt or mobility shifts in p56^{Lck}

Reactive oxygen intermediates are well known to induce activation of the ERK signaling pathway. We determined if oxidative stress-induced ERK activation had a role in Akt phosphorylation at either of these two residues. Inhibition of the ERK signaling pathway using PD98059 resulted in decreased phosphorylation of ERK in response to hydrogen peroxide (Fig. 7); however, it did not influence the phosphorylation of Akt on either S473 or T308. We also determined if ERK had any role in p56^{Lck} phosphorylation. PD98059 did not have any influence on the hydrogen peroxide-induced shifts in p56^{Lck} mobility. These results strongly indicate that the ERK signaling pathway is not involved in oxidative stress-induced Akt phosphorylation.

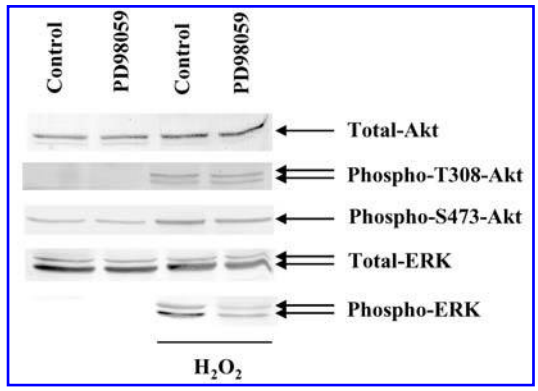

FIG. 7. PD98059 fails to block phosphorylation of Akt or mobility shifts in p56^{Lck}. Jurkat cells were washed and resuspended in RPMI 1640 containing 5% FBS at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with PD98059 (10 μ*M*) for 30 min. The cells were then stimulated with 10 m*M* hydrogen peroxide (H_2O_2) for 5 min. Cellular lysates were prepared and subjected to immunoblot analysis using antibodies against total-Akt, phospho-T308-Akt, phospho-S473-Akt, ERK, and phospho-ERK.

DISCUSSION

In agreement with our previous report (25), we found that Akt is phosphorylated in response to hydrogen peroxide on residue T308 and that this phosphorylation was rapid and transient (Fig. 1). We have now extended those findings and demonstrate that phosphorylation of Akt on S473 also occurred in response to oxidative stress (Fig. 1). A background of phospho-S473 staining could often be noted in unstimulated Jurkat cells. This could be due to the fact that Jurkat cells are PTEN-negative and may have some level of constitutive PI3K activation. The kinetics of the phosphorylation in HuT 78 cells was more transient than Jurkat cells in regards to residue T308. This also may be due to the expression of PTEN, but the reason for this difference is not known. Although phosphorylation of Akt is noted at both sites, it is possible that additional unknown modifications occur in response to hydrogen peroxide. Perhaps hydrogen peroxide induces phosphorylation at other residues that may potentially inhibit the activity of Akt. The results in Figs. 1 and 2 confirm that hydrogen peroxide can induce Akt phosphorylation/activation and are in agreement with the results of Salsman *et al*. (40).

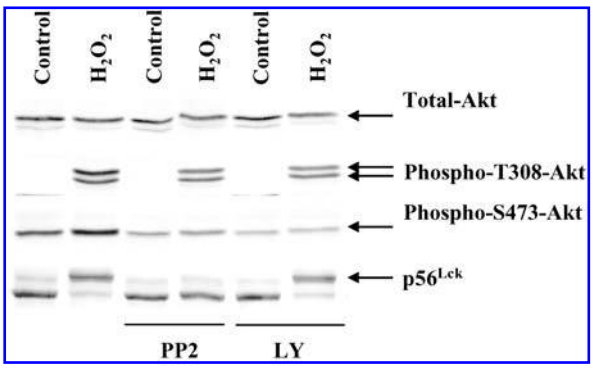

FIG. 6. PP2 and LY294002 decrease the hydrogen peroxide-induced phosphorylation of Akt at S473 in Jurkat cells. Jurkat cells were washed and resuspended in RPMI 1640 containing 5% FBS at 1.25 × 10^6 cells/ml. The cells were warmed to 37°C and treated with PP2 (10 μ*M*) or LY294002 (LY, 10 μ*M*) for 30 min. The cells were then stimulated with 10 m*M* hydrogen peroxide (H_2O_2) for 15 min (total-Akt, phospho-T308-Akt, p56^{Lck}) or 5 min (phospho-S473-Akt). Cellular lysates were prepared and subjected to immunoblot analysis using antibodies against total-Akt, phospho-T308-Akt, phospho-S473-Akt, and p56^{Lck}.

Hydrogen peroxide-induced phosphorylation of Akt on residue T308 was sensitive to inhibition by the CaM-K inhibitors KN-93 and K252a (Fig. 3). This is in agreement with the reports of others that demonstrated that both CaM-KK and CaM-KII could phosphorylate this residue (36, 48). In our previous studies, we did not measure the ability of KN-93 to inhibit the phosphorylation of S473 (25). The data in this report indicate that a CaM-K is required for the hydrogen peroxide-induced phosphorylation of Akt on both S473 and T308. Furthermore, these data suggest a role for the CaM-Ks in hydrogen peroxide-induced PI3K activation.

We previously published that ionomycin would induce shifts in the mobility of a src family kinase, p56^{Lck}, and that the ionomycin-induced shifts were dependent on the CaM-Ks (21). Other investigators have demonstrated Src involvement in PI3K activation (15, 43, 46). Hydrogen peroxide induces shifts in the mobility and increases in the kinase activity of p56^{Lck} (5, 24, 28, 31). Our data imply that p56^{Lck} is an important component leading to the phosphorylation of Akt on residue S473. The finding that S473 phosphorylation was decreased in p56^{Lck}-negative cells and that the phosphorylation of T308 remained largely intact suggested that phosphoryla-

tion of these two residues may be partially independent of each other.

Because the Src kinases have been proposed to have a role in PI3K activation (15, 43, 46) and because LY294002 also inhibited phosphorylation of S473 while phosphorylation of T308 was largely intact; it is suggested that hydrogen peroxide, in a CaM-K-dependent manner, activates a Src kinase, which leads to PI3K activation. We do not know at this time if this response is specific to the Src kinase $p56^{Lck}$. Although the J.CaM/Rep3 cell line does not express $p56^{Lck}$, it is reported to express other Src kinases such as $p66^{Src}$ (11), which do not appear to substitute for the $p56^{Lck}$ deletion in these cells.

A proposed model of hydrogen peroxide-induced Akt phosphorylation is presented in Fig. 8. Our data indicate that when PI3K or Src are inhibited, S473 phosphorylation is also prevented. The CaM-K inhibitors block the phosphorylation of Src, preventing the activation of PI3K. Blocking CaM-K activation also prevents the CaM-Ks from directly phosphorylating T308. However, inhibition of PI3K or Src activity leaves hydrogen peroxide-induced CaM-K activity and T308 phosphorylation intact.

We have mapped some of the signaling components that are involved in the phosphorylation of Akt in response to oxidative stress. It was reported that treatment of keratinocytes with hydrogen peroxide can increase their resistance to apoptotic stimuli through increased activation (12). Since many chemotherapeutic treatments (such as ionizing radiation, doxorubicin, and photodynamic therapy) generate oxygen radical production, understanding how oxidative stress can activate anti-apoptotic pathways could lead to a mechanism of sensitizing cells toward these different cancer treatments. We propose that the CaM-Ks may make good targets to sensitize cancer cells to certain cancer treatments that kill the cells by generating oxidative stress.

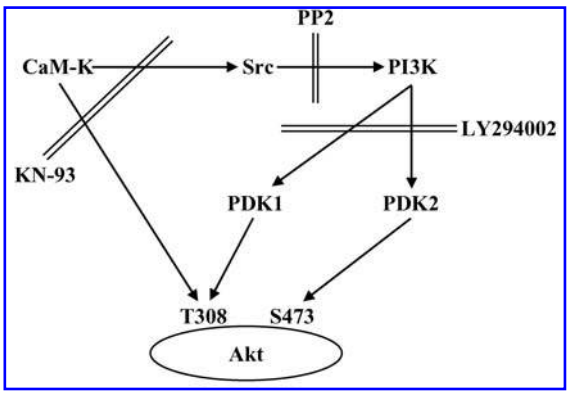

FIG. 8. The proposed pathways by which hydrogen peroxide leads to Akt phosphorylation on T308 and S473. The phosphorylation of T308 is independent of PI3K and a Src family kinase; however, it is dependent on a CaM-K. Phosphorylation of S473 is dependent on Src and PI3K and the activation of both of these pathways is downstream of a CaM-K. The Src/PI3K pathway likely mediates some T308 phosphorylation; however, phosphorylation of this residue is not dependent on either Src family kinase activity or PI3K.

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ABBREVIATIONS

AP, alkaline phosphatase; BCIP, 5-bromo,4-chloro,3-indolylphosphate; CaM, calmodulin; CaM-K, calcium/calmodulin-dependent kinases; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; GSK, glycogen synthase kinase; IgG, immunoglobulin G; ILK, integrin-linked kinase; IL1-β, interleukin 1 beta; NAC, *N*-acetyl cysteine; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NBT, nitroblue tetrazolium; O_2^-, superoxide ion; PDK, proline directed kinase; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tension homolog deleted from chromosome 10; S473, serine 473; T308, threonine 308; TBST, Tris buffered saline with Tween® 20; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor.

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