Caspase and Reactive Oxygen Species Generated by \( \text{H}_2\text{O}_2 \) Induce Caspase-3-independent Degradation of Akt/Protein Kinase B*

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This study was designed to elucidate the mechanisms leading to down-regulation of the Akt/protein kinase B (PKB) survival pathway during \( \text{H}_2\text{O}_2 \)-induced cell death. \( \text{H}_2\text{O}_2 \) produced early activation of Akt/PKB and also DNA damage that was followed by stabilization of p53 levels, formation of reactive oxygen species (ROS), and generation of ceramide through activation of a glutathione-sensitive neutral sphingomyelinase. These events correlated with long term dephosphorylation and subsequent degradation of Akt. A membrane-targeted active Akt version attenuated apoptosis but not necrosis induced by \( \text{H}_2\text{O}_2 \) and was more resistant to dephosphorylation and proteolysis induced by apoptotic concentrations of \( \text{H}_2\text{O}_2 \). Proteolysis of Akt was prevented by exogenous addition of glutathione, indicating a role of ROS and ceramide in Akt degradation. However, Akt was degraded similarly in cells transfected with wild type and dominant negative p53 mutant, indicating that degradation of Akt under oxidative injury may be p53-independent. Specific inhibitors of caspase groups I and III prevented proteolysis of Akt/PKB and poly(ADP-ribose) polymerase in cells submitted to apoptotic but not necrotic \( \text{H}_2\text{O}_2 \) concentrations. Surprisingly, in caspase-3-deficient MCF-7 cells Akt was more sensitive to \( \text{H}_2\text{O}_2 \)-induced degradation than the caspase-3 substrate poly(ADP-ribose) polymerase. Moreover, the Akt/PKB double mutant Akt(D108A,D119A), which is not cleaved by caspase-3, and a triple mutant (D453A,D455A,D456A), which lacks the consensus sequence for caspase-3 cleavage, were also degraded in \( \text{H}_2\text{O}_2 \)-treated cells. Our results suggest that strong oxidants generate intracellular ROS and ceramide which in turn lead to down-regulation of Akt by dephosphorylation and caspase-3-independent proteolysis.

Although molecular oxygen is critically required for aerobic life, mitochondrial respiration in higher organisms constantly generates low levels of potentially dangerous reactive oxygen species (ROS), which include superoxide anion. Mitochondrial and cytosolic superoxide dismutases convert superoxide into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), a non-radical molecule that generates highly toxic hydroxyl radicals via the Fenton reaction (1). Neurons of the central and peripheral nervous system are particularly sensitive to oxidative damage. Cell death derived from high ROS levels has been associated with a number of neurodegenerative disorders including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (2) as well as the general decline of the central nervous system function frequently associated with senescence (3).

In the nervous system, neurotrophins enhance tolerance to oxidative stress in both animal and cellular models (4, 5). A prominent mechanism involved in neurotrophin-induced cell survival includes activation of the PI3K and Akt/protein kinase B signaling pathway. The survival signal elicited by Akt proceeds through several mechanisms including inactivation of BAD and caspase-9, stimulation of nuclear factor-κB activity, inactivation of forkhead transcription factor, and phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) and glycogen synthase kinase 3 (GSK3) kinases (for recent reviews, see Refs. 6 and 7). Akt exerts protective actions against oxidative damage in central and peripheral neurons. For instance, neuregulin prevents \( \text{H}_2\text{O}_2 \) induction of ROS in a PI3K-dependent manner (8), and loss of oxidative stress tolerance with aging has been linked in part to reduced Akt kinase activity in old rats (9). Concerning neurodegeneration, we have reported previously the protective effect of active Akt1 against peptides of β-amyloid protein characteristic of senile plaques found in the brains of Alzheimer’s disease patients (10) and against the Parkinson-inducing toxin 1-methyl-4-phenylpyridinium (11).

When cells are exposed to oxidative injury they activate multiple signaling pathways that dictate whether those cells will ultimately tolerate or succumb to the insult. Akt/protein kinase B appears to be one such pathway that becomes activated by ROS-generating agents (10, 12) and by \( \text{H}_2\text{O}_2 \) (13–15). Activation of Akt by ROS may reflect an attempt of the damaged cells to survive the oxidative insult. On the other hand, the best characterized signaling pathways that are activated by oxidative injury are those leading to cell death. Stress-triggered apoptosis induces cytochrome c release from mitochondria and activation of caspase cascades that execute the apoptotic program (16). In addition, oxidative stress also produces EGFP, enhanced green fluorescent protein; 2×m, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; mter-, myristoylated; NGF, nerve growth factor; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; FQ, phycoerythrin; P13K, phosphatidylinositol 3 kinase; PKB, protein kinase B; ZVAD, benzoyloxycarbonyl-Ala-Val-Ala-Asp(Ome)-fluoromethyl ketone; YVAD, acetyl-Tyr-Val-Ala-Asp(Ome)-fluoromethyl ketone; DEVD, benzoyloxycarbonyl-Asp(Ome)-Glu(Ome)-Val-Ala-Asp(Ome)-fluoromethyl ketone.
genotoxic damage that results in stabilization of p53 levels and cell death (17).

The fact that oxidative stress activates contradictory signaling pathways of survival and death implies that there must be sophisticated cross-talk between these opposite signals that dictate cells fate. The regulation of the apoptosis machinery by Akt is being explored in detail and, as stated above, a number of Akt substrates have been identified as elements of the initiation and execution phases of apoptosis. In general, phosphorylation of these proteins leads to their inactivation. However, very little is known about the mechanism(s) employed by the apoptotic machinery to down-regulate the Akt survival signal. We and other groups have reported the enhanced dephosphorylation of Akt are more ill defined, but Akt appears to be a substrate of caspase-3 in vitro (21), and caspase-3-dependent cleavage of Akt may be relevant in detachment-induced and Fas ligand-induced cell death (22, 23).

Because cells submitted to prolonged oxidative stress ultimately die, despite the initial activation of Akt, the long term effects of these insults leading to down-regulation of the PI3K/Akt survival pathway are particularly relevant and require further investigation. We have addressed this question in PC12 cells submitted to H$_2$O$_2$, a well reported activator of Akt which induces oxidative stress, senescence, and cell death. Our findings indicate that Akt is activated rapidly in response to H$_2$O$_2$, and apoptosis resumes at a time when Akt activity has been completely depleted by dephosphorylation and proteolysis. These results are relevant to an understanding of oxidative stress-induced cell death and for devising strategies that might promote cell survival in the nervous system under the presence of oxidative insults.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Reagents**—PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7.5% fetal bovine serum, 7.5% heat-inactivated horse serum, and 80 μg/ml gentamicin. Neuro 2A and MCF-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and 80 μg/ml gentamicin. Neuro 2A and MCF-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 7.5% heat-inactivated horse serum, and 80 μg/ml gentamicin. Neuro 2A (18) hAkt1(D453A,D455A,D456A) (21), or with expression vectors pcDNA3-EGFP-Akt1 and pcDNA3HA-Akt1 (11), or with expression vectors pCEFL(X-)EGFP, pCEFL(X-)EGFP-Akt1, pCEFL(X-)myr-Akt1 (Qiagen). All reagents were purchased from Sigma except NGF, bacteria.

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Oxide, 50 mM NaCl, 51 mM imidazol, 1 mM EDTA, 12.5 mM MgCl\(_2\), 2 mg dithiothreitol, 0.7% glycerol, 70 \(\mu\)M \(\beta\)-mercaptoethanol, 500 \(\mu\)M ATP, 5 \(\mu\)Ci/100 \(\mu\)l [\(^{32}\)P]ATP, and 35 ng of diacylglycerol kinase was added to each sample. Reactions were incubated at 30 °C for 30 min and stopped by the addition of 170 \(\mu\)l of stop buffer (135 mM NaCl, 1.5 mM Hepes, pH 7.2) and 30 \(\mu\)l of 100 mM EDTA. Lipids were extracted again with 1 ml of chloroform, methanol, and 1 N HCl (100:100:1), resuspended in 40 \(\mu\)l of chloroform, spotted onto silica gel TLC plates (Whatman Inc.), and resolved using chloroform:methanol:acetic acid (65:15:5) as solvent. Samples were measured in an Instant-Imager (Packard, Canberra, Australia). Quantification of ceramide mass was obtained by comparison with a standard curve ranging from 0 to 1,000 pmol of purified ceramide (Sigma), which was processed in parallel to the samples.

Statistical Analyses—Student’s \(t\) test was used to assess differences in hydroethidine staining and accumulation of ceramide between control and \(H_2O_2\)-treated groups and between control and GSH-treated groups, respectively. A \(p < 0.01\) was considered significant. Unless indicated, all experiments were performed at least three times with similar results. The values in graphs correspond to the average of at least three samples. Bars indicate standard deviation.

RESULTS

Short Term Effects of \(H_2O_2\) on PC12 Cells: Activation of Akt and Induction of Genotoxic Damage—First we analyzed the effect of \(H_2O_2\) on Akt kinase activity and the signaling mechanisms leading to Akt activation under our experimental conditions. Serum-starved PC12 cells were pretreated with vehicle, wortmannin, or LY294002 for 15 min and then incubated with \(H_2O_2\) or NGF for 5 min. Akt activity was measured in \textit{in vitro} immunocomplex kinase assays using histone H2B as substrate. As indicated in Fig. 1A, \(H_2O_2\) and NGF stimulated Akt activity in PC12 cells, and the two structurally unrelated inhibitors of PI3 kinase, wortmannin and LY294002, dramatically prevented the activation of Akt by both inducers. Similar results were obtained when Akt activity was monitored as a function of phosphorylation at Ser473 or Thr308 with phospho-Akt-specific antibodies (data not shown). Therefore, our results indicate that \(H_2O_2\) activates Akt in a PI3K-dependent manner.

Then, Akt activation was monitored with phospho-Akt(T308)-specific antibodies in cells treated for 10 min and 3 h with \(H_2O_2\) and NGF. As shown in Fig. 1B, \(H_2O_2\) induced a stronger activation of Akt than NGF after a 10-min stimulation. By contrast, after a 3-h stimulation, phospho-Akt was hardly detectable in \(H_2O_2\)-treated cells, whereas NGF-treated cells exhibited about half the phospho-Akt observed at 10 min. Similar results were obtained when early Akt was monitored with an antibody against phospho-Ser473 (data not shown). The different kinetics of Akt activation may be caused, at least in part, by a faster down-regulation of Akt activity in \(H_2O_2\)-treated cells (data below) or by a quick degradation of \(H_2O_2\) by cell antioxidant enzymes that shut down this signal. Therefore, we determined the rate of \(H_2O_2\) degradation under our experimental conditions. 400,000 serum-starved PC12 cells were incubated with 0.5 mM \(H_2O_2\) for increasing time periods, and the \(H_2O_2\) concentration remaining in the medium was measured by a chemiluminescent assay (see “Experimental Procedures”). An initial concentration of 0.5 mM \(H_2O_2\) was reduced to less than 0.1 mM in 60 min (data not shown). We also tested the capacity of the \(H_2O_2\) concentration remaining in the medium to activate Akt. The conditioned medium from cells treated with 0.5 mM \(H_2O_2\) was rapidly used to restimulate new serum-starved cells for 10 min. As a control we tested the effect of Akt activation of control conditioned medium of parallel samples of \(H_2O_2\) which was added to cell-free plates. As shown in Fig. 1C, in cell-free medium 0.5 mM \(H_2O_2\) was degraded in about 2 h because conditioned media from longer incubations were unable to stimulate phosphorylation/activation of Akt. By contrast, in the presence of PC12 cells, \(H_2O_2\) exhibited a faster degradation kinetics, with lack of Akt stimulating activity in about 60 min. These results indicate that \(H_2O_2\) is metabolized quickly in the cell environment and establish a time frame to discriminate short term effects that are connected directly with the presence of \(H_2O_2\) and long term effects that come into view at a time when \(H_2O_2\) is no longer present.

![Fig. 1. Short term effects of \(H_2O_2\) on PC12 cells. A. \(H_2O_2\) activates Akt in a PI3K-dependent manner. The results of \textit{in vitro} kinase assays of Akt immunoprecipitated from serum-starved PC12 cells that were preincubated with vehicle, 100 nM wortmannin, or 40 \(\mu\)M LY294002 for 15 min and then stimulated with 20 ng/ml NGF or 0.5 mM \(H_2O_2\) for 5 min are shown. Assays were performed using histone H2B as substrate. B. Comparison of the Akt phosphorylation rates by NGF and \(H_2O_2\). Serum-starved PC12 cells were treated for 10 min and 3 h with 20 ng/ml NGF or 0.5 mM \(H_2O_2\) as indicated. Upper panel, immunoblot with anti-phi-spho-Akt(T308) antibodies; lower panel, immunoblot of the same cell lysates with anti-Akt antibody showing a similar amount of Akt lane. C. \(H_2O_2\) is catalyzed down to levels unable to activate Akt within the 1st h of incubation. Serum-free medium and serum-starved PC12 cells were incubated with 0.5 mM \(H_2O_2\) for the indicated times, and the medium from these plates, which contained the \(H_2O_2\) that was not catalyzed yet, was used to stimulate new serum-starved cells for 10 min. Upper panel, immunoblot with anti-phi-spho-Akt(T308) antibodies. Lower panel, immunoblot of the same cell lysates with anti-Akt antibody showing a similar amount of Akt lane. D, early genotoxic damage induced by \(H_2O_2\). Serum-starved cells were preincubated with vehicle of LY294002 for 15 min and then submitted to the indicated \(H_2O_2\) concentrations for 30 min, embedded in agarose, and electrophoresed according to the comet assay. The length of the comet tails reflects the extent of DNA damage (25). Plates show representative fields of DAPI-stained nuclei. E, comparison of the \(H_2O_2\)-induced genotoxic damage in control and LY294002-treated cells as determined from the comet tail moments. More than 200 comets/treatment were sorted visually into classes 0–4 representing increasing amounts of damage. The DNA damage index was calculated as the addition of the five classes and referred to 100 comets (25).
Despite the rapid elimination of most \( \text{H}_2\text{O}_2 \) cells underwent apoptosis and necrosis in the following 10–24 h (see below). Therefore we reasoned that in addition to activating Akt, \( \text{H}_2\text{O}_2 \) might also deliver a mark for long-term induction of cell death, such as DNA damage. To test this hypothesis, serum-starved PC12 cells were submitted for 30 min to 0.5 and 1 \( \text{mM H}_2\text{O}_2 \). DNA integrity was analyzed by the comet assay as shown in Fig. 1, D and E. Besides activating Akt, these \( \text{H}_2\text{O}_2 \) concentrations also produced strong genotoxic damage as evidenced by the long tails of electrophoresed chromatin. Moreover, pretreatment with LY294002 (Fig. 1, D and E) or with wortmannin (data not shown) did not significantly prevent nor enhance the DNA damage, suggesting that the PI3K/Akt pathway does not shield the cells from the initial genotoxic mark produced by \( \text{H}_2\text{O}_2 \).

**Cells Submitted to \( \text{H}_2\text{O}_2 \) Exhibit a Progressive Drop of GSH**—To test whether \( \text{H}_2\text{O}_2 \) could alter the cell redox status, we analyzed the intracellular level of GSH by the use of monochlorobimane (27). The amount of fluorescent GSH-monochlorobimane adduct that is generated by the enzymatic action of intracellular glutathione S-transferase was measured fluorometrically. Unspecific basal incorporation of the probe was determined in cells treated for 24 h with BSO, an irreversible inhibitor of \( \gamma \)-glutamylcysteine synthase, which is the rate-limiting enzyme in glutathione biosynthesis (29). Serum-starved PC12 cells were submitted to 0.5 \( \text{mM H}_2\text{O}_2 \) for the time periods indicated in Fig. 2A and incubated for the last 30 min with monochlorobimane. Within the first 3-h incubation, \( \text{H}_2\text{O}_2 \) depleted GSH to levels similar to those with a 24-h treatment with BSO. Because GSH is the major non-protein-reducing agent within the cell, these results indicate that \( \text{H}_2\text{O}_2 \) substantially reduces the cell capacity to maintain redox homeostasis.

Most GSH depletion took place at a time point when \( \text{H}_2\text{O}_2 \) was no longer present, at least at a sufficient amount to elicit Akt activation (Fig. 1C), suggesting that other secondary sources of oxidative stress might be responsible for the GSH loss. Therefore, we estimated mitochondria-generated oxygen free radicals. Mitochondrial transmembrane potential (\( \Delta \Psi m \)) was estimated by flow cytometry in cells stained with CM-H\(_2\)XROS, a reduced rhodamine that is concentrated by the active mitochondria and upon oxidation fluoresces red (30). As shown in Fig. 2B, when serum-starved PC12 cells were submitted to 0.5 \( \text{mM H}_2\text{O}_2 \), an increasing fraction of the cell population was refractory to CM-H\(_2\)XROS staining, indicating a gradual decay of \( \Delta \Psi m \). Interestingly, the increase in cells that were not stained by the probe inversely correlated with the progressive drop of GSH levels. We also analyzed the intracellular levels of ROS by flow cytometry of cells stained with hydroethidine, a fluorescent probe most sensitive for superoxide ion (31). As shown in Fig. 2C, when cells were submitted to 0.5 \( \text{mM H}_2\text{O}_2 \) for 3 h, they exhibited an almost 2-fold increase in intracellular ROS. Taken together these results suggest that after \( \text{H}_2\text{O}_2 \) induction of DNA damage, a secondary source of ROS production, including superoxide, is generated at least in part at the mitochondria.

**\( \text{H}_2\text{O}_2 \) Induces Long Term Accumulation of Ceramide**—Because ceramide is a lipid second messenger of apoptosis in neural cells, we determined ceramide levels after \( \text{H}_2\text{O}_2 \) treatment of serum-starved PC12 cells. As shown in Fig. 3A, a small increase in ceramide was detected during the first 30-min incubation with \( \text{H}_2\text{O}_2 \). Although this increase was not statistically significant in our assays, other groups have reported a similar increase in cells treated with other insults (32–34). Interestingly, ceramide levels increased much more significantly up to 4–6-fold over that of untreated cells after a 3-h incubation. We analyzed the accumulation of ceramide in the presence of fumonisin B1, an inhibitor of ceramide synthase. As shown in Fig. 3B, the \( \text{H}_2\text{O}_2 \)-induced accumulation of this lipid was similar to that in the absence of fumonisin B1, indicating that \textit{de novo} synthesis did not contribute significantly to the observed elevation of ceramide levels. The increase in ceramide ran parallel to the decrease of GSH levels (Fig. 2A), suggesting that the accumulated ceramide might come from the activation of a ROS-sensitive neutral sphingomyelinase as reported previously (35–37). To explore this possibility, cells were submitted to 1 \( \text{mM H}_2\text{O}_2 \) and after 30 min GSH was exogenously added to the cells. Ceramide levels were monitored 3 and 6 h after the addition of \( \text{H}_2\text{O}_2 \). As shown in Fig. 3C, exogenously added GSH prevented the late increase in ceramide which was induced by \( \text{H}_2\text{O}_2 \). These observations establish a direct correlation between the \( \text{H}_2\text{O}_2 \)-induced alteration in redox homeostasis and the activation of a redox-sensitive neutral sphingomyelinase resulting in the accumulation of the proapoptotic lipid ceramide.

**Active Akt Prevents \( \text{H}_2\text{O}_2 \)-induced Apoptosis but Not Necrosis**—We characterized the pattern of \( \text{H}_2\text{O}_2 \)-induced cell death in our cells. In addition, we also studied the protective effect of an active membrane-targeted version of this kinase against cell death induced by \( \text{H}_2\text{O}_2 \). PC12 cells were stably transfected with expression vectors for EGFP or for a myristoylated (myr) fusion
ROS and Ceramide Mediate Down-regulation of Akt/PKB by \( \text{H}_2\text{O}_2 \)

H\(_2\text{O}_2\) concentration increased. However, this decay was significantly lower in myr-EGFP-Akt1-transfected cells, particularly at low \( \text{H}_2\text{O}_2\) concentrations. Thus, the \( \text{H}_2\text{O}_2\) concentration at which we observed a 50% effect on MTT reduction was slightly higher in myr-EGFP-Akt1 cells (EC\(_{50}\) 0.75 mM) than in EGFP cells (EC\(_{50}\) 0.55 mM). Then, we analyzed the effect of \( \text{H}_2\text{O}_2\) on necrotic cell death as a function of membrane integrity determined by trypan blue exclusion. As shown in Fig. 4C, both EGFP and myr-EGFP-Akt1 cells excluded the dye at concentrations below 0.5 mM. Higher concentrations resulted in a similar sharp increase of trypan blue-stained control and myr-EGFP-Akt1 cells, indicative of necrotic cell death. Therefore, both control EGFP and myr-EGFP-Akt1 cells were similarly sensitive to \( \text{H}_2\text{O}_2\)-induced necrosis with an EC\(_{50}\) of 0.7 mM.

The effect of \( \text{H}_2\text{O}_2\) on apoptosis was monitored in annexin V-PE-stained cells. To discriminate early apoptosis from late apoptosis and necrosis, cells were stained simultaneously with the vital dye 7-AAD, which only stains cells lacking membrane integrity (38). Serum-starved EGFP and myr-EGFP-Akt1 cells were submitted to a 12-h incubation with 0.5 mM \( \text{H}_2\text{O}_2\), stained with annexin V-PE and 7-AAD, and analyzed by flow cytometry. As shown in Fig. 4, D and E, most untreated EGFP and myr-EGFP-Akt1 cells were annexin V-PE- and 7-AAD-negative, indicating that they were viable. After \( \text{H}_2\text{O}_2\) treatment about 50% of the EGFP cells were annexin V-PE-positive and 7-AAD-negative, indicating that this population was undergoing early apoptosis. By contrast, the fraction of myr-EGFP-Akt1 cells that were annexin V-PE-positive and 7-AAD-negative after the \( \text{H}_2\text{O}_2\) insult only increased to 23%.

As an additional estimation of apoptosis, we analyzed the levels of p53 in both cell lines after incubation with 0.5 mM \( \text{H}_2\text{O}_2\). As shown in Fig. 4, F and G, the p53 levels of EGFP cells dramatically increased within 3 h of \( \text{H}_2\text{O}_2\) treatment and remained elevated for at least 12 h. By contrast, myr-EGFP-Akt1 cells exhibited a lower increase in p53 levels. Taken together, these results suggest that membrane-targeted, active Akt attenuates the apoptotic but not the necrotic component of \( \text{H}_2\text{O}_2\)-induced cell death in PC12 cells.

\( \text{H}_2\text{O}_2\) Induces Long Term Down-regulation of Akt by Dephosphorylation and Proteolytic Degradation—We analyzed the kinetics of Akt phosphorylation/dephosphorylation and proteolysis after treatment with 0.5 mM \( \text{H}_2\text{O}_2\). As shown in Fig. 5, A and B, cells transfected with wild type EGFP-Akt1 exhibited a rise in phospho-EGFP-Akt1 within minutes of \( \text{H}_2\text{O}_2\) stimulation, but no phospho-EGFP-Akt1 was detected after 3 h. When we analyzed myr-EGFP-Akt1 cells, a drop in phospho-myr-EGFP-Akt1 levels was also observed, but, in contrast to control cells, a significant amount of phospho-Akt1 was maintained, even after 12 h of \( \text{H}_2\text{O}_2\) treatment. The time frame of Akt dephosphorylation was similar to the accumulation of ceramide, suggesting that Akt dephosphorylation is mediated by a ceramide-activated protein phosphatase. In fact, pretreatment of PC12 cells with C2-ceramide prevented the phosphorylation of Akt by either NGF or \( \text{H}_2\text{O}_2\) (data not shown). These results are in agreement with our previous observations (18; see also 19, 20), and therefore we focused our study on the down-regulation of the PI3K/Akt survival signal by proteolytic degradation of Akt as indicated below.

As shown in Fig. 5, A and B, EGFP-Akt1 cells exhibited a significant reduction of total Akt protein after a 3-h incubation with \( \text{H}_2\text{O}_2\), and EGFP-Akt1 was no longer detectable after 6 h. When we analyzed the Akt levels in the myr-EGFP-Akt1 cells a reduction of Akt was also observed after 3 h, but, in contrast to control cells, a significant amount of Akt was still detectable after a 6- and 12-h incubation. This fraction most likely corresponds to the phosphorylated Akt. Therefore, although \( \text{H}_2\text{O}_2\)
initially activated Akt, long term effects of H2O2 led to Akt dephosphorylation and proteolytic degradation, but membrane-targeted active Akt was more resistant to both effects.

Proteolytic Degradation of Akt by H2O2 Is Independent of p53 Stabilization and Is Mediated through a Change of Redox Status and Generation of Ceramide—The observed degradation of Akt correlated on one hand with the rise in ROS and ceramide (Figs. 2 and 3) and on the other with stabilization of p53 (Fig. 4F). To discriminate which of these signals leads to degradation of Akt, we performed the experiments shown in Fig. 6. First, serum-starved PC12 cells were submitted to 0.5 mM H2O2 for 30 min and then left untreated or incubated with 10 mM GSH for 12 h. Akt degradation and p53 stabilization were determined as indicated in Fig. 6A. Under these conditions, H2O2 induced the complete disappearance of immunodetectable Akt and a strong increase in p53 levels. By contrast, GSH reduced p53 stabilization close to basal levels and partially prevented the degradation of Akt. These results suggest that either the increment in ROS or the stabilization of p53 may be...
were incubated with 40 μM H2O2 and then left untreated or supplemented with 10 mM GSH. Cells were lysed 12 h after the addition of H2O2. B. Cells were incubated with 40 μM C2-ceramide for 30 min and then left untreated or supplemented with 10 mM GSH. Cells were lysed 12 h after the addition of C2-ceramide. Top panels, immunoblots with anti-Akt antibodies. Middle panels, immunoblots with anti-p53 antibodies. Bottom panels, immunoblots with anti-PDI antibodies showing a similar load of protein/lane. C. H2O2 promoted Akt degradation regardless of the functional state of p53. Neuro 2A cells that were transiently cotransfected with expression vectors for FLAG-Akt1 and either wild type His-p53 or mutant His-p53(S15A). After 6 h, cells were serum-starved for 8 h and then submitted to 0.5 mM H2O2 for 12 h as indicated. Upper panel, immunoblot with anti-FLAG antibodies. Lower panel, immunoblot with anti-His antibodies.

relevant to the degradation of Akt. To discern further whether p53-mediated DNA damage signaling is involved in H2O2-induced proteolytic degradation of Akt, or on the contrary, whether Akt degradation may proceed in a p53-independent manner, we used a genetic approach based on transfection of expression vectors for wild type and a p53 mutant containing an alanine substitution at the critical Ser15. This site is a target of several stress-sensitive kinases including ataxia telangiectasia mutated (ATM), DNA-PK, and p38 (24–31). p53-mediated Akt degradation is involved in H2O2-in-duced proteolysis. Moreover, p53 levels were not altered by C2-ceramide. Taken together these results suggest that cere-mide activates the proteolytic pathway leading to Akt degra-duation at a level that is independent of p53 stabilization and that runs parallel or downstream from redox alterations.

H2O2-induced Degradation of Akt Proceeds through Caspase-dependent and -independent Mechanisms—To identify the proteases responsible for H2O2-induced degradation of Akt, serum-starved cells were submitted to H2O2, and the pattern of Akt degradation was compared with that of PARP, which is a well-established substrate of caspase-3 in vitro (42). Two H2O2 concentrations were selected, 0.5 and 1 mM, because they induce different modes of cell death: apoptosis and necrosis, respectively (Fig. 4). As shown in Fig. 4A, both H2O2 concentrations led to PARP degradation. ZVAD, a generic caspase inhibitor, as well as DEVD, a specific inhibitor of caspase group III, prevented PARP degradation at 0.5 mM but not at 1 mM H2O2. By contrast, YVAD, a specific inhibitor of caspase group I, did not significantly block PARP degradation, in agreement with the notion that PARP is cleaved preferentially by caspase-3 in vivo during apoptosis (42). These results demonstrate further the existence of a wider array of proteases that become activated at high, necrotic H2O2 concentrations. Then we analyzed the effect of these inhibitors on Akt degradation. As shown in Fig. 7A, Akt was degraded at both 0.5 and 1 mM H2O2 concentrations. Interestingly, ZVAD, DEVD, and YVAD fully protected Akt from 0.5 mM H2O2, but not from 1 mM H2O2-induced proteolysis. These results evidence a complex mechanism of Akt degradation in the H2O2 paradigm which most likely reflects an apoptosis-related caspase-dependent component at 0.5 mM H2O2 and a necrosis-related, caspase-independent component at 1 mM H2O2.

Apoptotic Degradation of Akt May Progress in a Caspase-3-independent Manner—Some of us have demonstrated previously that Akt can be cleaved in vitro and in vivo by caspase-3 (21). To determine whether caspase-3 is responsible for the H2O2-induced apoptotic degradation of Akt we used the caspase-3-deficient cell line MCF-7. These cells have been widely reported to be deficient in caspase-3 because of a 47-bp deletion within exon 3 of the CASP-3 gene (43, 44). Because the sensitivity of these cells to H2O2 may differ from that of PC12 cells, we submitted serum-starved MCF-7 cells to increasing H2O2 concentrations for 12 h. As shown in Fig. 8A and B, PARP, a substrate of caspase-3, resisted H2O2-induced degradation at concentration below 2 mM. These results establish 2 mM H2O2 as the threshold concentration necessary in these cells to induce PARP proteolysis in a caspase-3-independent, probably necrotic manner. When we analyzed the effect of
H$_2$O$_2$ on Akt levels, we found that Akt was degraded at concentrations below 2 mM, despite the lack of caspase-3. Immunoreactive Akt dropped to 50% with 0.5 mM H$_2$O$_2$ and was barely detectable with 1 mM H$_2$O$_2$. These results suggest that the apoptotic degradation of Akt may proceed in a caspase-3-independent manner and suggest a role for other caspase group III proteases.

The residues Asp$_{108}$ and Asp$_{119}$ have been identified as specific sites for caspase-3 cleavage in vitro (21). In addition, Akt contains a putative sequence for caspase-3 cleavage close to the carboxyl terminus (453DQDD456). To determine whether these sites are the specific targets of H$_2$O$_2$-induced proteases and to determine further whether H$_2$O$_2$-induced degradation of Akt may proceed in a caspase-3-independent manner and suggest a role for other caspase group III proteases.

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the FLG-FLAG-Akt1(D453A,D455A,D456A) mutant were degraded to an extent similar to that of wild type FLG-FLAG-Akt1. Moreover, neither of the two antibodies used allowed the detection of cleaved Akt products (data not shown). These results do not discard a role of caspase-3 in H2O2-induced cell death; but because the caspase group III inhibitor DEVD prevented Akt degradation, other proteases downstream from this group must be also involved. Taken together, these results indicate because the caspase group III inhibitor DEVD prevented Akt degradation, other proteases downstream from this group must exist additional p53-independent ways to induce mitochondrial membrane potential (Fig. 2), production of ceramide. This in vitro results suggest that the accumulation of ceramide is an event that lies downstream from ROS generation. The long term accumulation of ceramide might result from increased de novo synthesis via activation of ceramide synthase or from sphingomyelin breakdown by acidic or neutral sphingomyelinases (46, 47). In our H2O2-treated PC12 cells, fumonisin B1, a specific inhibitor of ceramide synthase, did not significantly reduce the ceramide levels observed within the time frame of these assays. On the other hand, the addition of GSH prevented H2O2-induced accumulation of ceramide. A neutral sphingomyelinase is activated by oxidants (48, 49) or regulated by the cell redox state (50–52). In addition, GSH decreases the activity of a magnesium-dependent sphingomyelinase in vitro (35). Therefore, our results are in agreement with those findings and point to a neutral sphingomyelinase as responsible of H2O2-induced accumulation of ceramide downstream from ROS in PC12 cells.

Because genotoxic damage activates the p53 pathway, it is tempting to speculate that p53-mediated up-regulation of the proapoptotic Bcl-2 family member BAX might link the short term genotoxic damage induced by H2O2 with the later generation of mitochondrial ROS and production of ceramide. This appears to be the case for etoposide-induced apoptosis in glioma cells (53). In fact, we observed a similar kinetics for loss of mitochondrial membrane potential (Fig. 2), production of ceramide (Fig. 3), and stabilization of p53 (Fig. 4). However, there must exist additional p53-independent ways to induce mitochondrial release of ROS and further accumulation of ceramide because the ROS- and ceramide-induced proteolysis of Akt proceeded similarly in cells expressing wild type and dominant negative p53.

The identification of the proteases involved the H2O2-induced degradation of Akt is hampered by the complex cell death response to this oxidant, which includes apoptotic and necrotic features as stated under “Results.” Compared with PARP, a protein that in vitro is preferentially cleaved by caspase-3 during apoptosis (34) but also by other proteases during necrosis (42), we observed that a generic caspase inhibitor, ZVAD, protected Akt and PARP from degradation. Two previous reports have shown caspase-mediated cleavage of Akt in response to FAS ligand and anoikis in epithelial cells, and one of our groups has demonstrated in vitro degradation of Akt1 by caspases-3, -6, and -7 and generation of 44-kDa cleaved products (21). However, none of these studies including our present work could show the expected Akt-cleaved products. These results suggest that Akt is cleaved by several proteases in vivo, resulting in the generation of small peptides. Because inhibitors of two caspase groups, DEVD and YVAD, prevented Akt proteolysis but the caspase-3-insensitive Akt double mutant (D108A,D119A) and the triple mutant (D453A,D455A,D456A) were also degraded in H2O2-treated cells, we reasoned that such proteases must act downstream from the caspase cascade. We are currently investigating the role of other proteases.

We observed a retardation between loss of Akt activity, as determined by phosphorylation of Thr^308, and its proteolytic degradation. These results suggest that down-regulation of Akt during oxidative injury proceeds in two sequential phases: first, the inactivation of Akt which may be mediated by a ceramide-activated protein phosphatase and, second, caspase-dependent proteolytic degradation. In agreement with this model, cells overexpressing a membrane-targeted, active and phosphorylated Akt were more resistant to Akt proteolysis. Therefore, execution of apoptotic cell death programs may require previous inactivation of Akt, and the proteolysis of this kinase may serve as a reinforcement for the irreversible commitment to cell death.

The present study provides a better understanding of the mechanisms of down-regulation of Akt survival activity by oxidative damage and may help devise pharmacological or genetic strategies to prevent or delay age-related declines of the nervous system and to increase resistance to oxidative injury.

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Ceramide and Reactive Oxygen Species Generated by H$_2$O$_2$ Induce Caspase-3-independent Degradation of Akt/Protein Kinase B

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