Hydrogen peroxide controls Akt activity via ubiquitination/degradation pathways

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Abstract. Akt is a well-established protein that regulates cell growth, survival and anti-apoptotic mechanisms. In this study, we demonstrated that hydrogen peroxide (H_2O_2)-induced oxidative stress regulates the activity of the anti-apoptotic protein Akt via the ubiquitin-proteasome degradation system. H_2O_2 induced cytotoxicity in PC12 cells and decreased the cellular protein and phosphorylation levels of Akt in a concentration- and exposure time-dependent manner. This downregulation was blocked by the proteasome inhibitor MG132 and the Akt-specific inhibitor LY294002. In addition, an *in vivo* ubiquitination assay revealed that the degradation of Akt was mediated by the ubiquitin-mediated proteasome pathway and further demonstrated that this ubiquitination was dependent on the phosphorylation status of Akt. Furthermore, the exogenously overexpressed active form of Akt, but not its inactive form, induced resistance to H_2O_2-mediated cell death. These results suggested that H_2O_2-induced cytotoxicity was mediated by active Akt degradation.

Introduction

Reactive oxygen species (ROS) is a broad term encompassing hydroxyl ($^\bullet$OH), alkoxy ($RO^\bullet$), peroxyl ($ROO^\bullet$), superoxide ($O_2^\bullet$), and nitroxyl ($NO^\bullet$) radicals and nonradicals hydrogen peroxide (H_2O_2) and organic hydroperoxide ($ROOH$) (1). These ROS are byproducts of normal cellular metabolism that are generated by all aerobic organisms. ROS levels are strictly controlled by antioxidant enzymes and small antioxidant molecules

such as glutathione. If this control is lost due to high levels of ROS generation or impairment of the antioxidant defense mechanism, ROS can lead to oxidative stress, resulting in high cellular toxicity (2). However, ROS have been demonstrated to function in inflammation, antimicrobial defense, and apoptosis (3-5). Furthermore, ROS function as second messengers involved in the responses of receptor tyrosine kinases to radiation or alkylating agents (6-8).

H_2O_2 is considered one of the most toxic ROS. Although H_2O_2 itself is not highly reactive, it can readily diffuse across cellular membranes, after which it is converted into highly reactive hydroxyl radicals through the Fenton reaction in the presence of metal ions (9). ROS-mediated cell death may have a contributory role in the development of the oxidative stress associated with many diseases such as Alzheimer's disease, Parkinson's disease, and lung airway diseases (10-12).

Akt is a serine/threonine kinase that functions in cell survival pathways to suppress apoptosis (13). After growth factor stimulation by factors such as insulin, epidermal growth factor, and insulin-like growth factor I, Akt is recruited to the plasma membrane and activated through PI3K/PDK1-mediated phosphorylation at threonine 308, followed by full activation via phosphorylation by TORC2 at serine 473 (14,15). Once Akt is activated, several antiapoptotic downstream targets including Bad, caspase-9, glycogen synthase kinase-3-β (GSK-3β), and forkhead transcription factors are phosphorylated and inactivated, thereby inhibiting cell death (16-19). Akt1 knockout mice are more susceptible to apoptosis induced by several genotoxic stresses, and the life span of $Akt1^{-/-}$ mice is shorter than that of wild-type mice (20).

H_2O_2 modulates the activity of cellular Akt. Several reports demonstrated that a basal level of ROS induces the phosphorylation of Akt, thereby protecting cells from oxidative stress-mediated damage (21-23). The phosphorylation/activation of Akt precedes the inactivation of BAD, capase-9, forkhead transcription factors, and GSK-3β and the upregulation of nuclear factor-κB activity (16-19). In contrast, high levels of ROS induce the downregulation of the Akt signaling pathway, resulting in cell death. Excessive ROS levels induce the dephosphorylation/inactivation of Akt, resulting in Bax oligomerization, cytochrome c release from mitochondria, and the activation of caspase cascades. In addition to ROS-induced

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Akt dephosphorylation, two reports exist on ROS inhibited Akt activation through the degradation of cellular Akt (24,25). Two different Akt degradation mechanisms have been reported. Mann *et al* (25) demonstrated that ROS generated by arsenic trioxide decreased Akt protein levels in a caspase-dependent manner; however, Martin *et al* (24) reported that capase-3 is dispensable for Akt degradation induced by ROS. However, their experiments were performed in different cell lines, namely NB4 cells and PC12 cells. This suggests that Akt degradation mechanisms are cell-type dependent and that the other known mechanisms can induce Akt degradation. Here, we found that the ubiquitin-proteasome system is a novel mechanism involved in Akt degradation during cell death induced by H_2O_2.

Materials and methods

Plasmids. The entire coding region of Akt was generated by PCR from cDNA synthesized from HeLa cells. Akt cDNA was cloned into the pcDNA3.1-Myc/His (Invitrogen, Carlsbad, CA) vector. The dominant-negative Akt (DN-Akt: T308A and S473A) was created using the QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA) according to the manufacturer's instructions. A HA-ubiquitin plasmid, pMT123, was kindly provided by Dr Dirk Bohmann (University of Rochester, Rochester, NY).

Cell culture and transfection. PC12 cells were grown in Dulbecco's modified Eagle's medium (Gibco®, Invitrogen) containing 10% heat-inactivated horse serum (Gibco) and 5% fetal bovine serum (Gibco). Cells were transfected with the indicated plasmids using Hylimix (Dojindo, Kumamoto, Japan).

Antibodies and reagents. Primary antibodies against Akt, phospho-Akt (T473), and Myc tag were purchased from Cell Signaling Technology (Danvers, MA). HA tag antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH antibody was obtained from Abfrontier (Seoul, Korea). MG132 was purchased from Sigma-Aldrich (St. Louis, MO), and H_2O_2 (30%) was purchased from Merck (Darmstadt, Germany). HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PC12 cells were seeded onto 96-well plates (100 cells/mm²) and starved with serum-free DMEM overnight. The next day, cells were treated with H_2O_2 at the indicated concentrations and times. Cell viability was measured by the MTT assay according to the manufacturer's instructions (Sigma-Aldrich).

Western blot analysis. Cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM p-nitrophenyl phosphate, and a protease inhibitor cocktail) on ice for 30 min. After centrifugation at 14,000 x g for 20 min at 4℃, proteins in supernatants were separated on a 10 or 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated with primary antibodies (1:1000) overnight at 4℃. After washing with 0.5% Tween-20 in Tris-buffered saline, membranes were incubated with HRP-conjugated secondary antibodies (1:5000). Proteins were visualized using ECL reagents (Amersham Biosciences, Piscataway, NJ) and detected with the LAS-3000 imager (Fuji, Tokyo, Japan).

In vivo ubiquitination assay. Akt-Myc/His plasmids together with HA-ubiquitin were cotransfected into PC12 cells as indicated in individual experiments. Twenty-four hours after transfection, cells were treated with H_2O_2 at the indicated concentrations and 10 μM MG132 at various times. The cells were washed with PBS, lysed in 200 μl of denaturing lysis buffer (50 mM Tris-Cl, pH 7.4; 0.5% SDS, and 70 mM β-mercaptoethanol) by vortexing, and then boiled for 15 min at 95℃. The denatured lysates were diluted with 800 μl of buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) with protease inhibitor cocktail and MG132. The diluted lysates were incubated with Ni-NTA (Qiagen, Valencia, CA) beads overnight at 4℃. The beads were washed with buffer B (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0) five times. The proteins bound to the beads were eluted by boiling with SDS-PAGE sample buffer containing buffer C (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). The eluted proteins were immunoblotted with anti-HA antibody.

Statistical analysis. MTT assays were repeated six times. Data are expressed as mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer method for multiple comparisons. $P<0.05$ was considered statistically significant.

Results

H_2O_2 induces the apoptosis of PC12 cells and decreases the levels of Akt. We first examined the effect of H_2O_2 on cell viability. After seeding PC12 cells, the culture media was replaced with fresh serum free media, and cells were incubated overnight. Cells were then treated with H_2O_2 at the indicated concentrations for 24 h, after which cell viability was analyzed using the MTT assay. As shown in Fig. 1A, H_2O_2 decreased the viability of PC12 cells in a concentration-dependent manner (Fig. 1A). In addition, H_2O_2 induced cytotoxicity in PC12 cells in a time-dependent manner (Fig. 1B). To investigate whether the decrease in cell viability was associated with regulation of Akt protein stability, we investigated the cellular levels of Akt and its phosphorylation status by using a biochemical immunoblotting assay with the indicated antibodies in H_2O_2-treated PC12 cells. H_2O_2 significantly decreased both the levels of total Akt and phospho-Akt (Fig. 1C, lanes 1 to 5). Next, to determine whether this downregulation was related to proteasomal degradation, cells were treated with a proteasome inhibitor, MG132, in the same manner as described for previous experiments. Of note, the decreases in Akt protein levels were fully restored by treatment with MG132 (Fig. 1C, lane 6 to 10). It has been reported that ROS generated by H_2O_2 induce Akt activation in the early response and subsequent Akt degradation in the later response (24). Thus, we sought to
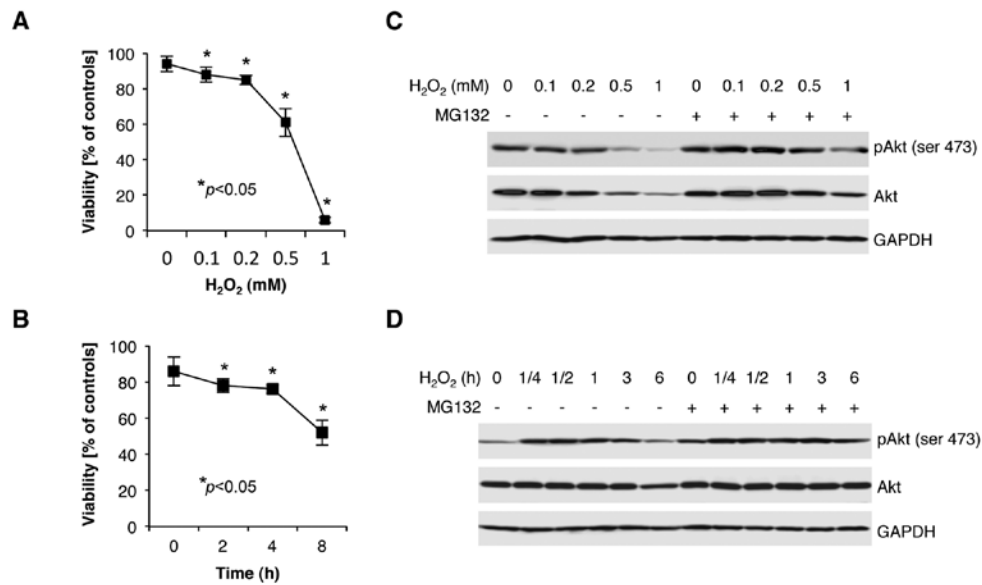
Figure 1. H_2O_2 induces apoptosis and decreases Akt protein levels. (A) H_2O_2 induced cytotoxicity in PC12 cells in a concentration-dependent manner. PC12 cells were cultured in the absence of serum overnight and treated with 100, 200, 500, and 1000 μM H_2O_2 for 24 h. (B) H_2O_2 induced cell death in an exposure time-dependent manner. PC12 cells were treated with 1 mM H_2O_2 for the indicated times. The number of live cells was estimated by the MTT assay. In (A) and (B), data are expressed as mean ± SD, and * indicates statistical significance compared with findings in control cells at each concentration and exposure time ($P<0.05$). (C) and (D) H_2O_2 induced Akt degradation via a proteasomal pathway. PC12 cells were treated with H_2O_2 at various concentrations (C) and for various lengths of time (D) and then additionally treated with 10 μM MG132 for 6 h. Protein levels of phospho-Akt, Akt, and GAPDH (as a loading control) were measured via immunoblotting with antibodies as indicated.
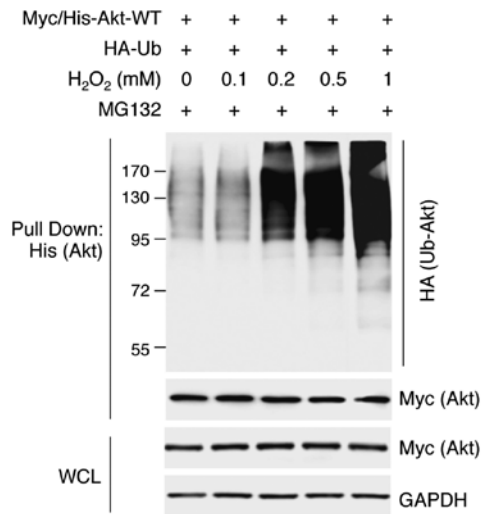

Figure 2. H_2O_2 induces Akt polyubiquitination. PC12 cells were cotransfected with plasmid for wild-type Akt (Myc/His-Akt-WT) together with the HA-ubiquitin plasmid as indicated. Twenty-four hours after transfection, cells were incubated in serum-free media and then treated with different concentrations of H_2O_2 and 10 μM MG132 for 6 h. Cell lysates were subjected to *in vivo* ubiquitination analysis with Ni-NTA beads. Ubiquitinated Akt was detected by immunoblotting with the anti-HA antibody.

was attenuated by MG132 treatment (Fig. 1D, lane 6 to 10). In particular, at the 6-h time point, phosphorylated and total Akt levels were dramatically decreased, and their degradation was correlated with the significantly decreased cell viability at the same time point in Fig. 1A and B, suggesting that H_2O_2-induced cytotoxicity was related with the proteasomal degradation of Akt.

H_2O_2 induces Akt polyubiquitination. Proteasomal degradation is mediated through both ubiquitin-dependent and ubiquitin-independent mechanisms (26). To examine whether the ubiquitin-mediated process is involved in the proteasomal degradation of Akt, H_2O_2-treated PC12 cell lysates were subjected to immunoprecipitation against Akt, followed by immunoblotting with ubiquitin antibody. As shown in Fig. 2, high-molecular-weight smear bands representing polyubiquitination were detected for immunoprecipitated Akt, and the levels of polyubiquitination were increased in an H_2O_2 concentration-dependent manner. In particular, the level of Akt ubiquitination was substantial after treatment with 1 mM H_2O_2, which was correlated with the degradation level in Fig. 1C. These results indicated that H_2O_2 induced the polyubiquitin-mediated proteasomal degradation of Akt in PC12 cells.

Activated Akt is a major target of H_2O_2-induced Akt ubiquitination. We further clarified the molecular details of H_2O_2-induced Akt ubiquitination in PC12 cells. According to previous data, a long exposure of cells to H_2O_2 induced ubiquitin-dependent degradation of phosphorylated and total Akt (24). These results suggested a relationship between phosphorylation and ubiquitination regarding H_2O_2-induced Akt degradation. We first examined the effect of LY294002, a
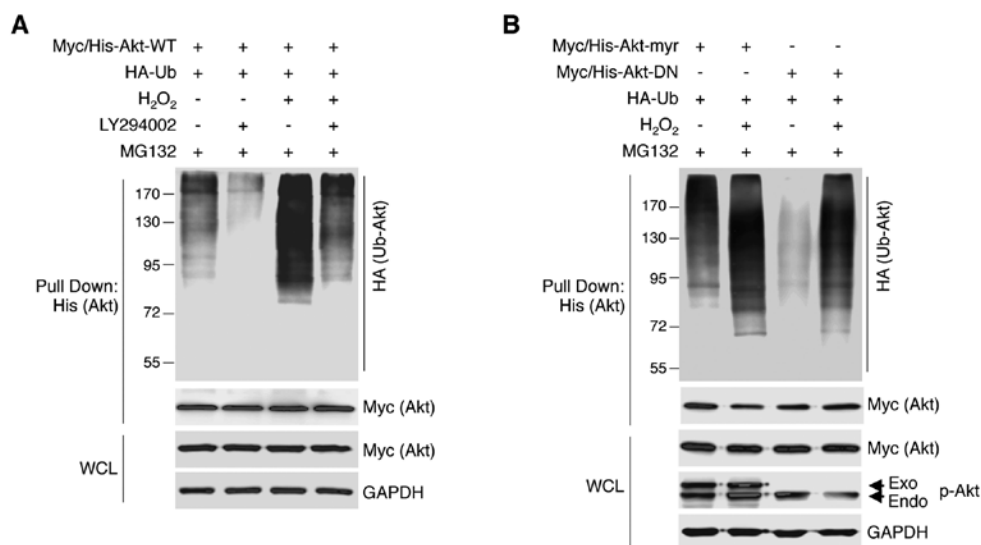
determine the relationship between phosphorylation-mediated activation and proteasomal degradation of Akt in PC12 cells. Cells were stimulated with 1 mM H_2O_2 for various lengths of time and then incubated with or without MG132, after which the levels of phosphorylation and degradation of Akt were monitored. As shown in Fig. 1D (lanes 1 to 5), H_2O_2 induced Akt phosphorylation within 15 min; however, this phosphorylation was decreased after 3 h. Moreover, the degradation of Akt

Figure 3. H$_2$O$_2$ induces ubiquitination of activated Akt. (A) H$_2$O$_2$-induced Akt ubiquitination was blocked by LY294002 treatment. PC12 cells were cotransfected with plasmids for wild-type Akt (Myc/His-Akt-WT) and the HA-ubiquitin plasmid as indicated. After 24 h of incubation, cells were starved overnight and pretreated with 10 μM LY294002, followed by stimulation with 1 mM H$_2$O$_2$ and 10 μM MG132 for 6 h. Equal amounts of lysate from the cells were subjected to an *in vivo* ubiquitination assay. Akt ubiquitination was examined by immunoblotting with anti-HA antibody. (B) Activated Akt, but not inactivated Akt, was efficiently ubiquitinated in response to H$_2$O$_2$ exposure. PC12 cells were cotransfected with Myc/His-Akt-Myr, Myc/His-Akt-DN, and HA-ubiquitin plasmids as indicated. After 24 h of incubation, the cells were treated LY294002, H$_2$O$_2$, and MG132 as indicated. Equal amounts of lysate from the cells were subjected to an *in vivo* ubiquitination assay. Akt ubiquitination was examined by immunoblotting with anti-HA antibody.
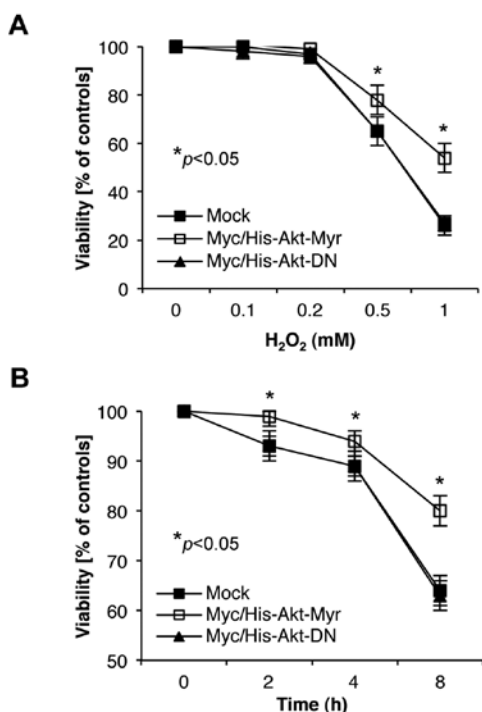

Figure 4. Akt activation prevents H$_2$O$_2$-induced cell death. Myc/His-Akt-Myr, Myc/His-Akt-DN, and control plasmids (Mock) were transfected into PC12 cells. After 24 h of incubation, PC12 cells were treated with H$_2$O$_2$ at the indicated concentrations (A) or with 1 mM H$_2$O$_2$ for the indicated lengths of time (B). The number of live cells was estimated by the MTT assay. In (A) and (B), data are expressed as means ± SD, and * indicates statistical significance in comparison with findings in control cells at each concentration and treatment time (P<0.05).

PI3K inhibitor that blocks Akt activation, on Akt ubiquitination in PC12 cells. As shown in Fig. 3A, H$_2$O$_2$ dramatically induced Akt ubiquitination relative to the control level;

however, this ubiquitination was significantly blocked by additional LY294002 treatment. In addition, constitutively active myristorylated Akt (Myc/His-Akt-Myr) was strongly ubiquitinated in response to H$_2$O$_2$ stimulation in PC12 cells relative to the ubiquitination of DN Akt (Myc/His-Akt-DN) (Fig. 3B). These data indicated that H$_2$O$_2$ preferably induced the ubiquitination of phosphorylated (activated) Akt in PC12 cells.

Akt activity prevents cell death due to H$_2$O$_2$ stimulation. We previously demonstrated that H$_2$O$_2$ stimulation induced cytotoxicity and the ubiquitination of phosphorylated Akt. To confirm the effect of Akt activation on cell viability under H$_2$O$_2$ stimulation, constitutively activated Akt and DN Akt were overexpressed in PC12 cells, and then the viability of these cells was investigated using the MTT assay. As shown in Fig. 4, Myr-Akt-expressing PC12 cells, but not DN-Akt-expressing cells, exhibited resistance to H$_2$O$_2$-mediated cytotoxicity in a concentration- and time-dependent manner. Overall, these results suggested that H$_2$O$_2$ stimulation induced the downregulation of Akt activity by ubiquitin-mediated proteasomal degradation and the loss of Akt activity decreased cell viability.

Discussion

The present study demonstrated that H$_2$O$_2$-induced Akt degradation occurs through the ubiquitin-proteasome pathway, and this ubiquitination depends on the phosphorylation status of Akt. In PC12 cells, H$_2$O$_2$ treatment induced cell death in a concentration- and exposure time-dependent manner. In our experimental condition, H$_2$O$_2$ induced total and phospho-Akt degradation, which was blocked by MG132 treatment. Furthermore, an *in vivo* ubiquitination assay revealed that H$_2$O$_2$

enhanced Akt ubiquitination, and the ubiquitination level was reduced by LY294002, a specific PI3K inhibitor that blocks Akt phosphorylation. Phosphorylation-dependent proteolytic degradation of Akt was also investigated using ectopic expression of Myc/His-Akt-Myr and Myc/His-Akt-DN. The *in vivo* ubiquitination assay using these constructs demonstrated that ubiquitination induced by H_2O_2 stimulation occurred more strongly on active Akt-Myr than on inactive Akt-DN *in vivo*. In addition, overexpression of active Akt-Myr, but not unphosphorylated inactive Akt-DN, induced resistance to H_2O_2-dependent cell death, which indicates that the ubiquitin-mediated proteasomal degradation of active Akt increases the sensitivity of cells to H_2O_2. Overall, our data indicate that H_2O_2 preferentially ubiquitinates phosphorylated Akt and promotes its degradation to induce cell death.

ROS generated by H_2O_2 regulates Akt activation via dephosphorylation and caspase-dependent and caspase-independent degradation pathways (24,25). Akt is phosphorylated and activated early during H_2O_2 stimulation, but it is dephosphorylated and inactivated via caspase-mediated cleavage and caspase-3-independent degradation during long-term exposure to H_2O_2 (24). This downregulation including dephosphorylation and cleavage of Akt is correlated with apoptosis. However, the mechanisms by which total Akt and phosphorylated Akt levels are decreased in response to H_2O_2 stimulation have not been fully demonstrated, as dephosphorylation only induced Akt inactivation but not its degradation, and in caspase-3-deficient MCF-7 cells, Akt is more susceptible to H_2O_2-induced degradation. Additionally, Akt mutants (D108A, D119A), which are not cleaved by caspase-3, are still degraded in H_2O_2-stimulated PC12 cells (24). While the precise mechanism remains unclear, we demonstrated that Akt degradation in H_2O_2-treated PC12 cells was mediated through the ubiquitin/proteasome degradation pathway.

Ubiquitin-proteasome dependent degradation is an irreversible pathway of protein degradation and is important for the regulation of many cellular functions, including cell cycle progression and cell growth (27). Polyubiquitination involves three components that participate in a cascade of ubiquitin transfer reactions: the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-target protein ligase (27). A previous study reported that H_2O_2 stimulates ubiquitin-conjugating activity and the expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes (28). That study revealed that the H_2O_2-mediated ubiquitin conjugation response required at least 4 h after exposure to H_2O_2 and persisted for at least 24 h. To confirm these findings, our study included exposure of cells to H_2O_2 for specific time points between 4 and 24 h (exposure times of 6 and 24 h). At these times, H_2O_2 significantly induced total Akt and phospho-Akt degradation after H_2O_2 stimulation, and their degradation was stimulated by polyubiquitination. Although the specific E3 ligase responsible for Akt ubiquitination in this condition was not investigated, the expression of the unknown E3 ligase gene might be induced by H_2O_2 exposure.

As Akt is phosphorylated quickly after H_2O_2 exposure, it suggests that Akt activation is one of the defense mechanisms against ROS toxicity. However, activated Akt levels are decreased during long-term exposure to H_2O_2 through ubiquitin-mediated degradation. We could not exclude the possibilities that activated Akt is dephosphorylated through ROS-induced phosphatase or that its levels are decreased via caspase in our experimental conditions. Phospho-Akt and total Akt levels were not completely recovered by MG132 treatment, suggesting that Akt activity is regulated through diverse mechanisms under ROS-induced apoptotic conditions. However, dephosphorylation is not a major event in the downregulation of Akt activity. Immunoblot assays in the present study and other studies demonstrated that total Akt levels were decreased by H_2O_2 exposure (24,25). In addition, we found that Akt degradation occurred in response to high levels of H_2O_2 and long-term exposure to H_2O_2. In particular, the decrease in Akt levels is due to ubiquitin-mediated degradation of active Akt. In correlation with the results, constitutive expression of activated Akt abrogated the effects of ROS-induced cell death (Fig. 4). This suggests that the Akt degradation represents an irreversible step towards cell death opposed to the dephosphorylation-Akt inactivation event.

In summary, we demonstrated that H_2O_2 induces the degradation of active Akt via a ubiquitination-dependent pathway and this degradation regulates cellular Akt function as a negative regulator of H_2O_2-mediated cell death. These results suggest that H_2O_2-induced Akt ubiquitin-dependent degradation might accelerate the oxidative stress inducing cell apoptosis. Moreover, these findings provide potential evidence that Akt activity is regulated by ubiquitination.

Acknowledgements

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