

Hydrogen peroxide inhibits mTOR signaling by activation of AMPK α leading to apoptosis of neuronal cells

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Oxidative stress results in apoptosis of neuronal cells, leading to neurodegenerative disorders. However, the underlying molecular mechanism remains to be elucidated. Here, we show that hydrogen peroxide (H₂O₂), a major oxidant generated when oxidative stress occurs, induced apoptosis of neuronal cells (PC12 cells and primary murine neurons), by inhibiting the mammalian target of rapamycin (mTOR)-mediated phosphorylation of ribosomal p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). *N*-acetyl-L-cysteine (NAC), a scavenger of reactive oxygen species (ROS), blocked H₂O₂ inhibition of mTOR signaling. Ectopic expression of wild-type (wt) mTOR, constitutively active S6K1 or downregulation of 4E-BP1 partially prevented H₂O₂ induction of apoptosis. Furthermore, we identified that H₂O₂ induction of ROS inhibited the upstream kinases, Akt and phosphoinositide-dependent kinase 1 (PDK1), but not the type I insulin-like growth factor receptor (IGFR), and activated the negative regulator, AMP-activated protein kinase α (AMPK α), but not the phosphatase and tensin homolog (PTEN) in the cells. Expression of a dominant negative AMPK α or downregulation of AMPK α 1 conferred partial resistance to H₂O₂ inhibition of phosphorylation of S6K1 and 4E-BP1, as well as cell viability, indicating that H₂O₂ inhibition of mTOR signaling is at least in part through activation of AMPK. Our findings suggest that AMPK inhibitors may be exploited for prevention of H₂O₂-induced neurodegenerative diseases.

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Oxidative stress elicits elevated levels of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydroxyl radical (HO[•]), which cause increased permeability of the blood–brain barrier, tubulin alterations, and perturbation in synaptic transmission,¹ and are known to have an important function in neuronal cell death.² Therefore, ROS induction becomes a prominent feature of many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Increasing evidence has further shown that under pathological conditions, excessive amounts of ROS can readily react with thiol groups of proteins, thereby disrupting the structure of cellular proteins and altering their functions,

and also activate related signaling pathways, leading to neuronal apoptosis or neurodegeneration.^{3–9}

The mammalian target of rapamycin (mTOR), a 289-kDa serine/threonine (Ser/Thr) protein kinase, lies downstream of insulin-like growth factor 1 (IGF-1) receptor (IGFR).¹⁰ In response to ligand binding, IGFR, a transmembrane tyrosine kinase, is activated via auto-phosphorylation of multiple tyrosine residues. Activated IGFR phosphorylates the insulin receptor substrates, which in turn triggers activation of phosphatidylinositol 3' kinase (PI3K). Activation of PI3K results in activation of PDK1, Akt, and mTOR, which is negatively regulated by phosphatase and tensin homolog (PTEN), a dual-specificity protein and lipid phosphatase.

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Activated mTOR further phosphorylates ribosomal p70 S6 kinase (S6K1), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), and Akt, controlling cell proliferation, growth, and survival.^{10,11} Activated PDK1 also positively regulates S6K1.¹⁰ Studies have placed tuberous sclerosis complex (TSC) 1/2 as a modulator between PI3K/Akt and mTOR.^{12–14} The TSC1/2 complex acts as a repressor of mTOR function.^{12–14} TSC2 has GTPase-activating protein (GAP) activity toward the Ras family small GTPase Rheb (Ras homolog enriched in brain), and TSC1/2 antagonizes the mTOR signaling pathway via stimulation of GTP hydrolysis of Rheb.^{15–20} Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38,²¹ though this remains controversial.²² The TSC can also be activated by energy depletion through the activation of AMP-activated kinase (AMPK).²³ AMPK responds both to changes in AMP concentration and to changes in the ratio of AMP to ATP.²⁴ An increased AMP to ATP ratio allows AMPK phosphorylation and activation.²⁴ This, in turn, activates the TSC, which catalyzes the conversion of Rheb-GTP to Rheb-GDP and thus inhibits mTOR.²³ AMPK can also be phosphorylated and activated by the calcium- and calmodulin-dependent protein kinase kinase- β (CaMKK- β) in response to alterations in intracellular calcium homeostasis.²⁵ In addition, AMPK may be activated by oxidative stress.²⁵

Recently, we have shown that H₂O₂ induces neuronal apoptosis, which is associated with the inhibition of protein phosphatase 2A and protein phosphatase 5, leading to activation of MAPK pathway.⁹ Here, we further show that H₂O₂-induced ROS inhibited mTOR-mediated phosphorylation of S6K1 and 4E-BP1 in PC12 cells and primary murine neurons. Furthermore, we identified that H₂O₂ induction of ROS inhibited the upstream kinases, Akt and PDK1, but not IGFR, and activated the negative regulator, AMPK α , but not PTEN in the cells. Expression of a dominant negative AMPK α or downregulation of AMPK α 1 conferred partial resistance to H₂O₂ inhibition of phosphorylation of S6K1 and 4E-BP1, as well as cell viability, indicating that H₂O₂ inhibition of mTOR signaling is at least in part through activation of AMPK.

MATERIALS AND METHODS

Materials

Hydrogen peroxide (30%) was purchased from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) was provided by Mediatech (Herndon, VA, USA). Horse serum and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA), whereas 0.05% Trypsin-EDTA, NEUROBASAL Media, and B27 Supplement were from Invitrogen (Carlsbad, CA, USA). Enhanced chemiluminescence solution was from Pierce (Rockford, IL, USA). CellTiter 96 AQueous One Solution Cell Proliferation Assay kit was from Promega (Madison, WI, USA). Caspase-3/7 Assay Kit (Cat.# 71118) was purchased from AnaSpec, Inc. (Fremont, CA, USA). The following antibodies were used: phospho-AMPK α (Thr172), acetyl-CoA carboxylase (ACC), phospho-ACC

(Ser79), phospho-mTOR (Ser2448), mTOR, phospho-Akt (Ser473), S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236) (all from Cell Signaling Technology, Beverly, MA, USA); hemagglutinin (HA), IGFR β subunit (IGFR β), Akt, AMPK α , phospho-S6K1 (Thr389), S6K1 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); 4E-BP1 (Zymed Laboratories, South San Francisco, CA, USA); Phosphotyrosine (BD Biosciences, San Jose, CA, USA); FLAG and β -tubulin (Sigma); goat anti-rabbit IgG-horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP (Pierce). *N*-acetyl-L-cysteine (NAC), Poly-D-lysine (PDL), papain, DNase1 and all the other chemicals were purchased from Sigma.

Cell Culture

Rat pheochromocytoma (PC12) and SH-SY5Y cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). PC12 cells were used for no more than 10 passages, and were in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS, whereas SH-SY5Y cells were grown in antibiotic-free DMEM supplemented with 10% FBS. Cells were maintained in a humid incubator (37°C, 5% CO₂).

To isolate primary neurons, CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in the Animal Resource Center at Louisiana State University Health Sciences Center-Shreveport under specific pathogen-free conditions. All procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee and were in compliance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animals. Fetal mouse cerebral cortexes of 14–18 days of gestation were used. Briefly, the pregnant mice were anaesthetized with pentobarbital and embryos were removed by a cesarean section under sterile conditions. Cerebral cortexes were isolated under dissection microscope and washed with ice-cold Ca²⁺/Mg²⁺-free Hank's-buffered salt solution (HBSS) supplemented with 1% glucose and 50 μ g/ml gentamicin. The isolated embryo cortexes were digested with papain solution [HBSS containing 500 μ g/ml papain, 50 μ g/ml DNase1, 1% glucose, 10 μ M CaCl₂, 5 mM EDTA] for 15 min at 37°C. Digested tissues were triturated into single cells using 1 ml pipette in NEUROBASAL Media supplemented with 2% B27 Supplement, 2 mM glutamine, 1 mM sodium pyruvate, 5 μ g/ml insulin, and 40 μ g/ml of gentamicin. Isolated cells were seeded at a density of 2 \times 10⁶ cells/well in a six-well plate coated with 10 μ g/ml PDL in the above culture medium, and grown in a humid incubator (37°C, 5% CO₂). Fresh medium was replaced every 3 days. The cells were used for experiments after 6 days of culture.

Recombinant Adenoviral Constructs and Infection of Cells

To generate recombinant adenoviruses expressing FLAG-tagged wild-type (wt) mTOR, wt-mTOR was excised from

expression vector PIRENeo-FLAG-mTOR²⁶ (gifts from Dr Jie Chen, University of Illinois, Urbana, IL, USA), and subcloned to pShuttle-CMV vector. The recombinant adenoviruses were generated and amplified using 'Ad-Easy system'.²⁷ Recombinant adenoviruses expressing HA-tagged constitutively active S6K1 (Ad-S6K1-ca) and the green fluorescence protein (GFP) (Ad-GFP) were generated and titrated, as described.²⁸ Recombinant adenovirus encoding HA-tagged dominant negative AMPK α 1 (Ad-dn-AMPK α)²⁹ was a gift from Dr Nicholas JG Webster (University of California at San Diego, La Jolla, CA, USA). For experiments, PC12 cells were grown in the growth medium, and infected with the individual adenovirus for 24 h at 5 of multiplicity of infection (MOI=5). Subsequently, cells were used for experiments. Cells infected with Ad-GFP alone served as a control. Expression of HA-tagged S6K1-ca or dn-AMPK α was confirmed by western blot with antibodies to HA. As S6 ribosomal protein and ACC are the substrates for S6K1 and AMPK α , the functions of S6K1-ca and dn-AMPK α in the cells were assessed by immunoblotting with antibodies to phospho-S6 ribosomal protein (Ser235/236) and phospho-ACC (Ser79), respectively.

Lentiviral shRNA Cloning, Production, and Infection

To generate lentiviral shRNA to human AMPK α 1, oligonucleotides containing the target sequences were synthesized, annealed, and inserted into FSIPPW lentiviral vector via the EcoR1/BamH1 restriction enzyme site. Oligonucleotides used were: sense: 5'-AATTCCCGTTGTGGCTCACCCAACTATGCAAGAGATAGTTGGGTGAGCCACAACCTTTTTG-3', anti-sense: 5'-GATCCAAAAAGTTGTGGCTCACCCAACTATCTCTGCATAGTTGGGTGAGCCACAACGGG-3'. Lentiviral shRNA construct targeting GFP (control) was described.²⁶ To produce lentiviral shRNAs, above constructs were co-transfected together with pMD2.G and psPAX2 (Addgene, Cambridge, MA, USA) to 293TD cells using Lipfectamine 2000 reagent (Invitrogen). Each virus-containing medium was collected 36 and 60 h post-transfection, respectively. For use, monolayer cells, when grown to about 70% confluence, were infected with above lentivirus-containing supernatant in the presence of 8 μ g/ml polybrene for 12 h twice at an interval of 6 h. Uninfected cells were eliminated by exposure to 2 μ g/ml puromycin for 48 h before use.

Assays for Cell Viability, Morphology, and Caspase-3/7 Activity

PC12 cells, infected with Ad-mTOR, Ad-S6K1-ca, Ad-dn-AMPK α , or Ad-GFP (control), or infected with lentiviral shRNA to AMPK α 1 or GFP, respectively, were seeded at a density of 1×10^4 cells/well in a flat-bottomed 96-well plate, precoated with PDL (0.2 μ g/ml). Next day, cells were treated with/without H₂O₂ (0.5 and 0.7 mM) for 24 h with six replicates of each treatment. After incubation, each well was added 20 μ l of one solution reagent (Promega) and incubated for 3 h. Cell viability was determined by measuring the optical density (OD) at 490 nm using a Wallac 1420 Multi-

label Counter (PerkinElmer Life Sciences, Wellesley, MA, USA). Caspase-3/7 activity was determined using Caspase-3/7 Assay Kit (AnaSpec, Fremont, CA, USA), following the instructions of the supplier. In addition, the cells were also seeded at a density of 5×10^5 cells/well in a PDL-coated six-well plate. Next day, cells were exposed to H₂O₂ (0.5 and 0.7 mM). After incubation for 24 h, images were taken with an Olympus inverted phase-contrast microscope (Olympus Optical Co., Melville, NY, USA) (200 \times) equipped with the Quick Imaging system.

Western Blot Analysis

After treatment, cells were briefly washed with cold PBS. On ice, cells were lysed in RIPA buffer [50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% SDS; 1% Triton-X 100; 10 mM NaF; 1 mM Na₃VO₄; protease inhibitor cocktail (1:1000, Sigma)]. Lysates were sonicated for 10 s and centrifuged at 14 000 rpm for 10 min at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as standard (Pierce). Equivalent amounts of protein were separated on 7.5–12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using enhanced chemiluminescence solution (Pierce). To check the amount of protein loaded, the immunoblots were treated with stripping solution (62.5 mM Tris buffer, pH 6.7, containing 2% SDS and 100 mM β -mercaptoethanol) for 30 min at 50°C and incubated with mouse monoclonal anti- β -tubulin antibody (Sigma) followed by horseradish peroxidase-coupled goat anti-mouse IgG (Pierce).

Detection of Phosphorylation of IGFR

PC12 cells (5×10^5 /ml) were seeded in 100-mm dishes, precoated with PDL (0.2 μ g/ml). The next day, cells were treated with H₂O₂ (0–1 mM), or were treated with/without H₂O₂ (0.5 and 0.7 mM) following 1 h of NAC (5 mM) preincubation. After incubation for 2 h, cells were washed once in ice-cold 1 \times PBS, followed by lysis in RIPA buffer. After clearing via centrifugation (14 000 rpm, 15 min, 4°C), 500 μ l supernatants of cell lysates were collected and incubated overnight at 4°C with 1 μ g of antibody to IGFR β and 30 μ l of protein A/G-agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were washed with RIPA buffer two times and twice with ice-cold PBS, followed by immunoblotting with antibodies to p-Tyr and IGFR β , respectively. IgG-heavy chain and light chain from each immunoprecipitated sample were used for loading control.

Statistical Analysis

Results were expressed as mean values \pm standard error (mean \pm s.e.). Statistical analysis was performed by Student's

t-test (STATISTICA, Statsoft Inc, Tulsa, OK). A level of $P < 0.05$ was considered to be significant.

RESULTS

Hydrogen Peroxide Induction of ROS Inhibits mTOR-Mediated Phosphorylation of S6K1 and 4E-BP1 in Neuronal Cells

We have shown that H₂O₂ induces apoptosis of neuronal cells.⁹ mTOR is a central regulator of cell survival.¹⁰ To determine whether H₂O₂ induction of neuronal cell death is associated with inhibition of mTOR signaling, PC12 cells and primary murine neurons were used. By western blotting analysis, we found that treatment of cells with different concentrations of H₂O₂ (0–2 mM) for 2 h decreased phosphorylation of mTOR, S6K1, and 4E-BP1 in a concentration-dependent manner, and at concentrations of >0.5 mM (for

PC12) or >1 mM (for neurons), H₂O₂ dramatically reduced or completely blocked phosphorylation of S6K1 or the substrate of S6K1, S6, as well as 4E-BP1 (Figure 1a). We also observed that H₂O₂ inhibited phosphorylation of S6K1 and 4E-BP1 in a time-dependent manner. As shown in Figure 1b, at 15-min post-experiment, H₂O₂ obviously reduced phosphorylation of S6K1 and 4E-BP1, and such effect was sustained for over 2 h. We also noticed that a complete abolishment occurred at 30 min (for PC12) or at 120 min (for neurons) post-exposure to H₂O₂. H₂O₂ did not markedly alter total protein levels of those proteins (Figure 1a and b). It should be mentioned that a considerable basal level of phosphorylation of 4E-BP1 was detected (Figure 1a and b) in PC12 cells and primary murine neurons. Phosphorylation state of 4E-BP1 was initially detected with an antibody to 4E-BP1. Phosphorylation of 4E-BP1 decreases its electrophoretic

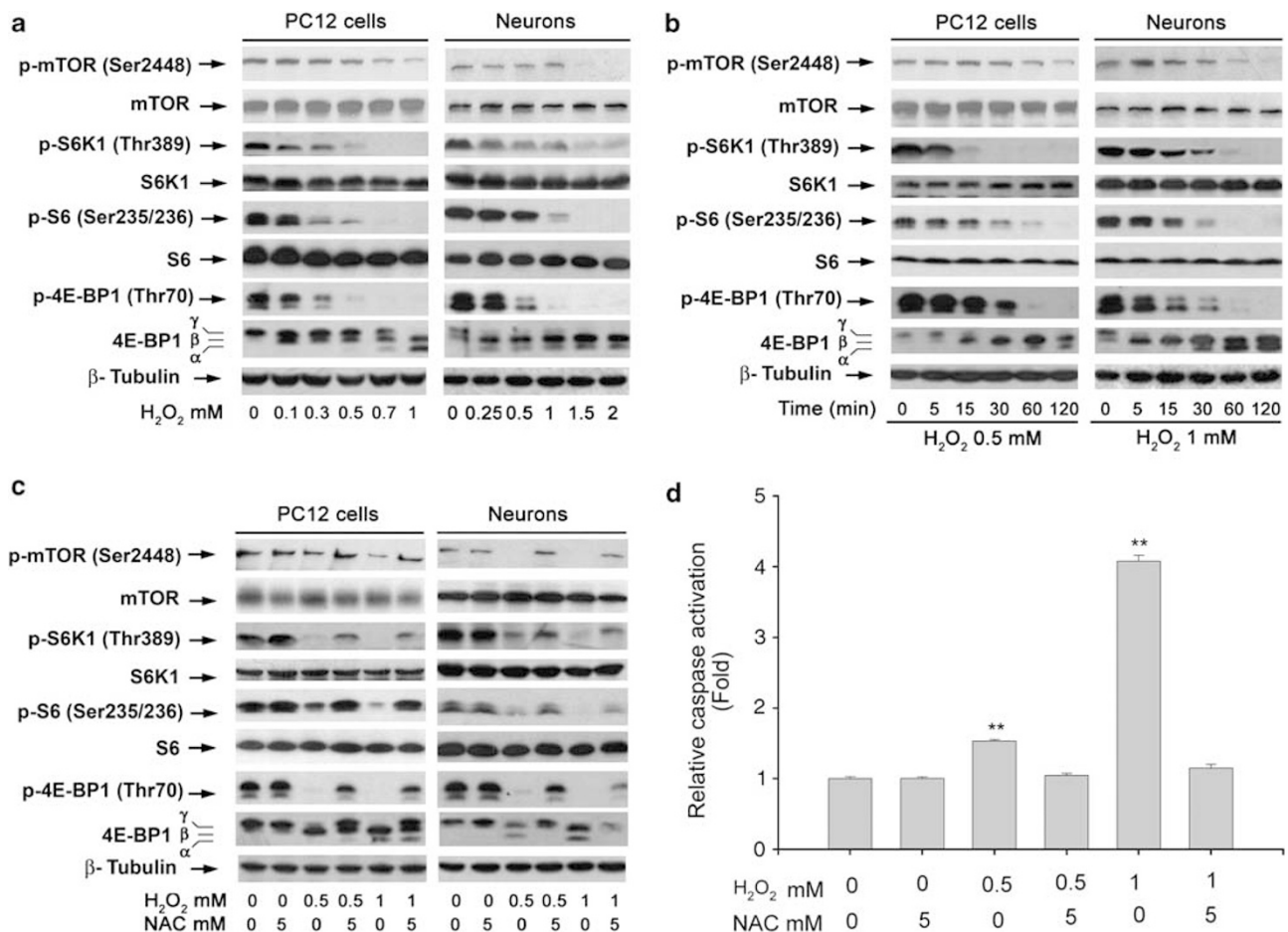


Figure 1 Hydrogen peroxide induction of ROS inhibits phosphorylation of mTOR-mediated S6K1 and 4E-BP1 in neuronal cells. (a) PC12 cells and primary neurons were treated with 0–2 mM H₂O₂ for 2 h, (b) treated with 0.5–1 mM H₂O₂ for indicated time (0–120 min), or (c) with 0.5 and 1 mM H₂O₂ for 2 h after pretreatment with NAC (5 mM) for 1 h. Total cell lysates were subjected to western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. H₂O₂ markedly decreased phosphorylation of S6K1 and 4E-BP1 in a concentration-dependent (a) and time-dependent (b) manner, which was attenuated by NAC (c). (d) PC12 cells, seeded in 96-well plates (black wall), were exposed to 0.5 and 1 mM H₂O₂ for 24 h after pretreatment with NAC (5 mM) for 1 h. Subsequently, 50 μl of caspase-3/7 substrate solution was added into each well. After incubation on a plate shaker at 100 rpm at room temperature for 1 h (avoiding direct light), the fluorescence intensity was measured at Ex/Em = 354 nm/422 nm, using a Wallac 1420 Multilabel Counter. Results are presented as mean ± s.e. ($n = 6$). ** $P < 0.01$, difference vs control group (H₂O₂ = 0 mM, and NAC = 0 mM).

mobility during SDS-polyacrylamide gel electrophoresis.³⁰ H₂O₂ also decreased phosphorylation of 4E-BP1 in a concentration- and time-dependent manner, as indicated by the decrease in the intensity of the uppermost band γ and by the increase in the higher mobility band α and β that corresponds to a less phosphorylated form of 4E-BP1 (Figure 1a and b). We also verified the finding using the antibodies against to phospho-4E-BP1 (Thr70) (Figure 1a and b). The findings indicate that H₂O₂ inhibits mTOR-mediated S6K1 and 4E-BP1 signaling pathways in the neuronal cells.

To unveil whether H₂O₂ inhibition of mTOR signaling is due to induction of ROS, PC12 cells were exposed to H₂O₂ (0.5 and 1 mM) for 2 h after pretreatment with NAC, a ROS scavenger, for 1 h. As shown in Figure 1c, H₂O₂ inhibited phosphorylation of mTOR, S6K1 and 4E-BP1, which was remarkably attenuated by NAC. Similar data were seen in primary neurons (Figure 1c). In addition, consistent with our earlier findings,⁹ we also found that NAC blocked H₂O₂ inhibition of cell viability, induction of cell roundup (data not shown), and activation of caspases 3/7 in PC12 cells (Figure 1d), suggesting that NAC prevented H₂O₂-induced apoptosis of the cells (Figure 1d). The findings support the notion that H₂O₂ induces ROS contributing to suppression of mTOR-mediated S6K1 and 4E-BP1 pathways, leading to neuronal cell death.

Ectopic Expression of Wild-Type mTOR or Constitutively Active S6K1, or Downregulation of 4E-BP1 Partially Prevents Hydrogen Peroxide-Induced Neuronal Cell Death

To further assess the role of mTOR in H₂O₂-induced neuronal cell death, PC12 cells were infected with Ad-mTOR

and Ad-GFP (control), respectively. Ectopic expression of FLAG-tagged wt-mTOR conferred considerable resistance to H₂O₂ inhibition of mTOR-mediated phosphorylation of S6K1 and 4E-BP1 (Figure 2a), as well as H₂O₂-induced cell death (Figure 2b), suggesting that H₂O₂-induced apoptosis is at least partially associated with inhibition of mTOR.

mTOR-mediated S6K1 and 4E-BP1 pathways are critical for cell survival.¹⁰ To evaluate the role of S6K1 pathway in H₂O₂ induction of neuronal cell death, PC12 cells were infected with recombinant adenoviruses expressing HA-tagged constitutively active S6K1 (Ad-S6K1-ca) or control virus encoding GFP alone. As shown in Figure 3a, expression of high levels of recombinant S6K1-ca was observed in the cells infected with Ad-S6K1-ca, but not in those infected with Ad-GFP, as detected by immunoblotting with antibodies to HA and S6K1. The basal level of phosphorylation of S6 ribosomal protein, a substrate of S6K1, was considerably high in the cells, which was not enhanced by expression of S6K1-ca. However, when the cells were exposed to H₂O₂ (0.5 and 0.7 mM) for 2 h, expression of S6K1-ca obviously rendered resistance to H₂O₂ inhibition of phosphorylation of S6K1 and S6 ribosomal protein. Of interest, expression of S6K1-ca also significantly increased cell viability in the presence of H₂O₂ (Figure 3b) or prevented H₂O₂-induced neuronal apoptosis (Figure 3c).

Next, we assessed the role of 4E-BP1 pathway in H₂O₂ induction of neuronal cell death. As 4E-BP1 functions as a suppressor of eIF4E,¹⁰ downregulation of 4E-BP1 would lead to loss of suppression of eIF4E. For this, 4E-BP1 was downregulated by >90% in PC12 cells using lentiviral

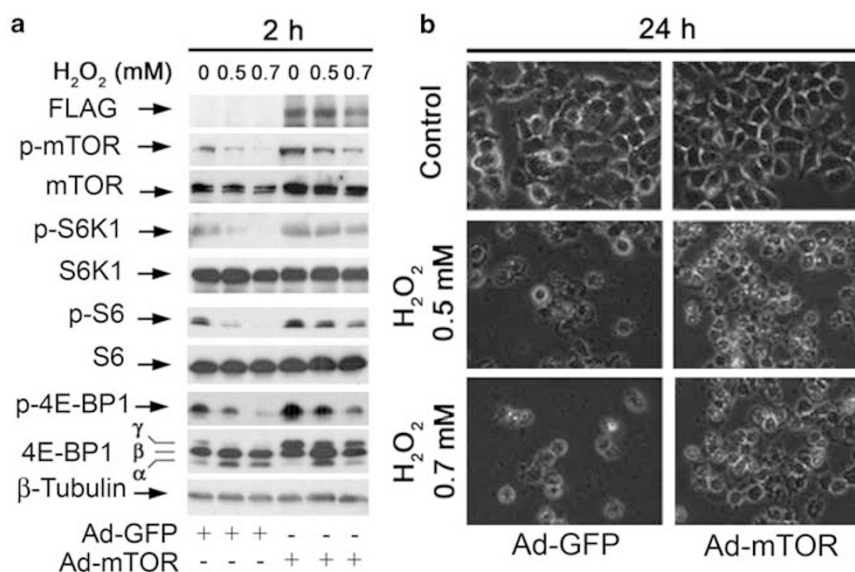


Figure 2 Expression of wt-mTOR partially prevents hydrogen peroxide inhibition of mTOR signaling and cell death. (a) PC12 cells, infected with replication-defective adenoviral recombinant expressing FLAG-tagged wt-mTOR (Ad-mTOR) or the control adenovirus (Ad-GFP), were exposed to H₂O₂ (0.5 and 0.7 mM) for 2 h, followed by western blot analysis using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. (b) PC12 cells, infected with Ad-GFP or Ad-mTOR, were exposed to H₂O₂ (0.5 and 0.7 mM) for 24 h, followed by morphological analysis using an Olympus inverted phase-contrast microscope (200 \times) equipped with Quick Imaging system.

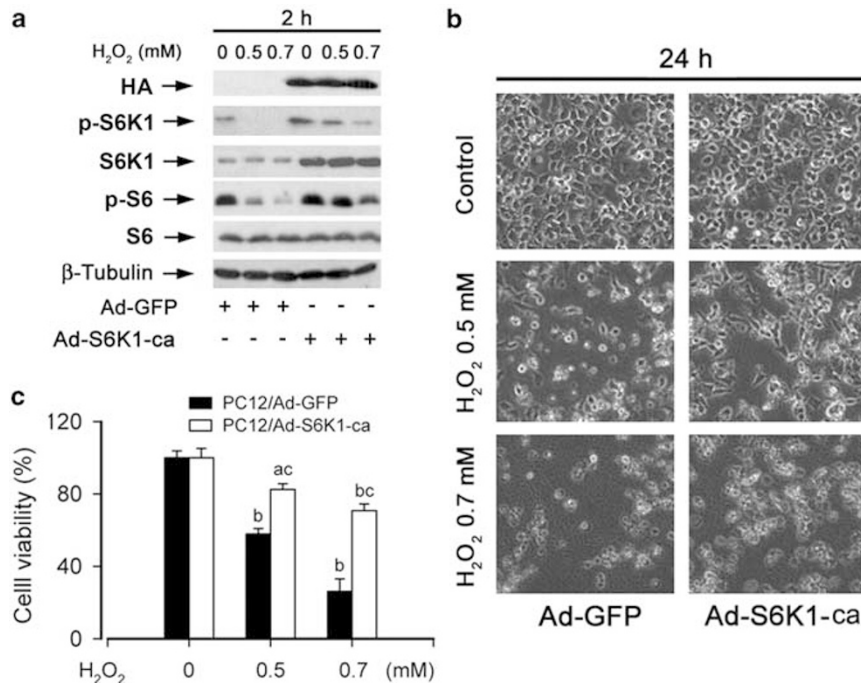


Figure 3 Expression of constitutively active S6K1 partially prevents hydrogen peroxide inhibition of S6K1 pathway and cell viability. **(a)** PC12 cells, infected with replication-defective adenoviral recombinant expressing constitutively active S6K1 (Ad-S6K1-ca) or the control adenovirus (Ad-GFP), were exposed to H₂O₂ (0.5 and 0.7 mM) for 2 h, followed by western blot analysis using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. **(b and c)** PC12 cells, infected with Ad-GFP or Ad-S6K1-ca, were exposed to H₂O₂ (0.5 and 0.7 mM) for 24 h, followed by **(b)** morphological analysis using an Olympus inverted phase-contrast microscope (200 \times) equipped with Quick Imaging system, and **(c)** cell viability assay using one solution reagent, respectively. Results are presented as mean \pm s.e. ($n = 6$). ^a $P < 0.05$, ^b $P < 0.01$, difference vs control group; ^c $P < 0.01$, Ad-S6K1-ca group vs Ad-GFP group. PC12 cells infected with an adenovirus expressing constitutively active S6K1 (Ad-S6K1-ca), but not a control virus (Ad-GFP), conferred partial resistance to H₂O₂ inhibition of phosphorylation of S6 ribosomal protein **(a)**, as well as cell viability **(b and c)**.

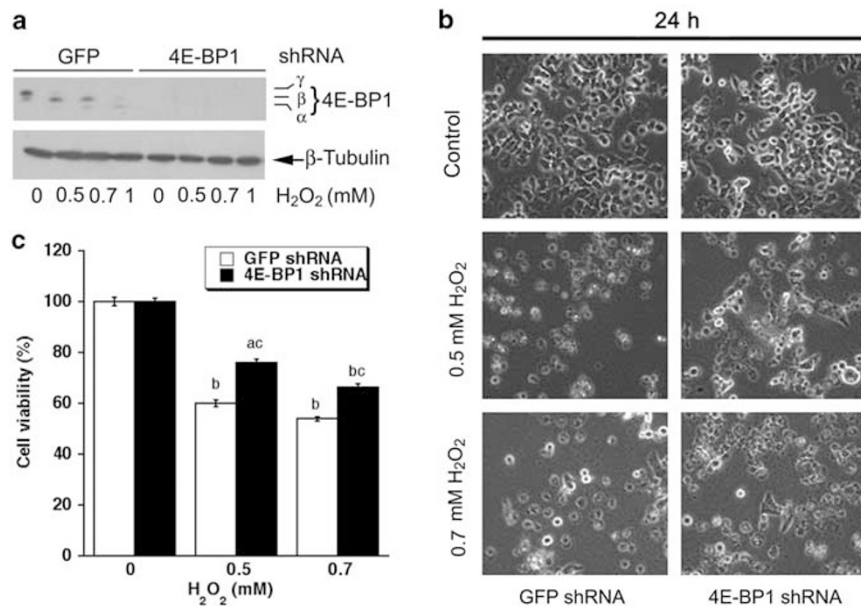


Figure 4 Downregulation of 4E-BP1 partially rescues hydrogen peroxide-induced neuronal cell death. **(a)** PC12 cells, infected with lentiviral shRNAs to 4E-BP1 and GFP (as control), were exposed to H₂O₂ (0.5, 0.7, and 1 mM) for 2 h, followed by western blot analysis with antibodies to 4E-BP1. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. **(b and c)** Morphology and cell viability of lentiviral shRNA-infected PC12 cells, treated with/without H₂O₂ (0.5 and 0.7 mM) for 24 h, were assessed using an Olympus inverted phase-contrast microscope (200 \times) equipped with Quick Imaging system, and using one solution assay, respectively. Results are presented as mean \pm s.e. ($n = 6$). ^a $P < 0.05$, ^b $P < 0.01$, difference vs control group; ^c $P < 0.01$, 4E-BP1 shRNA group vs GFP shRNA group.

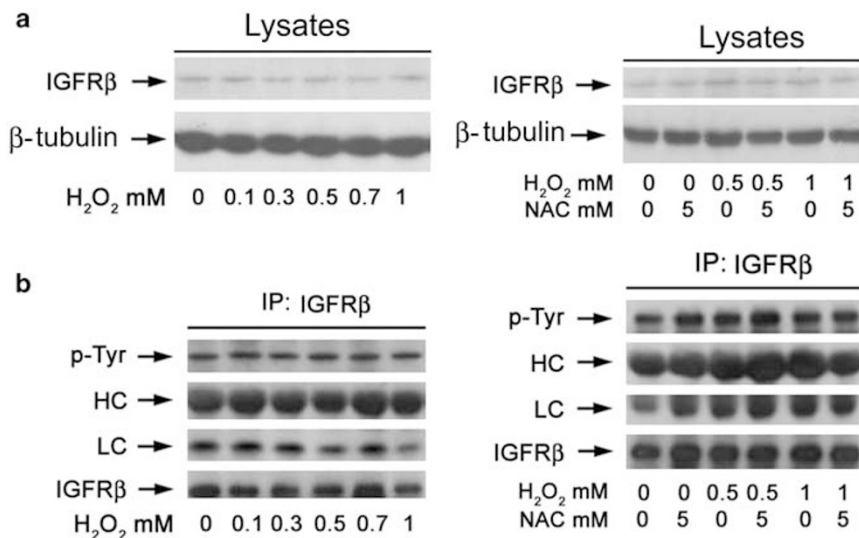


Figure 5 Hydrogen peroxide does not alter protein expression and phosphorylation of IGFR in neuronal cells. PC12 cells were treated with 0–1 mM H₂O₂ for 2 h, or with 0.5 and 0.7 mM H₂O₂ for 2 h after pretreatment with NAC (5 mM) for 1 h, followed by (a) western blot analysis with indicated antibodies, or (b) immunoprecipitation with antibodies to IGFR β subunit (IGFR β) plus A/G-agarose, and immunoblotting with antibodies to phosphotyrosine (p-Tyr) and IGFR β , respectively. HC, heavy chain of IgG; LC, light chain of IgG.

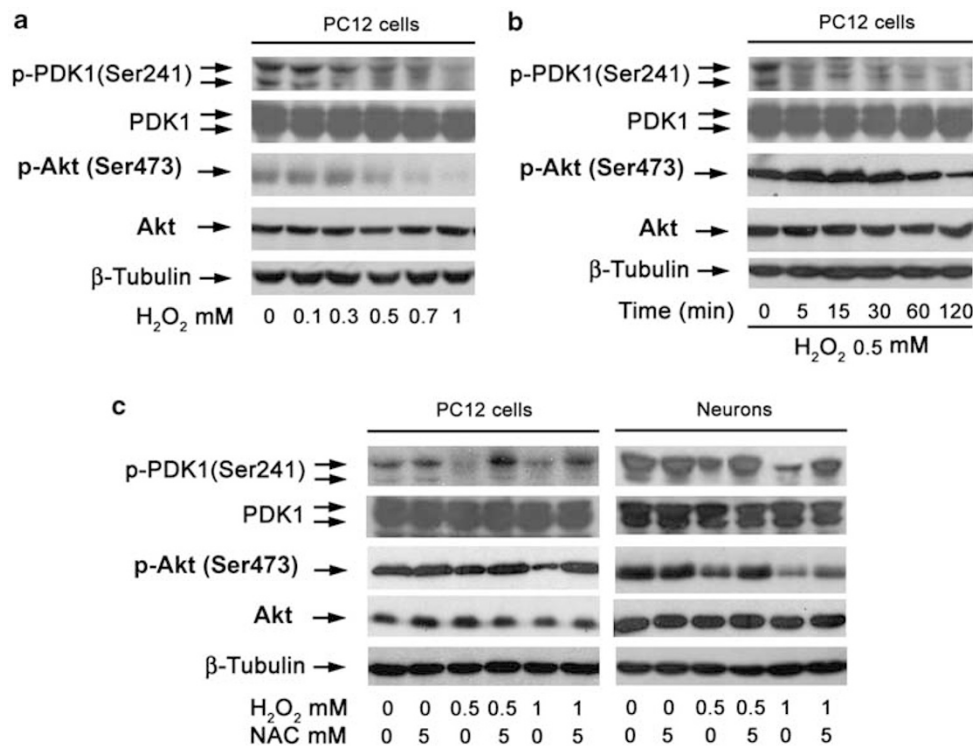


Figure 6 Hydrogen peroxide inhibits Akt and PDK1 phosphorylation in neuronal cells. (a) PC12 cells were treated with 0–1 mM H₂O₂ for 2 h, or (b) with 0.5 mM H₂O₂ for 0–120 min. Total lysates were subjected to western blot analysis using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. H₂O₂ inhibited phosphorylation of Akt and PDK1 in a concentration-dependent (a) and time-dependent (b) manner. (c) PC12 cells and primary neurons were pretreated with treated with/without NAC (5 mM) for 1 h, and then incubated with/without H₂O₂ at indicated concentrations for 2 h, followed by western blot analysis using indicated antibodies. NAC markedly reversed H₂O₂ inhibition of Akt or PDK1 activity.

shRNA to 4E-BP1 (Figure 4a). Downregulation of 4E-BP1 significantly increased cell viability in the presence of H₂O₂ (0.5 and 0.7 mM) (Figure 4b and c). Collectively, our results

indicate that H₂O₂ induced neuronal cell death at least in part through inhibition of mTOR-mediated S6K1 and 4E-BP1/eIF4E pathways.

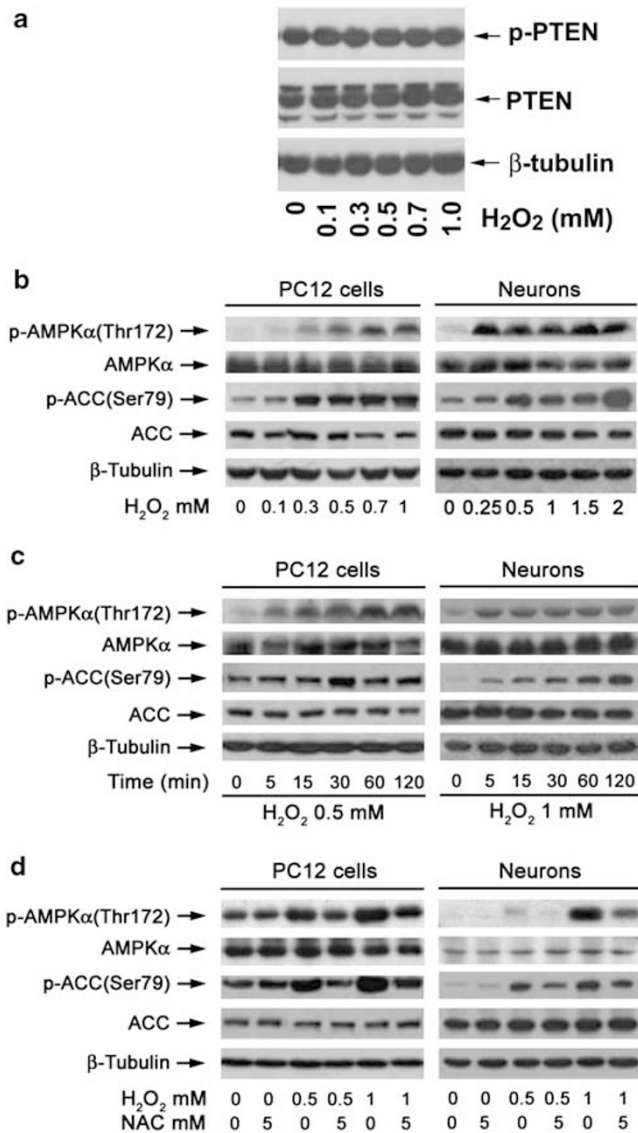


Figure 7 Hydrogen peroxide induction of ROS activates AMPK signaling in neuronal cells. (a) PC12 cells were treated with 0–1 mM H₂O₂ for 2 h, (b) PC12 cells and primary neurons were treated with 0–2 mM H₂O₂ for 2 h, (c) or treated with 0.5–1 mM H₂O₂ for indicated time (0–120 min), or (d) with 0.5 and 0.7 mM H₂O₂ for 2 h after pretreatment with NAC (5 mM) for 1 h. Total cell lysates were subjected to western blot analysis using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. H₂O₂ obviously increased phosphorylation of AMPK α as well as its substrate (ACC) in a concentration-dependent (b) and time-dependent (c) manner, which was remarkably blocked by NAC (d).

Hydrogen Peroxide Inhibits Phosphorylation of PDK1 and Akt, but not IGFR, in Neuronal Cells

IGFR, a transmembrane protein tyrosine kinase, lies upstream of mTOR.¹⁰ To determine whether H₂O₂ induction of ROS inhibits mTOR signaling by suppressing the activity of IGFR, PC12 cells were treated with H₂O₂ (0–1 mM) for 2 h, or exposed to H₂O₂ (0.5 and 1 mM) for 2 h following 1 h of NAC (5 mM) pretreatment, followed by immunoprecipita-

tion with antibodies to IGFR β subunit (IGFR β), and immunoblotting with antibodies to phosphotyrosine (p-Tyr) and IGFR β , respectively. We found that treatment with H₂O₂ or NAC did not obviously impact protein expression of IGFR β (Figure 5a). Furthermore, H₂O₂ failed to alter the basal or NAC-pretreated tyrosine phosphorylation of IGFR β , indicating that H₂O₂ did not affect IGFR activity (Figure 5b).

As activated Akt and PDK1 may positively regulate mTOR and S6K1, respectively,¹⁰ we next investigated whether H₂O₂ induction of ROS inhibits mTOR-mediated phosphorylation of mTOR and S6K1 by suppressing the activity of Akt and PDK1, respectively. To this end, PC12 cells were treated with different concentrations of H₂O₂ (0–1 mM) for 2 h, or with 0.5 mM H₂O₂ for 0–2 h. We found that a concentration-dependent decrease of phosphorylation of Akt and PDK1 was detected by western blot analysis, although H₂O₂ did not alter cellular protein levels of Akt and PDK1 (Figure 6a). H₂O₂ inhibited phosphorylation of PDK1 in a time-dependent manner. However, H₂O₂ transiently activated Akt within 5–30 min, and then inhibited phosphorylation of Akt in 1–2 h (Figure 6b). Treatment with NAC markedly prevented H₂O₂ inhibition of Akt and PDK1 phosphorylation in PC12 cells (Figure 6c). Similar results were also observed in primary neurons (Figure 6c), indicating that H₂O₂ induction of ROS inhibits Akt and PDK1 activity.

Hydrogen Peroxide Induction of ROS Activates AMPK, but not PTEN

PTEN and AMPK are two major negative regulators of mTOR signaling.^{10,23} To determine whether H₂O₂ inhibition of mTOR signaling is associated with activation of PTEN and/or AMPK, PC12 cells were exposed to 0–1 mM H₂O₂ for 2 h or to 0.5 mM H₂O₂ for different time (0–120 min). Our western blot analysis revealed that exposure to H₂O₂ did not obviously alter protein expression and phosphorylation of PTEN (Figure 7a). However, H₂O₂ increased phosphorylation of AMPK α and its substrate (ACC) in a concentration- and time-dependent manner, although there was no effect on cellular protein expression of AMPK α (Figure 7b and c). Similar data were observed in primary neurons (Figure 7b and c).

Next, we tested whether H₂O₂ activation of AMPK α is related to ROS induction. By western blot analysis, we found that pretreatment with NAC markedly attenuated H₂O₂ increase of phosphorylation of AMPK α and ACC in PC12 cells and primary neurons (Figure 7d). Our findings suggest that H₂O₂-induced oxidative stress activates AMPK α signaling.

Expression of Dominant Negative AMPK α or Downregulation of AMPK α 1 Partially Prevents Hydrogen Peroxide Inhibition of mTOR-Mediated Phosphorylation of S6K1 and 4E-BP1, as well as Neuronal Cell Death

To further determine whether H₂O₂ activation of AMPK α is correlated with its inhibition of mTOR-mediated phosphorylation of S6K1 and 4E-BP1, PC12 cells, infected with Ad-dn-AMPK α and Ad-GFP (as control), were exposed to H₂O₂ (0.5

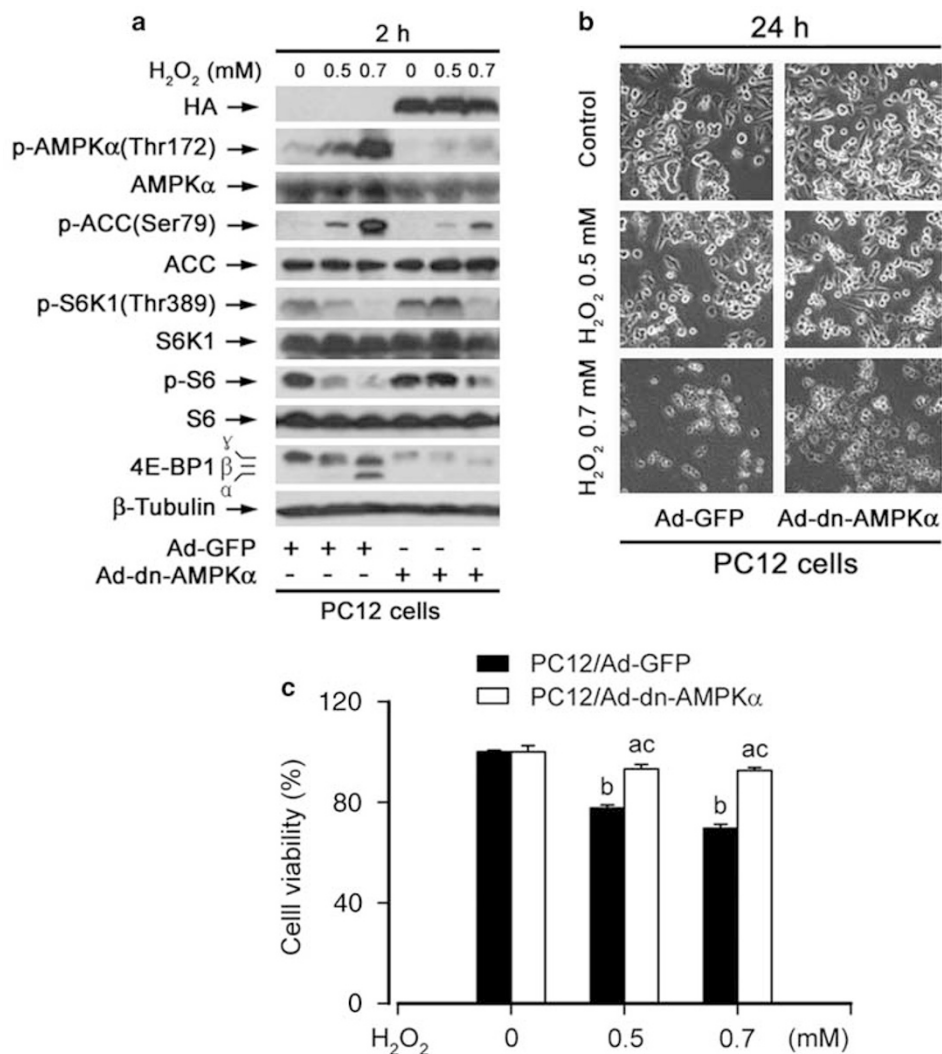


Figure 8 Expression of a dominant negative AMPK α partially prevents hydrogen peroxide inhibition of phosphorylation of S6K1 and 4E-BP1, as well as cell viability. **(a)** PC12 cells, infected with Ad-dn-AMPK α and Ad-GFP (as control), were exposed to H₂O₂ (0.5 and 0.7 mM) for 2 h, followed by western blot analysis using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. **(b and c)** PC12 cells, infected with Ad-dn-AMPK α and Ad-GFP (as control), were exposed to H₂O₂ (0.5 and 0.7 mM) for 24 h, followed by **(b)** morphological analysis using an Olympus inverted phase-contrast microscope (200 \times) equipped with Quick Imaging system, and **(c)** cell viability assay using one solution reagent, respectively. Results are presented as mean \pm s.e. ($n = 6$). ^a $P < 0.05$, ^b $P < 0.01$, difference vs control group; ^c $P < 0.01$, Ad-dn-AMPK α group vs. Ad-GFP group. Overexpression of a dominant negative AMPK α partially rescued cells from death induced by H₂O₂, by inhibiting phosphorylation of AMPK α and ACC and increasing phosphorylations of mTOR-mediated S6K1, S6, and 4E-BP1.

and 0.7 mM) for 2 h. Western blot analysis revealed that ectopic expression of dn-AMPK α obviously blocked H₂O₂-induced phosphorylation of AMPK α and its substrate, ACC (Figure 8a). Consistently, H₂O₂ inhibition of phosphorylation of S6K1, S6, and 4E-BP1 was apparently attenuated by expression of dn-AMPK α (Figure 8a). Furthermore, our morphological analysis and cell viability assay revealed that expression of dn-AMPK α also partially protected PC12 cells from apoptosis induced by H₂O₂ (Figure 8b and c). Therefore, our results suggest that H₂O₂ induces apoptosis of the neuronal cells at least in part through activation of AMPK α , leading to inhibition of mTOR-mediated phosphorylation of S6K1 and 4E-BP1.

To further show the role of AMPK in H₂O₂ inhibition of mTOR signaling and induction of neuronal cell death, expression of AMPK α 1 was silenced by RNA interference. As shown in Figure 9a, lentiviral shRNA to AMPK α 1, but not to GFP, downregulated AMPK α 1 expression by $\sim 85\%$ in SH-SY5Y cells. Downregulation of AMPK α 1 decreased the basal level of phosphorylation of ACC but increased the basal level of phosphorylation of mTOR. Of importance, silencing expression of AMPK α 1 obviously attenuated H₂O₂-induced phosphorylation of AMPK α and ACC, particularly at 0.5 mM. Consistently, downregulation of AMPK α 1 conferred partial resistance to H₂O₂ inhibition of mTOR signaling pathways (Figure 9a), as well as H₂O₂-induced cell death in SH-SY5Y cells (Figure 9b).

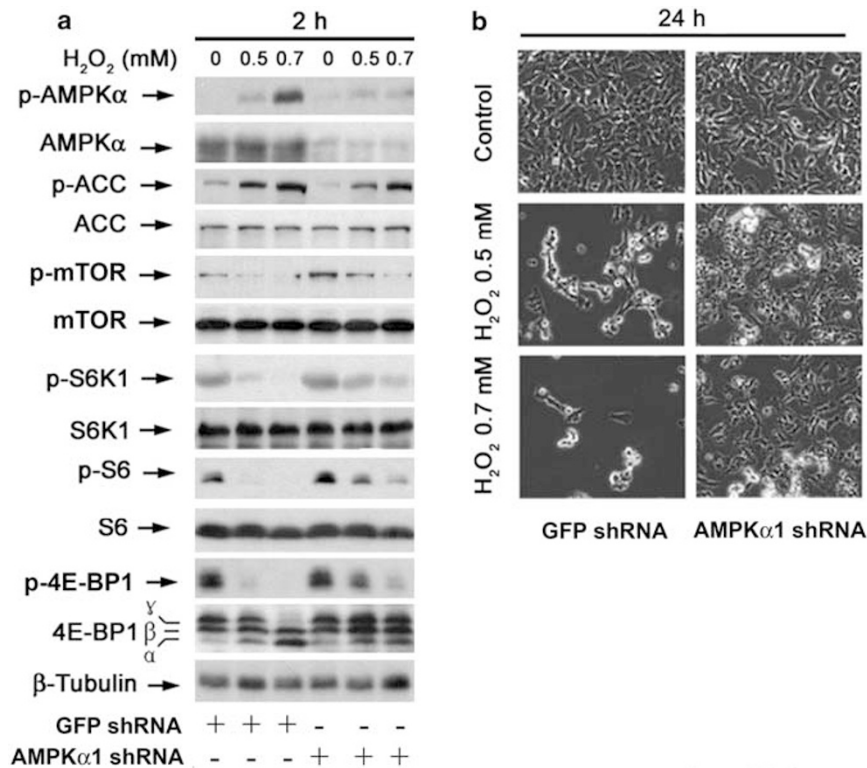


Figure 9 Downregulation of AMPK α 1 partially prevents hydrogen peroxide inhibition of mTOR signaling and induction of neuronal cell death. **(a)** SH-SY5Y cells, infected with lentiviral shRNAs to human AMPK α 1 and GFP (as control), were exposed to H₂O₂ (0.5, 0.7, and 1 mM) for 2 h, followed by western blot analysis with indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. **(b)** Morphology of lentiviral shRNA-infected SH-SY5Y cells, treated with/without H₂O₂ (0.5 and 0.7 mM) for 24 h, were assessed using an Olympus inverted phase-contrast microscope (200 \times) equipped with Quick Imaging system.

DISCUSSION

Increasing evidence has implicated oxidative stress as one of critical factors leading to neuronal cell death in many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.^{2,31-39} However, how oxidative stress causes neuronal cell death is not well understood. mTOR has been widely recognized as a central controller for cell proliferation, growth, and survival.^{10,23} Recently, we have shown that H₂O₂ induces apoptosis in PC12 cells and primary murine neurons.⁹ Here, we show that H₂O₂ induction of neuronal cell death is in part associated with inhibition of mTOR signaling pathway. This is strongly supported by the findings (Figures 1-4) that (1) H₂O₂ treatment dramatically inhibited phosphorylation of mTOR, S6K1, and 4E-BP1 in a concentration- and time-dependent manner; (2) the above event was blocked by NAC in PC12 cells and primary neurons; and (3) expression of wt-mTOR or constitutively active S6K1, or downregulation of 4E-BP1 conferred partial resistance to H₂O₂ induction of neuronal cell death. In addition, we also noticed that inhibition of mTOR with rapamycin (100 ng/ml) alone did not significantly induce apoptosis of PC12 cells, though rapamycin slightly enhanced H₂O₂-induced cell death (data not shown). The results suggest that H₂O₂-induced neuronal

cell death may involve more signaling pathways. At least JNK, Erk1/2, and p38 MAPK pathways have been described.⁹ Our results are in contrast to the findings in hepatoma (H4IIE) and epidermal (JB6) cells,⁴⁰⁻⁴⁴ but in agreement with the data from PC12 cells.⁴⁵ Treatment of H4IIE or JB6 cells with H₂O₂ or its inducers, such as arsenite and ultraviolet irradiation, increased phosphorylation of S6K1, which was blocked by NAC or catalase (a specific H₂O₂ scavenger).⁴⁰⁻⁴⁴ Ultraviolet irradiation-induced H₂O₂ contributes to tumorigenesis.⁴¹ However, treatment of PC12 cells with H₂O₂ inhibited phosphorylation of 4E-BP1 and initiation factor 4E (eIF4E), which was completely abolished by NAC.⁴⁵ The findings in this study and others⁴⁰⁻⁴⁵ imply that the effect of H₂O₂ on cell fate or mTOR signaling, to some degree, may be dependent on cell type.

In the studies, we observed that H₂O₂ inhibited phosphorylation of 4E-BP1 (Thr70), but increased expression of hypophosphorylated forms (α and β bands) of 4E-BP1 in PC12 and primary murine neurons. It has been reported that decreased phosphorylation of 4E-BP1 may increase the stability of 4E-BP1.⁴⁶ With a great possibility, H₂O₂ inhibition of phosphorylation of 4E-BP1 may increase stability of 4E-BP1, leading to increased expression of α and β bands of 4E-BP1.

mTOR signaling is positively regulated by IGFR/Akt, and negatively regulated by AMPK and PTEN.^{10,23} Besides, activated PDK1 may positively regulate mTOR-mediated S6K1.¹⁰ To identify the mechanism by which H₂O₂ inhibits mTOR signaling in neuronal cells, first of all, we focused on the upstream kinases, IGFR, Akt, and PDK1. Our data show that H₂O₂ did not impact protein expression and tyrosine phosphorylation of IGFR β (Figure 5), but inhibited phosphorylation of Akt and PDK1 in PC12 cells and primary neurons in a concentration-dependent manner (Figure 6). H₂O₂ inhibited PDK1 phosphorylation in a time-dependent manner, but activated Akt transiently within 10–30 min and then inhibited Akt in 1–2 h. The mechanism remains to be defined. Pretreatment with NAC, a ROS scavenger, dramatically prevented H₂O₂-reduced phosphorylation of Akt and PDK1 in the cells (Figure 6), revealing that H₂O₂ induction of ROS inhibits mTOR signaling is concurrently related to inhibition of Akt and PDK1, but not IGFR, in neuronal cells. PI3K is an upstream kinase of Akt and PDK1.²³ Whether this is due to inhibition of PI3K remains to be determined.

In this study, we also observed that H₂O₂ failed to alter protein expression and phosphorylation of PTEN, but dramatically increased the activity of AMPK (Figure 7). H₂O₂ treatment resulted in increase of phosphorylation of AMPK α and its substrate, ACC, in a dose- and time-dependent manner in PC12 cells and primary neurons. Pretreatment with NAC inhibited H₂O₂ activation of AMPK α . Clearly, NAC inhibited phosphorylation of AMPK α and ACC induced by H₂O₂ at 0.5 mM than at 1 mM more potently. Probably, at 1 mM, H₂O₂ activates AMPK through ROS-independent mechanism. Of importance, expression of dominant negative AMPK α or downregulation of AMPK α 1 partially prevented H₂O₂ inhibition of phosphorylation of mTOR, S6K1, and 4E-BP1, as well as induction of cell death (Figures 8 and 9). In particular, in response to a higher concentration (1 mM) of H₂O₂, the protective effect of dominant negative AMPK α or AMPK α 1 shRNA against H₂O₂-induced phosphorylation of ACC and apoptosis was marginal. We also noticed that overexpression of wild-type AMPK alone, like treatment with rapamycin alone, inhibited phosphorylation of mTOR and its downstream effector molecules, including S6K1 and 4E-BP1, but failed to induce significant apoptosis in PC12 cells (data not shown). However, overexpression of wt-AMPK did sensitize PC12 cells to H₂O₂-induced cell death (data not shown). The results are in agreement with the findings that AMPK/mTOR-independent pathways, such as JNK, Erk1/2, and p38 MAPK pathways,⁹ also contribute to H₂O₂-induced cell death. The above data indicate that H₂O₂ induction of ROS causes apoptosis of the neuronal cells at least in part through activation of AMPK α signaling, leading to inhibition of mTOR-mediated phosphorylation of S6K1 and 4E-BP1.

In summary, we have identified that oxidative stress by H₂O₂ inhibits mTOR-mediated phosphorylation of S6K1 and 4E-BP1, leading to apoptosis of neuronal cells. H₂O₂

induction of ROS inhibits Akt and PDK1, but not IGFR; activates AMPK, but not PTEN. Expression of dominant negative AMPK α or downregulation of AMPK α 1 conferred partial resistance to H₂O₂ inhibition of mTOR signaling and cell viability, indicating that H₂O₂ induction of ROS inhibits mTOR pathway at least in part by activating AMPK α . Our findings suggest that AMPK inhibitors may be exploited for prevention of oxidative stress (eg H₂O₂)-induced neurodegenerative diseases.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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