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Multi-mechanisms are involved in reactive oxygen species regulation of mTORC1 signaling

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ABSTRACT

The mammalian target of rapamycin complex 1(mTORC1) integrates diverse signals to control cell growth, proliferation, survival, and metabolism. Role of reactive oxygen species (ROS) on mTORC1 signaling remains obscure and mechanisms through which ROS modulate mTORC1 are not known. We demonstrate that low doses ROS exposure stimulate mTORC1 while high concentrations or long-term ROS treatment decrease mTORC1 activity *in vivo* and in a variety of cell lines. The dose/time needed for inhibition or activation are cell type-dependent. In HEK293 cells hydrogen peroxide (H_2O_2) stimulates phosphorylation of AMP-activated kinase (AMPK) (T172) and Raptor (S792), enhances association of activated AMPK with Raptor. Furthermore, AMPK inhibitor compound c inhibits H_2O_2-induced Raptor (S792) phosphorylation and reverses H_2O_2-induced de-phosphorylation of mTORC1 downstream targets p70-S6K1 (T389), S6 (S235/236) and 4E-BP1 (T37/46). H_2O_2 also stimulates association of endogenous protein phosphatase 2A catalytic subunit (PP2Ac) with p70-S6K1. Like compound c, inhibitor of PP2A, okadaic acid partially reverses inactivation of mTORC1 substrates induced by H_2O_2. Moreover, inhibition of PP2A and AMPK partially rescued cells from H_2O_2-induced cell death. High doses of H_2O_2 inhibit while low doses of H_2O_2 activate mTORC1 both in $TSC2^{-/-}$ $P53^{-/-}$ and $TSC2^{+/+}$ $P53^{-/-}$ MEFs. These data suggest that PP2A and AMPK-mediated phosphorylation of Raptor mediate H_2O_2-induced inhibition of mTORC1 signaling.

1. Introduction

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine protein kinase that plays a central role in controlling cell growth, size and metabolism [1–3]. It elicits its pleiotropic functions in the context of two functionally distinct signaling complexes termed as mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1, which contains mTOR, mLST8/GβL, Raptor, and PRAS40, is sensitive to immunosuppressive drug rapamycin [4,5]. mTORC2 shares mTOR and mLST8/GβL with mTORC1, but processes three unique components, rictor, mSin1, and PRR5/Protor [6–10]. Despite presence of mTOR, mTORC2 is not inhibited by acute treatment of rapamycin.

mTORC1 activity is regulated by a wide range of intracellular and extracellular cues, including growth factors, nutrient conditions, energy levels and stresses. Signaling activities triggered by these cues are channeled to mTORC1 by the TSC1–TSC2 complex, which functions as a GTPase activating protein (GAP) for the small GTPase Rheb, an activator of mTORC1. The TSC1–TSC2 complex stimulates the GTPase activity of Rheb and downregulates its activity, thus negatively regulates mTORC1 function. In response to insulin stimulation, TSC2 is phosphorylated by Akt, which reduces the GAP activity of the TSC1–TSC2 complex. In contrast, glucose deprivation promotes AMP-activated kinase (AMPK)-mediated phosphorylation of TSC2, which enhances the GAP activity [11–14].AMPK is a negative regulator of mTORC1. It elicits its inhibitory effect on mTORC1 through two different mechanisms. Activation under condition of low intracellular ATP (energy stress) by phosphorylating and activation of TSC2 leading to mTOR inhibition [15,16]. However, TSC2-deficient cells remain responsive to energy stress. Recent studies revealed that the phosphorylation of mTOR binding partner Raptor on S792 by AMPK is required for the inhibition of mTORC1 and cell-cycle arrest induced by energy stress [17,18].

Abbreviations: AMPK, AMP-activated protein kinase; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; 2,7-DCFH-DA, 2,7 Dichlorodihydrofluorescein diacetate; DTT, dithiothreito; EGFR, epidermal growth factor receptor; MDA, malondialdehyde; mSin1, mammalian stress-activated protein kinase-interacting protein 1; PAO, phenylarsine oxide; PDK1, phosphoinoside-dependent kinase 1; PI-3K, phosphatidylinositol kinase; PI, polyimide; PMSF, phenylmethyl sulfonylfluoride; PRR5, proline-rich repeat protein-5; Raptor, regulatory associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; TSC, tuberous sclerosis complex.

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mTORC1 exerts its role in cell size control mainly through controlling 4E binding protein 1 (4E-BP1) and the p70 ribosomal protein S6 kinase (p70-S6K), two key factors involved in translation initiation. mTORC1 phosphorylates and inactivates 4E-BP1, a translation repressor that binds to and inhibits the translation initiation factor 4E (eIF-4E). In contrast, mTORC1-directed phosphorylation activates p70-S6K1, which in turn phosphorylates and activates S6 protein, a component of the S40 ribosome subunit, thus facilitating protein translation [19,20].

Reactive oxygen species (ROS) are generated in the cells as a consequence of oxygen-based metabolism. ROS molecules such as hydroxyl radicals, superoxide anions and singlet oxygen are extremely reactive and can cause irreversible damage to intracellular molecules such as nucleic acids and proteins unless they are detoxified by antioxidant enzymes [21]. Growing evidence has demonstrated that ROS can act as secondary messengers in signaling processes. Although low levels of ROS produced by the mitochondria are usually detoxified quickly, an excessive accumulation of ROS caused by UV, ionizing irradiation, chemical insults, or aging may trigger various cellular responses. Depending on concentration of ROS, duration of the cell exposed to these agents and cell types, the responses may range from cell growth and proliferation to apoptosis or necrosis. In human, ROS-mediated signaling pathways have been linked to many diseases, including cancer, cardiac failure, arthero-sclerosis, diabetes, hypertension and osteoporosis [22–26].

ROS have been found to either activate or inhibit mTORC1 [27–33]. However, the molecular mechanisms by which ROS modulate mTORC1 signaling remain obscure. In this paper, we demonstrate that low doses and short-term ROS exposure stimulate mTORC1 while high concentrations or long-term ROS treatment inhibit mTORC1 activity, and most importantly, we found that protein phosphatase 2A and AMPK-mediated phosphorylation of Raptor (S792) contribute to ROS-induced inhibition of mTORC1 signaling.

2. Material and methods

2.1. Materials

Reagents, antibodies and plasmids were obtained from the following sources. Rose Park Memorial Institute (RPMI)1640, Dulbecco's modified Eagle's medium-high glucose (DMEM), alpha modified Eagle's medium (α-MEM), DPBS and fetal bovine serum (FBS) were from Gibco BRL Technology (Gaithersburg, MD, USA); Lipofectamine 2000 from Invitrogen (Carlsbad, CA); Compound c from MERK; 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), okadaic acid (OKA), dimethyl sulphoxide (DMSO) and the antibody against flag from Sigma-Aldrich (St. Louis, MO); LY294002 and antibodies against phospho-p70-S6K1 (T389), phospho-p70-S6K (T421/S424), 4E-BP1, phospho-4E-BP1 (T37/46), phospho-4E-BP1 (T70), phospho-4E-BP1 (S65), phospho-S6 (S235/236), Raptor, phospho-Raptor (S792) and phospho-AMPKα1/2 (T172) from Cell Signaling Inc (Beverly, MA); anti-S6, p70-S6K and AMPKα1/2 antibodies from Santa Cruz Biotech (Santa Cruz, CA); The PP2Ac antibody from Upstate. pcDNA3.1-flag-Rheb Q64L, pcDNA3.1-flag-Rheb wt and pcDNA3.1-flag were described previously [34]. pCMV-flag-TSC2 and PRK-7-HA-S6K1 were purchased from Addgene.

2.2. Cell culture and transient transfection

Mouse monocyte cell line RAW264.7 was cultured in RPMI 1640. Human breast cancer cell line MCF-7, human uterine cervix cancer cell line Hela, human osteosarcoma cell line MG63 , mouse embryo fibroblast cells (MEFs) $TSC2^{+/+}P53^{-/-}$, $TSC2^{-/-}$ $P53^{-/-}$ and human embryo kidney cell line HEK293 cells were cultured in high glucose DMEM. Mouse osteoblast cell line MC3T3-E1 and primary culture bone marrow stroma cell (BMSC) were cultured in α-MEM. The media were all supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg perstreptomycin in a humidified atmosphere of 5% CO_2. Cultures were trypsinized upon confluence and subcultured into 12-, 6-, or 96-well plates for further experiments. For transient transfection, HEK293 cells were seeded into 24-well plates or 6 cm dishes 24 h prior to transfection with plasmids of Rheb Q64L, Rheb wt and flag or flag-TSC2 and myc-Raptor using Lipofectamine 2000.

2.3. UV radiation and cellular H_2O_2 staining assays

Raw264.7 cells were cultured in 35 mm dishes until 90% confluence and treated with UVB radiation (25 mj/cm^2) for different intervals (0, 5, 15, 30 and 60 min). Cells were then washed three times with phosphate-buffered saline (PBS) and incubated in PBS containing DCFH-DA(10 μM) for 20 min at 37 °C. Cells were trypsinized, washed twice with PBS and subjected to FCM to detect the DCF fluorescent signals at 488 nm.

2.4. Alloxan treatment and detection of MDA and H_2O_2

One month old Kunming mice were intraperitoneally injected with alloxan (150 mg/kg) and normal saline after starvation for 24 h. 72 h after the injection, animals were sacrificed and brain, heart, kidney, liver and skeletal muscle were homogenized and ultrasonicated in ice-cold normal saline. After centrifugation at 12,000×*g* for 10 min, the supernatant were collected and stored at −70 °C. Protein concentration was determined by Protein Concentation Detection Kit (SHEN NENG BO CAI, Shanghai, China) and H_2O_2 and MDA concentrations were measured with the H_2O_2 and MDA detection kit from JIAN CHENG (Nanjing, China) following manufacturer's instruction.

2.5. Immunoprecipitation

To detect the association of Raptor with AMPK, HEK293 cells (3×10^6) grown in 6 cm dishes were rinsed once with PBS, lysed in 300 μl of ice-cold buffer A containing 40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, 1% Triton X-100, and 1× EDTA-free protease inhibitors from Roche. Cell lysates were incubated on ice for 30 min followed by centrifugation at 12,000×*g* for 10 min. Supernatants were incubated with 4 μg of anti-PP2A antibody for 3 h at 4 °C on a nutator followed by addition of 30 μl of a 50% slurry of protein G-Sepharose beads addition and incubation on nutator for another 2 h at 4 °C. Beads were washed four times with lysis buffer A and boiled in SDS sample buffer. Precipitated proteins were then subjected to SDS-PAGE and western blotting.

To detect the association of Raptor with P-AMPK, HEK293 cells were grown in 6 cm dishes and transfected with myc-Raptor plasmids. 24 h later, cells were treated with 1 mM H_2O_2 for 20 min, then harvested and lysed by incubating on ice for 30 min in the lysis buffer B (Buffer A containing 0.3% chaps instead of 1% Triton X-100). Lysates were precipitated with anti-myc antibody (4 μg) as described above.

To detect the association of TSC2 with AMPK, HEK 293 cells were transfected with flag-TSC2. After 30 h, transfected cells were treated with 1 mM H_2O_2 for 20 min and harvested. Cells were lysated in the buffer I (20 mM Tris-HCl [pH 7.5], 20 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM b-glycerophosphate, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and sonicated. The cell lysates were immunoprecipitated with the anti-flag antibodies and protein G-Sepharose beads. Immunocomplexes were washed twice with buffer I containing 0.5 M NaCl, twice with buffer II (10 mM HEPES [pH 7.4], 50 mM NaCl, 20 mM b-glycerophosphate, and 20 mM NaF) and then subjected to SDS-PAGE.

2.6. Western blot analysis

After treatment, cells were lysed immediately incubating in SDS sample buffer for 5 min at 95 °C. Cell lysates were subjected to SDS-PAGE followed by western blot analysis.

2.7. Detection and quantification of dead cells by PI staining

HEK 293 cells grown in 24 well plates were pretreated with compound c, OKA or drug vehicle for 30 min, and then incubated with 300 μM H_2O_2 for 12 h. At the end of treatment the cells were rinsed once with PBS and stained with PI using the Apoptosis/Death Detection Kit (JIAN CHENG, Nanjing, China).

2.8. Statistical analysis

Statistical analyses were performed by *T*-test(Fig. 1D and E) and one way ANOVA (Figs. 1B, 6A, B), and $p<0.05$ was considered statistically significant.

3. Results

3.1. ROS activates mTORC1 in vivo and in cells

Hydrogen peroxide (H_2O_2) represents an important type of ROS. Its effect on mTORC1 activity has been documented in many studies. Depending on cell types and concentrations of H_2O_2, the effect may

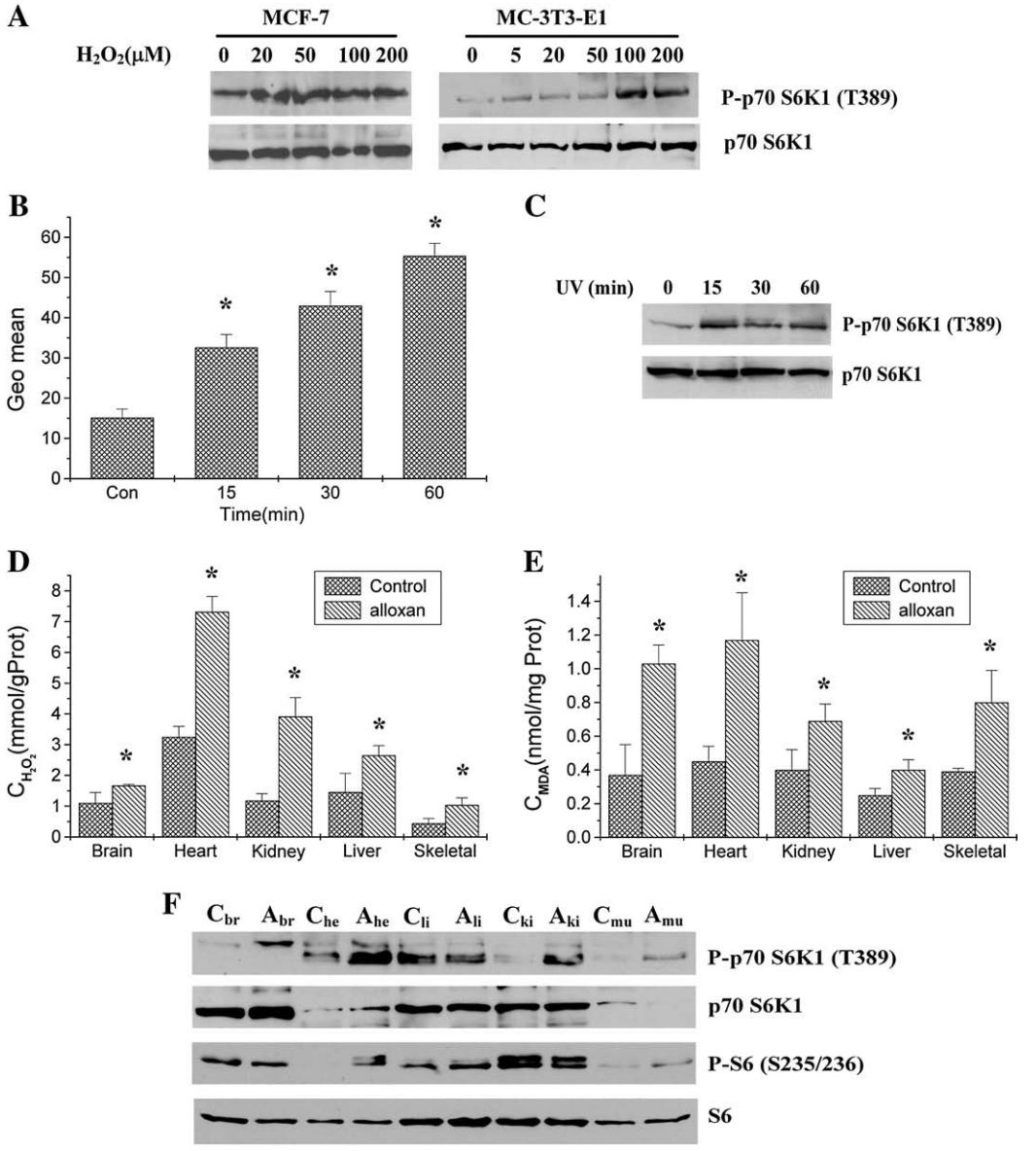

Fig. 1. Activation of mTORC1 *in vivo* and in cells. A) MCF-7 or MC3T3-E1 cells were incubated with 0–200 μM H_2O_2 for 30 min, then lysed and subjected to Western Blotting for P-p70-S6K1(T389) and p70-S6K1. B) Raw264.7 cells were treated with UVB radiation (25 mj/cm^2) for 0–60 min, incubated with DCF-DA (10 μM) and detected the DCF fluorescent signals at 488 nm by FCM. Data were analyzed by One Way ANOVA. Con, control; *significant differences compared to controls ($p<0.05$). C) lysed and subjected to SDS-PAGE before Western Blotting for P-p70-S6K1(T389) and p70-S6K1. D) The mice were treated with alloxan as described in Materials and methods, Levels of H_2O_2 and, E) MDA were determined. The data were analyzed by *T*-test and * is significant differences compared to controls ($p<0.05$). F) The tissues of brain, heart, kidney, liver and skeletal muscle were lysed immediately after sacrifice and subjected to SDS-PAGE before Western Blotting for P-p70-S6K1(T389), p70-S6K1, P-S6(S235/236) and S6.C_{br}, brain of control, A_{br}, brain of alloxan treatment, C_{he}, heart of control, A_{he}, heart of alloxan treatment, C_{li}, liver of control, A_{li}, liver of alloxan treatment, C_{ki}, kidney of control, A_{ki}, kidney of alloxan treatment, C_{mu}, skeletal muscle of control, A_{mu}, skeletal muscle of alloxan treatment.

vary from activation to inhibition [28–30,35,36]. We found that at low dose (5–200 μM), H_2O_2 increased phosphorylation of p70-S6K1 (T389) and S6 (S235/236) in multiple cell lines, including MCF-7, Raw264.7, MC3T3-E1, MEF, (Fig. 1A, and Supplemental Table A1).

Short term UV irradiation or arsenite exposure has been shown to increase the mTORC1-dependent phosphorylation of S6K and 4E-BP1 in several cell lines [31,32,37–39]. However, the underlying mechanism was unclear. Because ultraviolet (UV) irradiation or arsenite exposure is known to increase the amounts of ROS in cells [37], we surmised that the increased ROS in UV or arsenite treated cells is responsible for the increased mTORC1 activity. Indeed, we found that ROS levels increased from 2–4 fold in mouse macrophage RAW264.7 cells upon exposure to UV irradiation (Fig. 1B). Accompanying the elevated concentrations of ROS was an enhanced phosphorylation levels of p70-S6K1 (T389). (Fig. 1C). This finding is consistent with the notion that low dose ROS is stimulatory for mTORC1 activity.

While the effect of ROS on mTORC1 activity has been extensively studied in culture cells, how ROS affect mTORC1 activity *in vivo* is largely unknown. To investigate the effect of ROS *in vivo*, we employed an animal diabetes model induced by alloxan, a toxic glucose analogue which causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in animals. In the presence of intracellular thiols, alloxan generates ROS in a cyclic reaction with its reduction product, dialuric acid. We found that the levels of H_2O_2 and malondialdehyde (MDA) in homogenates of liver, kidney, brain, heart and skeletal muscle of the mice treated with alloxan monohydrate were significantly higher than those from animals treated with vehicle control (Fig. 1D and E). Accompanied with the increased ROS levels was an enhanced phosphorylation of p70-S6K1 (T389) and S6 (S235/236) in the tissues (Fig. 1F). These observations suggest that increased ROS levels stimulate mTORC1 activity *in vivo*.

3.2. High doses of ROS inhibit mTORC1

H_2O_2, at high concentrations, has been shown to inhibit mTORC1 [4,29,30]. Consistent with previous findings, we found that at concentrations higher than 200 μM, H_2O_2 inhibited mTORC1 activity in many types of cells, although the dose and treatment time needed to incur the inhibition varied from cell to cell (Fig. 2 and Supplemental Table A1). These results are summarized in Supplemental Table A1.

3.3. AMPK-mediated Raptor (S792) phosphorylation is involved in hydrogen peroxide-induced mTORC1 inhibition

It has been reported recently that AMPK is involved in H_2O_2-induced inhibition of mTORC1 in neuronal cells [30]. Previous studies have demonstrated that AMPK is able to inhibit mTORC1 through two different mechanisms, one depending on phosphorylation of TSC2, which increases its GAP activity toward Rheb and the other is mediated by phosphorylation of Raptor [15–18]. We thus examined the effect of H_2O_2 on these two processes.

As shown in Fig. 3A and B, we found that in HEK293 cells, H_2O_2 treatment increased phosphorylation of AMPK at position T172 at a dose- and time- dependent manner. Phosphorylation at this site has been shown to stimulate AMPK activity. Coincident with the increased AMPK phosphorylation was an enhanced phosphorylation levels of Raptor at position S792, a target site of AMPK in Raptor. To confirm that the increased phosphorylation of Raptor is caused by AMPK activation, we examined the Raptor phosphorylation in cells treated with compound c, an AMPK-specific inhibitor and found that the drug blocked H_2O_2-induced Raptor phosphorylation and reversed H_2O_2-induced de-phosphorylation of p70-S6K1 (T389), S6 (S235/236) and 4E-BP1 (T37/46). (Fig. 3C). This observation indicates that H_2O_2 induced Raptor phosphorylation was mediated by AMPK. Furthermore, using co-immunoprecipitation assay, we observed that H_2O_2 treatment augmented the association of AMPK with Raptor (Fig. 3D). Collectively, the above results suggest H_2O_2 inhibits mTORC1 through AMPK-dependent phosphorylation of Raptor.

3.4. TSC2/Rheb is not required for hydrogen peroxide-induced suppression of mTORC1

To assay the role of TSC2 in H_2O_2 mediated mTORC1 down-regulation, we examined the effect of H_2O_2 treatment on mTORC1 activity in TSC2 deficient cells. We observed that H_2O_2 treatment was able to decrease the phosphorylation of p70-S6K1(T389), S6 (S235/236) and 4E-BP1 (T37/46) in $TSC2^{-/-}$ $P53^{-/-}$ cells as it were in $TSC2^{+/+}$ $P53^{-/-}$ MEFs (Fig. 4A),

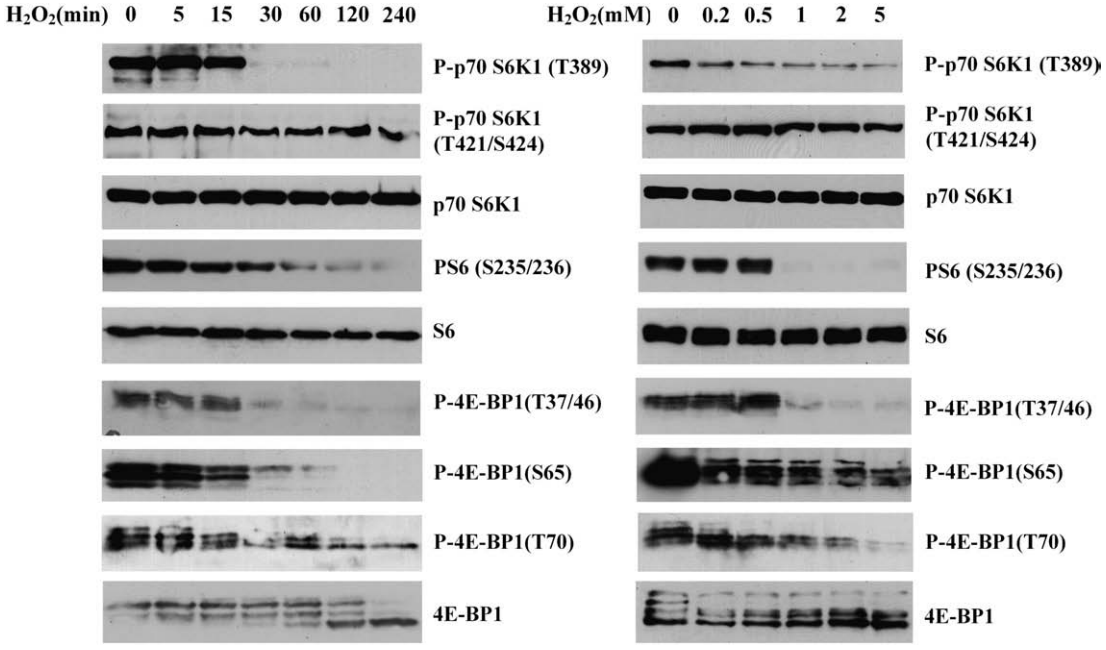

Fig. 2. Inhibition of mTORC1 in HEK293 cells. HEK 293 cells were treated with 1 mM H_2O_2 for indicated times or indicated doses of H_2O_2 for 30 min, then lysed and subjected to Western Blotting for levels of P-p70-S6K1 (T389), p70-S6K1 (T421/424), p70-S6K1, P-S6 (S235/236), S6, P-4EBP1 (T37/46), P-4EBP1 (S65), P-4EBP1 (T70) and 4EBP1.

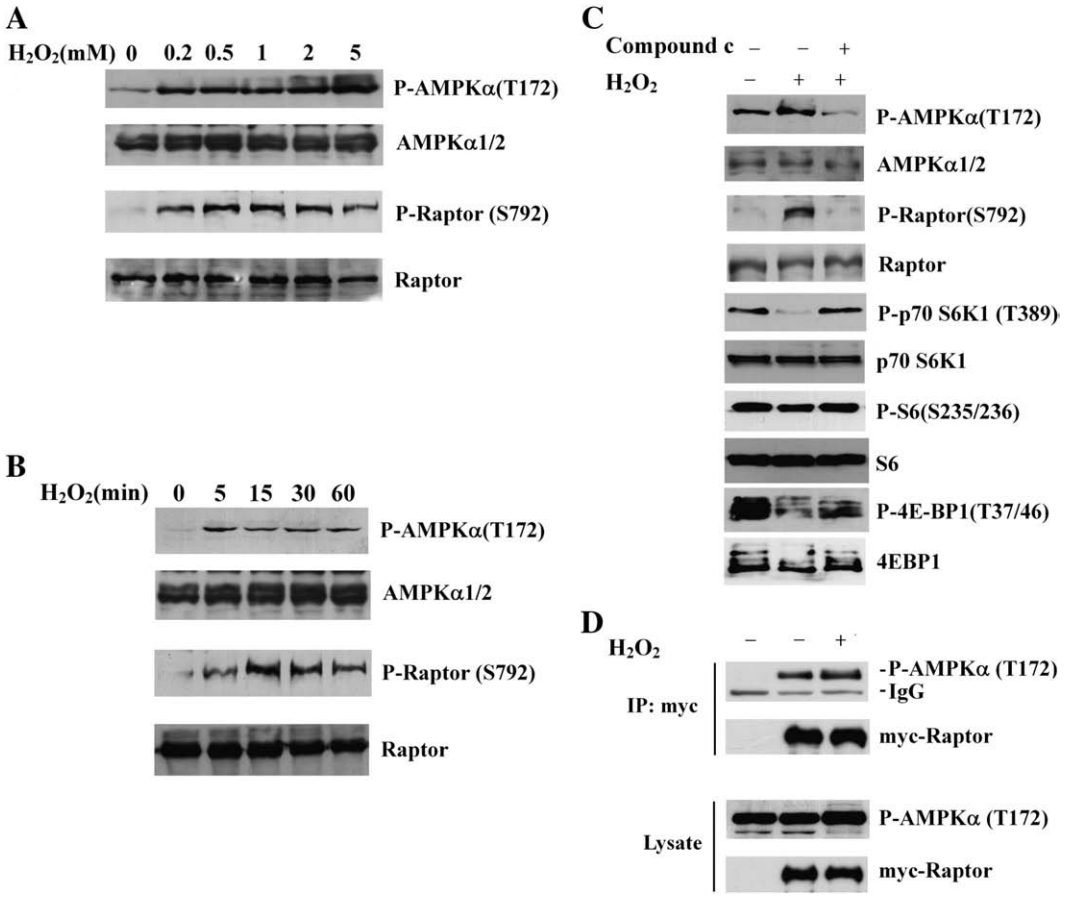

Fig. 3. AMPK-mediated Raptor (S792) phosphorylation is involved in H_2O_2-induced mTORC1 inhibition. A) HEK293 cells incubated with 0–5 mM H_2O_2 for 30 min or B) 1 mM H_2O_2 for 0–60 min, cells were lysed and subjected to Western Blotting for P-AMPKα (T172), AMPKα, P-Raptor (S792) and Raptor. C) HEK 293 cells were pretreated with 20 μM compound c or drug vehicle control for 30 min, followed by exposure to 500 μM of H_2O_2 for 30 min. Cells were lysed and the levels of P-AMPKα (T172), AMPKα, P-Raptor (S792), Raptor, P-p70-S6K1 (T389), p70-S6K1, P-S6 (S235/236), S6 and P-4EBP1 (T37/46) were determined by western blotting. D) HEK 293 cells transfected with myc-Raptor were treated with 1 mM H_2O_2 or vehicle control for 30 min and lysed. Cell lysates were immunoprecipitated with anti-myc antibody, and the levels of P-AMPKα (T172) and AMPKα in the precipitates were determined by Western Blotting.

suggesting that TSC2 is not required for the inhibitory effect of H_2O_2 on mTORC1. In addition, it has been reported that 2-deoxy-glucose (2-DG) activates AMPK and enhances interaction of AMPK with TSC2, leading to phosphorylation of TSC2 on S1345 and inhibition of mTORC1 [15,16]. We found that the association of AMPK with TSC2 was decreased but not increased by H_2O_2 treatment (Fig. 4B).

To examine the role of Rheb in regulation of mTORC1 by H_2O_2, wild type or active mutant (Q64L) of Rheb were transfected into HEK293 cells. As shown in Fig. 4C, although Rheb wt or Q64L overexpression partially reversed amino acid starvation-induced reduction of mTORC1 activity, they were unable to prevent H_2O_2-induced mTORC1 inhibition. These data suggest that TSC2/Rheb dependent mechanism is not involved in H_2O_2-induced mTORC1 inhibition.

3.5. Involvement of protein phosphatase 2A in hydrogen peroxide-induced inhibition of p70-S6K1, S6 and 4E-BP1.

Published studies have demonstrated the important role of protein phosphatase 2A (PP2A) in de-phosphorylation of p70-S6K1 [40]. In response to rapamycin treatment or amino acid-deprivation, PP2A interacts with p70-S6K1 and mediates mTORC1 inhibition-induced p70-S6K1 de-phosphorylation [40]. The possible role of PP2A in H_2O_2-induced suppression of p70-S6K1, S6 and 4E-BP1 are not known. As expected, H_2O_2 treatment stimulated the association of endogenous PP2A catalytic subunit (PP2Ac) with p70-S6K1 (Fig. 5A). Furthermore, OKA, an inhibitor of PP2A prevented the H_2O_2-induced de-phosphorylation of p70-S6K1(T389), S6(S235/236) and 4E-BP1(T37/46) (Fig. 5B). It is suggested that PP2A is involved in inhibition of mTORC1 downstream targets induced by H_2O_2.

3.6. Inhibition of PP2A and AMPK rescue hydrogen peroxide-induced cell death

We next examined the possible role of PP2A and AMPK in functional results of H_2O_2-treated cells. mTORC1 may play an important role in regulation of cell survival. Thus the effects of PP2A and AMPK on H_2O_2-induced cell death were determined in this study. As shown in Fig. 6A and B, long-term H_2O_2 exposure inhibits mTORC1 and induces cell death in HEK293 cells. Pre-treatment of OKA and compound c, reagents that inhibit PP2A and AMPK respectively, decreases H_2O_2-induced cell death significantly ($P<0.05$). It is suggested that inhibition mTORC1 signaling by PP2A and AMPK is involved in ROS-induced cell death.

3.7. PI-3K, but not TSC2, is required, for hydrogen peroxide-induced mTORC1 activation

The data above show some mechanisms through which ROS negatively regulate mTORC1. However, the cellular signals involved in ROS-induced mTORC1 activation are not fully studied. Previous studies using human skin keratinocytes and TSC2 knock-out MEFs ($TSC2^{-/-}$) have revealed important role of EGFR/PI-3K and TSC2 in UV and H_2O_2-induced p70-S6K1 activation [37]. However, in this study, we observed that 0–200 μM H_2O_2 treatment for 30 min dose-

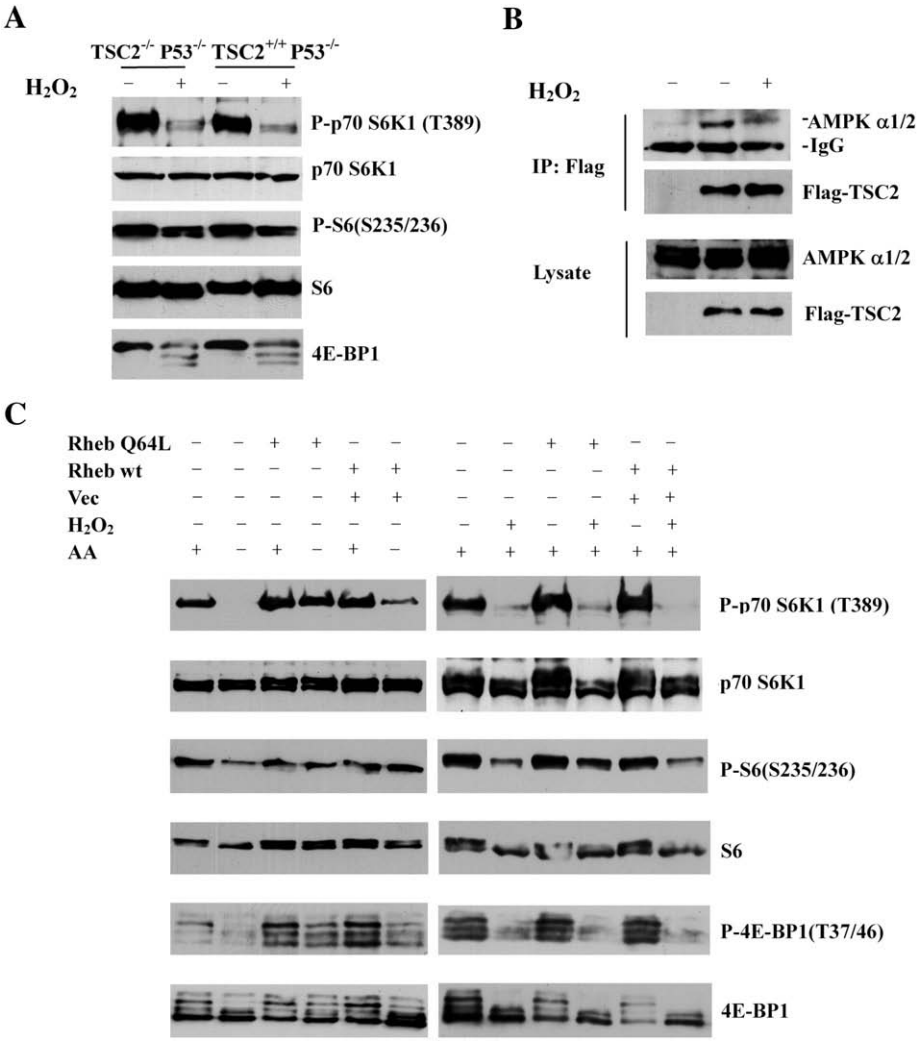

Fig. 4. TSC2/Rheb is not required for H_2O_2 inactivation of mTORC1. A) MEF TSC2$^{+/+}$ P53$^{-/-}$ and MEF TSC2$^{-/-}$ P53$^{-/-}$ cells were treated with 1 mM H_2O_2 for 30 min, lysed and subjected to Western Blotting for P-p70 S6K1(T389), S6K1, P-S6 (S235/236), S6 and 4EBP1. B) HEK293 cells transfected with flag-TSC2 were treated with 1 mM H_2O_2 or vehicle control for 30 min, and lysed. Lysates were immunoprecipitated with anti-flag antibody, and the levels of flag-TSC2 and AMPKα were determined by Western Blotting. C) HEK293 cells transfected with flag-Rheb wt and Q64L were starved for amino acid or treated with 1 mM H_2O_2 for 30 min. Cells were lysed and the levels of P-S6K1 (T389), S6K1, P-S6 (S235/236), S6 and P-4EBP1(T37/46) and 4EBP1 in the lysates were determined by western blotting.

dependently stimulated p70-S6K1 (T389) both in TSC2$^{+/+}$ P53$^{-/-}$ and TSC2$^{-/-}$ P53$^{-/-}$ MEFs (Fig. 7A). For involvement of PI-3K, as expected, we found that PI-3K specific inhibitor, Ly294002 blocked H_2O_2-stimulated p70-S6K1(T389) phosphorylation both in TSC2$^{-/-}$ P53$^{-/-}$ and TSC2$^{+/+}$ P53$^{-/-}$ MEFs (Fig. 7B). Our results suggest that PI-3K is required, TSC2 is not required for H_2O_2-induced mTORC1 activation.

4. Discussion

mTOR is regulated by a variety of stress signals, one of which is reactive oxygen species (ROS). ROS are well recognized for playing dual roles as both deleterious and beneficial factors. The "two-facet" character of ROS is substantiated by growing evidence that ROS can promote DNA, protein and lipid damage, and apoptosis but can also activate adaptive intracellular signaling pathways [41–44]. The effects of ROS on cellular functions are likely to depend on the location and concentration of ROS produced [45]. It has been reported that UV exposure stimulates p70-S6K activity, and pre-treatment with ROS scavengers or antioxidants prevents p70-S6K activation, suggesting that UV-induced ROS could activate mTORC1[32,37,46]. H_2O_2 can either activate [28] or inhibit [4] mTORC1, varying with exposure length, dose/concentration, and a particular cell type or mTORC1 downstream targets (readout, such as a specific phosphorylation site of p70 S6K1 or 4EBP1) tested. Thiol oxidants diamide and phenylarsine oxide(PAO) stimulate mTORC1 in HEK293 cells [47], while hypoxia-induced ROS inhibit 4EBP1 [36]. To further elucidate the effects of ROS on mTORC1 and explore mechanisms through which ROS regulate mTORC1, a variety of cells *in vitro*, different ROS origins (H_2O_2 or UV), and *in vivo* oxidative stress models induced by alloxan were used in our study. We provide evidence for the first time that ROS activate mTORC1 *in vivo* (Fig. 1D, E, F). Our data also showed that UV-induced ROS activate mTORC1, low doses of H_2O_2 stimulate mTORC1 while high concentrations or long-term H_2O_2 treatment decrease mTORC1 activity. The dose/time needed for inhibition or activation are cell type-dependent. Intracellular levels of radical scavenger enzymes and antioxidant vary from cell to cell. It is likely that effect of ROS on mTORC1 activity depends on the concentrations of antioxidant in different cells. Cells with low levels of antioxidant may respond to low doses and short term H_2O_2 exposure, but those with high concentration of antioxidant may have no reaction. as exogenous H_2O_2 is quickly scavenged by antioxidants.

How do high doses or long-term ROS inhibit mTORC1? TSC2 is an upstream negative regulator, which suppresses mTORC1 through inactivating GTP-bound Rheb when stimulated by insulin or growth factors [1,48–51]. The data presented here clearly show that TSC2 is

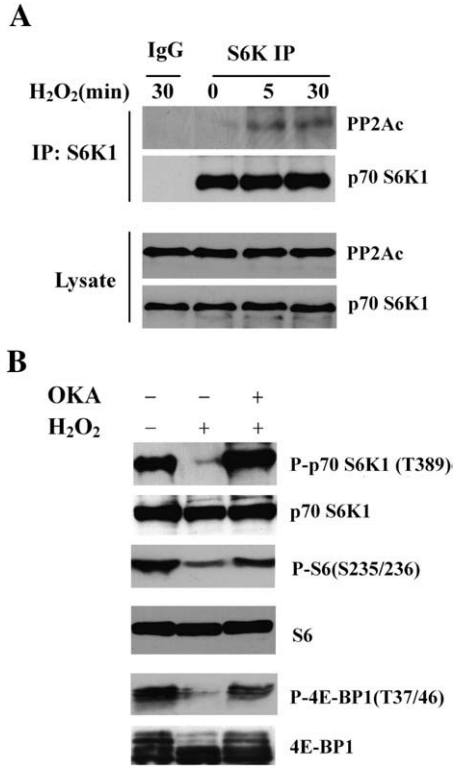

Fig. 5. Protein phosphatase 2A mediates H_2O_2 de-phosphorylation of S6K1, S6 and 4E-BP1. A) HEK293 cells were treated with 2 mM H_2O_2 for indicated times and lysed. Lysates were immunoprecipitated with anti-p70 S6K1 antibody, and the amounts of PP2Ac and S6K1 were determined by western blotting. B) HEK293 cells were treated with 50 nM OKA for 30 min, followed by 1 mM H_2O_2 exposure for 30 min. Cells were lysed and the levels of P-p70-S6K1 (T389), p70-S6K1, P-S6 (S235/236), S6 and P-4EBP1 (T37/46) and 4EBP1 in the lysates were determined by western blotting.

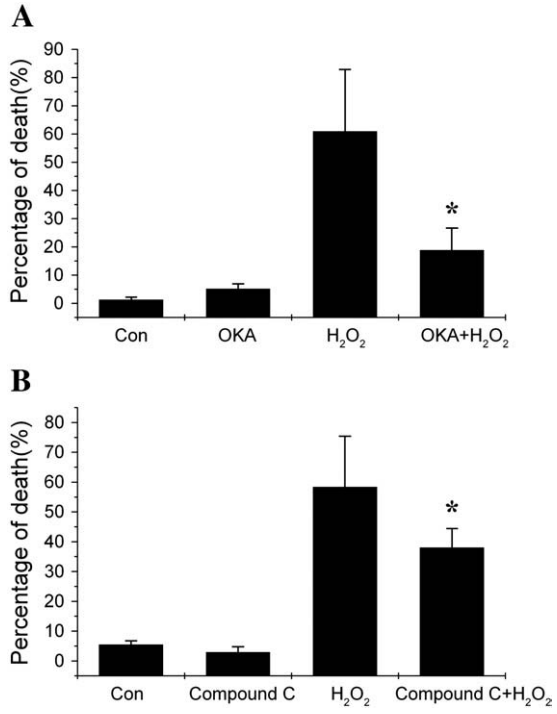

Fig. 6. Inhibition of PP2A and AMPK rescue hydrogen peroxide-induced cell death. HEK 293 cells were treated with 20 μM compound c A) or 10 nM OKA B) or drug vehicle control for 30 min, followed by exposure to 300 μM H_2O_2 for 12 h. The ratio of dead cells was determined. Data were analyzed by One Way ANOVA. Con, control; *significant differences compared to H_2O_2 treatment ($P<0.05$).

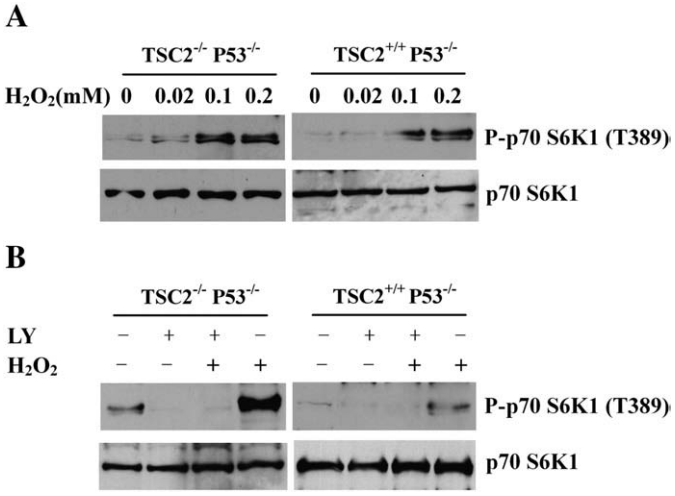

Fig. 7. PI-3K, but not TSC2, is required for H_2O_2-induced mTORC1 activation. A) MEF TSC2$^{+/+}$ P53$^{-/-}$ and MEF TSC2$^{-/-}$ P53$^{-/-}$ cells were treated with 100 μM LY294002 or vehicle control for 30 min , followed by exposure to 500 μM H_2O_2 for 30 min. Cells were lysed and the levels of P-p70-S6K1 (T389) and p70-S6K1 were determined by western blotting. B) MEF TSC2$^{+/+}$ P53$^{-/-}$ and MEF TSC2$^{-/-}$ P53$^{-/-}$ cells were treated with indicated concentrations of H_2O_2 for 30 min. Cells were lysed and lysates were subjected to Western Blotting to detect the levels of p70-S6K1(T389) and p70-S6K1.

not required for the effects of H_2O_2 on mTORC1 activity. Because we find that H_2O_2 still impairs mTORC1 signaling in cells that are devoid of TSC2. Moreover, AMPK has been shown to phosphorylate and activate TSC2, thus leading to mTORC1 inhibition under condition of low intracellular ATP (energy stress). 2-DG, an AMPK agonist, can enhance the interaction of TSC2 and AMPK and then phosphorylate TSC2 [15,16]. We observed that H_2O_2 stimulated AMPK and attenuated the interaction of AMPK and TSC2 instead of promoting the interaction, indicating that H_2O_2 do not exert their effects on mTORC1 signaling by activating TSC2. Our data substantially confirmed those of Smith et al. [33], who observed impairment of mTORC1 signaling in response to amino acid withdrawal and certain stresses in TSC2$^{-/-}$ cells. An active mutant of Rheb (Q64L) was also unable to prevent H_2O_2-induced mTORC1 inhibition. It is suggested that H_2O_2 inhibits mTORC1 in TSC/Rheb-independent pathway.

Recently, it has been reported that the phosphorylation of mTOR binding partner Raptor on S792 by AMPK is required for the inhibition of mTORC1 and cell-cycle arrest induced by energy stress [17]. We found that H_2O_2 stimulates both AMPK on T172 and Raptor on S792 in a dose- and time-dependant manner (Fig. 3A and B), indicating AMPK-mediated phosphorylation of Raptor (S792) may be involved in H_2O_2-induced mTORC1 inhibition. This notion was supported by further studies that AMPK inhibitor suppressed the phosphorylation of AMPK(T172) and Raptor(S792) by H_2O_2, and at the same time reversed H_2O_2-induced mTORC1 inhibition (Fig. 3C). Moreover, the association of P-AMPK(T172) with Raptor (S792) was also enhanced by H_2O_2 (Fig. 3D).

PP2A is composed of a catalytic subunit (PP2Ac), a structural subunit and one of many possible regulatory subunits that determine substrate specificity and cellular localization. PP2A has been shown to act in opposition to the mTORC1 and both enzymes control the phosphorylation status and, thereby, the activity of p70-S6K1 and 4E-BP1 [40,52]. In this study we revealed that H_2O_2 induced the association of PP2Ac with p70-S6K1 (Fig. 5A), and PP2A inhibitor reversed H_2O_2-induced dephoshorylation of p70-S6K1, S6 and 4E-BP1 (Fig. 5B), suggesting that PP2A is involved in this process. How does H_2O_2 stimulate PP2A activity? An early study by Peterson et al [40] has shown that mTOR controls 4E-BP1 and p70-S6k1 phosphorylation indirectly by restraining PP2A in Jurkat cells. They demonstrated that mTOR could phosphorylate and inhibit PP2A. Rapamycin or amino acid withdraw inactivate mTOR,

stimulate PP2A and induce its interaction with p70-S6K1 [40]. It is possible that H_2O_2 stimulates PP2A activity toward p70-S6K1 and 4E-BP1 by inhibiting mTORC1 and that PP2A mediates the inhibitory effect of H_2O_2 on mTORC1 downstream substrates.

Many studies have revealed that mTOR plays an important role in cell survival [53–55].Indeed, blockage of PP2A or AMPK signaling not only reversed high doses of H_2O_2 induced mTORC1 inhibition but also prevented high doses of H_2O_2 induced cell death (Fig.6). These results further support the important role of the two factors in regulation of mTORC1 activity in oxidative stress.

How do low doses of ROS stimulate mTORC1? We observed activation of p70-S6K (T389) by H_2O_2 in $TSC2^{-/-}$ MEFs, suggesting that TSC2 is not required for the process. This result is consistent with that of Smith et al. [33] using the same cell line ($TSC2^{-/-}$ $P53^{-/-}$ MEFs). But another study [37] using $TSC2^{-/-}$ MEFs demonstrated that TSC2 is required for H_2O_2-stimulated phosphorylation of p70 S6K1 (T389). The reasons behind this difference remain unclear. PI-3K specific inhibitor blocked H_2O_2-stimulated p70-S6K1(T389) in both $TSC2^{-/-}$ $P53^{-/-}$ and $TSC2^{+/+}$ $P53^{-/-}$ MEFs (Fig. 7). Cao et al [37] got the same results in human skin keratinocytes, which demonstrate that PI-3K is required for H_2O_2-induced mTORC1 activation. How PI-3K is activated and mediates the positive effect of H_2O_2 on mTORC1? Previous studies using skin keratinocytes have revealed an important role of EGFR in UV and H_2O_2-induced cellular signals [37,56]. It is possible that low doses of H_2O_2 stimulate EGFR and PI-3K, which may subsequently activate mTORC1 by PDK1, Akt or unknown mechanisms independent of TSC2/Rheb.

In conclusion, this study provides evidence that ROS from extracellular or intracellular may either inhibit or activate mTORC1 in vivo and in cells. Low doses of ROS exposure stimulate mTORC1 while high concentrations or long-term ROS treatment decrease mTORC1 activity. The dose/time needed for inhibition or activation are cell type-dependent. PP2A and AMPK-mediated phosphorylation of Raptor but not TSC2 or Rheb are involved in the inhibition of mTORC1 by high doses H_2O_2. PI3K is required, and TSC2 is not required for the activation of mTORC1 by low doses H_2O_2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.05.015.

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