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

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## ARTICLE INFO

## 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 with 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.

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## 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].

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, artherosclerosis, 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$_2$O$_2$ 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$_2$O$_2$

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$_2$O$_2$ and MDA concentrations were measured with the H$_2$O$_2$ 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$_3$VO$_4$, 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$_2$O$_2$ 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$_2$O$_2$ 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.