

Redox Regulation of the Nutrient-sensitive Raptor-mTOR Pathway and Complex*

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The raptor-mTOR protein complex is a key component of a nutrient-sensitive signaling pathway that regulates cell size by controlling the accumulation of cellular mass. How nutrients regulate signaling through the raptor-mTOR complex is not well known. Here we show that a redox-sensitive mechanism regulates the phosphorylation of the raptor-mTOR effector S6K1, the interaction between raptor and mTOR, and the kinase activity of the raptor-mTOR complex. In cells treated with the oxidizing agents diamide or phenylarsine oxide, S6K1 phosphorylation increased and became insensitive to nutrient deprivation. Conversely, the reducing reagent BAL (British anti-Lewisite, also known as 2,3-dimercapto-1-propanol) inhibits S6K1 phosphorylation and stabilizes the interaction of mTOR and raptor to mimic the state of the complex under nutrient-deprived conditions. Our findings suggest that a redox-based signaling mechanism may participate in regulating the nutrient-sensitive raptor-mTOR complex and pathway.

The mTOR (mammalian Target Of Rapamycin) pathway was discovered in studies into the mechanism of action of rapamycin, a lipophilic macrolide antibiotic (1–3). Rapamycin is used clinically as an immunosuppressant and to prevent restenosis after angioplasty and is under consideration as a potential anti-cancer agent (4–7). All evidence indicates that the pharmacological effects of rapamycin are due to its perturbation of the mTOR pathway.

Cell growth is the fundamental biological process through which cells accumulate mass and increase in size. The mTOR pathway is an important regulator of cell size that coordinates the activity of the cell growth machinery with the levels of energy and nutrients, such as amino acids and glucose. The mTOR pathway regulates growth through several downstream effectors including the 4E-BP1² (eukaryotic initiation factor 4E-binding protein) family of translational repressors and the S6K1 and S6K2 (S6 kinase 1 and 2) kinases. mTOR is a 289-kDa protein that contains a phosphatidylinositol-3-kinase-like domain. When part of a complex that also contains the proteins raptor (regulatory associated protein of mTOR) and GβL (G-protein β subunit-like) (8–11) mTOR

phosphorylates substrates like S6K1 and 4E-BP1. GβL binds strongly to mTOR near its kinase domain and is required for maximal mTOR activity (9). Raptor serves to target kinase substrates to mTOR and also regulates mTOR kinase activity in a poorly understood fashion (8, 10). Interestingly, within cells the stability of the interaction between mTOR and raptor is sensitive to nutrient and energy levels (8). Rapamycin, in a complex with its receptor FKBP12 (FK506-binding protein 12), binds to and inhibits the kinase activity of the raptor-mTOR-GβL complex (12) and when added to cells causes the dephosphorylation of S6K1 and 4E-BP1 and a decrease in cell size (8, 13–18). Recently, a rapamycin-insensitive mTOR complex has been identified that contains mTOR, GβL, and rictor (rapamycin insensitive companion of mTOR), but not raptor (19, 20). Rictor defines a distinct mTOR pathway that regulates the PKCα and Akt/PKB signaling networks (19, 21). For short, we refer to this complex as rictor-mTOR and the raptor-containing complex as raptor-mTOR.

Nutrients and cellular metabolism regulate mTOR effectors like S6K1 through the raptor-mTOR complex (8, 10). In cells growing under nutrient-rich conditions the raptor-mTOR interaction is in a low stability state associated with high mTOR kinase activity. In cells growing in nutrient-poor conditions the raptor-mTOR interaction is in a high stability state associated with a decrease in the kinase activity of mTOR. The two states of the raptor-mTOR interaction can be detected by measuring the amount of raptor bound to mTOR in cells cultured under different nutrient conditions. We find greater amounts of raptor bound to mTOR under nutrient-poor than nutrient-rich conditions (8). The molecular mechanisms regulating the nutrient-sensitive raptor-mTOR interaction are not known. Here, we provide evidence that a redox-sensitive mechanism contributes to the regulation of the interaction between raptor and mTOR.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources. Phenylarsine oxide (PAO), diamide, BAL (British anti-Lewisite, also known as 2,3-dimercapto-1-propanol), antimycin A, BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), 2-deoxyglucose, and TLCK (*N*^α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride) were from Sigma, protein G-Sepharose was from Pierce, [γ -³²P]ATP from PerkinElmer Life Sciences, mTOR, S6K1, and PKCα antibodies as well as horseradish peroxidase-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, phospho-S6K1 and phospho-PKCα antibodies were from Cell Signaling; leucine, RPMI, and RPMI without leucine were from U. S. Biologicals, and rapamycin and LY294002 were from Calbiochem. The rictor, raptor, and GβL antibodies were described previously (8, 9, 19).

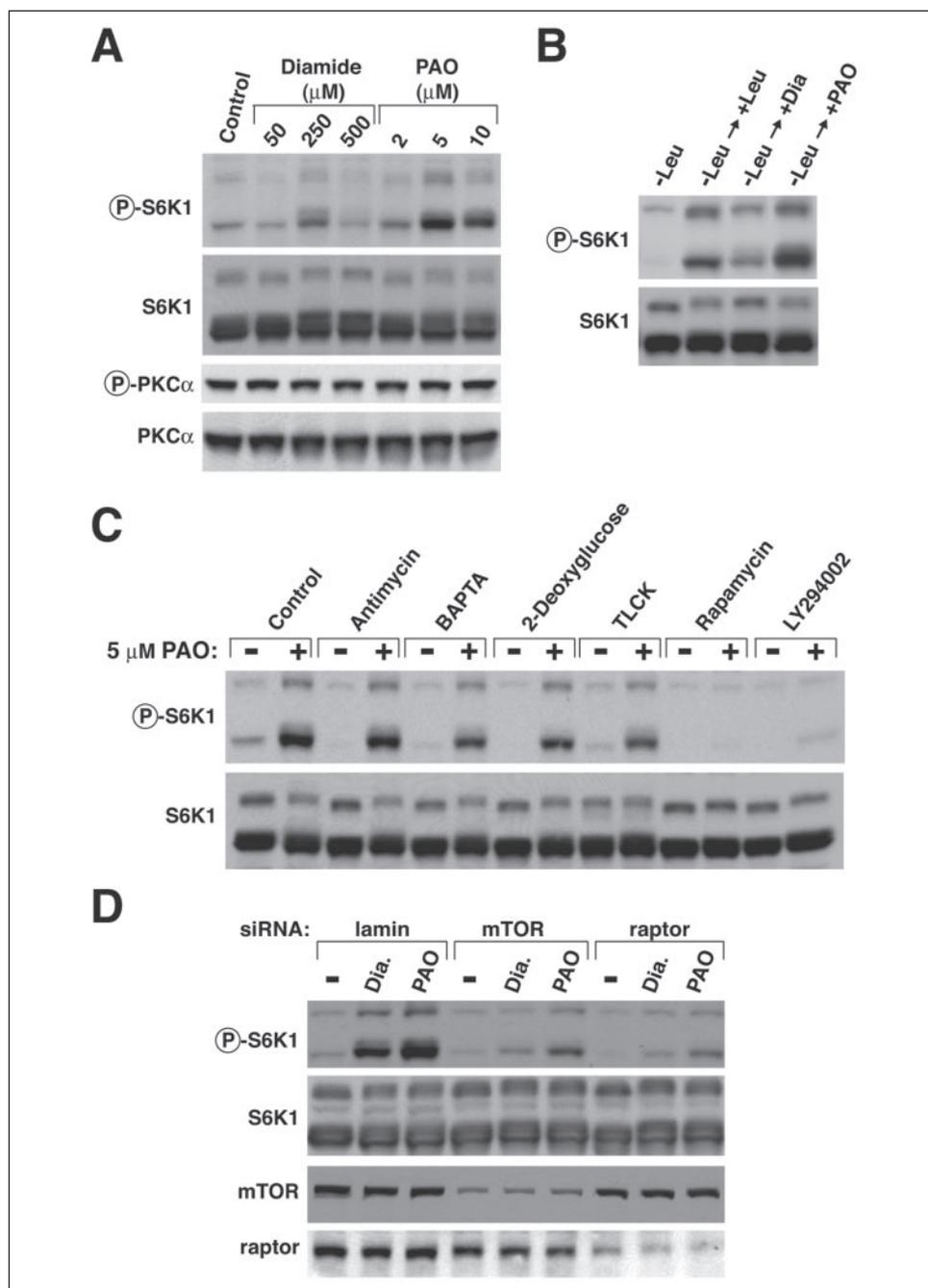
Cell Culture, Treatments, Lysis, and Immunoprecipitations—HEK293T cells were chosen for this study because the nutrient-sensing mTOR pathway is well characterized in this cell line (8). Before use in experiments 5×10^6 HEK293T cells were seeded in 10-cm dishes and

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² The abbreviations used are: 4E-BP1, eukaryotic initiation factor 4E-binding protein; S6K1, S6 kinase 1; FKBP, FK506-binding protein; PK, protein kinase; PAO, phenylarsine oxide; BAL, British anti-Lewisite, also known as 2,3-dimercapto-1-propanol; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; TLCK, *N*^α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; siRNA, small interfering RNA.

FIGURE 1. In the absence of nutrients the thiol oxidants diamide and PAO induce S6K1 phosphorylation in an mTOR- and raptor-dependent fashion. A, diamide and PAO activate S6K1 phosphorylation in a concentration-dependent fashion. Cell lysates were prepared from HEK293T cells growing in basal conditions and treated for 15 min with or without the indicated concentrations of diamide or PAO. Lysates were analyzed by immunoblotting for Thr(P)-389 S6K1, S6K1, phospho-PKC α , and PKC α . B, thiol oxidants induce a leucine-independent activation of S6K1. HEK293T cells were incubated for 1 h in media deprived of leucine (-Leu) or deprived of leucine and stimulated with leucine for 15 min (-Leu followed by +Leu) or deprived of leucine and treated with diamide or PAO for 15 min (-Leu followed by +Dia or -Leu followed by +PAO). Cell lysates were prepared and analyzed by immunoblotting for Thr(P)-389 S6K1 and S6K1. C, PAO prevents the effects of a variety of inhibitors on the mTOR pathway. HEK293T cells were pretreated for 5 min with or without PAO and incubated for an additional 15 min with 5 μ M antimycin A, 50 μ M BAPTA, 100 mM 2-deoxyglucose, 100 μ M TLCK, 50 nM rapamycin, or 20 μ M LY294002. Cell lysates were prepared and analyzed as in B. D, the stimulatory effect of the thiol oxidants on S6K1 is dependent on the expressions of mTOR and raptor. Cell lysates prepared from HEK293T cells transfected with siRNAs targeting lamin, mTOR, or raptor and incubated with or without diamide or PAO for 15 min were analyzed by immunoblotting for the indicated proteins.



grown overnight in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum. If not otherwise indicated, reagents were used at the following concentrations: 5 μ M PAO, 250 μ M diamide, 0.5 mM BAL, 5 μ M antimycin A. For leucine stimulation experiments, a 100 \times leucine stock in water (5.2 mg/ml) was added directly to cell media to a final concentration of 52 μ g/ml. After indicated treatments cells were rinsed once with cold phosphate-buffered saline and lysed on ice for 20 min in 1 ml of ice-cold lysis buffer (40 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, and EDTA-free protease inhibitors (Roche Applied Science)) containing 0.3% CHAPS. After centrifugation at 13,000 \times g for 10 min, 4 μ g of the mTOR antibody was added to the cleared supernatant and incubated under rotation for 90 min. 20 μ l of a 50% slurry of protein G-Sepharose was then added, and the incubation continued for 1 h. Captured immunoprecipitates were washed four

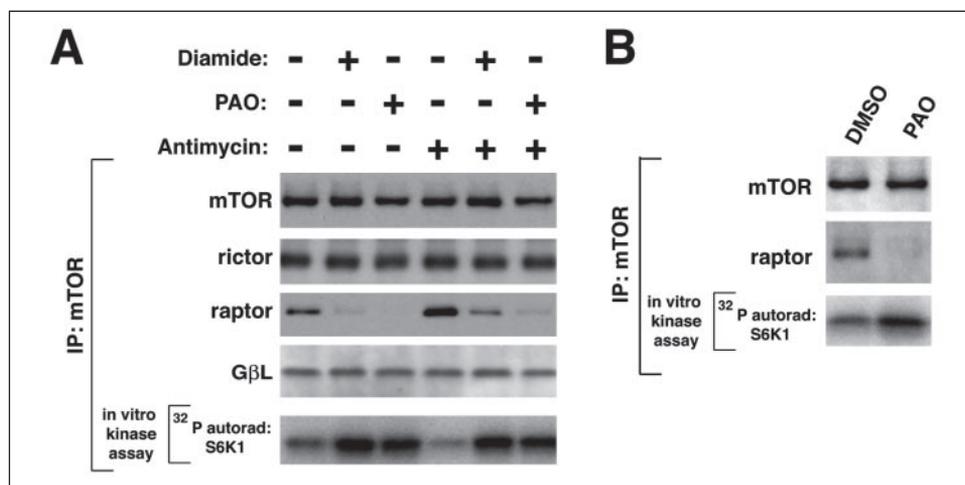
times with lysis buffer and once with wash buffer (50 mM Hepes, pH 7.5, 40 mM NaCl, and 2 mM EDTA). Cell lysates and immunoprecipitates were resolved by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane and visualized by immunoblotting as described. *In vitro* mTOR kinase assays were performed as described (8, 9).

siRNA Transfections—Effectene (Qiagen) was used to transfect 0.8 million HEK293T cells in 6-cm dishes with siRNA oligonucleotides as described previously (8, 9). 72 h after transfection, the cells were rinsed once with cold phosphate-buffered saline and lysed in the ice-cold lysis buffer and analyzed by immunoblotting as above. Sequences for synthetic siRNAs targeting lamin, mTOR, and raptor have been described (8, 9).

RESULTS AND DISCUSSION

How upstream signals such as nutrients regulate the raptor-mTOR complex is not well understood. Because of the dominant role of nutri-

FIGURE 2. The thiol oxidants destabilize the raptor-mTOR interaction and activate the mTOR kinase activity. *A*, HEK293T cells were pretreated with or without antimycin for 5 min and then treated with or without diamide or PAO for an additional 15 min. Cells were lysed, and mTOR immunoprecipitates were prepared and used to measure mTOR kinase activity toward S6K1 *in vitro* and to monitor the levels of mTOR, rictor, raptor, and G β L in the immunoprecipitates. *B*, PAO activates the mTOR kinase activity *in vitro*. mTOR immunoprecipitates isolated from HEK293T cells were incubated in lysis buffer with or without PAO for 15 min at room temperature, washed in lysis buffer, and then used to monitor the levels of mTOR and raptor and to measure mTOR kinase activity. DMSO, dimethyl sulfoxide.



ents and cellular metabolism in the regulation of the raptor-mTOR pathway, we hypothesized the involvement of a redox-sensitive mechanism in the regulation of the raptor-mTOR complex. This idea predicts a dependence of the raptor-mTOR pathway on oxidizing/reducing reactions of thiol-containing groups within a regulatory component of the pathway. To address this hypothesis, we perturbed the cellular redox state by treating cells with either of two oxidizing compounds, diamide or PAO. Both compounds are commonly used as cell-permeable oxidizing reagents that induce disulfide bonds between the thiol groups of cysteines in proteins (22–24). We assessed the effect of these structurally distinct oxidizing compounds on the mTOR pathway by analyzing the rapamycin- and nutrient-sensitive phosphorylation of threonine 389 of the raptor-mTOR substrate S6K1.

Treatment of HEK293T cells with diamide or PAO induced the phosphorylation of S6K1 in a concentration-dependent manner with PAO causing a more robust increase in phosphorylation than diamide (Fig. 1A). PAO induced maximal S6K1 phosphorylation at 5 μ M whereas a higher concentration of diamide (250 μ M) was required to activate S6K1. PAO is known to react simultaneously with the sulfhydryl groups of two closely spaced cysteines (so-called vicinal thiols) (24). The more potent stimulatory effect of PAO on S6K1 compared with diamide suggests the involvement of vicinal thiol-reactive groups in the regulation of the raptor-mTOR pathway. Diamide and PAO do not induce the hyperphosphorylation of all kinases because neither oxidant increased the basal phosphorylation state of PKC α (Fig. 1A, lower panel), although S6K1 and PKC α are structurally related AGC (protein kinases A, G, and C) family kinases.

Leucine deprivation inhibits the nutrient-sensitive raptor-mTOR pathway, and the readdition of leucine to deprived cells reactivates the phosphorylation of S6K1. Similar to leucine stimulation, incubation of leucine-deprived cells with the oxidizing reagents caused the hyperphosphorylation of S6K1. As above, PAO consistently caused a more robust effect than diamide (Fig. 1B). Thus, the oxidants activate the raptor-mTOR pathway even under nutrient-deprived conditions suggesting that the redox-sensitive mechanism acts downstream of the nutrient sensor.

The raptor-mTOR pathway is sensitive to a variety of factors besides nutrients, including antimycin (an inhibitor of mitochondrial function), BAPTA (a calcium chelator), 2-deoxyglucose (an inhibitor of glycolysis), TLCK (a protease inhibitor), rapamycin (a raptor-mTOR inhibitor), and LY294002 (inhibitor of kinases with phosphatidylinositol-3 kinase-like domains) (8, 25–28). To determine the generality of the effect of PAO, we assessed its capacity to increase S6K1 phosphoryla-

tion in cells treated with these inhibitors. PAO prevented the decrease in S6K1 phosphorylation caused by antimycin, BAPTA, 2-deoxyglucose, or TLCK treatment of cells (Fig. 1C). In contrast, direct inhibitors of raptor-mTOR, rapamycin and LY294002, prevented the stimulatory effect of PAO on S6K1 phosphorylation, indicating the dependence of the effect of PAO on mTOR kinase activity. The dominant stimulatory effect on S6K1 phosphorylation of PAO over a variety of raptor-mTOR pathway inhibitors suggests that PAO may act at the level of the raptor-mTOR complex instead of interfering with a distinct signaling cascade that also regulates S6K1. Consistent with this, siRNA-induced knock-downs in the expression of either mTOR or raptor diminished the increase in S6K1 phosphorylation caused by diamide and PAO (Fig. 1D). We have previously shown that siRNA-mediated reductions in the expression of raptor also lead to a small decrease in the expression of mTOR and vice versa (8). This likely reflects a decreased stability of the proteins in the absence of their partners.

We next asked if the raptor-mTOR complex itself is sensitive to diamide or PAO. As we had previously shown that the strength of the interaction between raptor and mTOR is regulated by nutrients and mitochondrial function (8), we analyzed the amount of raptor in mTOR complexes immunopurified from the cells treated with diamide or PAO. The amount of raptor recovered in the mTOR complexes decreased significantly in cells treated with diamide or PAO (Fig. 2A). Moreover, antimycin normally increases the amount of raptor that is recovered bound to mTOR, and the oxidizing reagents prevented this increase. The oxidants do not affect the interaction of mTOR with all proteins, as they do not perturb the interaction between mTOR and rictor or G β L (Fig. 2A). Consistent with the activating effects of the oxidants on the raptor-mTOR pathway in cells, mTOR immunoprecipitates isolated from diamide- or PAO-treated cells exhibited an increase in mTOR kinase activity *in vitro* toward S6K1 without affecting the levels of mTOR. The raptor-mTOR complex may be the direct target of the oxidizing reagents because incubation of the purified complex with PAO induced disassociation of the complex and increased mTOR kinase activity (Fig. 2B). The effects of the oxidizing reagents on the raptor-mTOR complex suggest a mechanism whereby they could induce the nutrient-independent phosphorylation of S6K1. Thus, the raptor and mTOR interaction may be destabilized by the thiol oxidants, resulting in activation of the raptor-mTOR kinase activity and phosphorylation of its substrate S6K1. The destabilizing effect of the oxidizing reagents on the raptor-mTOR interaction is similar to but stronger than those caused by nutrient stimulation of cells.

If PAO acts by forming disulfide bonds between the vicinal thiol

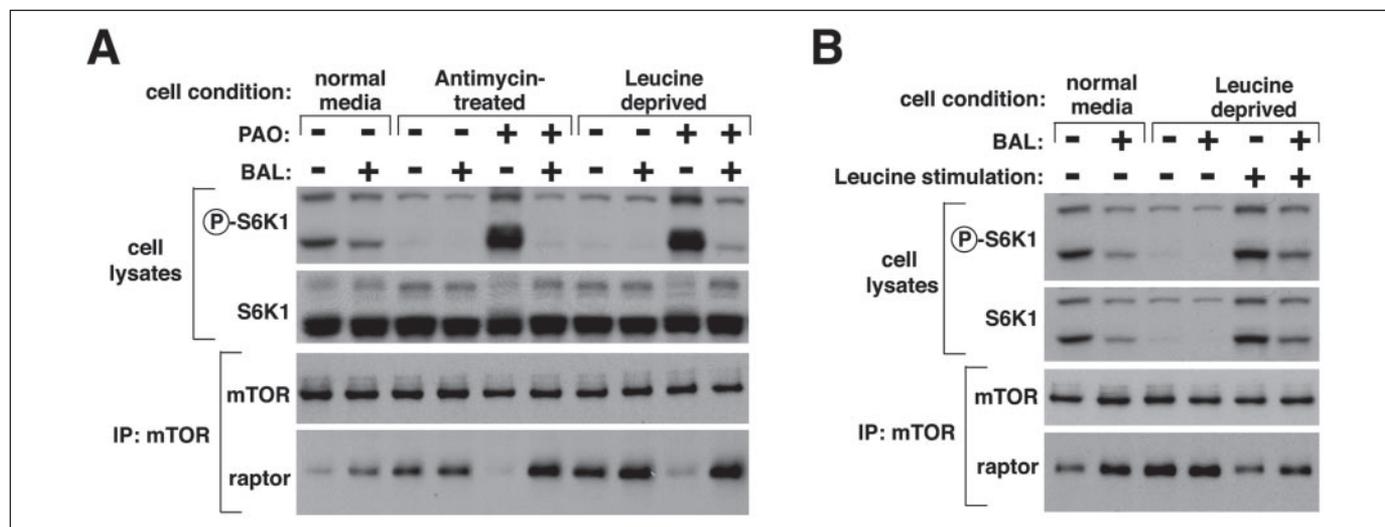


FIGURE 3. The reducing reagent BAL inhibits nutrient-activated raptor-mTOR signaling and stabilizes the raptor-mTOR interaction. *A*, BAL eliminates the effects of PAO on S6K1 phosphorylation and the raptor-mTOR interaction. Cells treated with antimycin for 10 min or deprived of leucine for 1 h were incubated with or without PAO for 10 min. After the treatment with PAO, BAL was added to the cells for 15 min where indicated. The effects of BAL were also tested in control, antimycin-treated, and leucine-deprived cells. Cell lysates and mTOR immunoprecipitates were analyzed by immunoblotting for the indicated proteins. *B*, the inhibitory effect of BAL on S6K1 phosphorylation correlates with a stabilization of the raptor-mTOR interaction. BAL was added to cells in normal media for 20 min. Cells were incubated in leucine-deprived media for 1 h and stimulated with leucine for 10 min. Where indicated BAL was added for 10 min prior to stimulation of cells with leucine. Cell lysates were analyzed as in *A*.

groups of proteins, its effects should be reversed by a reducing agent. For this study we selected the cell-permeable reducing reagent BAL because of its known high efficiency in reversing the oxidation reactions induced by PAO (29, 30). In cells treated with antimycin or in leucine-deprived cells, PAO caused a strong hyperphosphorylation of S6K1 that correlated with a decreased association of the raptor-mTOR interaction (Fig. 3A). The effects of PAO were completely reversed by treating the cells with BAL (Fig. 3A). The addition of BAL resulted in a significant dephosphorylation of S6K1 and was associated with an increase in the amount of raptor recovered with mTOR as is seen in cells in nutrient-deprived conditions (Fig. 3A). If the oxidizing compounds are mimicking an endogenous oxidant that normally activates the raptor-mTOR pathway, the reducing reagent should inhibit pathway activation caused by nutrients. Incubation with BAL of HEK-293T cells growing in nutrient-rich media caused a decrease in S6K1 phosphorylation that correlated with an increase in the amount of raptor recovered with mTOR (Fig. 3B). We also observed similar effects of BAL on the raptor-mTOR complex and S6K1 phosphorylation in leucine-deprived cells simulated with leucine. Thus, the normal nutrient-sensing mechanism that activates raptor-mTOR signaling is susceptible to a reducing environment just as is the artificial activation caused by the diamide and PAO oxidizing reagents.

In this study we found that the raptor-mTOR complex can be regulated by a redox-sensitive mechanism. Thiol oxidants induce activation of the raptor-mTOR pathway that correlates with a destabilization of the raptor-mTOR interaction, whereas a reducing reagent inhibits the pathway, and this correlates with a stabilization of the interaction. Our finding suggests that a redox-sensitive switch may contribute to the regulatory mechanism that controls the raptor-mTOR pathway. In this model, mitochondrial metabolism fueled by nutrients would alter the redox environment of the cell and activate the nutrient-sensing raptor-mTOR pathway by modifying a redox-sensitive mechanism on raptor or mTOR. Interestingly, recent work reveals that in yeast TOR the FATC domain may contain a redox sensor (31), suggesting that mTOR itself may contain the redox-sensitive mechanism. The identification of the thiol-reactive groups involved in regulating the raptor-mTOR complex is an important future goal to test this hypothesis.

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