mTOR ensures increased release and reduced uptake of the organic osmolyte taurine under hypoosmotic conditions in mouse fibroblasts

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Abstract

Mammalian target of rapamycin (mTOR) ensures increased release and reduced uptake of the organic osmolyte taurine under hypoosmotic conditions in mouse fibroblasts. Am J Physiol Cell Physiol 306: C1028–C1040, 2014. First published April 2, 2014; doi:10.1152/ajpcell.00005.2014.—Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that modulates translation in response to growth factors and alterations in nutrient availability following hypoxia and DNA damage. Here we demonstrate that mTOR activity in Ehrlich Lettré ascites (ELA) cells is transiently increased within minutes following osmotic cell swelling and that inhibition of phosphatidylinositol-3-phosphatase (PTEN) counteracts the upstream phosphatidylinositol kinase and potentiates mTOR activity. PTEN inhibition concomitantly potentiates swelling-induced taurine release via the volume-sensitive transporter for organic osmolytes and anion channels (VSOAC) and enhances swelling-induced inhibition of taurine uptake via the taurine-specific transporter (TauT). Chronic osmotic stress, i.e., exposure to hypotonic or hypertonic media for 24 h, reduces and increases mTOR activity in ELA cells, respectively. Using rapamycin, we demonstrate that mTOR inhibition is accompanied by reduction in TauT activity and increase in VSOAC activity in cells expressing high (NIH3T3 fibroblasts) or low (ELA) amounts of mTOR protein. The effect of mTOR inhibition on TauT activity reflects reduced TauT mRNA, TauT protein abundance, and an overall reduction in protein synthesis, whereas the effect on VSOAC is mimicked by catalase inhibition and correlates with reduced catalase mRNA abundance. Hence, mTOR activity favors loss of taurine following hypoosmotic cell swelling, i.e., release via VSOAC and uptake via TauT during acute hypotonic exposure is potentiated and reduced, respectively, by phosphorylation involving mTOR and/or the kinases upstream to mTOR. Decrease in TauT activity during chronic hypotonic exposure, on the other hand, involves reduction in expression/activity of TauT and enzymes in antioxidative defense.

Introduction

Recently, we have shown that it is the eicosanoid leukotriene D4 that, via inhibition of the phosphatidylinositol phosphatase (PTEN) that counteracts the cell growth-promoting phosphoinositide 3-kinase (PI3K) (8, 29). PTEN is a phosphatase domain that removes a phosphate group from the lipid second messenger phosphatidylinositol phosphate in the plasma membrane, a C2 domain that is critical for PTEN binding to the membrane, and a COOH-terminal domain that, upon phosphorylation of a cluster of Ser/Thr residues, interacts with the C2 domain-diminishing membrane affinity and hence catalytic activity of PTEN (1). The phosphatase required for PTEN activation has not been identified, and it is also unknown whether PTEN activity is affected by osmotic cell swelling. Abrogation of the volume-regulatory anion channel

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process in EATC involves PKC-mediated phosphorylation and desensitization of the CysLT1 receptor (18). VSOAC has not been cloned although data indicates that it is different from volume-sensitive Cl⁻ channels (27, 29) and Ca²⁺-sensitive Cl⁻ channels (19).

Recent investigations indicate that TauT transcription as well as TauT plasma membrane abundance/activity are favored by mTOR, and it was suggested that the reduced placental amino acid transport activity seen in intrauterine growth restriction could partly be caused by reduced mTOR activity (46, 47). mTOR is a serine/threonine kinase that forms the catalytic subunit of the complexes mTORC1 and mTORC2, which are distinguished by their regulatory scaffolding proteins, regulatory associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor), respectively. Binding of the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) and the p70-S6 kinase (S6K) to Raptor is a prerequisite for mTOR-mediated phosphorylation of 4E-BP1 and S6K (10, 53). 4E-BP1 will in its unphosphorylated state inhibit cap-dependent translation by binding to the eukaryotic translation initiation factor 4E (eIF4E). However, as 4E-BP1 becomes phosphorylated by mTOR, the elf4E4E will dissociate from 4E-BP1 and facilitate cap-dependent translation. It is assumed that rapamycin disrupts the Raptor mTOR interaction and hence the ability of mTOR to phosphorylate downstream substrates. mTORC1 activity is regulated by growth factors (insulin, insulin-like growth factors) and nutrient signals (amino acids), cellular AMP/AMP-activated kinases (AMPK) following cell stress (hypoxia, DNA damage) (60). Growth factor-induced mTOR stimulation involves phosphorylation by PI3K, which is counteracted by PTEN, as well as phosphorylation by the serine/threonine kinase Akt (protein kinase B). Activation of Akt elicits phosphorylation and inactivation of the GTPase-activating protein sclerosis complex (TSC1-TSC2), which in turn leads to an increased fraction of the monomeric GTP-binding protein Rheb in its Rheb-GTP and hence mTOR-activating form (10). Nutrients, such as amino acids, are suggested to promote Raptor/mTOR substrate interaction and hence increased substrate accessibility to mTOR in a process that involves signaling through the Ras-related GTP-binding (Rag) proteins or recruitment of mTOR to intracellular organelles, e.g., lysosomes (10, 21, 23, 48). Glucose deprivation leads to increased AMP levels, which promote AMPK activity and hence inhibition of mTORC1 either indirectly through AMPK-mediated phosphorylation and activation of TSC2 or more directly through phosphorylation of Raptor (10, 50). Although mTORC1 is usually inhibited when the availability of oxygen is low (50), Elorza and coworkers (3) have recently demonstrated that activation of the hypoxia-inducible factor 2α actually increases mTORC1 activity in a process involving upregulation of the Na⁺-independent, high-affinity amino acid transporter SLC7A5 (3).

Mammalian cells release organic osmolytes as part of the volume-regulatory process that follows osmotic cell swelling, and it has been shown that swelling-induced reduction in the taurine content in NIH3T3 mouse fibroblasts reflects an increased loss of taurine via VSOAC and a concomitant reduction in taurine uptake via TauT (7, 27). The reduction in TauT activity following reduction in the extracellular osmolality occurs independently of TonEBP (7). As mTOR inhibition reduces TauT gene transcription and TauT activity in primary human trophoblasts (46), we initiated the present project to test whether osmotic perturbation affects mTOR activity and whether mTOR exerts coordinated regulation of TauT and VSOAC. Regulation of mRNA translation is a dynamic process broadly affected by changes in cell physiology (49), and translation is primarily regulated at the step of initiation, where the major regulatory mechanism involves phosphorylation of eIF2α by one of four known eIF2α kinases (9). To illustrate the role of mTOR in the regulation of TauT/VSOAC, we used the clinically used agent rapamycin and tested its effect on mTOR activity (phosphorylation of the mTOR substrate 4E-BP1), gene transcription (qPCR), translation/protein synthesis (phosphorylation of elf2α), and taurine uptake/release (tracer technique). We have used nonmalignant NIH3T3 fibroblasts and Ehrlich Lettré tumor cells to test whether the effect of mTOR on taurine transporters, in congruence with the effect of the CK2 (8), occurs independently of the oncogenic status of the mouse fibroblasts.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), [2,2-³H(N)]-taurine, α-[1-¹⁴C]-methyl aminoisobutyric acid (meAIB), l-[³⁵S]-cysteine, and the scintillation fluid Ultima Gold were from PerkinElmer (Waltham, MA). Primary antibodies against mTOR (rabbit Ab), elf2α/Phospho-eIF2α (rabbit Ab), PTEN/phospho-PTEN (rabbit mAb) and 4E-BP1/Phospho-4E-BP1 (rabbit mAb), histone (rabbit Ab), and β-actin were all from Cell Signaling Technology (Cambridge, UK). TauT antibody (rabbit Ab) was from Yorkshire Biosciences (York, UK). Materials for cell culturing, real-time PCR, and Western blotting were from Invitrogen (Paisley, UK) and Agilent Technologies (Hörsholm, Denmark). Rapamycin stock solution (400 μM) was prepared in dimethyl sulfoxide.

Cell cultures. NIH3T3 mouse fibroblasts (clone 7) and ELA cells (ATCC) were grown in 75-cm² culture flasks (Cellstar, Frickhausen, Germany) at 37°C, 5% CO₂, 100% humidity as monolayer cultures in DMEM and RPMI-1640, respectively. Both media contained 10% heat-inactivated fetal bovine serum and 1% antibiotics (penicillin, streptomycin). Cells were split every 3–4 days using 0.5% Trypsin (Invitrogen) in PBS to detach cells.

Inorganic media. PBS contained 137 mM NaCl, 2.6 mM KCl, 6.5 mM Na₂HPO₄, and 1.5 mM KH₂PO₄. Isosmotic NaCl medium for NIH3T3 cells contained 141 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgSO₄, and 10 mM HEPES. Isosmotic NaCl medium for NIH3T3 cells contained 159 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 1 mM MgSO₄, and 10 mM HEPES. Hypoosmotic NaCl solution was prepared by reduction of the NaCl concentration in the isosmotic solutions to 50% without reducing the other components. Isosmotic N-methyl-D-glucamine-Cl (NMDGCl) and KCl media were prepared by substitution of NMDGCl or KCl for NaCl, respectively. pH was in all media adjusted to 7.4.

Radioactive tracer technique for amino acid uptake and release. [2,2-³H(N)]-taurine and α-[1-¹⁴C]-meAIB were used to characterize amino acid uptake via TauT and System A, respectively, as well as release via the VSOAC. Uptake and release were determined at room temperature using cells grown to 80% confluence in six-well polyethylene plates (9.6 cm² per well) in their respective growth medium and following previously published procedures (13). For determination of influx, cells were washed and incubated with isosmotic medium. Five wells in a six-well plate were used for determination of accumulation of labeled amino acid, using 3-, 6-, 9-, 12-, and 15-min exposure, and one well was used for a representative determination of the protein content in the wells using the Lowry method (36) and BSA as standard. Influx was initiated by washing cells twice in isotonic NaCl.
medium, followed by addition of isotope and terminated by aspiration of the medium followed by rapid addition/aspiration of ice-cold MgCl₂ (115 mM). For determination of influx under hyposmotic conditions, isosmotic medium was substituted with hyposmotic medium containing the isotope and incubated in the time intervals indicated. Following cell lysis with 96% ethanol, labeled isotope was extracted with ddH₂O and transferred to scintillation vials, and activity was determined by β-scintillation counting (Ultima Gold). Cellular amino acid activity [in counts per minute (cpm)/well] was converted to nmol/g protein, using the extracellular specific activity (cpm/nmol) and the protein content (mg protein/well), and finally plotted vs. time. Influx (nmol·g protein⁻¹·min⁻¹) was determined by linear regression. In cells pretreated in growth medium supplemented with 100 mM taurine or 100 mM sucrose, the wash of cells and experiment were performed with standard NaCl medium supplemented with 100 mM sucrose to preserve the toxicity. Two six-well plates were used for calculation of taurine transport kinetics, i.e., 10 wells for variation of the extracellular Na⁺ concentration in the range 0 to 159 mM (NaCl substituted isosmotically by NMDGCl) and determination of taurine [³H]-taurine uptake within 15 min and two wells for quantification of protein. [³H]-taurine influx for sodium kinetics (cpm/g protein per 15 min) was plotted vs. the extracellular Na⁺ concentration ([Na⁺]), and the curves were fitted to a Hill type equation: \[ Y = \left( V_{\text{max}} \times \left[ \text{Na}^+ \right]/(K_m + \left[ \text{Na}^+ \right]) \right) \], where \( V_{\text{max}} \) is the maximal taurine uptake, \( K_m \) is the Na⁺ concentration required for half maximal taurine uptake, and \( n \) is the number of Na⁺ ions required for initiation of the uptake of one taurine.

For determination of efflux, cells were preloaded for 2 h in growth medium containing [³H]-labeled taurine or [¹⁴C]-labeled meAIB, the medium was aspirated, and cells were washed three times and left with isosmotic NaCl medium. Taurine efflux was executed by sampling/exchanging the extracellular medium every 2 min with isosmotic medium in the first 8 min and hyposmotic medium in the subsequent experimental time period. Cells were lysed at the end of the efflux experiment by addition of NaOH (1 M), and total [³H]-taurine in each well, representing a single experimental setup, was estimated as the sum of activity in all the efflux samples, the NaOH lysate plus two washouts with ddH₂O. The natural logarithm to the fractional rate constant (\( k_{\text{eff}} \)) was plotted vs. time. The fractional rate constant for the taurine efflux (per minute), at a given sampling time point, was calculated from the logarithmic plot as the slope between the time point and its proceeding time point.

Real-time polymerase chain reaction. Real-time PCR was used to quantify mRNA levels for PTEN, mTOR, TauT, and the actin-related protein (ARP) (house-holding gene) as described previously (52). RNA was extracted from cells and grown in Petri dishes (90% confluence) using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany). Reverse transcription was performed on 1 μg RNA (determined from the 260 nm/280 nm absorbance ratio) and incubated first at 65°C for 5 min in nucleotide solution (dNTP mix) plus oligo dT primer and subsequently at 42°C for 2 min with strand buffer plus dithiothreitol. Superscript II reverse transcriptase was added and mixture incubated at 42°C for 50 min. The reaction was inactivated by raising the temperature to 70°C for 15 min. For quantification, we used the Stratagene Mx3000P real-time PCR system and the method for influx with the following exceptions: 106 cells were washed gently in ice-cold PBS and lysed in lysis buffer containing 1% SDS, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 0.5% Triton X-100, 1 mM NaN₃, and 1% protease inhibitor mix before sonication. Protein content was estimated by a DC assay (Bio-Rad, Hercules, CA), and lysates with equivalent amount of protein were mixed with sample buffer (10% SDS, 1% bromophenol blue, 10% glycerol, 5 mM Tris-HCl, pH 6.8) and proceeded for SDS PAGE gel electrophoresis (NuPAGE precast 10% Bis-Tris gels) in NOVEX chambers under reducing and denaturing conditions (NuPAGE MOPS SDS running buffer, Invitrogen). Benchmark protein ladder was used for indication of molecular weight. NuPAGE transfer buffer (Invitrogen) was used for protein transfer to nitrocellulose membranes, and protein transfer was verified by Ponceau staining. Membranes, blocked at 37°C in Tris-buffered saline with Tween (TBST) (0.01 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk incubated overnight at 4°C with primary antibodies, were washed in TBST and subsequently incubated with secondary antibodies for 1 h. Following wash in TBST, membranes were developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (KPL, Gaithersburg, MD). Membranes were scanned and bands quantified using UN-SCAN-IT (Silk Scientific, Orem, UT).

Protein synthesis. Protein synthesis was measured according to the method for influx with the following exceptions: 1) cells in wells 1 to 5 in the six-well plate were exposed to [¹⁴C]-labeled cysteine for the time periods 5, 15, 30, 60, and 120 min, and 2) following termination of the cysteine uptake/incorporation process and cell lysis, the dried ethanol precipitated protein in each well was washed once with isosmotic medium containing unlabeled cysteine (1 mM), hydrolyzed with NaOH (24 h), and the [¹⁴C] activity in hydrolysate plus two washouts with ddH₂O was used as an indication of [¹⁴C]-cysteine incorporation (cpm/well). Values were converted (cpm/mg protein), using the protein content (mg protein/well) in well 6 (see Radioactive tracer technique for amino acid uptake and release), and plotted vs. time. Cellular protein incorporation (cpm-mg protein⁻¹·min⁻¹) was determined by linear regression.

Statistics. In bar and scatter plots, the error bars indicate means ± SE. Statistical significance was analyzed by Student’s t-test and one-way ANOVA test.
RESULTS

Rapamycin-induced inhibition of mTOR reduces TauT mRNA abundance, TauT expression, and TauT activity. Inhibition of mTOR has previously been shown to reduce TauT mRNA abundance as well as TauT activity in primary human trophoblasts (46). In the present investigation, we used the mTOR inhibitor rapamycin to test whether inhibition of mTOR affects TauT activity to the same extent in cells expressing different levels of mTOR. From Fig. 1A, it is seen that ELA express ∼50% less mTOR compared with nonmalignant NIH3T3 mouse fibroblasts. However, qPCR technique revealed that mTOR mRNA abundance was twice as high in ELA compared with NIH3T3 cells (Fig. 1B). This can be explained by either lower translation of mTOR mRNA or increased degradation of mTOR protein in ELA cells, and hence a considerable capacity for upregulation of mTOR expression and activity exists.

From Fig. 2A, it is seen that 24-h incubation under hypoosmotic conditions reduced mTOR activity in ELA cells, which was observed as a reduction in phosphorylation of the mTOR substrate 4E-BP1, i.e., the phospho-4E-BP1/4E-BP1 ratio was reduced. On the other hand, mTOR activity increased significantly following 24-h exposure to hypertonic conditions (Fig. 2A). TauT transport activity in mouse fibroblasts was previously shown to decrease following osmotic cell swelling (7, 11) and to increase following osmotic cell shrinkage (27).

Hence, TauT activity correlates with mTOR activity following prolonged exposure to hypotonic and hypertonic tonicity. Ehrlisch cells release amino acids in response to hypoosmotic cell swelling (11), and, as mTOR is sensitive to the cellular level of amino acids (50), we tested whether a shift in the cellular taurine content would affect mTOR activity. Preincubation of ELA cells with growth medium supplemented with 5 mM β-alanine, which depletes cells for taurine (52), had no effect on mTOR activity, i.e., the phospho-4E-BP1/4E-BP1 ratio was from seven sets of experiments determined to 0.82 ± 0.14 and 0.78 ± 0.04 in normal and taurine-depleted cells, respectively. Hence, reduction in mTOR activity following 24-h osmotic cell swelling is not explained by the concomitant net loss of taurine. ELA is an adherent subline of the nonadherent EATC, and we have previously shown that osmotic cell swelling in EATC is accompanied by depolarization of the plasma membrane due to a significant increase in the Cl− conductance, which exceeds the concomitant increase in the K+ conductance (31). We found that hyperpolarization of the plasma membrane by preincubation in isotonic Na+-free NMDGCl medium significantly increased mTOR activity 5.5 ± 1.4-fold, whereas depolarization of the membrane by preincubation in isotonic KCl medium significantly reduced mTOR activity to 43 ± 7% of the control value. Hence, neither membrane depolarization nor amino acid loss during osmotic cell swelling seems to explain downregulation of mTOR activity following prolonged hypoosmotic exposure.

To mimic the effect of reduced mTOR activity following long-term hypoosmotic conditions, we exposed ELA and NIH3T3 cell to the mTOR inhibitor rapamycin. From Fig. 2B, it is seen that exposing ELA cell to 50 nM rapamycin for 24 h reduced mTOR activity significantly under isotonic conditions as well as under hypertonic conditions, whereas rapamycin had no effect under hypotonic conditions (Fig. 2B). Hence, mTOR activity in ELA cells was reduced to the same extent following long-term hypoosmotic exposure and rapamycin-induced inhibition. Figure 2 also shows that rapamycin-induced inhibition of mTOR was accompanied by a reduction in protein synthesis, which was observed as an increased phosphorylation of eIF2α (Fig. 2C) and reduced incorporation of [35S]-labeled cysteine into protein (Fig. 2D). Similarly, we find that exposure to rapamycin reduced protein synthesis in NIH3T3 cells (Fig. 2E), which express a high level of mTOR (Fig. 1A). From Fig. 3, it is seen that mTOR favors amino acid uptake in ELA cells under isotonic conditions, i.e., 24-h pretreatment with rapamycin reduced uptake of taurine (Fig. 3A) as well as meAIB (Fig. 3B), which is a synthetic tracer for the alanine-preferring amino acid transporter (System A). Four-hour rapamycin preexposure had no significant effect on taurine influx in ELA cells, i.e., taurine influx was 0.20 ± 0.01 nmol·g protein−1·min−1 (n = 3) in rapamycin-treated cells compared with 0.18 ± 0.01 nmol·g protein−1·min−1 (n = 13) in control cells. Adding rapamycin at the time of the initiation of the influx experiment, likewise, had no effect on taurine influx in ELA either (data not shown). The reduced TauT activity observed in ELA cells exposed to rapamycin for 24 h correlates with a reduced TauT mRNA abundance (Fig. 3C) and protein expression (Fig. 3D). Consistently, we found that rapamycin-induced inhibition of mTOR reduced taurine uptake via TauT in NIH3T3 cells (Fig. 3E) and that the reduced taurine uptake correlates with reduced maximal transport ca-
pacity (V\textsubscript{max}), i.e., a reduction in the number of functional transporters in the membrane and not a shift in the affinity of TauT toward the cosubstrate Na\textsuperscript{+}/H\textsuperscript{+} or the Na\textsuperscript{+}/H\textsuperscript{+}:taurine stoichiometry for translocation of taurine via TauT (Fig. 3F). Hence, reduction in mTOR activity, induced by prolonged exposure to hypotonic conditions or addition of rapamycin, is taken to reduce TauT activity through reduction of TauT mRNA and/or protein abundance and/or translocation of TauT to the plasma membrane.

We have previously shown that accumulation and transport capacity of TauT is reduced reversibly following long-term exposure to a high extracellular taurine concentration (56). This is confirmed in Fig. 4A, where it is shown that 24- or 48-h exposure to growth medium supplemented with 100 mM taurine almost eliminated taurine influx in ELA cells (compare control in Fig. 3A with Tau-24h/Tau-48h in Fig. 4A) and that TauT activity recovered in ELA cells when the 24-h preexposure with 100 mM taurine was followed by 24-h exposure to growth medium supplemented with 100 mM sucrose (compare Tau/Suc with Tau-24h/Tau-48h in Fig. 4A). Recovery of TauT activity was not complete, as taurine influx only reached a value equivalent to 50% of the value seen in cells exposed to growth medium supplemented with 100 mM sucrose for 48 h (Fig. 4A). From Fig. 4B, it is seen that rapamycin treatment reduced taurine uptake to the same extent irrespective of the magnitude of the prevailing TauT activity. From Fig. 4C, it is seen that the reversible, substrate-induced downregulation of TauT transport activity reflects a reduced TauT mRNA level, i.e., TauT mRNA abundance was reduced significantly by 48-h pretreatment with 100 mM taurine in the growth medium. TauT mRNA increased when extracellular taurine was substituted by 100 mM sucrose and approached 50% of the amount seen in cells exposed for 24 h to growth medium supplemented with 100 mM sucrose. Figure 4D shows that mTOR inhibition reduced TauT mRNA abundance to the same extent in control cells and cell preexposed to sucrose. However, rapamycin does not seem to reduce TauT mRNA level in cells exposed for 24 h with 100 mM taurine followed by 24 h with 100 mM sucrose, presumably because upregulation of gene transcription following removal of extracellular taurine balances the concomitant rapamycin-induced inhibition. Taken together the data in Figs. 1–4 indicate that mTOR under isotonic conditions ensures...
accumulation of taurine and other organic osmolytes in non-
malignant and carcinoma mouse fibroblasts by stimulating
transcription of genes coding for the transporters TauT and
System A, translation of the mRNA to functional protein, as
well as expression of the transporters in the plasma membrane.
Conversely, prolonged hypotonic exposure was accompanied
by reduced mTOR activity (Fig. 2A) and a concomitant reduc-
tion in TauT mRNA abundance and TauT activity (7), which
will add to restoration of cell volume by limiting reuptake of
osmolytes.

Acute swelling-induced stimulation of mTOR is accompa-
nied by reduction in TauT activity. Cell swelling has previously
been reported to activate PI3K in Swiss 3T3 fibroblasts (4), and
accordingly we find that mTOR activity was significantly
increased by 30% in ELA within 6 min following exposure to
hypotonic conditions and that inhibition of PTEN, which
counteracts the effect of increased PI3K activity, potentiated
the effect of osmotic cell swelling on mTOR activity (Fig. 5A).
However, PTEN activity was unaltered by acute hypotonic
exposure, i.e., the ratio between phosphorylated (inactive)
PTEN and total PTEN was 0.22 ± 0.03 under isotonic condi-
tions and 0.20 ± 0.004 following 6-min hypotonic exposure (5
sets of paired experiments). This indicates that swelling-in-
duced increase in mTOR activity is secondary to the increased
PI3K activity. Acute osmotic cell swelling in mouse fibroblasts
is accompanied by reduction in TauT activity (7), and, from
Fig. 5B, it is seen that taurine influx under hypotonic conditions
in ELA cells was significantly reduced in the presence of HOpic and that this inhibition was amplified by rapamycin.
Hence, swelling-induced reduction in TauT activity correlates
with the increase in PI3K and mTOR signaling. As inhibition
of mTOR leads to reduction in taurine uptake (Fig. 3), one
would expect that increased mTOR activity following acute
osmotic exposure should increase taurine uptake. This is not
the case, indicating that downregulation of TauT within the
initial minutes following hypoosmotic exposure is most likely
mediated by direct phosphorylation involving the protein ki-
nases upstream to mTOR. The more pronounced effect of acute
osmotic cell swelling on TauT in rapamycin-treated cells most
probably reflects the contribution from rapamycin-induced
reduction in TauT mRNA abundance. The opposing effect of
short-term (6 min) and long-term (24 h) hypotonic exposure on
mTOR activity most probably reflects a general downregula-
tion of mTOR activity-mediated signaling, elicited by pro-
longed hypotonic conditions.

Rapamycin-induced inhibition of mTOR potentiates swell-
ing-induced taurine release. Cell swelling elicits a transient
release of taurine to restore cell volume and hence functionali-
ty (27, 32). From the time trace in Fig. 6A, where the fractional
rate constant for release of taurine is plotted with 2-min time

intervals under isotonic and hypotonic conditions, it is seen that 24-h pretreatment with 50 nM rapamycin amplified swelling-induced taurine release in ELA cells although the time-frame for activation and inactivation of the volume-sensitive pathway was unaffected. Using the maximal fractional rate constant obtained after the shift to hypotonic conditions, i.e., 6 min after hypotonic exposure, as indication for VSOAC activity, it was determined that rapamycin-induced inhibition of mTOR increased swelling-induced taurine release 1.4-fold in ELA cells (Fig. 6C) and 1.5-fold in NIH3T3 cells (Fig. 6D). Taurine release under isotonic conditions was not affected by rapamycin, i.e., the respective rate constants for taurine release in control and rapamycin-treated cells were 0.0013 ± 0.002 cells/min (n = 16) and 0.0014 ± 0.003 cells/min (n = 10) for ELA cells and 0.0027 ± 0.007 cells/min (n = 14) and 0.0028 ± 0.008 cells/min (n = 10) for NIH3T3 cells. Adding rapamycin at the time of initiation of the efflux experiment had no effect on swelling-induced taurine release, i.e., the respective maximal rate constants in control and rapamycin-treated ELA cells were 0.054 ± 0.035 cells/min (n = 16) and 0.0578 ± 0.077 cells/min (n = 4), respectively. Thus, long-term rapamycin-induced inhibition of mTOR increases VSOAC activity.

PI3K, PTEN, and the glycogen synthase kinase 3 (GSK3) are all upstream to mTOR (60), and, in congruence with previous findings for NIH3T3 (8, 26), data with ELA cells showed that volume-sensitive taurine release was potentiated in the presence of the PTEN inhibitor HOpic and reduced in the presence of the PI3K inhibitor wortmannin (Fig. 6B). As protein synthesis according to Fig. 2 is reduced in rapamycin treated cells, an increased VSOAC activity could be caused by a reduced PTEN expression or PTEN activity. However, as seen in Fig. 6C (ELA cells) and Fig. 6D (NIH3T3 cells), 24-h pretreatment with 50 nM rapamycin amplified swelling-induced taurine release under conditions where VSOAC activity was impaired by inhibition of PI3K with wortmannin and GSK3 with SB216763 or boosted by inhibition of PTEN with HOpic. Furthermore, although PTEN mRNA abundance (Fig. 6E) and PTEN protein expression (Fig. 6F) were significantly increased following 24-h rapamycin exposure, the PTEN activity (Fig. 6G) was not concomitantly affected. Hence, it
by oxidation of a cysteine in the catalytic site, whereas vanadate is a phosphate analog that acts as a competitive inhibitor of the protein tyrosine phosphatases (14). Hence, reduction in expression of enzymes involved in elimination of ROS could contribute to rapamycin-induced potentiation of VSOAC activity. Mouse fibroblasts express the antioxidant enzymes SOD1, catalase, and GPX (Fig. 7C), and, from Fig. 7, D–F, it is seen that SOD1/2 and GPX mRNA abundance is higher in ELA compared with NIH3T3, whereas catalase mRNA is larger in NIH3T3 compared with ELA. From Fig. 7G, it is seen that reduction in catalase activity in ELA cells with 3-amino-1,2,4-triazole (5 mM, 2-h preincubation) and hence elimination of ROS increased the maximal rate constant for the swelling-induced taurine release in ELA cells 1.2-fold and that preconditioning of ELA cells with N-acetyl cysteine (precursor for glutathione synthesis), which enhances cellular glutathione content (52) and presumably GPX activity, reduced swelling-induced taurine release to 65% of the control value. Hence, in agreement with previous data, modulation of the antioxidative capacity affects swelling-induced release of taurine (26). In Fig. 7H, it is shown that rapamycin-induced inhibition of mTOR reduced catalase mRNA abundance significantly, whereas SOD1 mRNA and GPX mRNA abundance was unaffected. Thus reduced catalase capacity following inhibition of mTOR could contribute to reduced antioxidative capacity and explain the concomitant potentiation of VSOAC activity. It is noted that we are not able to test whether rapamycin affects expression of the protein tyrosine phosphatase and VSOAC genes, as they are not identified.

**DISCUSSION**

**mTOR-taurine homeostasis following osmotic cell swelling.** In mouse fibroblasts, restoration of cell volume following osmotic cell swelling and hence protection of cellular function is ensured by transient activation of the release pathway for ions and organic osmolytes VSOAC as well as a concomitant downregulation of the taurine-accumulating transporter TauT (7, 27, 29). Conversely, osmotic cell shrinkage elicits inactivation of VSOAC and upregulation of TauT expression and activity (27–29, 56). Mouse fibroblasts also release ions and amino acids during the initial phase of apoptosis, which leads to cell shrinkage (apoptotic volume decrease) and hence initiation of the cell death process (12, 32, 43). ROS are produced following osmotic cell swelling as well as osmotic cell shrinkage (2, 5, 13, 26, 39, 55). We have recently demonstrated that acute exposure to ROS reduces taurine uptake as a result of modulation of TauT transport kinetics (7) and concomitantly potentiates swelling-induced taurine release (13, 26, 39), presumably through potentiation of 5-lipoxygenase activity and/or oxidation/inhibition of protein tyrosine phosphatases (13, 26). Hence, ROS favor net loss of organic osmolytes under hypoosmotic conditions. Here we demonstrate that mTOR activity is transiently increased following osmotic cell swelling. Within minutes following exposure to hypoosmotic conditions (acute phase), the increased mTOR activity correlates with potentiation of taurine loss via VSOAC and reduction in taurine uptake via volume-sensitive transporter for organic osmolytes and anion channels (VSOAC). Values are means from 5 sets of experiments. #Significantly reduced compared with hypotonic control and hypotonic plus HOpic, respectively (ANOVA test).

seems reasonable to exclude that rapamycin-induced potentiation of the volume-sensitive taurine release is secondary to rapamycin-induced inhibition of PTEN activity. Similar to the effect of lipid phosphatase (PTEN) inhibition in Fig. 6B and in accordance with previously published data (28), Fig. 7A shows that volume-sensitive taurine release from ELA cells is potentiated by ROS (H_2O_2) and exposure to the protein phosphatase inhibitor vanadate. From Fig. 7B, it is seen that 24-h pretreatment with 50 nM rapamycin amplified swelling-induced taurine release in the presence of H_2O_2 and vanadate and to an even larger extent compared with cells treated with rapamycin alone. ROS are assumed to block protein tyrosine phosphatases by oxidation of a cysteine in the catalytic site, whereas vanadate is a phosphate analog that acts as a competitive inhibitor of the protein tyrosine phosphatases (14). Hence, reduction in expression of enzymes involved in elimination of ROS could contribute to rapamycin-induced potentiation of VSOAC activity. Mouse fibroblasts express the antioxidant enzymes SOD1/2, catalase, and GPX (Fig. 7C), and, from Fig. 7, D–F, it is seen that SOD1/2 and GPX mRNA abundance is higher in ELA compared with NIH3T3, whereas catalase mRNA is larger in NIH3T3 compared with ELA. From Fig. 7G, it is seen that reduction in catalase activity in ELA cells with 3-amino-1,2,4-triazole (5 mM, 2-h preincubation) and hence elimination of ROS increased the maximal rate constant for the swelling-induced taurine release in ELA cells 1.2-fold and that preconditioning of ELA cells with N-acetyl cysteine (precursor for glutathione synthesis), which enhances cellular glutathione content (52) and presumably GPX activity, reduced swelling-induced taurine release to 65% of the control value. Hence, in agreement with previous data, modulation of the antioxidative capacity affects swelling-induced release of taurine (26). In Fig. 7H, it is shown that rapamycin-induced inhibition of mTOR reduced catalase mRNA abundance significantly, whereas SOD1 mRNA and GPX mRNA abundance was unaffected. Thus reduced catalase capacity following inhibition of mTOR could contribute to reduced antioxidative capacity and explain the concomitant potentiation of VSOAC activity. It is noted that we are not able to test whether rapamycin affects expression of the protein tyrosine phosphatase and VSOAC genes, as they are not identified.

**DISCUSSION**

**mTOR-taurine homeostasis following osmotic cell swelling.** In mouse fibroblasts, restoration of cell volume following osmotic cell swelling and hence protection of cellular function is ensured by transient activation of the release pathway for ions and organic osmolytes VSOAC as well as a concomitant downregulation of the taurine-accumulating transporter TauT (7, 27, 29). Conversely, osmotic cell shrinkage elicits inactivation of VSOAC and upregulation of TauT expression and activity (27–29, 56). Mouse fibroblasts also release ions and amino acids during the initial phase of apoptosis, which leads to cell shrinkage (apoptotic volume decrease) and hence initiation of the cell death process (12, 32, 43). ROS are produced following osmotic cell swelling as well as osmotic cell shrinkage (2, 5, 13, 26, 39, 55). We have recently demonstrated that acute exposure to ROS reduces taurine uptake as a result of modulation of TauT transport kinetics (7) and concomitantly potentiates swelling-induced taurine release (13, 26, 39), presumably through potentiation of 5-lipoxygenase activity and/or oxidation/inhibition of protein tyrosine phosphatases (13, 26). Hence, ROS favor net loss of organic osmolytes under hypoosmotic conditions. Here we demonstrate that mTOR activity is transiently increased following osmotic cell swelling. Within minutes following exposure to hypoosmotic conditions (acute phase), the increased mTOR activity correlates with potentiation of taurine loss via VSOAC and reduction in taurine uptake via TauT. This acute regulation of the taurine transporters mimicked by the presence of rapamycin, results in a reduced
transcription of genes coding for antioxidative enzymes (catalase) and TauT as well as reduced TauT expression and incorporation in the plasma membrane. RNA-mediated silencing of Raptor, i.e., mTORC1 inhibition, is similarly shown to reduce basal System A and System L amino acid transport activities in human trophoblast cells by modulation of cell surface abundance of the transporters (47). In ELA cells, reduction in antioxidative capacity will boost VSOAC activity, mimicked by addition of \( \text{H}_2\text{O}_2 \), and concomitantly inhibit TauT activity (7, 29). Regulation of TauT and VSOAC by mTOR under acute and prolonged hypoosmotic exposure is summarized in Fig. 8.

Activation of PI3K-Akt signaling in response to volume perturbation has previously been reported (22, 37, 45, 57, 59), and here we demonstrate that acute osmotic cell swelling is accompanied by activation of mTOR and that inhibition of PTEN, which counters the action of PI3K, potentiates swelling-induced mTOR activity. Increased PI3K-Akt-mTOR signaling favors similar to ROS (7, 29) net loss of organic osmolytes under hypotonic conditions by potentiation of taurine loss via VSOAC. Accordingly, inhibition of protein kinase activity coupled to the epidermal growth factor receptor, which is upstream to PI3K and mTOR, inhibits volume-sensitive taurine release, whereas addition of an epidermal growth factor potentiates volume-sensitive taurine release (26). We have previously shown that addition of the phospholipase A2 activator melittin evokes taurine release via VSOAC in NIH3T3 cells under isotonic conditions by means of the same signaling pathway as evoked by osmotic cell swelling (26). Melittin-induced taurine release from NIH3T3 cells is doubled under hyperpolarized conditions (Na\(^{+}\)/H\(^{+}\)-free NMDGCl medium) and reduced to about 30% under depolarized conditions (Na\(^{+}\)-free...
KCl medium) (40). Similarly, we demonstrate that mTOR activity is increased by hyperpolarization and reduced by depolarization of the plasma membrane. Hence, the sensitivity of melittin-induced taurine release to the membrane potential could reflect an effect on mTOR activity. A hypothesis, which has to be tested, is that shift in the membrane potential affects association of Akt to phosphatidylinositol bisphosphate/phosphatidylinositol triphosphate at the inner side of the plasma membrane and hence activation of Akt by other protein kinases (PDK1, autophosphorylation) and subsequently mTOR by Akt.

It has been shown that the plasma membrane depolarizes within the first minutes following hypoosmotic cell swelling in EATC attributable to a swelling-induced increase in anion conductance that exceeds a concomitant increase in cation conductance (31). However, the swelling-induced depolarization would, if anything, reduce mTOR activity and hence taurine release. It is noticed that swelling-induced mTOR activation is transient, i.e., mTOR activity is significantly increased following 6-min hypotonic exposure (Fig. 7A) but reduced following 4-h (data not shown) and 24-h hypotonic exposure (Fig. 2A). mTOR activity is reported to sense nutrient availability, in a process that, according to Kim and coworkers (20, 21), involves the mTOR regulator Raptor and G protein β-subunit-like proteins. However, as shown in RESULTS, depleting ELA cells for taurine has no effect on mTOR activity, i.e., even though prolonged osmotic cell swelling reduces cellular

Fig. 7. Effect of 

\[ H_2O_2 \] exposure and inhibition of protein phosphatase activity on rapamycin-induced stimulation of the volume-sensitive taurine release. Taurine release was determined as indicated in Fig. 6. mRNA abundance was determined by qPCR. A: time trace for taurine release in ELA cells under isotonic and hypotonic conditions (\( \Delta \)) or the protein phosphatase inhibitor Nαvanadate (Van, 50 \( \mu \)M, present during the efflux experiment, ⌧). Curves represent 19 (control), 4 (H2O2), and 4 (Vanadate) sets of experiments. B: rapamycin induced upregulation of swelling-induced taurine release from ELA following hypoosmotic exposure in control cells and cells treated with H2O2 or Vanadate. Maximal rate constants for taurine release were determined at 6 min after hypoosmotic exposure. Data are given relative to the respective control conditions with no rapamycin and represent 13, 4, and 4 sets of experiments for control, H2O2, and Vanadate, respectively. *Significantly increased compared with the respective controls (Student’s t-test). C: expression of antioxidative enzymes. mRNA was isolated from ELA cells and RT-PCR performed using mouse-specific primers for superoxide dismutase 1 (SOD1, 91 bp, lane 1), SOD2 (96 bp, lane 2), glutathione peroxidase (GPX, 96 bp, lane 3), and catalase (Cat, 99 bp, lane 4) as indicated in MATERIALS AND METHODS. M indicates the band sizes of the DNA ladder used in the present experiment. D–F: SOD1 and SOD2 mRNA abundance (D), catalase mRNA abundance (E), and GPX mRNA abundance (F) in NIH3T3 (shaded bars) and ELA (solid bars). Values are given relative to ARP mRNA and represent mean of 3 sets of experiments. G: effect of catalase inhibition (3AT, 5 mM during 2-h preincubation, shaded bar) and GPX boosting (20 mM of N-acetyl cysteine, NAC, added 24 h before the experiment, solid bar) on swelling-induced taurine release from ELA cells. Maximal rate constants for taurine release were determined at 6 min after hypoosmotic exposure. Values are given relative to control and represent 8 and 3 sets of paired experiments for 3AT and NAC, respectively. H: effect of rapamycin-induced mTOR inhibition (50 nM, 24 h) on SOD1, catalase, and GPX mRNA abundance. mRNA expression in control and rapamycin-exposed cells was determined relative to ARP mRNA. Values for rapamycin-exposed cells are given relative to control cells, which were not exposed to rapamycin and represent 6 (SOD1), 6 (catalase), and 8 (GPX) sets of experiments. #Significant difference between control and rapamycin-treated cells (Student’s t-test).
Fig. 8. mTOR-mediated regulation of taurine accumulation via TauT and taurine release via the volume-sensitive leak pathway VSOAC. For acute hypotonic exposure, phospholipase A2 (PLA₂) and 5-lipoxygenase (5-LO) activities are required for swelling-induced activation of VSOAC. Once activated, VSOAC activity is potentiating by reactive oxygen species (ROS), produced by a volume-sensitive NADPH oxidase (NOX), and acting by inhibition of protein tyrosine phosphatase (PTP) activity and hence potentiating of protein tyrosine kinases (PTK) activity. ROS concomitantly reduce TauT activity. PI3K, Akt, and mTOR activities are activated within minutes following osmotic cell swelling, and PTK antagonizes the effect. An increased mTOR or protein kinase upstream to mTOR activity favors net loss of taurine through potentiation of VSOAC and a concomitant inhibition of TauT. For chronic hypotonic exposure, prolonged exposure to hypotonic conditions or rapamycin leads to reduced mTOR activity, reduced catalase mRNA/TauT mRNA abundance, as well as reduced TauT expression/incorporation in the plasma membrane. Reduced catalase capacity is accompanied by potentiation of VSOAC activity and reduction in TauT activity.

taurine concentration dramatically (11), the concomitant reduction in mTOR activity seems not to be secondary to intracellular taurine restriction. Treating ELA cells with rapamycin did not increase the effect of prolonged hypotonic exposure on mTOR activity (Fig. 2B), and, as the potentiating effect of the rapamycin on volume-sensitive taurine release was the same irrespective of the prevailing VSOAC activity, i.e., under control conditions and following inhibition of PI3K/PTEN and GSK3 signaling, we assume that inhibition of mTOR following prolonged hypotonic exposure similar to rapamycin-induced mTOR inhibition increases VSOAC activity through limitation in the antioxidative capacity.

We have previously shown that TauT mRNA abundance and TauT activity are significantly reduced under hypotonic conditions in NIH3T3 mouse fibroblasts (7). As acute stimulation of PI3K/mTOR following osmotic cell swelling correlates with inhibition of TauT, and not a potentiation of TauT as expected from the rapamycin data, it is suggested that the acute regulation of TauT involves phosphorylation of protein kinases upstream to mTOR or an unrelated mechanism. On the other hand, in accordance with the rapamycin data, reduction of TauT following prolonged hypotonic conditions most probably involves reduction in expression and translocation of amino acid transporters or regulators of the transporters to the plasma membrane. As rapamycin exposure is accompanied by a reduction in catalase mRNA abundance, it is suggested that an increased ROS availability contributes to reduction in TauT activity under hypotonic conditions.

**mTOR-taurine homeostasis following osmotic cell shrinkage.** Osmotic cell shrinkage normally elicits net uptake of KCl and organic osmolytes to reverse cell shrinkage in a process entitled regulatory volume increase (RVI) and hence restore normal cell function (12, 29). Cells protect themselves from chronic hypotonic exposure through phosphorylation and activation of the cytosolic transcription regulator NFAT5/TonEBP, which, via its translocation to the nucleus and binding to toxicity-responsive enhancer TonE, facilitates transcription of genes encoding transporters for taurine, myoinositol, and betaine and subsequent increase in the net accumulation of organic osmolytes (29). mTOR facilitates, according to Ortells and coworkers (38), transcription of several osmotic stress response genes including TonEBP by increasing histone H4 acetylation and hence recruitment of RNA-polymerase II. Thus increased mTOR activity seen in ELA cells exposed to prolonged hypertonic exposure (Fig. 2A) could well reflect an increased mTOR-dependent TonEBP activity.

During apoptosis, loss of KCl and amino acids exceeds the capacity of the RVI process, which results in severe loss of osmotic substances, cell shrinkage (apoptotic volume decrease), induction of caspase activity, and hence apoptosis (12, 33, 43). In multidrug- and cisplatin-resistant EATC, the permeabilities toward taurine, K⁺, and Cl⁻ are only moderately increased, and the unet of cisplatin-induced apoptosis was prevented and/or delayed following cisplatin exposure compared with cisplatin-sensitive EATC (43). Furthermore, prevention of taurine loss protects human epithelium lung cells against hypoxia-induced apoptosis (13), and functional TauT expression protects cells against cisplatin-induced apoptosis, presumably through an increased cellular preservation of organic osmolytes and limitation in the initial drug-induced cell shrinkage (6, 13, 43, 52). In congruence with the observation with human trophoblasts by Roos and coworkers (46), we demonstrate that mTOR ensures TauT mRNA abundance, TauT expression, and TauT activity in nonmalignant NIH3T3 and carcinoma ELA fibroblasts. As the chemotherapeutic drug cisplatin ensures a significant activation of mTOR within 3 h in human ovarian cancer cells (41), it is possible that increase in taurine release, cell shrinkage, and hence initiation of the apoptotic process, which in EATC occurs within the same time frame (43), involves mTOR. Targeting mTOR with rapamycin would, not only prevent growth factor-induced cell growth along the growth factor receptor, PI3K, Akt, and mTOR axis, but also limit expression of functional TauT in the plasma membrane and hence favor initiation of apoptosis.

In conclusion, mouse fibroblasts reduce the cellular content of taurine following osmotic cell swelling by releasing taurine via VSOAC and concomitantly reducing taurine uptake via TauT. During acute hypotonic exposure, i.e., within minutes
following osmotic exposure, the shift in VSOAC and TauT activities correlates with increased mTOR activity and most probably reflects the effect of phosphorylation by mTOR and/or areas upstream to mTOR kinases. During chronic hypertonic exposure, shift in VSOAC and TauT activities correlates with reduced mTOR activity and consequently reduction in expression/activity of TauT and enzymes involved in anti-oxidative defense. Under isotonic conditions and during chronic hypertonic exposure, where VSOAC is inactive, mTOR ensures tauine uptake by warranting transcription of the TauT gene, translation of TauT mRNA, and/or translocation of TauT to the plasma membrane.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: I.H.L. and P.A.P. conception and design of research; I.H.L. and J.V.J. performed experiments; I.H.L. and J.V.J. and P.A.P. analyzed data; I.H.L. and P.A.P. interpreted results of experiments; I.H.L. and P.A.P. prepared figures; I.H.L. drafted manuscript; I.H.L., J.V.J., and P.A.P. revised manuscript; I.H.L., J.V.J., and P.A.P. approved final version of manuscript.

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