Autophagy-Dependent Metabolic Reprogramming Sensitizes TSC2-Deficient Cells to the Antimetabolite 6-Aminonicotinamide

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Abstract

The mammalian target of rapamycin complex 1 (mTORC1) is hyperactive in many human cancers and in tuberous sclerosis complex (TSC). Autophagy, a key mTORC1-targeted process, is a critical determinant of metabolic homeostasis. Metabolomic profiling was performed to elucidate the cellular consequences of autophagy dysregulation under conditions of hyperactive mTORC1. It was discovered that TSC2-null cells have distinctive autophagy-dependent pentose phosphate pathway (PPP) alterations. This was accompanied by enhanced glucose uptake and utilization, decreased mitochondrial oxygen consumption, and increased mitochondrial reactive oxygen species (ROS) production. Importantly, these findings revealed that the PPP is a key autophagy-dependent compensatory metabolic mechanism. Furthermore, PPP inhibition with 6-aminonicotinamide (6-AN) in combination with autophagy inhibition suppressed proliferation and prompted the activation of NF-κB and CASP1 in TSC2-deficient, but not TSC2-proficient cells. These data demonstrate that TSC2-deficient cells can be therapeutically targeted, without mTORC1 inhibitors, by focusing on their metabolic vulnerabilities.

Implications: This study provides proof-of-concept that therapeutic targeting of diseases with hyperactive mTORC1 can be achieved without the application of mTORC1 inhibitors. Mol Cancer Res; 12(1); 48–57. ©2013 AACR.

Introduction

Autophagy is a catabolic process leading to the degradation of cytoplasmic content in lysosomal compartments, thereby providing an endogenous source of nutrients for energy production under stress conditions and allowing dysfunctional organelles, including mitochondria, to be "recycled", (1). Tumor cells often upregulate autophagy to promote survival and drug resistance (2). The role of autophagy in cancer therapy is an area of active clinical investigation.

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Tuberous sclerosis complex (TSC) is an autosomal dominant syndrome caused by germline mutations of the TSC1 and TSC2 tumor-suppressor genes (3). Patients with TSC develop histologically benign but life-threatening proliferative lesions in multiple organs, including the brain, heart, lung, and kidney. The TSC1 and TSC2 gene products form a complex that suppresses mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a major regulator of protein translation, autophagy, and metabolism. Hyperactive mTORC1 occurs in the tumors that develop in patients with TSC and in many human cancers, leading to differential metabolic requirements in the setting of low autophagy levels (4, 5).

Autophagy inhibition has been shown to suppress the growth of tumor cells (6). We previously found that constitutive activation of mTORC1 in TSC2-null cells leads to decreased levels of basal and stress-induced autophagy, and increases their vulnerability to further autophagy inhibition (7), yet the metabolic consequences of autophagy inhibition in the context of hyperactive mTORC1 are not fully understood. Moreover, the metabolic pathways used by tumor cells to maintain growth and survival when autophagy is inhibited are poorly characterized. We report here that the metabolic reprogramming induced by autophagy inhibition leads to pentose phosphate pathway (PPP) “addiction” selectively in Tsc2-deficient cells. We also provide proof-of-concept that the metabolic consequences of mTORC1 hyperactivation can be therapeutically targeted without
inhibiting mTORC1 itself, thereby revealing novel therapeutic strategies for TSC.

**Experimental Procedures**

**Cell lines**  
Tsc2+/−/p53+/− and Tsc2−/−/p53−/− mouse embryonic fibroblasts (MEF) were the gift of D.J. Kwiatkowski (Harvard Medical School, Boston, MA) and were confirmed to exhibit Tsc2 deficiency and constitutive activation of mTORC1 by immunoblotting of tuberin. Tsc2 deficiency and constitutive activation of mTORC1 were confirmed by immunoblotting of tuberin and phospho-S6, respectively. The novel Tsc2-null cystadenoma cell line, 105K, was derived from a Tsc2+−/− C57Bl/6 mouse renal tumor and isolated in the laboratory of Dr. Elizabeth Henske. These cells were confirmed to have loss of the second allele of Tsc2 by PCR, loss of tuberin expression, and increased phospho-S6 levels by immunoblotting. All cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin.

**Drugs and shRNA**  
Chloroquine diphosphate salt, bafilomycin A, and 6-amiononocotinamide (6-AN) were obtained from Sigma-Aldrich. Short hairpin RNAs (shRNA) against Atg5 (TRCN0000099434) and Nonicotinamide (6-AN) were obtained from Sigma-Aldrich. Drugs and shRNA were normalized by total proteins and then uploaded in metabolanlyst (http://www.metaboanalyst.ca/MetaboANAlyst/) for subsequent data processing and analyses. Raw and normalized/processed data are reported in Supplementary Table S1. Metabolites that were not detected in at least one sample were excluded from the analysis. Data were normalized to the median (per sample) and processed through log transformation. Heat map and hierarchical clustering was generated using Pearson correlations and the Ward method. Metabolite Set Enrichment Analysis (MSEA) was performed using Metaboanalyst that exploits the Kyoto Encyclopedia of Genes and Genome (KEGG) (http://www.genome.jp/kegg/pathway.html) pathway database. Fifty-eight metabolites were confirmed to be increased in Tsc2−/− MEFs with chloroquine treatment compared with vehicle (P < 0.05, calculated by the Student t test with univariate analysis and equal group variation) were used for MSEA analysis. For reference metabolites, a total of 235 metabolites were used. Metabolite sets containing at least 5 compounds were used in the analysis. MSEA calculates hypergeometric test score based on cumulative binomial distribution. Only 3 metabolites were found increased in Tsc2+/+/+ MEFs upon chloroquine treatment.

**Nutrient consumption**  
Metabolite levels in the medium were measured using the Yellow Springs Instruments (YSI) 7100 as previously described (9).

**Glucose and fatty acid oxidation**  
Cells were seeded in 12-well plates, treated for 24 hours, and incubated for 3 hours at 37°C with either 1 μCi/mL of [14C(U)]-labeled D-glucose or [14C(U)]-labeled palmitate (American Radiolabelled Chemicals Inc.). Then, 3 mol/L of perchloric acid was added to the culture media and the dishes were sealed with a phenylthylamine (Sigma-Aldrich)-saturated Whatman filter paper to capture 14C-CO2, as previously described (10). Following 3-hour incubation at room temperature on a gentle shaker, the filter paper was removed, placed into Ultima Gold F Scintillation Fluid (PerkinElmer Inc.), and radioactivity counts were read in a 1600 liquid scintillation analyzer (Packard).
A microscopic tumor score was observed in the control group compared to the treated group. The cell proliferation percentage also increased significantly in the treated group over time. The macroscopic tumor score was higher in the control group than in the treated group. The phospho-S6 expression was lower in the treated group compared to the control group. The protein biosynthesis and RNA transcription were both decreased in the treated group. The Log2 fold change of the pentose phosphate pathway, aspartate metabolism, pyrimidine metabolism, and amino sugar metabolism were all decreased in the treated group. The Log2 fold change of the pyruvate metabolism, RNA transcription, and protein biosynthesis were also decreased in the treated group. The Log2 fold change of the 2-Isopropyl-5-Methyl-1-cysteine acetylphosphate, pyrophosphate, cytosine, phenylalanine, glycerophosphate, dTDP, and Coenzyme A were also decreased in the treated group.
Oxygen consumption
Oxygen consumption was measured under basal conditions or in the presence of the Trifluorocarbonylcyanamide phenylhydrazone (FCCP) using the Seahorse Bioscience XF24 analyzer as previously described (7). Levels of oxygen consumption were normalized to cell number.

ROS production
Mitochondrial reactive oxygen species (ROS) were measured by using MitoSoX (Invitrogen) staining (5 mmol/L for 15 minutes at 37°C). Cells were washed with PBS, trypsinized and resuspended in PBS containing 1% FBS. Data were acquired with a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo analytical software (Treestar; ref. 11).

Crystal violet staining
Cells were plated into 96-well plates (1,000 cells/well). After treatment, cells were fixed with 10% formalin for 5 minutes, stained with 0.05% crystal violet in distilled water for 30 minutes, washed 2 times with tap water, and drained. Crystal violet was solubilized with 100 μL of methanol and the plate was read with a BioTek plate reader [optical density (OD) 540].

Antibodies and immunoblot analysis
Phospho-S6 (Ser235/236), phospho-S6K (Thr 389), total S6, total S6K, phospho-4E-BP (Thr 37/46), total p65, β-actin, and phospho-p65 antibodies were obtained from Cell Signaling Technology, tuberin antibody from Abcam, Beclin-1 and caspase-1 antibodies from Santa Cruz, LC3 antibody from Novus Biologicals, Arg5 antibody from Sigma, and LAMP2A antibody from Invitrogen. For immunoblot analyses, cells were washed with PBS and harvested in a lysis buffer containing Nonidet P-40. Whole-cell lysates were resolved by electrophoresis, and proteins were transferred onto polyvinylidene difluoride membrane (Immobilon P; Millipore), blocked in Tris-buffered saline Tween-20 and probed with the indicated antibodies in this buffer.

IL-6 measurement
IL-6 levels were measured in the conditioned media using a Luminex LX100 instrument.

Statistical analysis
One- and two-sample t tests and the Mann-Whitney test were used for in vitro and in vivo studies (GraphPad Prism version 5.04 for Windows; GraphPad Software, www.graphpad.com). Significance was defined as P < 0.05. Data are shown as means ± SD.

Results
Autophagy inhibition suppresses proliferation and tumorigenesis of Tsc2-null cells and leads to metabolic reprogramming
To investigate the impact of autophagy suppression on the proliferation and metabolism of cells with hyperactive mTORC1 signaling, we used chloroquine, which raises lysosomal pH and inhibits the fusion between autophagosomes and lysosomes, to treat Tsc2-deficient MEFs in monolayer culture. Chloroquine partially suppressed cell proliferation (38% after 96 hours of treatment, P < 0.05; Fig. 1A and Supplementary Fig. S1A) in a TSC2-dependent manner (Fig. 1A). Next, we treated Tsc2+/− mice, which develop spontaneous renal cystadenomas, with chloroquine (50 mg/kg/day, intraperitoneally, 5 days/week) for 4 months. Chloroquine decreased the number of macroscopic and microscopic renal lesions by approximately 50% (Fig. 1B). Chloroquine treatment did not affect body weight (Supplementary Fig. S1B).
To identify compensatory metabolic mechanisms activated upon suppression of autophagy, metabolic profiling of Tsc2-null MEFs treated with chloroquine (5 μmol/L) was performed. Chloroquine induced extensive alterations, including changes in the glucose, amino acid, and nucleotide utilization/biosynthesis pathways (Fig. 1C and Supplementary Table S1). Importantly, mTORC1 activity was not affected by chloroquine as assessed by the phosphorylation of ribosomal protein S6 (Fig. 1C). MSEA applied to metabolites whose levels increased in chloroquine-treated Tsc2-null cells identified the PPP as the most enriched metabolic pathway (Fig. 1C and D). In contrast, metabolites of the PPP were either unchanged or decreased upon treatment with chloroquine in Tsc2+/− cells (Supplementary Fig. S2 and Supplementary Table S1).

Autophagy inhibition enhances glucose uptake and glucose oxidative metabolism via non-mitochondrial pathways
We next measured uptake of glucose and secretion of lactate in Tsc2+/− and Tsc2+/+ MEFs treated for 24 hours with chloroquine. Interestingly, glucose uptake was significantly increased by chloroquine in Tsc2-null cells (P < 0.05; Fig. 2A) but not Tsc2+/− cells. The amount of lactate
in the media was unchanged, indicating that the chloroquine-induced glucose uptake in Tsc2−/− cells is not associated with increased aerobic glycolysis. Tsc2−/− MEFs in which the autophagy gene Atg5 or Beclin 1 (Atg6) was inhibited by shRNA also exhibited significantly higher glucose consumption relative to control shRNA, with no increase in lactate secretion (Fig. 2A and Supplementary Fig. S3), consistent with the chloroquine results.

### Figure 2.

**Autophagy inhibition enhances glucose uptake and suppresses oxygen consumption.**

A, glucose uptake and lactate secretion measured by YSI in Tsc2−/− and Tsc2+/+ MEFs treated with chloroquine (CQ, 5 μmol/L) or control for 24 hours (left) or infected with shRNA against the autophagy genes Atg5 (Atg5-1 and Atg5-2) or Beclin1 (right). Bars, average of 4 independent samples ± SD; *, P < 0.05; **, P < 0.01. B, intact cellular respiration measured using the Seahorse Bioscience XF24 analyzer, under basal conditions or in the presence of FCCP in Tsc2−/− MEFs treated with chloroquine (5 μmol/L) for 24 hours. Levels of oxygen consumption were normalized to cell number. Bars, average of 3 independent experiments ± SD. *, P < 0.05.

### Figure 3.

**Autophagy inhibition enhances glucose oxidative metabolism in mTORC1-dependent manner.**

A, glucose oxidation measured by 14C-CO2 production in Tsc2−/− MEFs following 24-hour treatment with chloroquine (CQ, 5 μmol/L) and 3-hour labeling with D[U-14C]glucose (left). Bars, average of 5 independent experiments ± SD. **, P < 0.01. B, fatty acid oxidation measured by 14C-CO2 production in Tsc2−/− MEFs following 24-hour treatment with chloroquine (5 μmol/L) and 3-hour labeling with [U-14C]palmitate (right). Bars, average of 4 independent experiments ± SD. *, P < 0.05. C, glucose oxidation measured by 14C-CO2 production in Tsc2−/− MEFs following 24-hour treatment with chloroquine (5 μmol/L), rapamycin (20 nmol/L), or both and 3-hour labeling with D[U-14C]glucose. Bars, average of 3 independent experiments ± SD. *, P < 0.05; **, P < 0.01. D, glucose and fatty acid oxidation measured by 14C-CO2 production in Tsc2+/+ MEFs following 24-hour treatment with chloroquine (5 μmol/L) and 3-hour labeling with D[U-14C]glucose (left) or [U-14C]palmitate (right). Bars, average of 3 independent experiments ± SD.
To define the metabolic routes to which glucose is directed when autophagy is inhibited, we assayed oxidative metabolism. First, we measured the oxygen consumption rate of cells treated with chloroquine. After 24 hours treatment with chloroquine, Tsc2−/− MEFS displayed a significant suppression of both basal and maximal (FCCP-induced) oxygen consumption rate (Fig. 2B), consistent with our previous results in Tsc2-null rat leiomyoma ELT3 cells (7). A concomitant increase in mitochondrial ROS was observed in Tsc2−/− MEFS treated with chloroquine for 24 hours (Supplementary Fig. S4), suggesting progressive accumulation of damaged mitochondria.

Next, we dissected changes in oxidative utilization of individual nutrients by measuring 14C-CO2 release from cells labeled for 3 hours with D[U-14C]glucose or [U-14C]palmitate. Consistent with the metabolomic results, chloroquine induced an increase in glucose oxidation selectively in Tsc2-null cells (P < 0.05; Fig. 3A and D). In addition, fatty acid oxidation decreased in chloroquine-treated Tsc2-null cells (P < 0.05; Fig. 3B and D), consistent with the reduction in oxygen consumption rate. Treatment with rapamycin markedly reduced glucose oxidation even in the presence of chloroquine (P < 0.05; Fig. 3C), suggesting mTORC1 dependence.

Together, these results suggest that autophagy inhibition in Tsc2-null cells leads to increased glucose utilization via the PPP, coupled with decreased oxygen consumption, in an mTORC1-dependent manner.

**Autophagy inhibition sensitizes Tsc2-null cells to 6-AN**

On the basis of the above results, we hypothesized that Tsc2-null cells become dependent on the PPP under autophagy inhibition. To test this hypothesis, we used the antimetabolite 6-AN, a competitive inhibitor of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), the two key enzymes of the oxidative branch of the PPP. G6PD catalyzes the conversion of glucose-6-phosphate into 6-phosphogluconolactone, which is the rate-limiting step of the pentose phosphate shunt, and 6PGD the subsequent oxidation of 6-phosphogluconolactone. Cells were treated with chloroquine, 6-AN, or both drugs for 96 hours. Strikingly, the chloroquine–6-AN combination selectively inhibited the proliferation of Tsc2−/− cells (Fig. 4A and B). No consistent effect on mTORC1 signaling was noted (Supplementary Fig. S5A). To validate these results, we used a mechanistically distinct inhibitor of autophagy, spautin-1, which promotes the proteasomal degradation of Beclin 1 (12). Consistent with the
chloroquine results, treatment with the combination of spautin-1 and 6-AN selectively inhibited the proliferation of Tsc2−/− cells (Fig. 4C). In addition, 6-AN treatment of cells infected with either Atg5 or LAMP2a (a chaperone-mediated autophagy gene) shRNA decreased proliferation of Tsc2−/− cells compared with control shRNA (Fig. 5). The addition of rapamycin did not further affect proliferation in the presence of chloroquine and 6-AN (Supplementary Fig. S5B). The PPP generates ribose-5-phosphate and NADPH. Interestingly, supplementation with 0.1 mmol/L of NADPH (13) was sufficient to sustain the proliferation of Tsc2−/− cells during treatment with the combination of chloroquine and 6-AN (Fig. 4D), suggesting that production of NADPH via PPP is essential to the proliferation of Tsc2−/− cells.

Figure 5. Genetic inhibition of macro- or chaperone-mediated-autophagy sensitize Tsc2-null cells to 6-AN. A, proliferation of Tsc2−/− MEFs infected with control shRNA or shRNA against Atg5 (Atg5-1 and Atg5-2) or Lamp2A and treated with 6-AN (10 μmol/L) for 4 days (crystal violet staining). Time points represent average of 3 independent experiments ± SD. **, P < 0.05; **, P < 0.01. B, Immunoblot analysis of Atg5 (left) and Lamp2A (right) in shRNA-infected Tsc2−/− MEFs. C, autophagy flux analysis in Tsc2−/− MEFs infected with control shRNA or shRNA against Atg5 (Atg5-1 and Atg5-2) or Lamp2A. Immunoblot analysis of LC3 following 6 hour treatment with BafilomycinA 20 nmol/L.

Combined autophagy and PPP inhibition leads to activation of NF-kB and inflammasome pathways

We found that chloroquine treatment led to an increase in ROS levels in Tsc2−/− MEFs (Supplementary Fig. S4), which, together with cytosolic release of mitochondrial DNA due to impaired mitophagy (14), may activate NF-kB/Rel activity (15). Consistent with this reasoning, phosphorylation of p65/RelA was induced at 96 hours by the combination of chloroquine and 6-AN (Fig. 6A), associated with increased secretion of IL-6, a known transcriptional target of NF-kB (Supplementary Fig. S7). IL-6 was not detectable in Tsc2+/+ control cells at baseline or after treatment with chloroquine/6-AN (Supplementary Fig. S7). Furthermore, we observed accumulation of cleaved caspase-1, a marker of inflammasome activation, under the combined chloroquine/6-AN treatment at 96 hours (Fig. 6B). Supplementation with 0.1 mmol/L of NADPH was sufficient to prevent accumulation of cleaved caspase-1 under chloroquine/6-AN treatment for 96 hours (Fig. 6C), consistent with the rescue of cell proliferation by NADPH (Fig. 4D).

To test the hypothesis that activation of NF-kB is necessary for the inhibition of cell proliferation by chloroquine and 6-AN, we treated the cells with parthenolide, which
inhibits both p65/RelA and caspase-1 via alkylation (16). Parthenolide restored proliferation in cells treated with chloroquine and 6-AN (Supplementary Fig. S8). These data suggest that the combination of chloroquine and 6-AN inhibits the proliferation of Tsc2-null cells via ROS-dependent activation of NF-kB and caspase-1.

Discussion

Chloroquine and its analog hydroxychloroquine, which block lysosome–autophagosome fusion and lysosomal function thus suppressing macroautophagy and chaperone-mediated autophagy, can inhibit cancer cell survival under stress conditions (1). We report here that prolonged (4 months) treatment with chloroquine suppressed the number and size of renal tumors in Tsc2<sup>-/-</sup> mice, consistent with the decreased spontaneous renal tumors we previously observed in Tsc2<sup>-/-</sup>/Beclin1 double heterozygous mice (7). We hypothesized that metabolic compensatory mechanisms are activated when autophagy is suppressed by chloroquine in Tsc2-deficient cells, thereby preventing a more complete therapeutic response. Metabolite profiling revealed striking changes in the metabolome of Tsc2-null cells treated with chloroquine, including increased levels of five PPP intermediate metabolites. Isotope labeling experiments revealed an increase in glucose oxidation under chloroquine treatment, which may reflect activation of the oxidative branch of the PPP. The pentose phosphate shunt, which is often upregulated in cancer, has both biosynthetic and oxidative functions, representing the main source of NADPH via its oxidative branch and supplying ribose-5 phosphate for nucleotide synthesis.

In parallel with the PPP upregulation, we found that chloroquine induced ROS production at a higher rate in Tsc2-null cells than in cells with intact TSC2. This could be due to impaired mitophagy and subsequent accumulation of dysfunctional mitochondria, which would increase the cell requirement for reducing power to buffer-elevated ROS production. TSC2-deficient cells may be particularly vulnerable to loss of reducing power because they are known to produce elevated amounts of ROS at baseline (17) and to be highly sensitive to increased ROS (18).

Therefore, these data suggest that the PPP becomes a crucial metabolic survival mechanism in TSC2-null cells when they encounter the increase in oxidative stress due to further autophagy suppression. To test this hypothesis, we used the antimetabolite 6-AN, which inhibits the PPP-dependent NADPH supply (19), in combination with chloroquine. The chloroquine/6-AN combination dramatically suppressed the proliferation of Tsc2-null cells, which was rescued by supplementation of NADPH. Similar results were seen with spautin-1 (12), a recently reported inhibitor of Beclin-1, and with downregulation of Atg5 and Lamp2a genes, supporting the hypothesis that the effect of chloroquine in these experiments is autophagy-mediated. Spautin-1 has been found to induce cell death under glucose deprivation conditions in breast cancer cells (12), further underscoring the links between the metabolic status of tumor cells and their response to autophagy inhibition.

Notably, the combination of chloroquine and 6-AN triggered activation of the NF-kB and inflammasome pathways in a TSC2-dependent manner, and parthenolide (16), which inhibits both NF-kB and caspase-1, restored proliferation under these conditions. Inflammasomes are...
multiprotein complexes that mediate the cleavage and activation of caspase-1, thereby stimulating an inflammatory response and/or programmed cell death (20). Multiple factors can activate the inflammasome, including autophagy inhibition, mitochondrial DNA release, increased ROS, and activation of NF-kB (15, 21), thus affecting cell survival/proliferation. Recent work has linked the NF-kB pathway to the immune response and cellular senescence and has suggested that NF-kB may serve a tumor-suppressor function in some circumstances. Together, our results lead us to the conclusion that the inhibition of autophagy and the PPP leads to depletion of NADPH, increased ROS production, and activation of NF-kB/Rel. We propose that this ultimately suppresses cell proliferation via activation of the inflammasome (Fig. 6D).

Our results point toward novel therapeutic strategies for neoplasms with mTORC1 activation by targeting their intrinsic dependence on autophagy and the metabolic compensatory mechanisms triggered by autophagy inhibition. Thus, in TSC and related diseases, including lymphangioleiomymatosis (LAM), our data provide a first proof-of-concept that therapeutic targeting of cells with hyperactive mTORC1 can be accomplished by targeting their metabolic vulnerabilities without the utilization of mTORC1 inhibitors. We note that the autophagy inhibitors chloroquine and hydroxychloroquine (Plaquenil) are currently being tested in clinical trials for LAM. Our data provide a conclusion that therapeutic targeting of cells with hyperactive mTORC1 activation may have broad clinical impact.

Disclosure of Potential Conflicts of Interest
D.J. Kwiatkowski is a consultant/advisory board member of Novartis. No potential conflicts of interest were disclosed by the other authors.

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