mTORC1/NF-κB axis controls amino acid catabolism by regulating the expression of the key enzymes in human hepatocytes

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Abstract

Background

In addition to serving as building blocks for protein synthesis, amino acids also provide energy and precursors that are used by cells through catabolism. Mechanistic target of rapamycin complex 1 (mTORC1) is a central coordinator of cellular metabolism. However, little is known regarding the function of mTORC1 in amino acid catabolism. The aims of this study were to explore the mechanism by which mTORC1 controls the conversion of glutamate to α-ketoglutarate and ornithine to putrescine, and mTORC1 regulates the expression amino acid catabolism-related genes in hepatocyte.

Methods

HL-7702 hepatocytes were treated with glutamate, ornithine, rapamycin or SC75741, alone or in combination; the plasmids pRNAT-U6.1/Neo-shRaptor and pIRES2-EGFP-Rheb were transfected into HL-7702 cells to silencing Raptor or overexpressing Rheb. The intracellular content of glutamate, oxaloacetate, α-ketoglutaric acid, and aspartic acid, and the intracellular level of aspartate aminotransferase (AST), ornithine decarboxylase (ODC), glutamate dehydrogenase (GDH), and glutamic acid decarboxylase (GAD) were measured by ELISA. The concentrations of intracellular ornithine and putrescine were measured by HPLC. The mRNA level of amino acid catabolism-related genes was detected by qRT-PCR, and the protein level of mTORC1 and NF-κB was investigated by western blot.

Results

Our data showed that rapamycin inhibits the utilization of glutamate and ornithine in HL-7702 hepatocytes. mTORC1 regulates the expression of AST and ODC through the transcription factor NF-κB in response to glutamate or ornithine. Further, inactivated mTORC1 by Raptor silencing downregulated the expression of AST, ODC, GDH and GAD, while enhanced mTORC1 by Rheb overexpression upregulated NF-κB activation and the indicated genes expression in hepatocytes. Inhibited NF-κB by inhibitor SC75741 decreased the AST, ODC, GDH, and GAD expression.

Conclusions
Our results demonstrate that mTORC1 regulates amino acid catabolism by inducing the expression of AST, ODC, GDH, and GAD, which is mediated by NF-κB. This finding constitutes a novel mechanism by which amino acid catabolism is regulated in hepatocytes.

Introduction
The availability of nutrients and energy is critical for cellular growth and proliferation. Cells must coordinate anabolism and catabolism with environmental inputs. Mechanistic (formerly mammalian) target of rapamycin (mTOR) complex 1 (mTORC1) is a nutrient-sensitive multi-protein complex that links nutrient and energy signals and functions as the master regulator of cell growth and metabolism in specific energy- and nutrient-consuming processes [1-3]. The availability of amino acids serves as a signal that can initiate mTORC1 signaling. mTORC1 is sensitive to the availability of amino acids, which recruits mTORC1 to the lysosomal surface, where it is activated by the small GTPases Rheb and Rags [4-7]. mTORC1 uses distinct mechanisms to sense several types of amino acid in lysosomal and cytosolic has been demonstrated [8-11]. However, the function of mTORC1 in amino acid catabolism remains poorly understood.

To achieve cellular homeostasis, cells must efficiently utilize available extracellular and intracellular nutrients (e.g., fatty acids and amino acids) to provide energy when nutrients are scarce [12]. The main amino acid catabolic pathways include transamination, oxidative deamination, and decarboxylation, which are catalyzed by specific enzymes: aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), glutamic acid decarboxylase (GAD), and ornithine decarboxylase (ODC). Although little is known of the expression patterns of the genes that are involved in amino acid catabolism, a few reports related to the role of mTORC1 in the expression and activation of amino acid catabolic enzymes.

Rapamycin can reduce plasma levels of alanine aminotransferase (ALT) and AST in cirrhotic rats [13]. Inhibition of mTOR activity by rapamycin or blocking S6 expression by siRNA inhibited GDH and GLS activity in ovarian cancer cells [14]. ODC mRNA is stabilized in an mTORC1-dependent manner in Ras-transformed rat intestinal epithelial (RIE-1) cells [15]. At present, the relationship between GAD and mTOR signaling is unclear.
The transcription factors NF-κB (nuclear factor κB) is best known as a central regulator of inflammation, and have recently attracted attention as functioning in metabolic disorders [16–18]. Cooperative NF-κB/STAT signaling regulates lymphoma metabolic reprogramming and aspartate transaminase (GOT2) gene expression [19]. Several lines of evidence support a crucial role for NF-κB in governing energy homeostasis and mediating metabolic reprogramming in cancer cells [20]. Thus, it is believed that NF-κB functions in cell metabolism.

Compared with the regulation of protein synthesis, little is known of the function of mTORC1 in controlling amino acid catabolism processes. The purpose of this study was to determine the role and mechanism of mTORC1 in amino acid catabolism in hepatocytes. The results demonstrate that mTORC1 regulates amino acid catabolism by inducing the expression of AST, GDH, GAD, and ODC, which is mediated by NF-κB. This study provides a possible mechanism of amino acid catabolism.

Materials And Methods

Cell lines and culture conditions

HL-7702 hepatocytes were maintained in 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were cultured in humidified air with 5% CO₂ at 37°C.

Reagents and antibodies

Rapamycin (Gene Operation, Ann Arbor, MI, USA) was dissolved in ethanol (Sigma-Aldrich, Inc., USA) to a stock concentration of 50 mg/ml and stored at -20°C, and was diluted to target final concentrations with culture medium before use. SC75741 (America Selleck Biotechnology Co., Ltd[] Houston[]Texas[]USA) was dissolved in DMSO to a stock concentration of 50 mM, and was diluted to target final concentrations with culture medium before use. The concentration of ethanol in the final solution did not exceed 0.5% (v/v) in any experiment. The following primary antibodies were purchased from Cell Signaling Technology, Inc. (Beverley, MA, USA): anti-NF-κB p65, anti-phospho-NF-κB p65 (Ser536), anti-p-4EBP1 (Thr37/46), anti-p-S6 (Ser240/244), and anti-Raptor. anti-S6 primary antibody was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). anti-4EBP1, anti-p-mTOR (Ser2448), and anti-mTOR were purchased from Abcam (plc 330 Cambridge Science Park, Cambridge, UK). Anti-β-actin primary antibodies were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).
ECL Anti-Rabbit IgG-HRP and ECL Anti-Mouse IgG-HRP were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

**ELISA**

HL-7702 cells were seeded in 6-well plates at $1 \times 10^6$ cells per well and were incubated until 80% confluence, respectively. To determine the intracellular concentrations of glutamate, oxaloacetate, α-ketoglutaric acid and aspartic acid, HL-7702 cells were cultured in serum-free medium for 13 hours, followed by amino acid starvation for 1 hour, and were then incubated in the presence of glutamate for 1 hour. During serum starvation, cells were pretreated with 100 nM rapamycin for 8 hours, or with 10 μM SC75741 for 12 h, respectively. To determine the intracellular concentrations of glutamate, oxaloacetate, α-ketoglutaric acid and aspartic acid, three groups—control, glutamate, and glutamate with rapamycin—were established. To determine the intracellular concentrations of AST, GDH, ODC, and GAD, HL-7702 cells were treated with rapamycin or SC75741 or were transfected with pRNAT-U6.1/Neo-shRaptor or pIRES2-EGFP-Rheb, respectively. Treated HL-7702 cells were harvested with trypsin and were centrifuged to remove cell culture supernatants. Cell lysates were prepared by 5 freeze–thaw cycles and standardized with the same protein concentration in control groups and treatment groups by adjusting the volume of the protein lysate. Equal volume of protein lysates were measured for glutamate, oxaloacetate, α-ketoglutaric acid and aspartic acid, or for AST, GDH, ODC, and GAD using enzyme-linked immunosorbent assay (ELISA) kits (Wuhan XiqidiBiological Technology Co. Ltd. Wuhan, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm and 630 nm on a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA). The absorbance values were measured three times per sample, and the mean value of the 3 independent measurements was used in the statistical analyses.

**HPLC analysis**

High-performance liquid chromatography (HPLC) was performed using an Agilent 1260 liquid chromatography system (Agilent Technologies Inc. Santa Clara, CA, USA) and DAD (Diode Array Detector). HL-7702 cells were cultured with serum-free medium for 13 hours, followed by amino acid starvation for 1 hour, and were then incubated with ornithine for 1 hour. During serum starvation,
cells were pretreated with 100 nM rapamycin for 8 hours. Three groups—control, ornithine, and ornithine with rapamycin—were established. HL-7702 cells were collected and dissolved in 1 mL of lysis buffer, and protein concentration was determined.

For the analysis of putrescine, the protein samples were treated with n-hexane to remove lipids, and the mixture was extracted with N-butanol/trichloromethane (1:1 v/v ratio), which the extracting agent was removed by aspiration and evaporation to dryness under a stream of nitrogen at 40°C. Samples were then dissolved in 0.1 mM HCl for derivatization. For derivatization, the mixture was combined with dansyl chloride, which was then aspirated and evaporated to dryness under a stream of nitrogen at 40°C. Samples were then dissolved in 1 mL methanol for HPLC analysis with a C18 column (150 mm × 4.6 mm, 5 μm) at 30°C. The mobile phase contained A (methanol) and B (water), which was used according to the following program: 0 min, 55% A; 7 min, 65% A; 14 min, 70% A; 20 min, 70% A; 27 min, 90% A; 30 min, 100% A. The flow rate was 1.5 ml/min, and the injection volume was 20 μL. Putrescine was tentatively identified by comparing its retention time with that of authentic standards under identical analysis conditions at 254 nm.

For the detection of ornithine, 200 μL of each protein sample was mixed with 10 μL of 1.0 mg/mL norleucine, 100 μL of 1 mM triethylamine-acetonitrile solution, and 100 μL of 0.1 mM phenyl isothiocyanate-acetonitrile solution and let stand at room temperature for 1 hour. Next, 400 μL n-hexane was added, and the mixture was left standing for 10 min, and the lower clear solution was passed through a 0.45-μm filter. Next, 2.0 μL of each sample was injected into the HPLC system with a DIONEX Acclaim 120 C18 column (250 mm × 4.6 mm, 5 μm) at 40°C (Thermo Fisher Scientific Inc., Waltham, MA, USA). The mobile phase contained A (0.2 mM sodium acetate-acetonitrile solution, v/v = 93:7) and B (water-acetonitrile solution, v/v = 20:80), per the following program: 0 min, 0% B; 5 min, 3% B; 14 min, 11% B; 17 min, 21% B; 29 min, 34% B; 41 min, 100% B. The flow rate was 1.0 ml/min. Ornithine was tentatively identified by comparing its retention time with that of authentic standards under identical analysis conditions at 254 nm.

**Western blot analysis**

Cells were harvested with trypsin, washed with cold phosphate-buffered saline, and lysed in cell lysis
buffer. The cells were then placed on ice for 10 min and centrifuged at 10625 g RCF at 4°C for 10 min. Lysate protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, USA). Equal amounts (40 μg) of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels (w/v)), transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with the primary antibody overnight at 4°C. Membranes were then incubated with the peroxidase-conjugated secondary antibody for 1 hour at room temperature. Enhanced chemiluminescence (ECL) reagent (Amersham) was used with the Western Blotting System (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) to detect proteins of interest. Protein bands were quantified on a Gel-Pro Analyzer 4.0 (Media Cybernetics, USA).

**RT-qPCR analysis**

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was used to determine mRNA levels of **AST**, **GDH**, **ODC**, and **GAD** and the **Raptor**, **Rheb** in HL-7702 cells of treatment and control groups. Total RNA from the untreated and treated cells was reverse-transcribed with an oligo (dT)12–18 primer using the AMV 1st Strand cDNA Synthesis Kit (Takara Co. Ltd., China). cDNA sequences were amplified with the primers shown in Table S1. The reactions were run using the KAPA SYBR® FAST qPCR Kit optimized for LightCycler® 480 (KAPA BIOSYSTEMS, Inc, Boston, Massachusetts, USA) according to the manufacturer’s instructions. One microliter of cDNA was amplified in a 25-μL reaction that contained 10 μM forward primer (0.5 μL), 10 μM reverse primer (0.5 μL), SYBR Premix Ex Taq (12.5 μL), and nuclease-free water (10.5 μL). Cycling conditions consisted of an initial denaturation step at 95°C for 5 min, then 40 cycles at 95°C for 5 sec, 54°C for 30 sec, and 72°C for 20 sec, followed by a final extension at 72°C for 10 min. Three technical replicates were performed per sample. \( 2^{-\Delta\Delta CT} \) values were calculated to determine expression levels, and the qPCR results were analyzed by student’s t-test to compare expression levels between untreated and treated groups. 3 independent experiments were performed.

Table S1 the primers for amplifying cDNA sequences of target genes
| Gene Name | Gene ID | Forward primer (5'-3') | Reverse primer (5'-3') |
|-----------|---------|------------------------|-----------------------|
| AST       | 26503   | CCTTCGTATGCTGGTATCCT    | TTGTACTTCACCTTTGGCG   |
| GDH       | 2746    | GCTGGAGGAGTGACAGTATCTT  | TGGAACTCTGCGTGGGTA    |
| GAD       | 2571    | GGCAATCTCCAAGAACCT      | TGATGAAAGTCCAGCACCT   |
| ODC       | 4953    | TGTGGGTGATGGATGCTC      | GGCTGCTCTGTCGCGGTTT   |
| Rheb      | 6009    | GTTGTTGGAATAAGAAAGAC    | CACATCACCGAGCATGAAGACT|
| Raptor    | 57521   | GAGCAGGTGACTAAGGAAGAC   | CAGGTGCCGAGAGTGAAAG    |

**DNA Constructs**

Short hairpin (shRNA) Raptor RNA-silencing constructs (shRaptor) with the sequence 5′-aaGCTCTGCACGTCTTACGTTTCAAGAGAACGTAAGGACGTGCAGAGCtt-3′ were designed and synthesized to construct pRNAT-U6.1/Neo-shRaptor. Rheb cDNA was amplified using the forward primer 5′-GTTGGTTGGGAATAAGAAAGAC-3′ and the reverse primer 5′-CACATCACCGAGCATGAAGACT-3′, which were based on the human Rheb sequence (GenBank Accession number NM_005614). The Rheb PCR fragment was inserted into pIRES2-EGFP (Clontech Laboratories, Inc., Mountain View, CA, USA) to construct pIRES2-EGFP-Rheb.

**In vitro transfection**

The plasmids pRNAT-U6.1/Neo-shRaptor and pIRES2-EGFP-Rheb were transfected into HL-7702 cells using Lipofectamine TM2000 (Invitrogen, Carlsbad, New Mexico, USA) per the manufacturer’s instructions. Transfectants were selected by culturing cells in the presence of G418 (Hyclone Laboratories, Inc. Logan, Utah, USA) for 48 hours and were imaged using a ZEISS AX10 fluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY, USA), and then cells were collected. For ELISA assay, cell lysates were prepared by 5 freeze–thaw cycles; for western blot analysis, cells lysates were prepared by lysed in cell lysis buffer.

**Statistical Analyses**

Statistical analyses were conducted using SPSS PASW Statistics for Windows, v18.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using standard parametric statistics, one-way ANOVA, followed by Tukey’s method. Data are expressed as mean ± SD. Results are presented as the average of at
least 3 independent experiments unless stated otherwise. Statistical significance was accepted when $p \leq 0.05$.

Results

Rapamycin inhibits catabolism of glutamate and ornithine in HL-7702 cells

To determine whether mTORC1 regulates the conversion between substrate and product in amino acid catabolism, the inhibitory effect of rapamycin on glutamate or ornithine catabolism was examined in HL-7702 cells. HL-7702 cells were starved, which were then divided into 3 groups: control, glutamate, and glutamate with rapamycin. The concentration of intracellular glutamate, oxaloacetate, $\alpha$-ketoglutaric acid, and aspartic acid was measured by ELISA. As shown in Fig. 1a, the intracellular glutamate concentration in the glutamate group was significantly higher than that in the control group ($p < 0.01$). The intracellular glutamate concentration of the glutamate with rapamycin group was significantly higher versus the glutamate group ($p < 0.05$). These data indicate that exogenous glutamate was absorbed by starved cells and that rapamycin reduced their utilization of glutamate.

Furthermore, the intracellular oxaloacetate concentration of the glutamate group was significantly lower than that in the control group ($p < 0.01$). The intracellular oxaloacetate concentration of the glutamate with rapamycin group was significantly higher compared with the glutamate group ($p < 0.01$). These data indicate that intracellular oxaloacetate was utilized and that rapamycin blocked this utilization. The intracellular concentration of $\alpha$-ketoglutaric acid and aspartic acid was higher in the glutamate group compared with the control group ($p < 0.01$) and no change in the glutamate with rapamycin group ($p > 0.05$). These results suggest that rapamycin prevents the accumulation of $\alpha$-ketoglutaric acid and aspartic acid.

Ornithine is converted to polyamine by ODC in cancer cells [21]. To characterize the inhibitory effects of rapamycin on ornithine catabolism, HL-7702 cells were starved, which were then divided into 3 groups: control, ornithine, and ornithine with rapamycin. The concentrations of intracellular ornithine and putrescine were measured by HPLC. The intracellular ornithine concentration of the ornithine group was significantly higher than that in the control group (Fig. 1b) ($p < 0.05$) and the ornithine
content of the ornithine with rapamycin group was significantly higher than that in the ornithine group (p < 0.05). These data indicate that exogenously added ornithine was absorbed by starved cells and that rapamycin blocked ornithine utilization.

Glutamate and ornithine promote AST and ODC expression via activation of mTORC1 and NF-κB in HL-7702 cells

Transamination between glutamate and α-ketoglutaric acid can be catalyzed by AST to produce oxaloacetate and aspartic acid, and the decarboxylation of ornithine to putrescine can be catalyzed by ODC; moreover, NF-κB has recently attracted attention as functioning in metabolic disorders [16, 17], thus, we speculated that AST and ODC expression is regulated by mTORC1 via NF-κB. To evaluate whether glutamate or ornithine can promote AST and ODC expression by mTORC1 via NF-κB, we first examined the effect of glutamate or ornithine on the activation of mTORC1 signaling and on NF-κB. Glutamate or ornithine was added to starve HL-7702 cells, and phosphorylation of S6 and 4EBP1 was assessed, which are phosphorylated in an mTORC1-dependent manner. Phosphorylation of NF-κB was also measured. The results showed that glutamate significantly increased the phosphorylation of S6, 4EBP1, and NF-κB p65 (Fig. 2a and 2b) compared with the starved group. Ornithine increased the phosphorylation of mTOR, 4EBP1, and NF-κB (Fig. 2c and 2d). As a result, expression of AST and ODC was increased by glutamate or ornithine in mRNA and protein levels (Fig. 2e-2 h) (p < 0.05). These results suggest that mTORC1 signaling is responsible for glutamate- and ornithine-induced upregulation of AST and ODC, and this process is probably through NF-κB in HL-7702 cells.

Inactive mTORC1 downregulates activation of NF-κB and expression of amino acid metabolic genes in HL-7702 cells

To verify the regulatory function of mTORC1 in the expression of amino acid metabolic genes via NF-κB, the effect of rapamycin on NF-κB activation and on AST, GDH, GAD, and ODC expression in HL-7702 cells was examined. Cells were treated with 100 nM rapamycin for 8 hours, and the relative abundance of AST, GDH, GAD, and ODC mRNA and corresponding intracellular enzymes were determined. The results showed that rapamycin significantly inhibited activation of mTORC1 and NF-
κB (Fig. 3a and 3b) (p < 0.05), and the mRNA level of AST, GDH, GAD, and ODC and the corresponding intracellular enzyme levels were significantly decreased by rapamycin (Fig. 3c and 3d) (p < 0.01). These data suggest that mTORC1 and NF-κB are associated with the expression of these catabolic genes.

To further evaluate the effect of rapamycin on AST, GDH, GAD, and ODC expression, the degree of mTORC1 activation was reduced by knocking down Raptor, a critical component of mTORC1, using targeting shRNA (Fig. S1) in HL-7702 cells, and then phosphorylation of S6, 4E-BP1, and NF-κB was measured. The phosphorylation of all targets was inhibited by Raptor silencing (Fig. 4a, 4b). The levels of metabolic gene expression and intracellular enzymes were also measured. The expression pattern of the genes was similar to that in the rapamycin-treated group (Fig. 4c, 4d) (p < 0.01). These results further demonstrate that mTORC1 and NF-κB are involved in catabolic gene expression.

To conform the function NF-κB in the expression of AST, GDH, GAD, and ODC, we used SC75741, a specific inhibitor of NF-κB, to inhibit NF-κB activation in HL-7702 cells, and then the levels of metabolic gene expression and intracellular enzymes were measured. The results showed that NF-κB activation was inhibited by the inhibitor (Fig. 5a), and the levels of AST, GDH, GAD, and ODC mRNA, and the corresponding intracellular enzyme were significantly decreased (Fig. 5b and 5c, respectively) (p < 0.01), suggesting that NF-κB directs the expression of AST, GDH, GAD, and ODC.

Enhanced mTORC1 activation upregulates amino acid metabolic genes expression in HL-7702 cells

To complement the results that Raptor silencing decreases mTORC1 activation and expression of AST, GDH, GAD and ODC, we cloned and overexpressed Rheb, an upstream positive effector of mTORC1, in HL-7702 cells to enhance mTORC1 activation (Fig. S2). We also measured AST, GDH, GAD and ODC expression and the concentration of the corresponding enzymes. Rheb overexpression upregulated mTORC1 signaling (Fig. 6a) and NF-κB phosphorylation (Fig. 6b), and the expression of these catabolic genes was enhanced both in mRNA level (Fig. 6c) (p < 0.01) and in protein level (Fig. 6d) (p < 0.01). These results indicate that the expression of these catabolic genes and NF-κB activation are increased by greater activation of mTORC1.

To further verify mTORC1 regulates the expression of AST, GDH, GAD and ODC through NF-κB, the
Rheb-over expressed HL-7702 cells were treated with 10 µM SC75741 for 12 h, and then NF-κB phosphorylation and expression of the amino acid catabolic genes were determined. The results showed that NF-κB activation was enhanced by Rheb over expression and inhibited by SC75741 (Fig. 7a), and the expression pattern of the genes was similar to phosphorylation of transcription factor NF-κB (Fig. 7b). These results indicate that mTORC1 controls the expression of these catabolic genes via NF-κB in HL-7702.

Discussion
Amino acid catabolism supplies energy and precursors for the synthesis of macromolecules and to support cellular function, for which key enzymes play an important role. In this study, we demonstrated that mTORC1 regulates the expression of amino acid-catabolic genes in HL-7702 cells. Two recent reports showed that plasma AST levels are regulated by mTORC1 in rats [13, 22]. GDH activity is related to mTORC1 activity in ovarian cancer cells [14], and prolactin induces ODC expression via mTOR signaling in mink uterine epithelial cells [23]; however, the relationship between GAD and mTOR signaling has not been reported. In our study, the expression of AST, GDH, GAD, and ODC was inhibited by rapamycin and Raptor silencing. Additionally, intracellular levels of AST, GDH, GAD, and ODC were regulated by mTORC1. Further, the conversion of glutamate to α-ketoglutarate and ornithine to putrescine were controlled by mTORC1.

A recent report showed that cooperative NF-κB/STAT3 signaling functions in lymphoma metabolic reprogramming and aspartate transaminase (GOT2) gene expression [19]. In our previous study, we found that mTORC1 regulates peptidoglycan-induced inflammation via NF-κB in murine macrophages [24]. In the present study, we focused on the regulation of NF-κB in amino acid catabolic gene expression. We found that rapamycin or Raptor silencing inhibited the expression of amino acid catabolic genes and the activation of NF-κB in HL-7702 cells. The serum and amino acid starvation significantly decreased the activation of NF-κB, but the intracellular agonist glutamate and ornithine, or Rheb overexpression, significantly enhanced its activation. The data from hepatocytes HL-7702, which were based on active and inactive forms of mTORC1 and phosphorylation or dephosphorylation of transcription factor NF-κB, indicate that the expression of AST, GDH, GAD, and ODC is regulated by
mTORC1/NF-κB axis. Thus, we conclude that mTORC1 regulates the expression of amino acid catabolic genes is via NF-κB.

In the present study, ornithine induced ODC expression, which was regulated by mTORC1, and the utilization of ornithine in HL-7702 cells was significantly blocked by rapamycin. However, the content of putrescine has no significant difference between ornithine group and glutamate with rapamycin group, meaning rapamycin has no effect on putrescine accumulation. In fact, putrescine can stimulate mTORC1 signaling and protein synthesis in porcine trophectoderm cells [25], thus, the inhibitory effect of rapamycin on ODC expression may be neutralized the activation of mTORC1 by putrescine, and resulted in no change in putrescine content.

Conclusions
In conclusion, this work examined the function of mTORC1 in amino acid catabolism in HL-7702 hepatocytes, demonstrating that mTORC1 regulates amino acid metabolism. mTORC1 governs the expression of amino acid metabolic genes, including AST, GDH, GAD, and ODC, through NF-κB. Furthermore, mTORC1 signaling is responsible for glutamate and ornithine, increasing the expression of AST and ODC through NF-κB, and controls the conversion of glutamate to α-ketoglutarate and ornithine to putrescine in HL-7702 hepatocytes. mTORC1 functions in amino acid catabolism via NF-κB in hepatocytes. This study provides a possible mechanism of amino acid catabolism.

Abbreviation
mTORC1, mechanistic target of rapamycin complex 1; AST, aspartate aminotransferase; GDH, glutamate dehydrogenase; GAD, glutamic acid decarboxylase; ODC, ornithine decarboxylase; ALT, alanine aminotransferase; NF-κB, nuclear factor-kappa B; 4EBP1, eukaryotic initiation factor 4E binding protein 1; S6, ribosomal protein S6; shRNA, short hairpin RNA

Declarations
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Author contributions
All authors read the manuscript and provided feedback. Z.W. and F.Q. conceived of the project, designed the study, and conducted the experiments. M.Z., Y.F. and Y.C. performed experiments. Y.M.,
Z.G., Y.W., and H.H. analyzed the data. Y.C. and M.Z. interpreted the data and wrote the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Additional Files

Supplementary file 1------Figure S1, S2

Fig. S1 Silencing of Raptor with shRNA in HL-7702

(a) pRNAT-U6.1/Neo-Raptor-shRNA-transfected HL-7702 cells expressing EGFP.

(b) Raptor mRNA levels were decreased in HL-7702 cells transfected with pRNAT-U6.1/Neo-Raptor-
shRNA.

(c) Raptor protein levels were decreased in HL-7702 cells transfected with pRNAT-U6.1/Neo-Raptor-shRNA. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, ** p<0.01)

**Fig. S2** Overexpression of Rheb in HL-7702 cells

(a) pIRES2-EGFP-Rheb-transfected HL-7702 cells expressing EGFP.

(b) Rheb mRNA levels are increased in HL-7702 cells transfected with pIRES2-EGFP-Rheb.

(c) Overexpression of Rheb in HL-7702 cells transfected with pIRES2-EGFP-Rheb. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, ** p<0.01)

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Figures
Figure 1

Rapamycin inhibits transamination of glutamate and decarboxylation of ornithine in HL-7702 cells. (a) Rapamycin inhibits transamination between glutamate and α-ketoglutaric acid to produce oxaloacetate and aspartic acid in HL-7702 cells. The content of intracellular glutamate, oxaloacetate, α-ketoglutaric acid, and aspartic acid was measured by ELISA. The intracellular content of glutamate in adding glutamate group is more than control, and that in glutamate with rapamycin group is more than adding glutamate group, meaning that the exogenous glutamate was absorbed by starved cells and that rapamycin reduced the utilization of glutamate. The intracellular α-ketoglutaric acid was accumulated as products of AST action and this action was attenuated by rapamycin. (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01) (b) Rapamycin inhibits ornithine decarboxylation to produce putrescine in HL-7702 cells. The content of intracellular ornithine, and putrescine was measured by HPLC. The intracellular content of ornithine in adding ornithine group is more than control, and that in ornithine with rapamycin group is more than adding ornithine group, meaning that the exogenous ornithine was absorbed by starved cells and that rapamycin reduced the utilization of ornithine. (The values represent the means ± SD, n = 2, * p<0.05)
Glutamate and ornithine stimulate the activation of mTORC1 signaling and NFκB, and promote expression of AST and ODC in HL-7702 (a) The activation of mTORC1 signaling was promoted by glutamate. (b) The activation of transcription factor NFκB was promoted by glutamate. (c) The activation of mTORC1 signaling was promoted by ornithine. (d) The activation of NFκB was promoted by ornithine. (e) The relative abundance of AST mRNA was upregulated by glutamate. (f) The intracellular content of AST was upregulated by glutamate. (g) The relative abundance of ODC mRNA was upregulated by ornithine. (h) The intracellular content of ODC was upregulated by ornithine. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)
Rapamycin inhibits the expression of AST, GDH, GAD, and ODC, and attenuates mTORC1 signaling and NFκB activation in HL-7702 cells. (a) Phosphorylation of mTOR, S6, and 4EBP1 was decreased by rapamycin. (b) Phosphorylation of NFκB was decreased by rapamycin. (c) Relative mRNA levels of AST, GDH, GAD, and ODC were decreased by rapamycin. (d) Intracellular content of AST, GDH, GAD, and ODC was decreased by rapamycin. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)
Raptor silencing decreases the expression of AST, GDH, GAD, and ODC, and impaired mTORC1 signaling and NF-κB activation in HL-7702 cells. (a) Phosphorylation of mTOR, S6, and 4EBP1 was reduced by Raptor silencing. (b) Phosphorylation of NFκB was reduced by Raptor silencing. (c) Relative mRNA levels of AST, GDH, GAD, and ODC were decreased by Raptor silencing. (d) Intracellular content of AST, GDH, GAD, and ODC was decreased by Raptor silencing. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)
Inhibited NF-κB by inhibitor SC75741 downregulates the expression of AST, GDH, GAD, and ODC in HL-7702 cells. (a) Phosphorylation of NFκB was inhibited by SC75741. (b) Relative mRNA levels of AST, GDH, GAD, and ODC were decreased by SC75741. (c) Intracellular content of GDH, GAD, and ODC was decreased by SC75741. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)
Rheb overexpression increases the expression of AST, GDH, GAD, and ODC, and enhanced mTORC1 signaling and NFκB activation in HL-7702 cells. (a) Phosphorylation of mTOR, S6, and 4EBP1 was promoted by Rheb overexpression. (b) Phosphorylation of NFκB was promoted by Rheb overexpression. (c) Relative mRNA levels of AST, GDH, GAD, and ODC were increased by overexpression of Rheb. (d) Intracellular content of AST, GDH, GAD, and ODC was increased by overexpression of Rheb. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)
Figure 7

SC75741 inhibites amino acid metabolic genes expression in Rheb overexpressed HL-7702 cells. Rheb-over expressed HL-7702 cells were treated with 10 μM SC75741 for 12 h, and then NF-κB phosphorylation and expression of the amino acid catabolic genes were determined. (a) NF-κB activation was enhanced by Rheb overexpression and inhibited by SC75741. (b) The expression of the amino acid catabolic genes was upregulated by Rheb over expression and inhibited by SC75741. Protein bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, Inc., Rockville, MD, USA). (The values represent the means ± SD, n = 3, *p<0.05, ** p<0.01)

Supplementary Files

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