Original Research

Interleukin-10 regulates starvation-induced autophagy through the STAT3-mTOR-p70s6k axis in hepatic stellate cells

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Impact Statement

Activation of hepatic stellate cells (HSCs) is recognized as a vital step in liver fibrosis that may result in liver cirrhosis or carcinoma. The level of autophagy in HSCs is closely related to the progression of liver fibrosis. Our previous studies showed that interleukin-10 (IL-10), a member of the Th2-type cytokine family, has strongly inhibitory effects on liver fibrosis. However, little is known about the effect of IL-10 on the autophagy in HSCs. The present study showed that IL-10 suppressed starved-induced autophagy in HSCs at the initial stage. Furthermore, the suppressive effect of IL-10 against autophagy was mediated by activation of the STAT3-mTOR-p70s6k axis. Finally, blockade of either the mTOR or STAT3 pathway reversed the inhibitory effect of IL-10 on starvation-induced autophagy in HSCs. In conclusion, the present study provides new experimental data supporting the antifibrotic effect of IL-10.

Abstract

The degree of activation of hepatic stellate cells (HSCs) is closely related to the level of autophagy in HSCs. We previously showed that interleukin-10 (IL-10) strongly inhibits HSC activation in rat fibrotic liver. However, little is known about the effect of IL-10 on HSC autophagy. For investigation of the effect of IL-10 on starvation-induced autophagy in immortal rat hepatic stellate cells (HSC-T6) and the molecular mechanism, HSC-T6 cells were incubated with serum-free DMEM for different periods and treated with IL-10 at different concentrations. Transmission electron microscopy (TEM), analysis of autophagic flux and Western blotting (WB) assays were used to observe changes in autophagosome morphology and number and autophagy-related protein expression in HSC-T6 cells and to evaluate the regulatory effect of IL-10 on starvation-induced autophagy. Cryptotanshinone (CPT) and rapamycin (Rapa) were used to block activation of the signal transducer and activator of transcription 3 (STAT3) and mTOR signaling pathways, respectively. STAT3-mTOR-p70s6k signaling pathway proteins were analyzed by WB to assess the signaling pathway by which IL-10 regulates autophagy. WB showed an increased LC3II/I ratio, increased Beclin1 expression, and decreased p62 expression in HSC-T6 cells starved for 3 h ($p < 0.05$). IL-10 inhibited the increases in the LC3II/I ratio and Beclin1 expression and upregulated p62 expression ($p < 0.05$), and the optimal IL-10 concentration was 20 ng/mL. TEM and double-labeled immunofluorescence analysis showed that IL-10 inhibited autophagosome formation and autophagic flux, as indicated by the decreased numbers of double-membrane autophagosomes and yellow autophagic puncta. Further examination of signaling pathway molecules showed that phosphorylation of the mTOR, STAT3, and p70s6k proteins was significantly decreased during starvation-induced autophagy, but IL-10 could increase mTOR, STAT3, and p70s6k protein phosphorylation ($p < 0.05$). Blocking either the mTOR or STAT3 pathway reversed the inhibitory effect of IL-10 on starvation-induced autophagy in HSC-T6 cells ($p < 0.05$). IL-10 suppresses starvation-induced autophagosome formation through activation of the STAT3-mTOR-p70s6k axis in HSC-T6 cells.

Keywords: autophagy, STAT3-mTOR-p70s6k, signaling axis, interleukin-10, liver fibrosis, hepatic stellate cells

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Introduction

Autophagy refers to a process by which cells maintain cell homeostasis by degrading and recovering aggregated proteins or damaged organelles under physiological or pathological conditions.[1] Studies have shown that the level of autophagy is closely related to liver fibrosis. An increase in autophagy can be observed during the activation of hepatic stellate cells (HSCs). Reducing the occurrence of autophagy can inhibit the activation and proliferation of HSCs. A study found that HSCs in which the autophagy-related gene Atg2A was knocked out could not spontaneously transform into myofibroblasts in cell culture,[2] suggesting that the level of HSC autophagy affects the activation of HSCs and

the progression of liver fibrosis. Interleukin-10 (IL-10) is a member of the Th2-type cytokine family with strong anti-inflammatory and immunosuppressive effects.[3] IL-10 has been shown to directly or indirectly participate in regulating physiological or pathological autophagy in human embryonic fibroblasts (MRC5 cells), cardiomyocytes, skeletal muscle cells, and hepatocytes.[4–7] However, little is known about the regulatory effect of IL-10 on HSCs. Our previous studies have shown that IL-10 can inhibit the activation of HSCs and antagonize the progression of liver fibrosis induced by carbon tetrachloride (CCL_4) and porcine serum, so we speculated that the anti-fibrotic effect of IL-10 may be related to its effect on the autophagy level in HSCs. Starvation is a classic method used to study cell autophagy.[8] During autophagy, the autophagy-associated protein LC3 is cleaved by autophagy-associated gene 4 to produce the cytoplasmic fragment LC3-I (18 kD). LC3-I covalently binds phosphatidylethanolamine to form LC3-II (16 kD), and an increase in the LC3II/I transformation rate is the classic index currently used to determine the level of autophagy.[9] In this study, an autophagy model was induced in HSC-T6 cells by serum deprivation, and the cells were treated with IL-10 at different concentrations. The inhibitory effect of IL-10 on HSC-T6 cell autophagy was verified by Western blotting (WB), transmission electron microscopy (TEM), and immunofluorescence analysis. Furthermore, cryptotanshinone (CPT) and rapamycin (Rapa), specific inhibitors of signal transducer and activator of transcription 3 (STAT3) and mTOR, respectively, were used to explore the role of the STAT-mTOR-p70s6k signaling pathway in the regulatory effect of IL-10 on autophagy in HSC-T6 cells subjected to starvation. The results provide a new perspective on the anti-fibrotic effect of IL-10.

Materials and methods

Reagents and antibodies

Immortal rat HSCs (HSC-T6) were purchased from Nanjing Kangbai Biotechnology Company (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone Company and GE Healthcare Life Sciences (Logan, UT, USA). IL-10 was purchased from PeproTech Company (New Jersey, USA). Rabbit anti-p-STAT3, anti-STAT3, anti-p-mTOR, anti-mTOR, anti-p-p70s6k, anti-p70s6k, anti-p62, anti-GAPDH, Rapa, and CPT were purchased from Cell Signaling Technology (Danvers, USA). Beclin1 was purchased from Abcam (Hong Kong, China). Microtubule-associated protein 1 light chain 3 (LC3)B was purchased from Sigma (St Louis, USA). A double-labeled adenovirus used to detect autophagy (mRFP-GFP-LC3) was purchased from Hanheng Biological Company (Shanghai, China). A primary and secondary antibody diluent, an enhance BCA protein assay kit, an enhanced chemiluminescence kit and an anti-fluorescence quenching sealing solution were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture

HSC-T6 cells were cultured in DMEM containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. The medium was changed every other day. When the cells grew to approximately 80% confluence, 2.5 g/L trypsin was used to digest and passage the cells.

Induction of an autophagy model in HSC-T6 cells by starvation

HSC-T6 cells were seeded at 4×10^5 per well in six-well plates for 24 h and then divided into two groups: the control group and starvation group. HSC-T6 cells in the starvation group were incubated with serum-free DMEM for 3 h, 6 h, or 9 h. WB to determine expression of the autophagy marker proteins Beclin1 and p62 and the LC3II/I ratio in HSC-T6 cells was used to evaluate the optimal duration of starvation for the HSC-T6 cells.

Effect of IL-10 on starvation-induced autophagy in HSC-T6 cells

After HSC-T6 cells were seeded at 4×10^5 per well in six-well plates and grown for 24 h, they were divided into two groups: the starvation control group and the IL-10 intervention group. Cells in the starvation control group were cultured with serum-free DMEM for 3 h. Cells in the IL-10 intervention group were incubated with IL-10 at different concentrations (2 ng/mL, 20 ng/mL, or 200 ng/mL) for 3 h. At the end of the experiment, WB of autophagy marker protein expression was performed to determine the optimal concentration of IL-10 for intervention.

Examination of the STAT3 and mTOR signaling pathways

HSC-T6 cells were seeded at 4×10^5 per well in six-well plates for 24 h and then divided into five groups: the (1) blank control group (control), (2)starved control group (starved), (3) IL-10 intervention group, (4) inhibitor control group, and (5) inhibitor experimental group. Among them, groups 1–3 were treated as described above. For group 4, the inhibitor control group, HSC-T6 cells were cultured with serum-free DMEM for 3 h with 1 nM Rapa or 10 μM CPT, and for group 5, the inhibitor experimental group, after HSC-T6 cells were cultured in serum-free DMEM containing 1 nM Rapa or 10 μM CPT for 30 min, 20 ng/mL IL-10 was added to culture for an addition 3 h of incubation. At the end of the intervention period, the LC-3II/I ratio; expression of the autophagy marker proteins Beclin1 and p62; and the p-mTOR/mTOR, p-p70s6k/p70s6k, and p-STAT3/STAT3 ratios between pathway-related proteins were detected by WB.

Western blot assay

WB was performed as previously described.[10] In brief, the total proteins were extracted from the different groups of HSC-T6 cells. The protein concentration was evaluated with an enhanced BCA protein assay kit. Samples containing equal amounts of total protein (30 μg) were separated on an 8–15% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. After blocking with 5% skim milk (diluted in Tris-buffered saline/Tween-20) for 1 h at room temperature, the membranes were incubated with primary

Table 1. Antibody information.

Antibody	Concentration	Product number	Company
Anti-LC-3B	1:1000	L7543	Sigma
Anti-β-actin	1:10000	A5441	Sigma
Anti-Beclin 1	1:1000	ab62557	Abcam
Anti-P62	1:1000	39749 S	Cell Signaling Technology
Anti-p-mTOR	1:1000	5536	Cell Signaling Technology
Anti-mTOR	1:1000	2983	Cell Signaling Technology
Anti-p-STAT3	1:1000	9145	Cell Signaling Technology
Anti-STAT3	1:1000	12640	Cell Signaling Technology
Anti-p-p70s6k	1:1000	9234	Cell Signaling Technology
Anti-p70s6k	1:1000	9202	Cell Signaling Technology
Anti-GAPDH	1:10000	2118	Cell Signaling Technology
Goat anti-rabbit IgG	1:5000	ZB-2301	Nakasi Jinqiao, Beijing
Goat anti-mouse IgG	1:5000	ZB-2305	Nakasi Jinqiao, Beijing

antibodies at 4°C overnight, washed and incubated with secondary antibody for 1 h at room temperature. Signals were visualized using an enhanced chemiluminescence kit. The band densities were determined by densitometry and quantified using a ChemiDoc™ Touch imaging system with Image Lab™ Touch software version 5.2. Antibody information is shown in Table 1.

TEM analysis

Ultrathin sections of HSCs were processed using conventional methods. The sections were examined and imaged using a PHILIP SEM 208 transmission electron microscope at an accelerating voltage of 80 kV.

Autophagic flux assay

A double-labeled adenovirus used to assess autophagy (MOI 300) was added to HSC-T6 cells after the cell density had reached approximately 20–30% over 20 h. After 2 h, the virus-containing medium was replaced with DMEM containing 10% fetal bovine serum. After 31 h of culture (at which point the cell density of each group was approximately 70%), the cells were subjected to starvation and IL-10 intervention according to the appropriate grouping: for the starvation group, the medium was replaced with DMEM, and for the IL-10 group, the medium was replaced with DEME to which 20 ng/mL IL-10 had been added. After 3 h, the experiment was terminated, and the cells in each well were fixed with 4% paraformaldehyde for 15 min. Then, 0.3% Triton X-100 was used to render the cells permeable, and 4′,6-diamidino-2-phenlindole dihydrochloride (Roche Diagnostics, Indianapolis, IN, USA) nuclear staining was performed. Twenty-five cells were randomly selected for examination under a laser confocal microscope. While the GFP fluorescence signal will be quenched due to the decrease in pH after GFP enters the lysosome, the fluorescence group mRFP can still emit fluorescence.[11] Therefore, yellow fluorescence indicates that the mRFP-GFP-LC3 fusion protein is in an autophagosome. When only red fluorescence is observed in the cell, the mRFP-GFP-LC3 fusion protein is located in a lysosome or autophagolysosome.[12]

Statistical analyses

All experimental data are presented as the mean ± SD and were analyzed with GraphPad Prism statistical software. The significance of differences was determined by one-way ANOVA with Dunnett's post hoc test or the unpaired *t* test. Each experiment was performed 3 times independently. Values of $P < 0.05$ or less were considered to indicate statistical significance.

Results

Induction of an autophagy model in HSC-T6 cells by starvation

In addition to the LC3II/LC3I transformation rate, which is the classic index currently used to determine the level of autophagy, enhanced autophagy is often accompanied by increased expression of the autophagy-associated protein Beclin1 and decreased expression of the p62 protein.[13] Therefore, in the present experiment, WB was used to detect changes in the expression of autophagy marker proteins at different time points to judge the effect of time on the intensity of autophagy induced by starvation, after which a suitable HSC starvation autophagy model was prepared. The WB results showed that the LC-3II/I ratio was significantly increased after starvation from 3 h to 9 h compared with that in non-starved controls ($P < 0.05$), and the LC-3II/I ratio peaked at 6 h (Figure 1(a)). An increase in Beclin1 expression was initiated in HSC-T6 cells after starvation for 3 h ($P < 0.05$), and beclin1 expression did not increase significantly with prolonged starvation (Figure 1(b)). However, p62 expression was downregulated in a time-dependent manner, especially upon starvation for 9 h (Figure 1(c)). These results suggest that autophagic activity was significantly increased after starvation for 3 h. To further confirm the effect of starvation on autophagy in HSC-T6 cells, WB was used to detect autophagic activity in HSC-T6 cells after starvation for 3 h. The results showed that the LC-3II/I ratio (Figure 1(d)) and Beclin1 expression (Figure 1(e)) in HSC-T6 cells were increased in the starvation group compared with the control group, while the protein expression of p62 (Figure 1(f)) was decreased ($P < 0.05$). Taken together, these results suggest

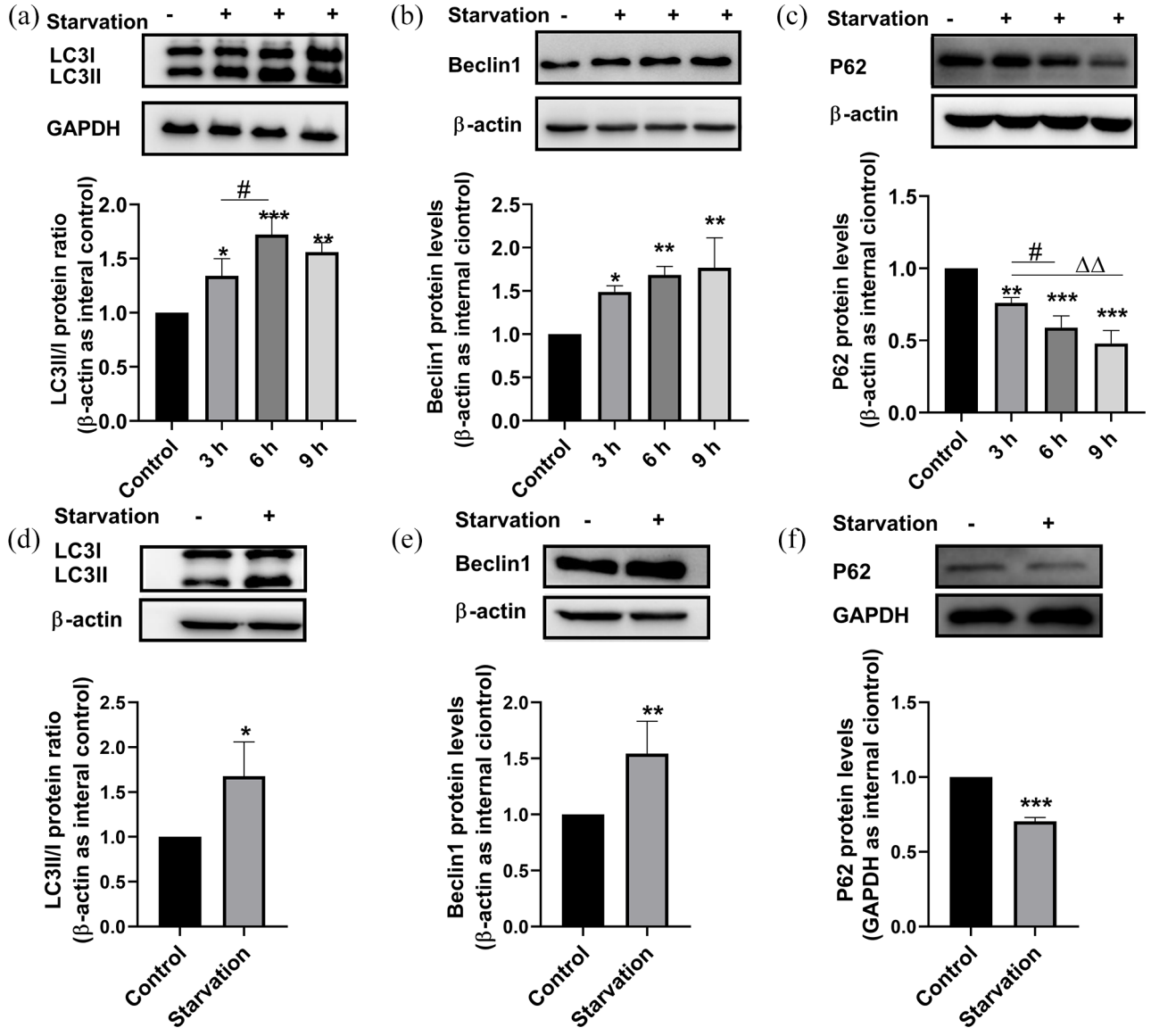

Figure 1. Establishment of an autophagy model in HSCs by starvation. HSC-T6 cells were starved by culturing in serum-depleted medium for 3 h, 6 h, or 9 h, and then, Western blotting was used to detect the expression of autophagy-related proteins in HSC-T6 cells at each time point. (a) Representative LC-3II/I ratio, (b) Beclin1 expression, and (c) p62 expression. Western blotting was used to confirm the expression of autophagy-related proteins in HSC-T6 cells after starvation for 3 h. (d) The LC-3II/I ratio, (e) Beclin1 expression, and (f) p62 expression. Data are expressed as the mean ± SD. $N = 3$ replications. Compared with the control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $^{\#}p < 0.05$, $^{\Delta}p < 0.05$.

that a starvation-induced autophagy model had been successfully constructed in HSC-T6 cells.

IL-10 inhibited starvation-induced autophagy in HSC-T6 cells

To investigate whether IL-10 could affect starvation-induced autophagy activity in HSC-T6 cells, HSC-T6 cells were treated with IL-10 at 2, 20, or 200 ng/mL for 3 h during the starvation period, after which the effects of IL-10 at different concentrations on starvation-induced autophagy in HSC-T6 cells was observed. WB showed that compared with the starvation group, the IL-10 group exhibited a reduced LC-3II/I ratio, reduced Beclin1 protein expression, and increased p62 protein expression ($P < 0.05$), which indicated that IL-10 could inhibit starvation-induced autophagy in HSC-T6 cells, and the inhibitory effect of IL-10 on autophagy increased in a dose-dependent manner (Figure 2(a) to (c)). Our previous studies have showed that IL-10 (20 ng/mL) inhibits the activation of HSC-T6 cells.[14] Therefore, 20 ng/mL was selected as a suitable concentration for intervention in the present study. The results of WB showed that IL-10 intervention inhibited starvation-induced autophagy, as indicated by the decreased LC-3II/I ratio, downregulated Beclin1 expression, and increased p62 expression ($P < 0.05$) (Figure 2(d) to (f)). Furthermore, the formation and number of autophagic bodies in the HSC-T6 cells in each treatment group were observed by TEM. The results of TEM showed significantly more round, vacuolar autophagic bodies in HSC-T6 cells than in control

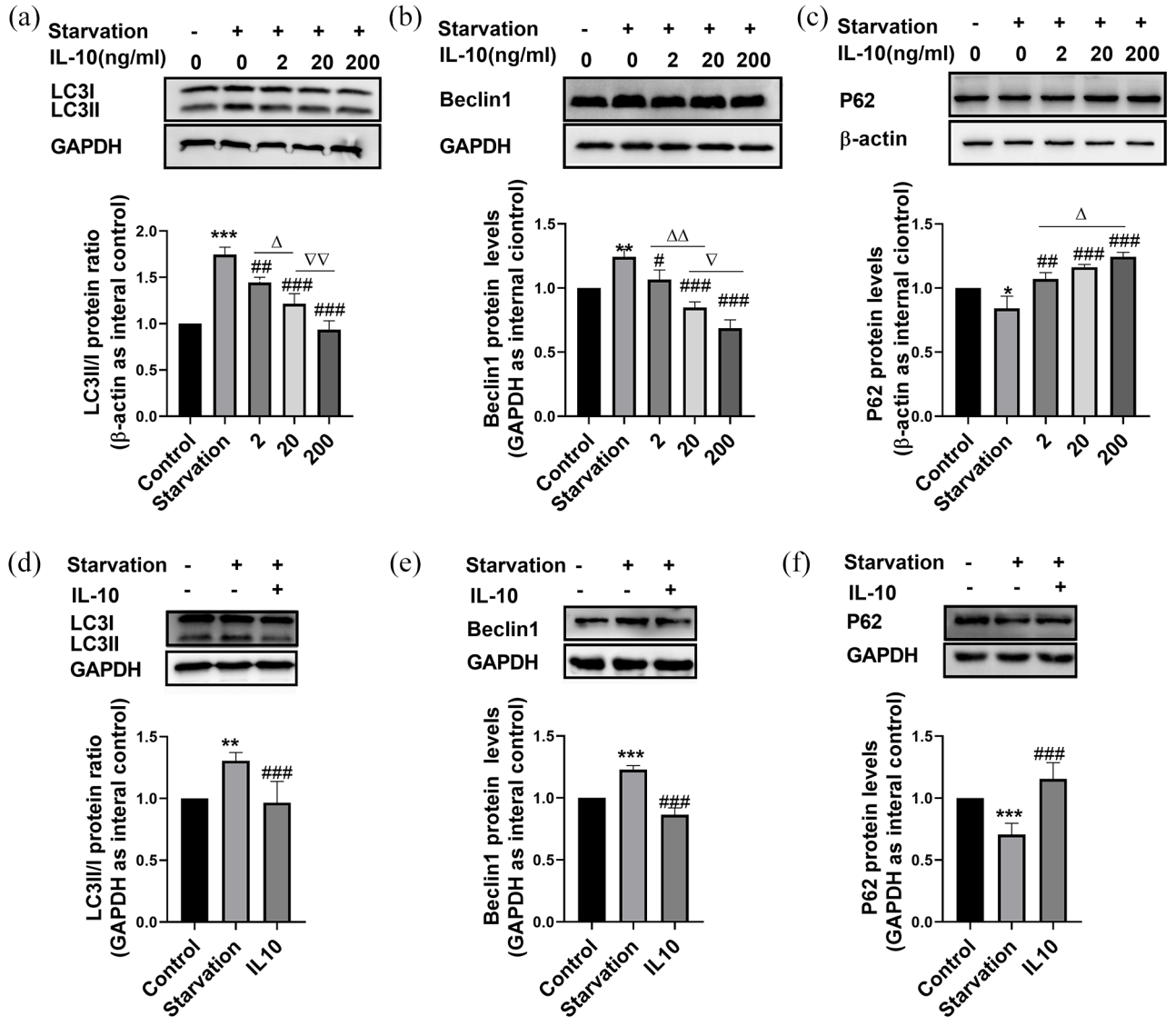

Figure 2. IL-10 inhibited autophagy-related protein expression in HSC-T6 cells after starvation. HSC-T6 cells after starvation for 3 h were treated with IL-10 at different concentrations, and then, Western blotting was used to detect the expression of autophagy-related proteins, (a) The LC-3II/I ratio, (b) Beclin1 expression, and (c) p62 expression. Western blotting was used to evaluate the inhibitory effect of IL-10 (20 ng/mL) on starvation-induced autophagy. (d) The LC-3II/I ratio, (e) Beclin1 expression, and (f) p62 expression. Data are expressed as the mean ± SD. $N = 3$ replications. Compared with the control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with the starvation group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; $^{\Delta}p < 0.05$, $^{\nabla}p < 0.05$.

cells after starvation for 3 h ($P < 0.05$), and the number of autophagic bodies in the IL-10 treatment group was significantly lower than that in the starvation group ($P < 0.05$). These results suggested that starvation induced the initiation and formation of autophagosomes in HSC-T6 cells, which was inhibited by IL-10 intervention (Figure 3(a) and (b)). Finally, autophagic flux was measured using tandem fluorescent-tagged LC3 (mRFP-GFP-LC3), which assesses the stage of the autophagy process. Observation under a laser confocal microscope showed that the number of yellow puncta in the cytoplasm was significantly increased in HSC-T6 cells in the starvation group compared with those in the control group ($P < 0.05$), suggesting that the production of autophagosomes was increased. After IL-10 treatment, the number of yellow puncta in the cytoplasm was significantly lower than that in the starvation group ($P < 0.05$), indicating that IL-10 inhibited the production of autophagosomes induced by starvation in HSC-T6 cells (Figure 3(c) and (d))

Blockade of the mTOR pathway weakened the inhibitory effects of IL-10 on autophagy

The mTOR signaling pathway is crucial for the initiation of autophagy. The level of mTOR phosphorylation influences the formation of autophagic complexes and is an important factor that affects the formation of autophagic vesicles.[15] Therefore, changes in autophagy induced by starvation in HSC-T6 cells treated with IL-10 were detected with or without mTOR inhibitor treatment. As shown in Figure 4(a)–(c), the WB results showed that phosphorylation of the signaling molecules mTOR, p70s6k, and STAT3 was lower in HSC-T6

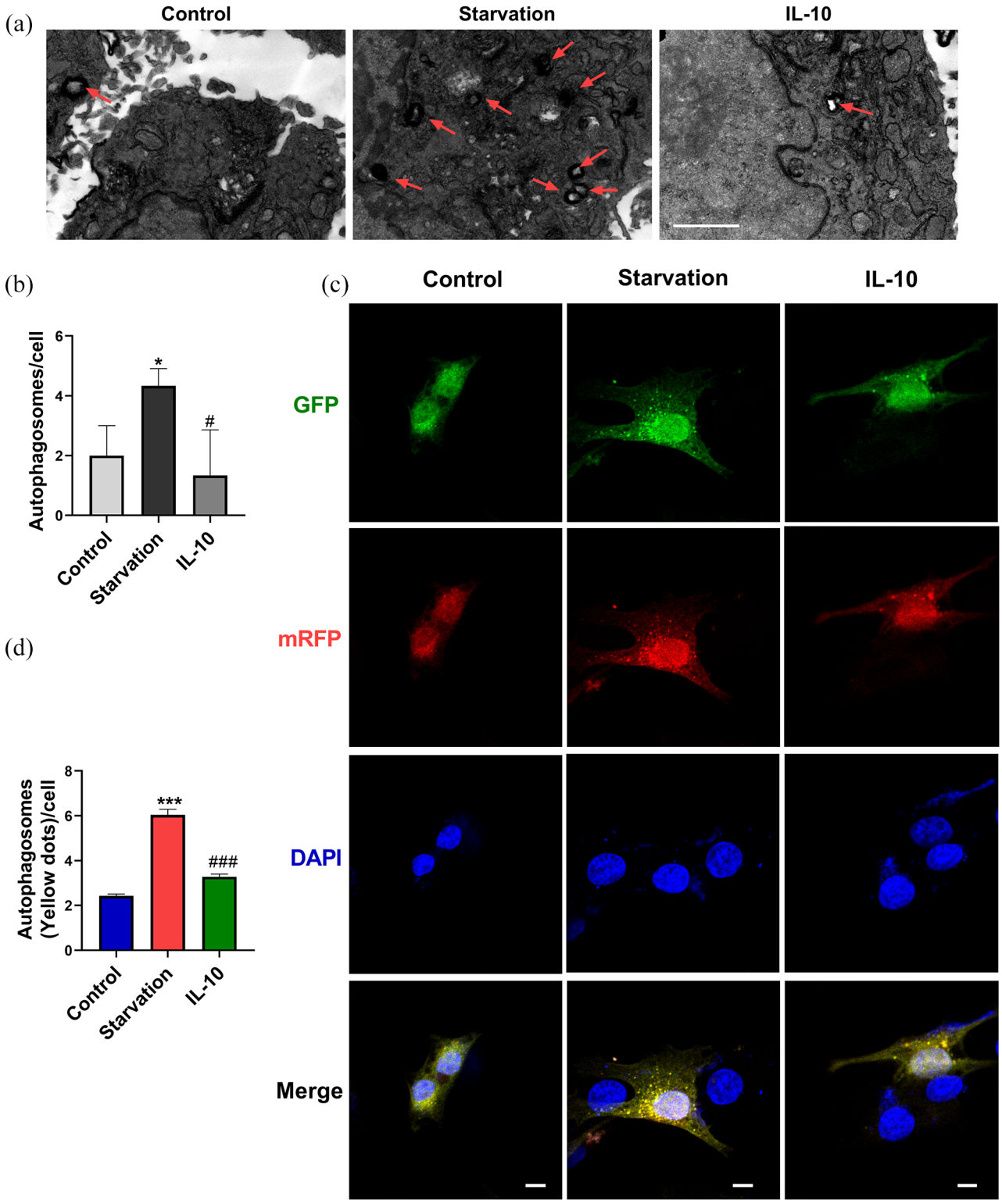

Figure 3. IL-10 inhibited autophagosome formation in HSC-T6 cells after starvation. HSC-T6 cells were treated with or without serum-depleted medium in the presence or absence of IL-10 (20 ng/mL). (a) Transmission electron microscopy was used to observe autophagosomes in HSC-T6 cells. Red arrows indicate autophagosomes or autolysosomes. Scale bar = 1 μm. (b) Numbers of autophagosomes or autolysosomes per HSC-T6 cells by ImageJ analysis. (c) Tandem fluorescent-tagged LC3 (mRFP-GFP-LC3) was used to measure autophagic flux in HSC-T6 cells after different treatment. Autophagosomes (yellow dots) and autolysosomes (red dots). Scale bar = 10 μm. (d) Numbers of autophagosomes per HSC-T6 cells by ImageJ analysis. Data are expressed as the mean ± SD. Compared with the control group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with the starvation group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. (A color version of this figure is available in the online journal.)

cells in the starvation group than those in the control group ($P < 0.05$). Compared with their phosphorylation in the starvation group, IL-10 significantly enhanced phosphorylation of the signaling molecules mTOR, p70s6k, and STAT3 and inhibited the expression of autophagy marker proteins in HSC-T6 cells ($P < 0.05$). To further verify the effect of the

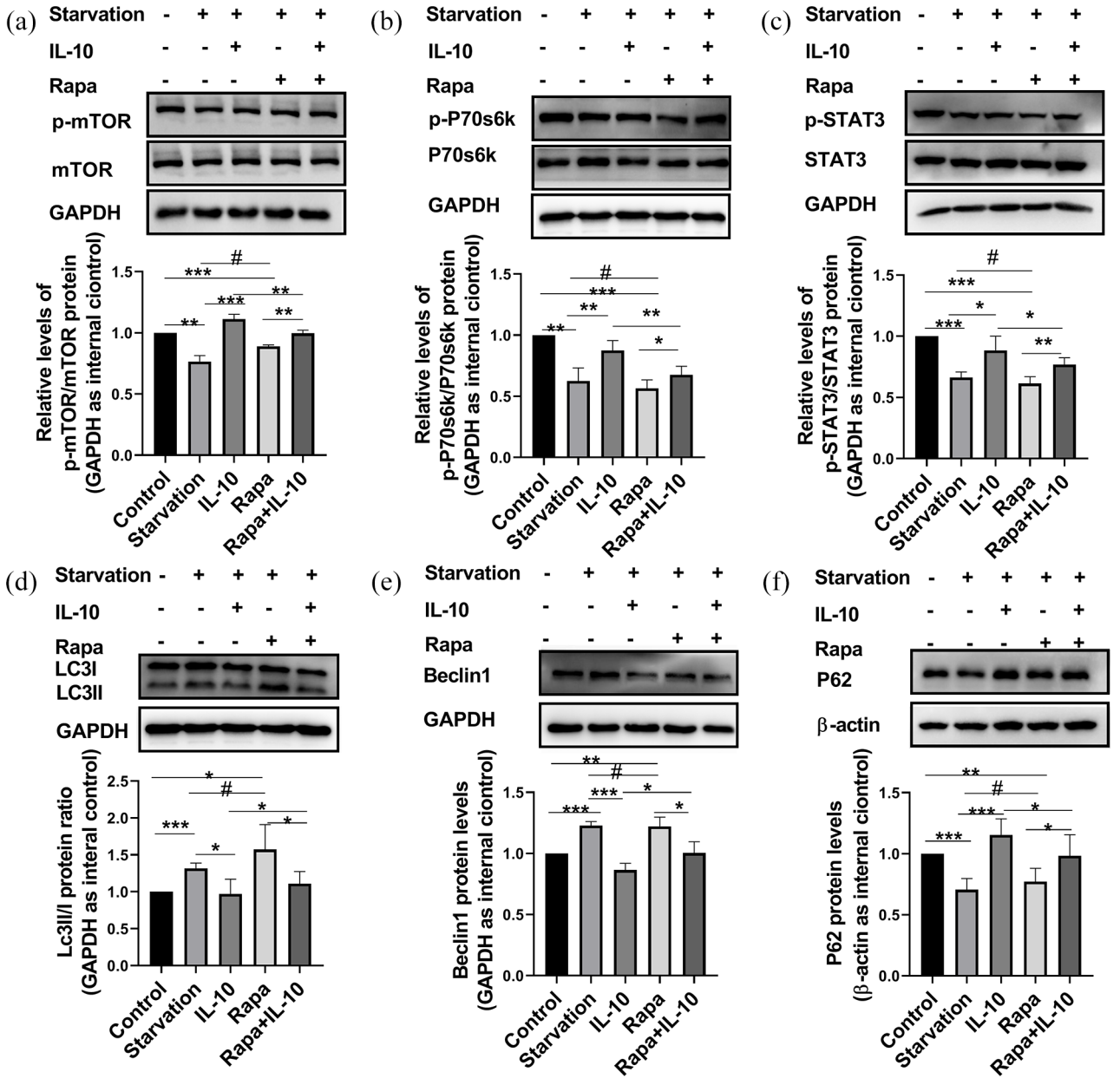

Figure 4. Blockade of the mTOR pathway weakened the inhibitory effects of IL-10 on autophagy. HSC-T6 cells were treated with or without serum-depleted medium in the presence or absence of IL-10, Rapa, or both. (a to c): Western blotting was used to detect the key signaling pathway proteins in HSC-T6 cells after different treatment, (a) Expression of p-mTOR/mTOR, (b) Expression of p-p70s6k/p70s6k, and (c) Expression of p-STAT3/STAT3. (d to f): Western blotting was used to detect the expression of autophagy-related proteins in HSC-T6 cells after different treatments, (d) The LC-3II/I ratio, (e) Beclin1 expression, and (f) p62 protein expression. Data are expressed as the mean ± SD. $N = 3$ replications. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p > 0.05$.

mTOR/p70s6k signaling pathway in the effects of IL-10 on starvation-induced autophagy in HSC-T6 cells, the mTOR-specific inhibitor Rapa was used to block mTOR phosphorylation. The results of WB showed that the phosphorylation of p70s6k, a downstream molecule, was significantly decreased when Rapa was used to inhibit mTOR phosphorylation ($P < 0.05$). As shown in Figure 4(d)–(f), autophagy was enhanced after treatment with Rapa, as the LC3II/I ratio and Beclin1 expression were upregulated, while p62 expression was downregulated compared with the levels in the control group ($P < 0.05$), but autophagy was not further enhanced compared with the level of autophagy in the starvation group ($P > 0.05$). Compared with their phosphorylation in the IL-10 group, Rapa intervention reduced phosphorylation of the signaling molecules mTOR, p70s6k, and STAT3, and Rapa decreased the inhibitory effect of IL-10 on autophagy ($P < 0.05$). Therefore, we speculate that the mTOR-p70s6k pathway is involved in or partly involved in the inhibitory effect of IL-10 on autophagy.

Blockade of the STAT3 pathway weakened the inhibitory effects of IL-10 on autophagy

STAT3, which transduces intracellular and extracellular signals, plays an important role in regulating gene expression, coordinating cell proliferation, survival, and immunosuppression and is a key downstream transcription factor of IL-10[7]. To explore the role of the STAT3 signaling pathway in

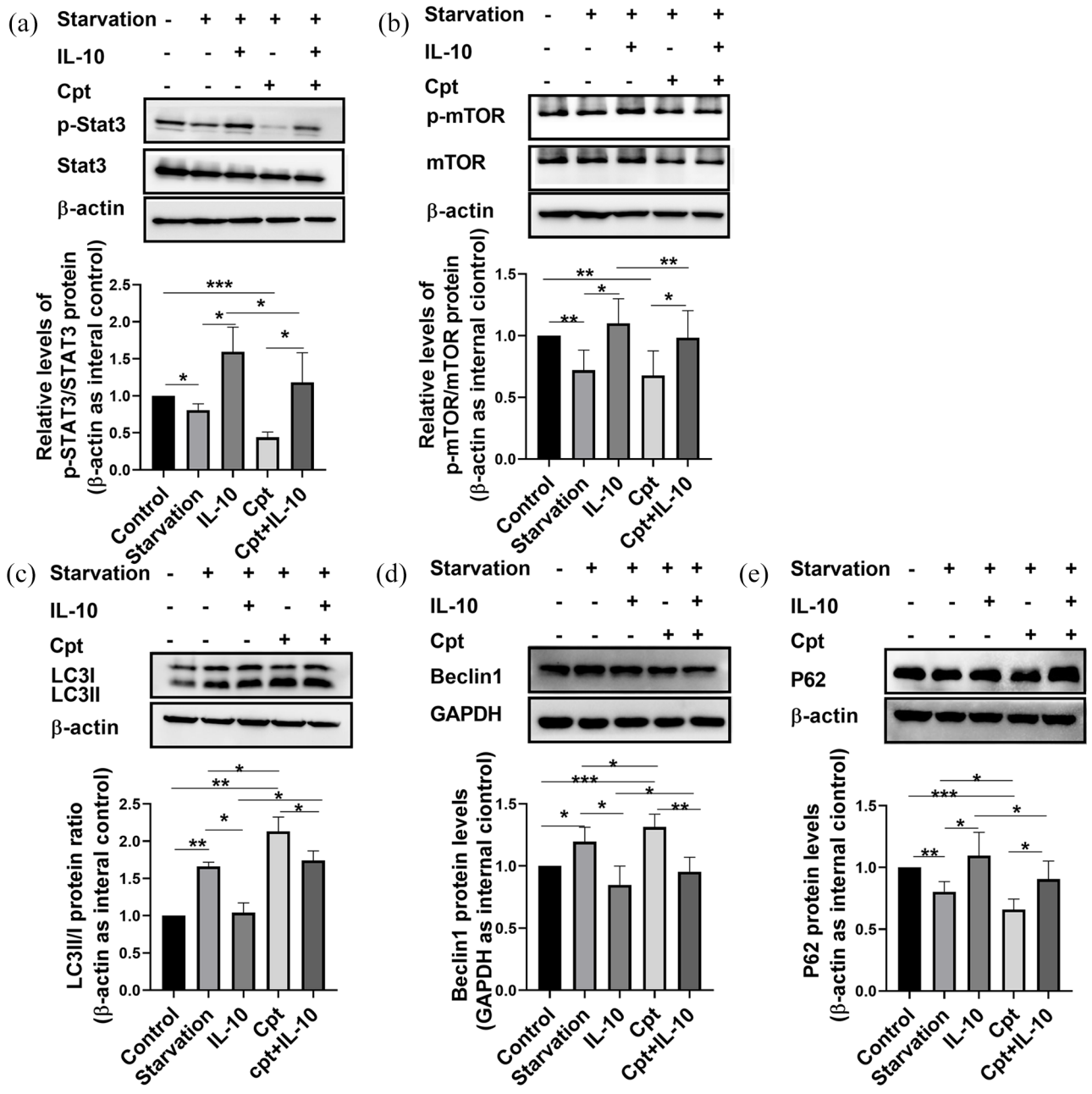

Figure 5. Blockade of the STAT3 pathway weakened the inhibitory effects of IL-10 on autophagy. HSC-T6 cells were treated with or without serum-depleted medium in the presence or absence of IL-10, CPT, or both. (a and b): Western blotting was used to detect the key signaling pathway proteins in HSC-T6 cells after different treatments, (a) Expression of p-STAT3/STAT3, (b) Expression of p-mTOR/mTOR. (c to e): Western blotting was used to detect the expression of autophagy-related proteins in HSC-T6 cells after different treatments, (c) The LC-3II/I ratio, (d) Beclin1 expression, and (e) p62 expression. Data are expressed as the mean ± SD. $N = 3$ replications. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the inhibitory effect of IL-10 on autophagy, WB was used to detect changes in autophagy-associated proteins and STAT3 signaling molecules in HSC-T6 cells subjected to starvation and treated with IL-10. As shown in Figure 5(a) and (b), the results of WB indicated that the p-STAT3/STAT3 and p-mTOR/mTOR ratios were lower in the starvation group than in the control group ($P < 0.05$). Furthermore, phosphorylation of the signaling molecules mTOR and STAT3 was significantly enhanced after IL-10 treatment ($P < 0.05$). These results showed that IL-10 increased the phosphorylation of mTOR and STAT3. Furthermore, CPT, a specific inhibitor of STAT3, was used to block the phosphorylation of STAT3. The WB results showed that STAT3 phosphorylation was significantly inhibited. Then, we detected the expression of autophagy-associated proteins to judge the role of the STAT3 signaling pathway in the inhibition of autophagy by IL-10. As shown in Figure 5(c)–(e), IL-10 inhibited autophagy in starved HSCs, and autophagy was significantly enhanced after CPT treatment; that is, the LC3II/I ratio and Beclin1 expression were upregulated, and p62 expression was downregulated ($P < 0.05$). Taken together, these findings show that CPT treatment inhibited the regulatory effect of IL-10 on autophagy and phosphorylation of the signaling molecules mTOR and STAT3, which confirmed that the

STAT3 signaling pathway is a key link in the effect of IL-10 on autophagy induced by starvation in HSC-T6 cells.

Discussion

Autophagy is a cellular process in which intracellular substances are transported to lysosomes or vacuoles for degradation. It is an important mechanism of cell self-renewal and a common physiological phenomenon. In addition, autophagy is simultaneously involved in a variety of pathological processes, such as the progression of liver fibrosis. Deng *et al.*[16] proved that hypoxia inducible factor-1α could promote HSC activation by regulating autophagy. Fu *et al.*[17] confirmed that transforming growth factor-β (TGF-β) could reduce HSC-T6 cell apoptosis by inducing autophagy. Duran *et al.*[18] found that p62/sequestosome-1 inhibited HSC activation and fibrosis by promoting Vitamin D-dependent (VDR) signal transduction, that is, inhibiting HSC autophagy to inhibit fibrosis. Therefore, inhibition of autophagy in HSC-T6 cells may be a new anti-fibrotic strategy.

Cytokines play an important role in the formation and prognosis of hepatic fibrosis. Through autocrine or paracrine signaling, cytokines participate in the activation of HSC-T6 cells and maintain balance in the extracellular matrix (ECM).[19] IL-10 is a multipotent cytokine that has significant anti-inflammatory effects and antagonistic effects against fibrosis.[20] Our previous studies confirmed that IL-10 had an antagonistic effect against rat liver fibrosis induced by chemical or immune injury, as it not only inhibited the inflammatory response of the liver and the activation of HSC-T6 cells but also significantly induced senescence of activated HSCs and reduced the degree of fibrosis.[20–22] Therefore, we wondered whether the inhibition of HSC-T6 cell activation and induction of HSCs senescence by IL-10 are related to the regulation of autophagic activity in HSC-T6 cells. Recent studies have found that IL-10 is also involved in the regulation of autophagy in a variety of physiological and pathological conditions, and IL-10 was found to directly or indirectly inhibit autophagy in mouse placental trophoblasts,[23] cardiomyocytes,[6] and hepatocytes.[7] In view of the inhibitory effect of IL-10 on autophagy in other cell lines, we speculated that IL-10 may also inhibit activated HSC-T6 cell autophagy. In this study, HSC-T6 cells were selected as the research object, and autophagy was induced by starvation before the cells were treated with IL-10 at different concentrations. The occurrence autophagy and changes in autophagy were detected by WB, immunofluorescence, and TEM. The experimental results suggested that IL-10 can inhibit the degree of HSC-T6 cell autophagy induced by starvation, and with increasing IL-10 concentration, the degree to which autophagy was inhibited increased, indicating its positive correlation with the concentration of IL-10.

The regulation of autophagy involves changes in the expression of molecules in multiple signaling pathways, among which the PI3K-Akt-mTORC1 signaling pathway, which is responsible for cell survival, energy metabolism, and protein synthesis, is the most important.[24] mTORC1 inhibits the formation of autophagosomes by inhibiting ULK complex formation.[24] In this experiment, IL-10 and Rapa were used alone or in combination to treat starved HSC-T6 cells, and the results showed that IL-10 inhibited starvation-induced autophagy and significantly increased the phosphorylation of mTOR, p70s6k, and STAT3, while Rapa intervention decreased the phosphorylation of mTOR, p70s6k, and STAT3; inhibited activation of the mTOR-p70s6k signaling pathway by IL-10; and blocked the inhibitory effect of IL-10 on autophagy. These findings suggest that the inhibitory effect of IL-10 on starvation-induced autophagy may be related to activation of the mTOR-p70s6k signaling pathway, which is consistent with Kishore's[6] report that IL-10 inhibited cardiomyocyte pathological autophagy induced by angiotensin II by upregulating the AKT-mTORC1 signaling pathway.

Other studies suggest that IL-10 participates in the regulation of differentiation, maturation, proliferation, apoptosis, and autophagy in many kinds of cells mainly through the IL-10-IL-10R-JAK-STAT intracellular signal transduction pathway.[4] In addition, crosstalk between IL-10 and the mTOR-p70s6k pathway is also an important way that IL-10 regulates cell proliferation, differentiation, and apoptosis.[25] For example, IL-10 could inhibit autophagy in starvation-induced hypertrophic scar (HS)-derived fibroblasts (HSFs) through activation of the IL-10-IL-10R-STAT3 pathway and direct/indirect activation of the AKT-mTOR pathway.[9] Therefore, we used IL-10 and CPT alone or in combination to treat HSC-T6 cells to verify the role of the STAT3 pathway in IL-10-mediated inhibition of starvation-induced HSC-T6 cell autophagy. The results showed that CPT reversed the effect of IL-10 on starvation-induced changes in the LC3-II/I ratio and expression of the autophagy-related proteins p62 and Beclin1, decreased the level of STAT3 phosphorylation, and decreased the expression of p-mTOR to some extent. These findings proved that STATs and mTOR signaling molecules are the key molecules by which IL-10 regulates the autophagy pathway. Inhibition of mTOR or STAT3 can affect both the mTOR-p70s6k and IL-10R-STAT3 pathways, and these pathways interact and promote IL-10-mediated autophagy inhibition.

In conclusion, autophagy is an important process by which HSC-T6 cell activation is maintained. Activated HSC-T6 cells are the main source of ECM in hepatic fibrosis. Inhibition of HSC-T6 cell activation or induction of HSC senescence can inhibit the formation of hepatic fibrosis. In this study, through generation of a starvation-induced HSC-T6 cell autophagy model and intervention with IL-10, we explored the inhibitory effect of IL-10 on starvation-induced HSC-T6 cell autophagy at the protein and suborganelle levels. These experiments were combined with the use of specific inhibitors to block signal transduction, and the role of the STAT-mTOR-p70s6k axis in the inhibitory effect of IL-10 on HSC-T6 cell autophagy was verified at the protein level, providing new experimental evidence for the antifibrotic mechanism of IL-10.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. HYH, CFL, and WXZ conceived the research ideas and supervised the project; material preparation, data collection, and analysis were performed by CDM, CJB, and CYZ. The first draft of the manuscript was written by CDM, and the previous versions of the manuscript were commented on by HYH. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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DATA AVAILABILITY

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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