Salvianolic acid B ameliorates hepatocyte lipid droplet accumulation via stimulation of autophagy

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Abstract

Salvianolic acid B (Sal B) the most abundant bioactive member in Salvia miltiorrhiza and has been reported lots of benefits on the treatment of cardio-cerebral vascular diseases and metabolic diseases. Lipid droplets are dynamic organelles, excessive lipid droplets accumulation in liver caused fatty liver disease. In this study, we are interested in the effect of Sal B on hepatic lipid accumulation and its possible mechanism. We found Sal B treatment significantly decreased lipid accumulation and TG level in primary hepatocytes. Meanwhile, we discovered Sal B significantly stimulated hepatic autophagy. Then we used autophagy inhibitor 3-MA and reveled that Sal B showed little effect on improving hepatic lipid accumulation when autophagy was inhibited, which indicated Sal B reduced cellular lipid accumulation through activating autophagy. This study demonstrates Sal B ameliorate hepatic lipid accumulation through activation of autophagy. These findings contribute to its benefit on liver disease related to hepatic lipid accumulation and hepatic autophagy.

Introduction

The storage and release of lipid droplets in liver plays an important role in maintaining the homeostasis of lipid metabolism. Excessive accumulation of hepatic lipids caused fatty liver is one of the main manifestations of metabolic syndrome. The amount of lipid that can be exported from the liver is dependent on synthesis as well as the availability of triglycerides (TGs) that are stored within the hepatocyte in the lipid droplets (LDs). [1] Although hepatocytes have a huge capacity of storing lipids, excessive LDs accumulation in hepatocytes is reported a lipotoxicity which can cause cellular inflammation and cell death. [2–4] Therefore, reduction of the accumulation of lipid droplets in the liver plays an important role in improving metabolic syndrome, especially insulin resistance.

Recent studies have reported that autophagy plays an important role in regulating hepatic lipid homeostasis. [5] Autophagy is an adaptive response of cells to metabolic stress and environmental changes which maintains the cellular energy homeostasis. [6] Autophagosome formation requires the localization of phosphatidylethanolamine conjugated microtubule-associated protein 1 light chain 3 (LC3) to the autophagosomal membrane which indicates the autophagosome occurrence. [7] The evidence of connection between autophagy and hepatic lipid metabolism has been confirmed that impaired autophagy in hepatocyte may promote triglycerides accumulation in liver and interfered with subsequent mitochondrial β-oxidation. [8] Since then, many studies supported that lipids could be degradated by macroautophagy, then fused with lysosomes. [9, 10] Thus, autophagy has been considered as a new cellular target for abnormalities in lipid metabolism and accumulation. [9, 10]

Salvianolic acid B (Sal B) is widely used for clinically treating cardio-cerebral vascular diseases. It is the most abundant and bioactive component in Salvia miltiorrhiza polyphenols. [11, 12] Recent studies also reported a potential implication of Sal B in the treatment of insulin resistance, obesity, and type 2 diabetes. [13–16] In hepatocytes, Sal B has been reported a strong ability to protect cells from injury

induced by oxidative stress, inflammation and enhance hepatic differentiation. [17–19] However, the effect of Sal B on improving hepatic lipid disorder is rarely reported. In this study, we interestingly found that Sal B could stimulated hepatic autophagy while reduced lipid accumulation in hepatocyte, which indicated a possible mechanism for Sal B improved lipid disorder and contributed to metabolic syndrome.

Materials And Methods

Primary culture of hepatocytes and treatment

Isolation of primary mouse hepatocytes was performed by nonrecirculating perfusion with collagenase. Briefly, a male C57BL/6 mouse (6-week old) was anaesthetized, sterilized and opened the abdominal cavity to expose the liver and inferior vena cava. Perfusate 1 (mixture of Krebs solution 50 mL and 50 mM EGTA solution 0.1 mL) was infused from the inferior vena cava puncture intubation for 3–5 minutes after an injection of 1 mL heparin. Then 30 mL perfusate 2 (mixture of Krebs solution 30 mL, 2M $CaCl_2$ 41.2μL and collagenase I 15 mg) was infused from inferior vena cava within 6 minutes. Liver was transferred on ice and cell isolation was performed by washing with pre-cooled1640 medium on a sieve. The cell was placed and obtained sedimentation. After discarding the supernatant, cells were resuspending in pre-cooled1640 medium without serum and centrifuged to get sedimentation. Then cells was resuspended in 1640 medium with 10% fetal serum 1% penicillin-streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Sal B, an effective water-soluble compound of Radix Salviae miltiorrhizae, was obtained from Tianjin Tasly LTD CO. Group Pharmaceutical. Sal B was dissolved in physiological saline to prepare concentration gradient then added into hepatocytes for 24 hours. Meanwhile, autophagy inhibitor 3-methyladenine (3-MA) in concentration gradient was added into cultured hepatocytes respectively according to research purpose for 24 hours.

Western blotting analysis

Western blotting was performed as previously described. Protein lysates were subjected to SDS-PAGE, transferred to hybond-PVDF membranes then incubated with specific primary antibodies against LC3 (Sigma, USA), p62, (abcam, USA), Beclin-1 (ImmunoWay Biotechnology Company, USA). Equal loading was checked by incubated the membrane with monoclonal antibody against β-actin (ZSGB, China) or GAPDH (ImmunoWay Biotechnology Company, USA). After washing, the membrane was incubated with anti-mouse or anti-rabbit secondary antibodies (ZSGB, China) for 2 h at room temperature. ECL was purchased from Millipore.

Bodipy staining

For neutral lipid drop staining [20], Primary hepatic cells grown on coverslips were treated with Sal B or 3-methyladenine (3-MA) combined with SalB for 24 h, and then were incubated with BODIPY 493/503 (Life Technologies, 20 μg/ml) to label lipid drops for 20 min and hoechst33342 (Sigma, 5 mg/mL) to stain

nucleus for 5 min at 37 °C and washed cells with PBS three times, after fixed and mounted on glass slides, and images were acquired using Olympus microscope (Olympus BX53, Japan).

Oil red O staining

The intracellular lipid accumulation was determined by Oil Red O staining. The cells were washed twice with PBS (pH = 7.4) and then fixed in 4% paraformaldehyde for 30 min, stained with Oil Red O solution (0.5 g of Oil Red O powder was dissolved in 60% isopropanol) at room temperature for 30 min, washed instantaneously with 60% isopropanol and counter-stained with hematoxylin. After twice washing with PBS, the cells were photographed using Olympus microscope (Olympus BX53, Japan). For quantification, Oil red O was dissolved by adding isopropropanol (100%) to the samples, 100 µl of supernatant was added to a 96-well plate and measured at 510 nm using pan-wavelength microplate reader (SynergyMx; BioTek Instruments, USA).

Measurement of TG content

Triglyceride content was assessed by GPO-PAP Enzymatic kit according to manufactory instruction (Nanjing Jiancheng Bioengineering Institute). In brief, cells were collected and washed by PBS (pH = 7.4), then 200 mL homogenate was prepared by PBS and sonicated on ice. 2.5µL ddH2O, standard solution and sample solution was mixed with 250µL working buffer and cultured in 37∘C for 10 minutes. The OD value was analyzed by automatic biochemical detector at absorption wave 510 nm. The final concentration was calculated according to the formula provide by manufactory instruction.

Statistical analysis

Statistical analyses were performed by ANOVA and means compared by Fisher's protected least-significant difference using StatView software from SAS Institute Inc. (Cary, NC). The data were summarized as mean ± S.D. p-Value < 0.05 was considered statistically significant.

Results

Sal B inhibited lipid accumulation in primary cultured hepatocytes

As the main component of neutral lipids of cell, triglyceride was extracted and further tested. Our result showed Sal B decreased triglyceride (TG) content in primary hepatocytes at the concentration of 100 and 250 µM (Fig. 1a). And neutral lipids droplet staining with Bodipy 493/503 revealed that control cells have lots of dispersed spherical lipid drop (LD) structures with strong fluorescence throughout the cytoplasm, while cells treated with Sal B of 100 µM weakened the staining of LD (Fig. 1b), further analysis of total fluorescence of bodipy showed that Sal B decreased LD accumulation in hepatocytes to nearly a third of the control cells (Fig. 1c).

Sal B stimulated autophagy in primary cultured hepatocytes

In our study, we found Sal B concentration from 50 nM to 200 nM significantly stimulated LC3B expression but inhibited P62 level in hepatocyte (Fig. 2a). We then selected 100 μM of Sal B as an experimental concentration, and a significant higher expression of LC3B and Beclin1 in Sal B treated hepatocyte were observed whereas a decreasing expression of P62 compared to control group (Fig. 2b). It suggested Sal B activated autophagy and stimulated autophagosome formation.

Inhibition of autophagy induced lipid accumulation in hepatocytes

Many studies reported a central role of hepatic autophagy in regulating glycogen and triglycerides metabolism. 3-Methyladenine (3-MA) can block autophagy through the action of phosphoinositide 3-phosphate kinase (PI3K), and the activity of PI3K is necessary for the early formation of autophagy cell nucleation and assembly. To further investigate the role of autophagy in regulating hepatic lipid accumulation, we used 3-MA to inhibit autophagy formation. As shown in our result, 3-MA concentration from 5 to 10 mM significantly reduced expression of LC3B whereas increased P62 expression (Fig. 3a and 3b). Meanwhile, oil red staining result showed that 10 mM 3-MA obviously stimulated lipid accumulation in hepatocyte (Fig. 3c). The absorbance of the isopropanoldissolved Oil-Red stain increased 50% as determined from the absorbance for 3-MA-treated cells compared to untreated cells (Fig. 3d). These data confirm the view that impaired autophagy might lead to hepatic lipid accumulation.

Sal B inhibited lipid accumulation through activated autophagy in hepatocytes

In our study, we found a stimulation of autophagy and inhibition of lipid accumulation in hepatocyte by Sal B. Then we investigated whether the inhibition of lipid accumulation by Sal B was stimulated via autophagy. Our results showed that Sal B inhibited lipid accumulation was significantly abolished by treatment of 3-MA (Fig. 4a and 4b). However, stimulation of lipid accumulation by 3-MA was not affected by extra Sal B (Fig. 4a and 4b). Meanwhile, the inhibition of TG level by Sal B was also prevented after administration of 3-MA, but stimulation of TG content in the hepatocytes by 3-MA was not affected by SalB. It suggested a central role of autophagy in Sal B inhibited hepatic lipid accumulation.

Discussion

In this study, we found Sal B significantly inhibited lipid accumulation in hepatocytes should be a possible explanation for its benefit on improving metabolic syndrome. Excessive LDs accumulation in hepatocytes can result in lipotoxicity, patients with hepatic lipid accumulation are at risk of serious complications, including progression to NAFLD, steatohepatitis, type 2 diabetes, fibrosis, cirrhosis, liver failure and hepatocellular carcinoma, currently, the only effective treatment option for NAFLD is weight loss, there are no pharmaceutical treatments approved. [21−23] our finding provided its clinical value of Sal B by decreasing lipid drop accumulation of hepatocytes.

Autophagy is beneficial to the metabolism and decomposition of nutrients, therefore it is very important for the regulation of nutrients metabolism and the maintenance of intracellular balance. Study reported an important role of autophagy involved in regulating lipid metabolism. The impairment of autophagy caused accumulation of lipid in cells leads to obesity, dyslipidemia and fatty liver disease. [24]

LDs undergoes degradation partly through autophagy lysosomal pathway. Lipid accumulation could stimulate autophagy. The endoplasmic reticulum forms the early autophagic structure, encapsulates the newly formed lipid droplets and transports them to lysosomes. [25] Our result indicated an increasing autophagy by upregulation of LC3B and Beclin1expression whereas a decreasing P62 expression in hepatocytes, which suggested the increasing autophagy stimulated by Sal B might be related to improvement of hepatic lipid drop accumulation.

We also found inhibition of autophagy by 3-MA induced intracellular lipid droplet accumulation and increased TG level in hepatocytes. Meanwhile, Sal B reduced LD accumulation and TG level in hepatocytes was abrogated by 3-MA. Although Sal B has been reported a variety of beneficial metabolic effects including ameliorated the histopathological alterations of pancreas, increased muscle glycogen content, increased p-AMPK protein expression in skeletal muscle and liver; increased protein expressions of PPARαand p-ACC in liver. [17, 18] Our study showed that Sal B reduced intracellular accumulation of LDs and TG level whereas stimulated autophagosome formation by increasing expression of autophagosome formation marker LC3 II and decreasing autophagy substrates marker p62 expression in hepatocytes. Meanwhile, Beclin-1, another aytophagy biomarker which mainly activated autophagy by formation of class III PI3-kinase complex to stimulate ATG proteins, was found significantly higher expression in Sal B treated hepatocytes. It suggested Sal B activated autophagy and stimulated autophagosome formation. Previous studies reported pharmaceutical inhibition of autophagy significantly increased LD accumulation in hepatocytes. [30] We also found inhibition of autophagy by 3-MA induced intracellular lipid droplet accumulation and increased TG level in hepatocytes. Meanwhile, Sal B reduced LD accumulation and TG level in hepatocytes was abrogated by 3-MA, which indicated Sal B inhibited hepatic lipid accumulation through stimulation of autophagy.

In conclusion, our study exhibited another function of Sal B on decreasing lipid drop accumulation in hepatocyte, and provided a possible mechanism of Sal B regulating hepatic lipid metabolism through activation of autophagy, that may contribute to its benefit on liver disease related to hepatic lipid accumulation.

Declarations

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Yanan Shi and Biao Mu contributes to the financial support of the experiments and paper supervision. Jihong Yuan contributes to experiment design and data analysis. Lihui Yan contributes to the molecular biologic experiment. Yajin Liu contributes to primary cells culture. Da Pan contributes to the staining. All authors read and approved the final manuscript

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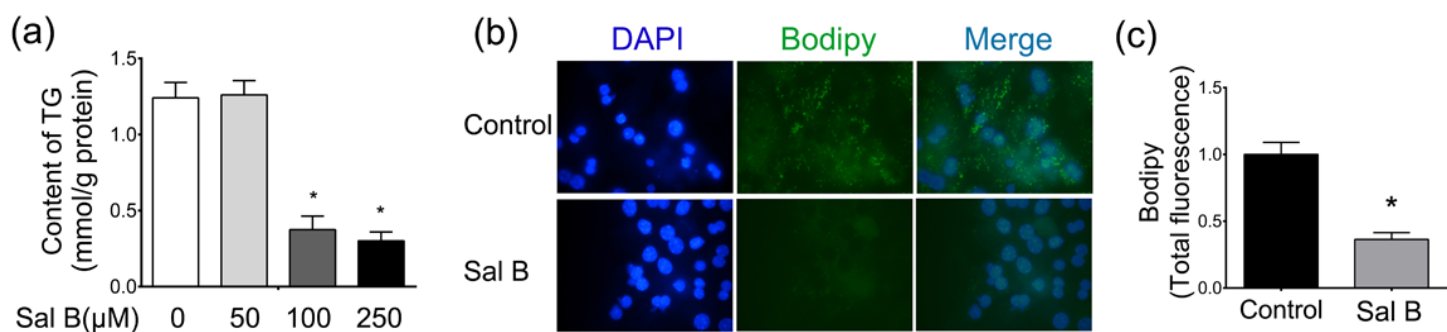
Figures

Figure 1

The effect of Sal B on content of TG and neutral lipid droplet in primary mice hepatocytes. TG content in primary hepatocytes was decreased by Sal B in different content (a). Neutral lipid droplet in the cytoplasm of hepatocyte was stained with bodipy (b). (c), Fluorescence of bodipy was compared between control and Sal B-treated hepotocyte. *p<0.05, compared to control cells.
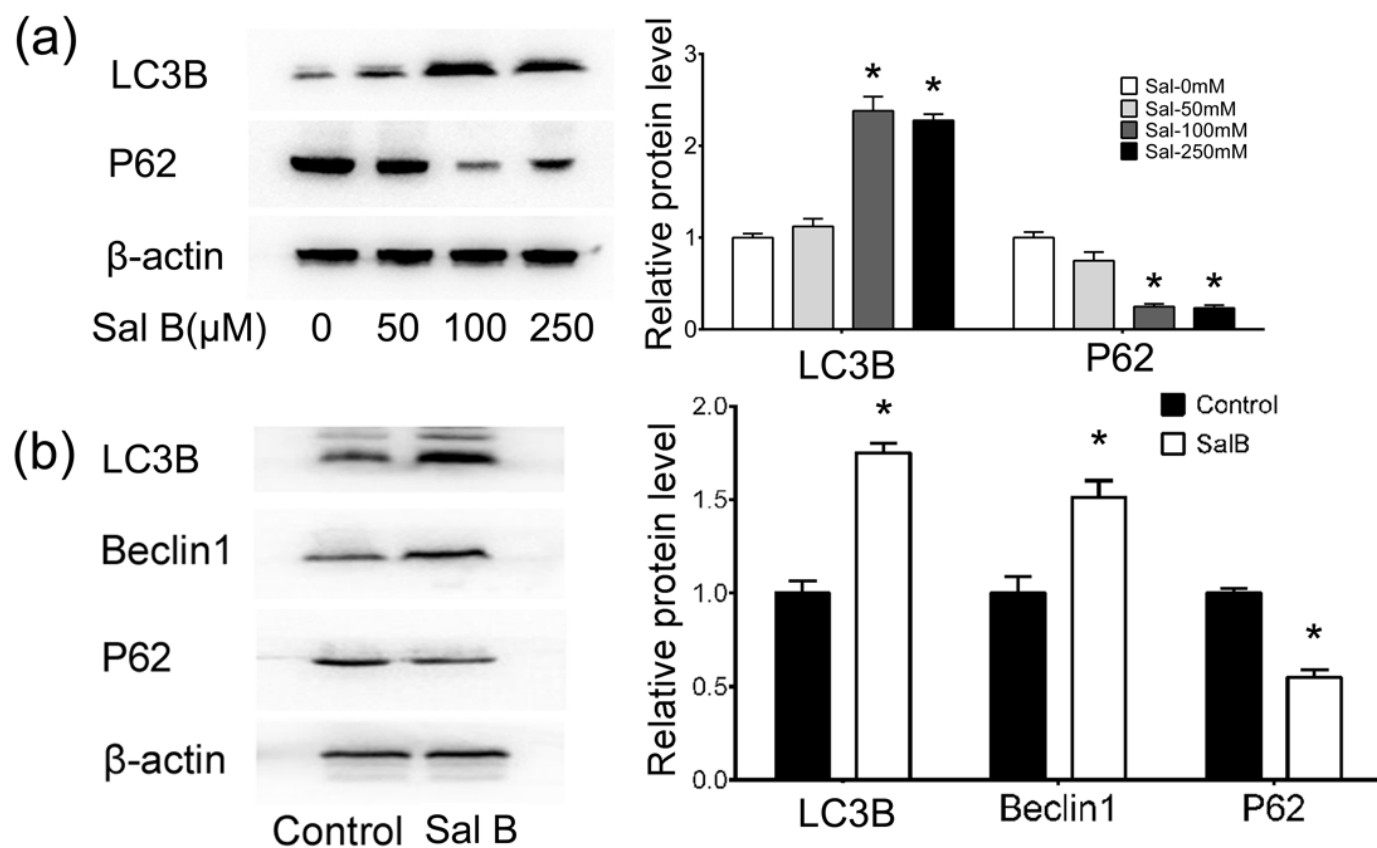
Figure 2

The effect of Sal B on the level of autophagy-related protein in primary mice hepatocytes. (a) LC3B and P62 were measured in primary hepatocytes treated with Sal B of different concentration. (b) The effect of Sal B on the level of LC3B, Beclin1 and P62 in primary mice hepatocytes. *p<0.05, compared to control cells.
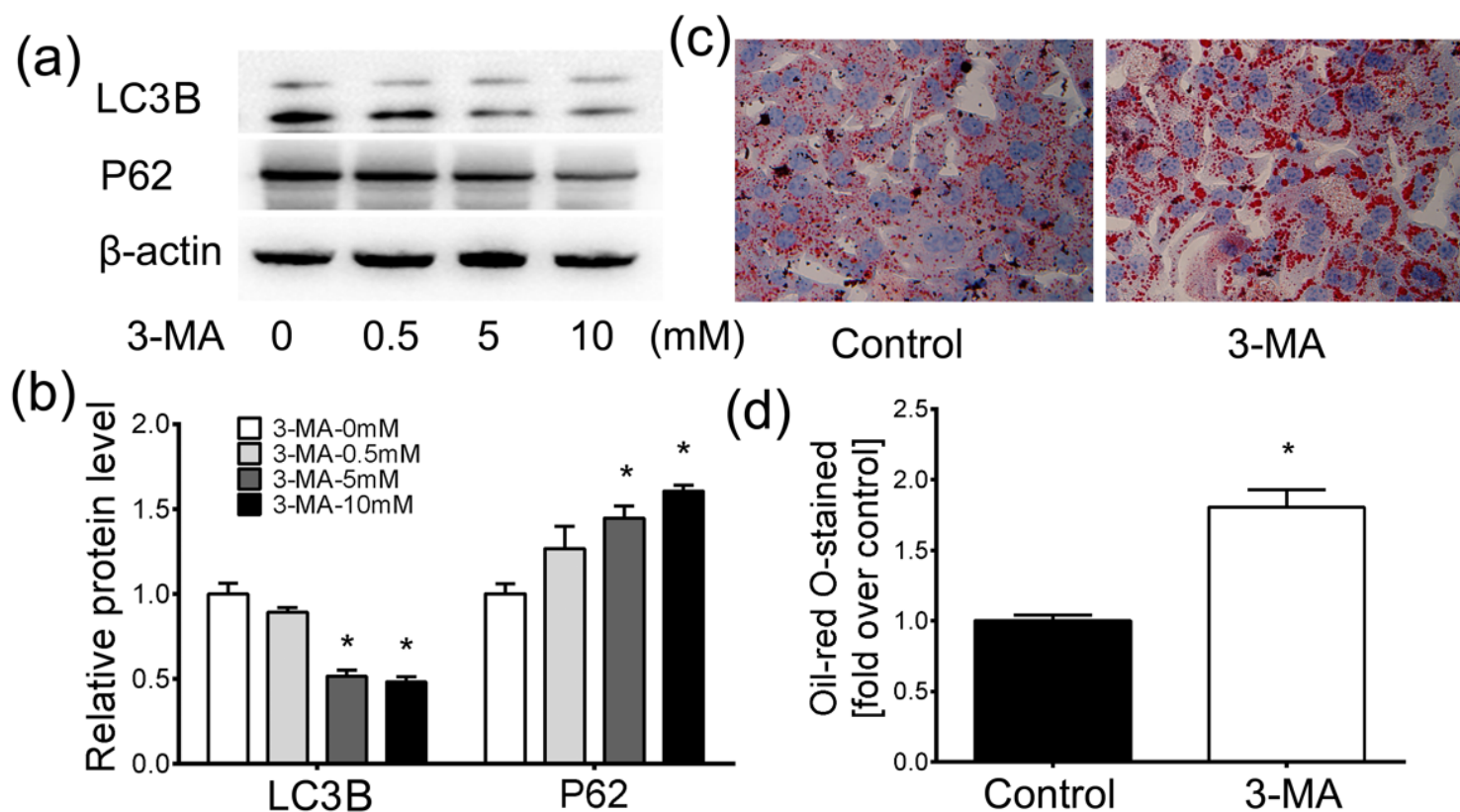
Figure 3

The effect of 3-MA on the level of autophagy-related protein and neutral lipid droplet in primary mice hepatocytes. Western blot (a) and gray value (b) of LC3B and P62 were measured in primary hepatocytes treated with 3-MA of different concentration. (c) The effect of 3-MA on neutral lipid droplet in the cytoplasm of hepatocyte was stained with oil red. (d) Fluorescence of oil red was compared between control and Sal B-treated hepotocyte. *p<0.05, compared to control cells.
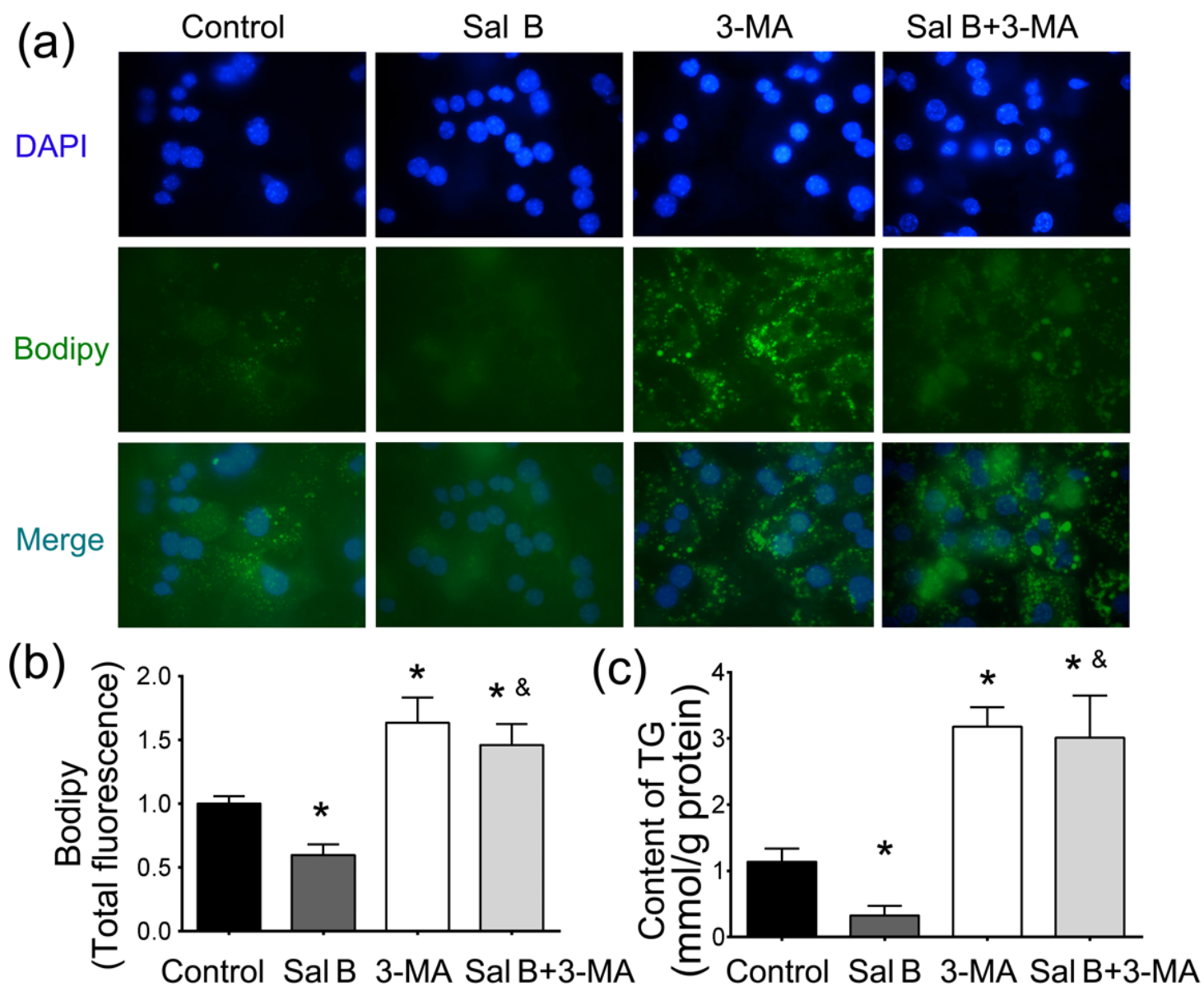
Figure 4

The effect of Sal B on neutral lipid droplet and TG content when autophagy was inhibited with 3-MA in primary mice hepatocytes. Bodipy staining (a), total fluorescence of bodipy (b) and TG content (c) were measured in primary hepatocytes treated with Sal B, 3-MA and Sal B combined with 3-MA. *p<0.05, compared to control cells, & p<0.05, compared to Sal B-treated cells.