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**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, this is the first demonstration for the effect of Sal B on lipid droplets. Meanwhile, a stimulation of Sal B on autophagy of hepatocytes was discovered, further we used autophagy inhibitor 3-MA and revealed that after administration of 3-MA Sal B showed little effect on improving hepatic lipid accumulation, this means the effects of Sal B on lipid drop depend on 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.

Key words: Salvianolic acid B, autophagy, hepatocyte, lipid drop.

Background

Lipid droplets are dynamic organelles that store neutral lipids during times of energy excess and serve as an energy reservoir during deprivation. Excessive lipid droplets accumulation in liver caused fatty liver disease is a hepatic manifestation 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]. Hepatocytes have the greatest capacity to store TGs in the form of small LDs [2]. Excessive LDs accumulation that occurs in hepatocytes can result in lipotoxicity with consequences of inflammation and subsequent cell death and is likely due to disruption in LDs packaging and/or secretory [3-4]. Recent study has reported that autophagy plays an important role in regulating hepatic lipid homeostasis [5].

Autophagy is an intracellular catabolic process with an essential function in the maintenance of cellular and 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]. It is an evolutionarily conserved lysosomal pathway tasked with degrading long-lived, unnecessary, or damaged proteins and organelles to maintain intracellular homeostasis [8]. This catabolic process also responds to various stresses including nutrient depletion/starvation, oxidative stress, and infection by viral and bacterial pathogens to confer cytoprotection [9]. Impaired autophagy in hepatocyte may promote triglycerides accumulation in liver and interfered with subsequent mitochondrial β -oxidation, which provided the first concrete evidence for a connection between autophagy and hepatic lipid metabolism. [10]. Since then, many studies supported the point of view that lipids could also undergo degradation by macroautophagy, by sequestration into autophagosomes that then fused with lysosomes, thus, autophagy has been considered as a new cellular target for abnormalities in lipid metabolism and

accumulation [11, 12].

Salvianolic acid B (Sal B) is the most abundant and bioactive member of polyphenolic compounds in *Salvia miltiorrhiza*. It is the most common medicine that is widely used in the treatment of cardio-cerebral vascular diseases [13, 14]. Recent studies also reported a potential implication of Sal B in the treatment of insulin resistance, obesity, and type 2 diabetes [15-17]. In vivo studies demonstrated that Sal B improved glycemic control, dyslipidemia, and insulin sensitivity in high fat diet and streptozocin-induced type 2 diabetic rats as well as in db/db mice [17, 18], which indicated a contribution of Sal B on improving glucose and lipid disorder. In hepatocytes, Sal B has been reported a strong ability to protect cells from injury induced by oxidative stress, inflammation and enhance hepatic differentiation [19-21]. 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.

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 fully exposed its abdominal cavity. The inferior vena cava was separated carefully and injected in 1mL heparin then perfused with perfusate 1 (mixture of Krebs solution 50mL and 50mM EGTA solution 0.1mL) for 3-5 minutes. After that, perfusate 2 (mixture of Krebs solution 30mL, 2M CaCl₂ 41.2μL and collagenase I 15mg) was injected through inferior vena cava for 3-6 minutes then transferred liver on ice. The liver was then isolated cells by washing with DMEM medium on a sieve. The cells were washed with Hanks' balanced salt solution (HBSS), followed by added to DMEM medium containing 10% fetal calf serum, 1% penicillin-streptomycin and re-suspended at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

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 2h at room temperature. ECL was purchased from Millipore.

Bodipy staining

For neutral lipid drop staining [22], Primary hepatic cells grown on coverslips were treated with Sal B or 3-methyladenine (3-MA) combined with SalB for 24h, and then were incubated with BODIPY 493/503 (Life Technologies, 20 μ g/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 methanol-free 4% paraformaldehyde for 30 min. After fixation, cells were stained with Oil Red O solution (0.5 g of Oil Red O powder was dissolved in 60% isopropanol) for 30 min at room temperature. Then it was washed instantaneously in 60% isopropanol and was

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 isopropanol (100%) to the samples, 100 μ l 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 200mL homogenate was prepared by PBS and sonicated on ice. 2.5 μ L ddH₂O, standard solution and sample solution was mixed with 250 μ L working buffer and cultured in 37°C for 10 minutes. The OD value was analyzed by automatic biochemical detector at absorption wave 510nm. 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 \pm 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 μ M (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 μ M 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 50nM to 200nM significantly stimulated LC3B expression but inhibited P62 level in hepatocyte (Fig. 2a). We then selected 100 μ M of Sal B as an experimental concentration, and a significant higher expression of LC3B and Beclin1 in Sal B treated hepatocyte were observed (Fig. 2b) whereas a decreasing expression of P62 (Fig. 2b) compared to control group. 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 10mM significantly reduced expression of LC3B whereas increased P62 expression (Fig. 3a and b). Meanwhile, oil red staining result showed that 10mM 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 b). However, stimulation of lipid accumulation by 3-MA was not affected by extra Sal B (Fig. 4a and b). 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 [24-26], our finding provided its clinical value of Sal B by decreasing lipid drop accumulation of hepatocytes.

Further, we measured decreased autophagy by the decrease of LC3B and Beclin1 but increase of P62 in hepatocytes treated with Sal B. In the liver, autophagy ensures the proper function of hepatocytes and regularity in the glycogen and triglycerides metabolism, animal model studies impaired hepatic autophagy show an accumulation of altered and deformed mitochondria, lipid droplets, and an increased number of ubiquitinated protein aggregates [27]. In adipose tissue, autophagy ensures the differentiation of adipocytes: impaired autophagy in adipocytes causes abnormal accumulation of lipids, and abnormal transformation of white into brown adipose tissue [28]. More and more evidences confirmed a central role of autophagy in metabolic syndrome, which indicates autophagy a promising therapeutic strategy to fight this disease through caloric restriction and intermittent fasting, physical exercise and, if necessary, pharmacological treatment [29]. Based on these, the improvement of autophagy might be related to hepatic lipid drop accumulation by Sal B, these will

be benefit for people with hepatic lipid 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 PPAR α and 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 autophagy 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 and hepatic autophagy.

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Figure legends:

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 hepatocyte. * $p < 0.05$, 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 hepatocyte. * $p < 0.05$, 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.

Figures

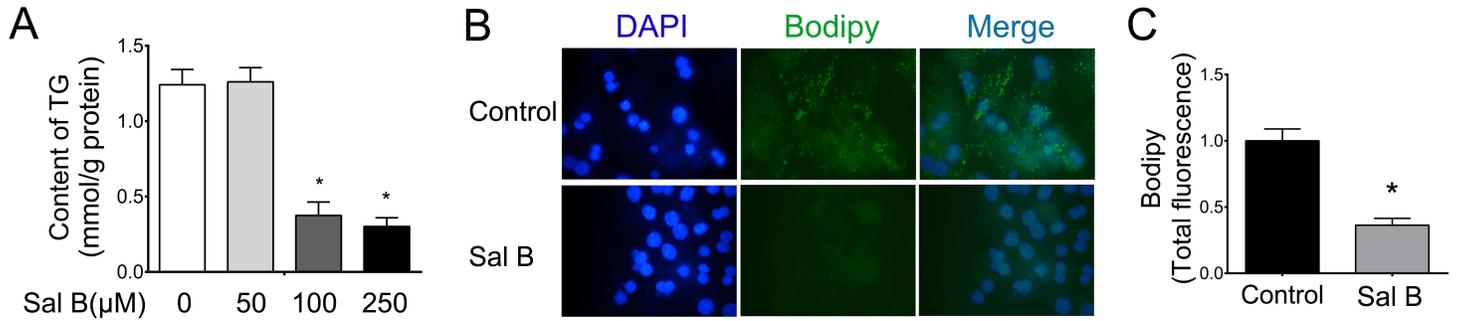


Figure 1

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 hepatocyte. * $p < 0.05$, compared to control cells.

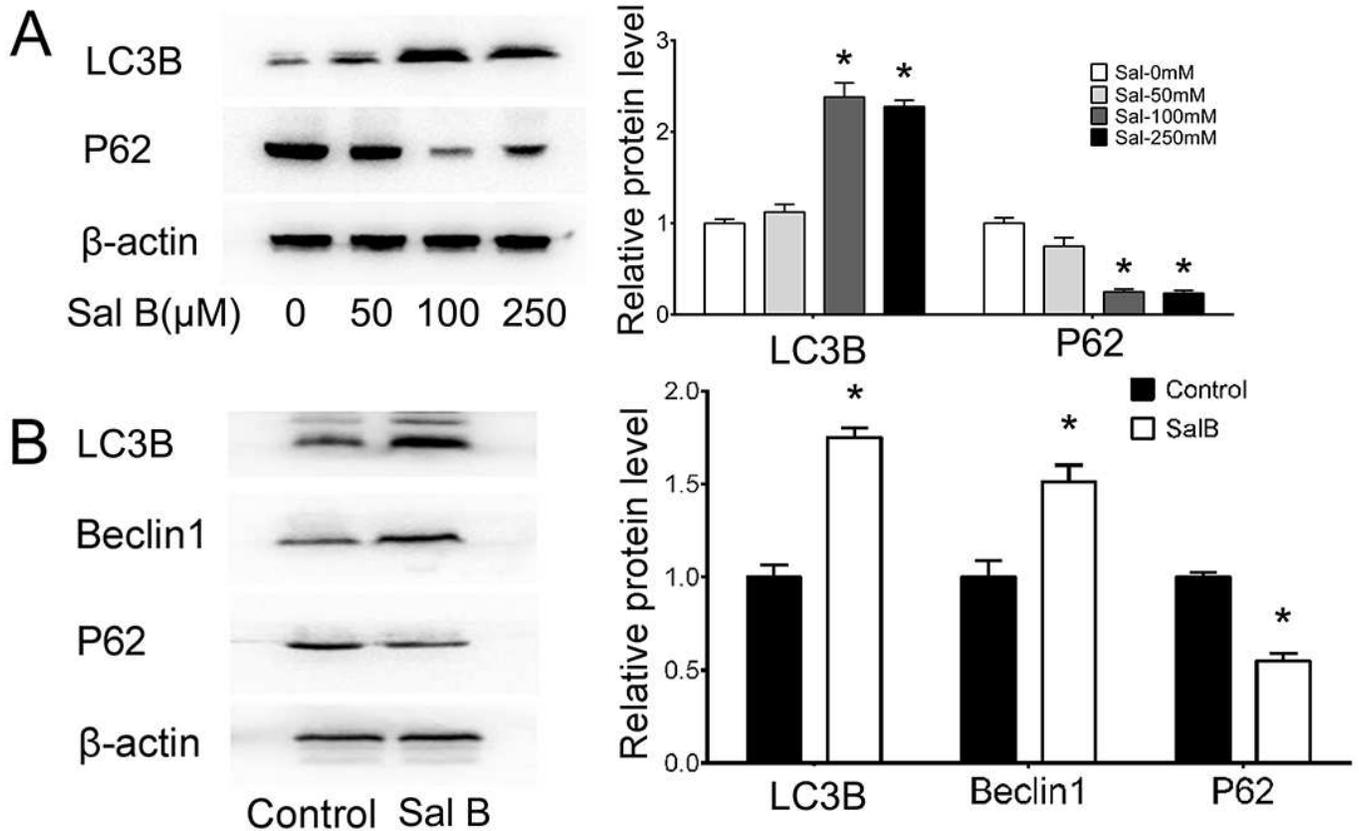


Figure 2

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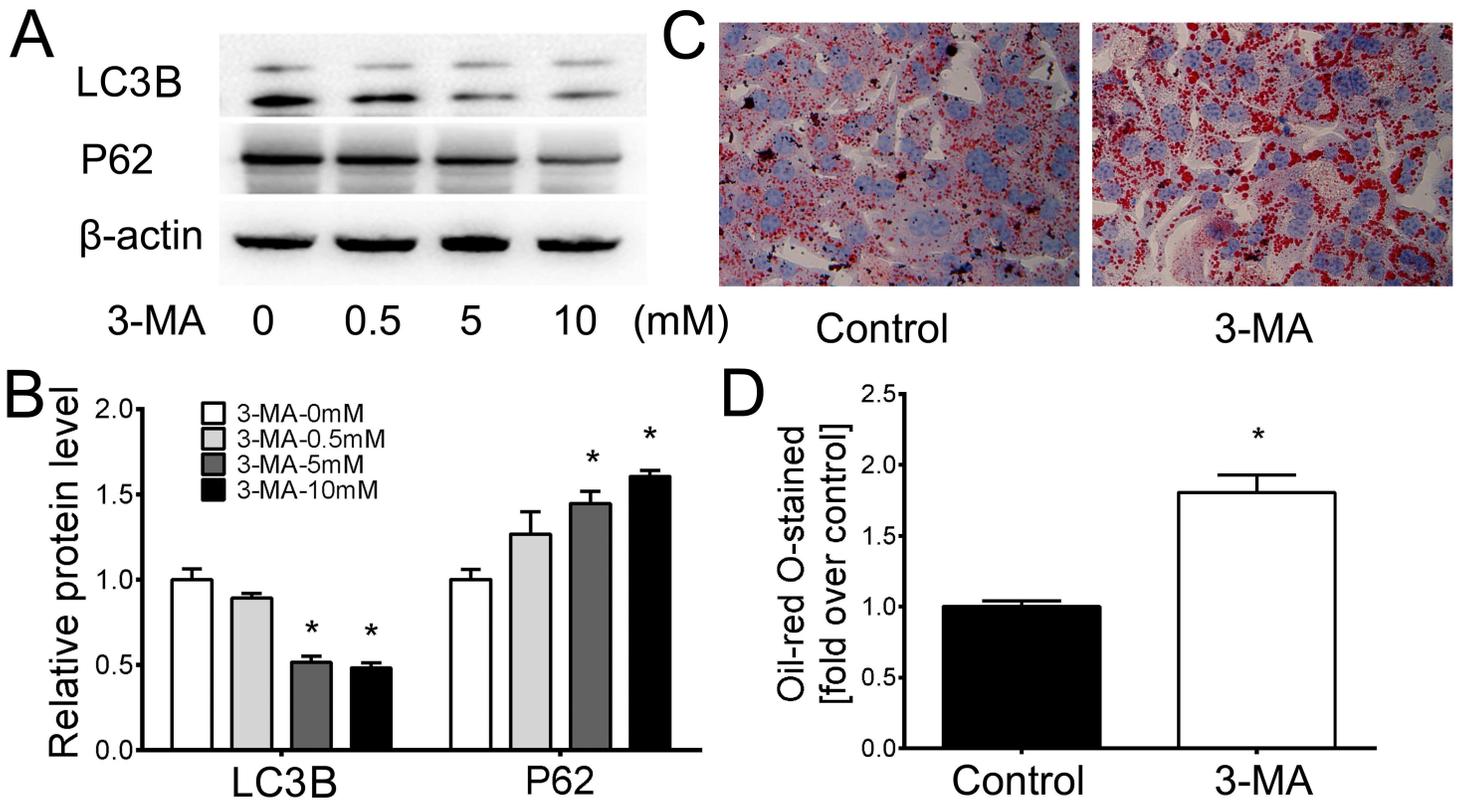


Figure 3

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 hepatocyte. * $p < 0.05$, compared to control cells.

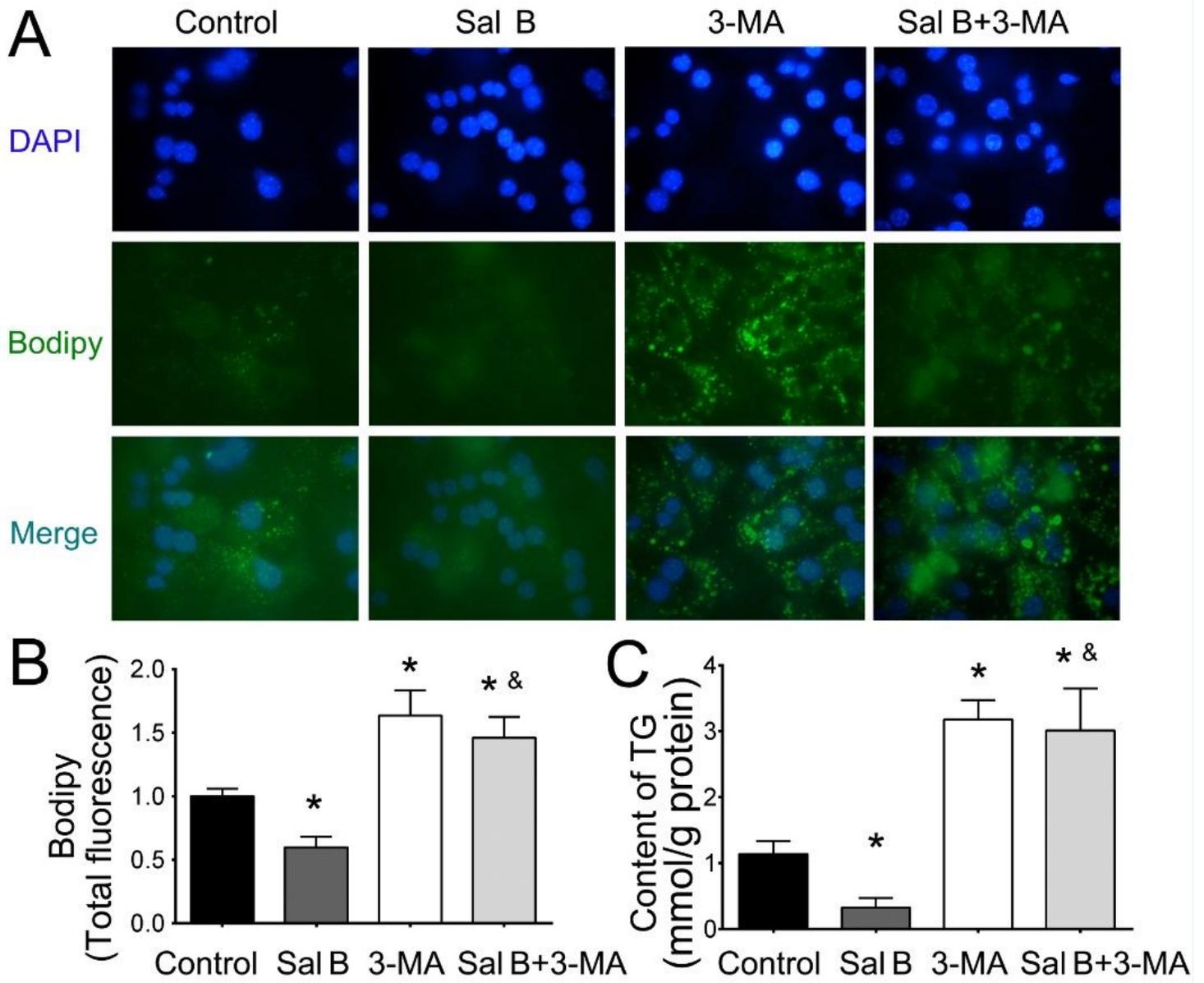


Figure 4

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.