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# Bromelain activates the AMP-activated protein kinase-autophagy pathway to alleviate hepatic lipid accumulation

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## Abstract

Bromelain, a cysteine protease found in pineapple, is known to exert protective effects against non-alcoholic fatty liver disease (NAFLD); however, the underlying mechanism is unclear. In this study, we aimed to investigate the molecular mechanisms underlying the beneficial effects of bromelain using *in vivo* and *in vitro* models. C57BL/6 mice were fed a high-fat diet (HFD) with or without bromelain (20 mg/kg/day) for 12 weeks. We found that treatment with bromelain alleviated hepatic lipid accumulation accompanied by the activation of AMP-activated protein kinase (AMPK) and autophagy flux, as evidenced by the elevated levels of phosphorylated AMPK, ATG5, ATG7, LC3-II, and lysosome-associated membrane protein 2 (LAMP2), and the decreased levels of p62 in the liver of HFD-fed mice. In human hepatoma Huh 7 cells, bromelain prevented oleic acid (OA)-induced lipid accumulation and increased the levels of phosphorylated AMPK, ATG5, ATG7, LC3-II, and LAMP2 but decreased the levels of p62. Inhibition of AMPK and autophagy flux by specific inhibitors or small interfering RNAs suppressed bromelain-mediated protective effect on lipid accumulation. Moreover, inhibition of AMPK activity abolished the activation of autophagy flux in OA-treated hepatocytes. Collectively, these findings suggest a new molecular mechanism involving the AMPK-autophagy pathway through which bromelain confers protection against the deregulation of lipid metabolism in the liver.

**Keywords:** AMPK, Autophagy, Bromelain, NAFLD

## 1. Introduction

Bromelain is a proteolytic enzyme that can be extracted from the bark, stem, and leaves of pineapple (*Ananas comosus*) [1–3]. It was first manufactured as a pharmaceutical product in 1956 and has been used as a clinical medication for decades [1,2]. The clinical indications for use of bromelain include post-surgical inflammation and osteoarthritis [2,3]. Apart from these indications, many other beneficial effects have been investigated, including antimicrobial activity, anticancer activity, and protective effect against bronchitis, sinusitis,

and thrombophlebitis [2–4]. However, the effects of bromelain on metabolic syndrome have not been thoroughly explored.

Metabolic syndrome is highly associated with obesity and inflammation [5]. Some known metabolic syndromes are glucose intolerance, obesity, hypertension, and dyslipidemia [5–7]. Diseases related to metabolic syndrome include cardiovascular disease, type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD) [5,8]. Recently, the importance of NAFLD in metabolic syndrome has received increased attention [9–11]. NAFLD is defined as a disease in which hepatic lipids

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accumulate to over 5%–10% of the liver weight [11]. When NAFLD worsens, it may develop into non-alcoholic steatohepatitis (NASH). NASH may progress to cirrhosis and hepatocellular carcinoma [12,13].

The global prevalence of NAFLD has been increasing over the years [14,15]. It is the most common liver disease, with a worldwide prevalence of approximately 25% [14,15]. The rising prevalence has led to increased research on curing NAFLD [16,17]. Currently, there are no specialized medications or therapies that focus on treating NAFLD [17]. Dietary control or bariatric surgery is mostly used to slow NAFLD progression [16,17]. Recent studies showed autophagy and AMP-activated protein kinase (AMPK) activity as potential therapeutic targets against NAFLD [18,19]. AMPK is a crucial regulator of lipid metabolism, and its activation has been shown to be beneficial for treating NAFLD [17,18]. Phosphorylation at threonine-172 of AMPK has been reported to increase AMPK activity and exert beneficial effects on lipid metabolism [18,19]. Additionally, AMPK activation is important for the initiation of autophagy flux, an intracellular degradation system that is important for cellular homeostasis under various stress conditions [18,19]. Many key regulators, including autophagy-related (ATG) proteins, microtubule-associated protein 1A/1B-light chain 3 (LC3), and p62, are involved in this process [20–22]. It is well established that autophagy activation is a potent therapeutic strategy for the treatment of NAFLD [20,21].

Our previous study revealed that bromelain has a protective effect against NAFLD development in mice [23]. We demonstrated that bromelain attenuates hepatic lipid accumulation in a high-fat diet (HFD)-fed mouse model [23]. However, the molecular mechanism underlying bromelain-conferred protection against NAFLD is not clear. In this study, we aimed to clarify the role of the AMPK-autophagy signaling pathway in the bromelain-mediated protective effect against the deregulation of hepatic lipid metabolism and NAFLD. We first investigated the effect of bromelain on the activation of AMPK and autophagy flux in the liver of HFD-fed mice, and then explored whether the AMPK-autophagy signaling pathway is involved in the lipid-lowering effect of bromelain in hepatocytes. Our findings demonstrate that activation of AMPK-autophagy signaling is required for the protective effect of bromelain against the deregulation of hepatic lipid metabolism.

## 2. Materials and methods

### 2.1. Reagents

Bromelain, oleic acid (OA), acridine orange, the autophagy inhibitors chloroquine (14194) and wortmannin (10010591), and the AMPK inhibitor compound C (11967) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Rabbit antibodies against LC3 (4108), p62 (5114), and phospho-AMP-activated protein kinase (p-AMPK, 2535) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit antibody for AMPK (A12718) was purchased from ABclonal (Woburn, MA, USA), and rabbit antibody for beclin-1 (sc-11427), control small interfering RNA (siRNA), and AMPK siRNA were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Lipofectamine RNAiMAX transfection reagent was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit antibody for autophagy protein 5 (ATG5, NB110-53818) and mouse antibody for lysosome-associated membrane protein 2 (LAMP2, NBP2-22217) were purchased from Novus Biologicals (Centennial, CO, USA). Rabbit antibody for autophagy protein 7 (ATG7, ab133528), DAPI (ab104139) were obtained from Abcam (Cambridge, UK). Horse anti-rabbit IgG antibody (H + L), DyLight® 488 (DI-1088) was purchased from Vector Laboratories (Burlingame, CA, USA). Oil red O (O0625-25G) and Nile Red (72485-100 MG) were purchased from Merck (Darmstadt, Germany). The AMPK inhibitor, wz4003 (S7317) was obtained from Selleck Chemicals (Houston, TX, USA). Phosphatase inhibitor cocktail II and III, eosin Y were obtained from Sigma–Aldrich (St. Louis, MO, USA). Hematoxylin was obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Mounting gel was purchased from MUTO Pure Chemicals (Tokyo, Japan).

### 2.2. Animals

This study conformed to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, eighth edition, 2011), and all animal experiments were approved by the Animal Care and Utilization Committee of National Yang-Ming University (No. 1070314). Eight-week-old wild-type C57BL/6 male mice were randomly divided into two groups: the vehicle (fed with PBS) and bromelain-treated (20 mg/kg) groups. Mice in both groups were fed with HFD (60% of calories from fat) for 12 weeks and daily administered with PBS or bromelain. All mice were housed in a 12h

light/12h dark cycle room where humidity was maintained at 40–60% and temperature at 22 °C. After 12 weeks, the mice were euthanized with CO<sub>2</sub>. The livers were isolated, weighed, and then subjected to histological analysis or stored at –80 °C. Frozen livers were homogenized, and the lysates were subjected to Western blot analysis.

### 2.3. Cell culture

Human hepatoma Huh 7 cells and Huh 7.5 cells stably expressing GFP-conjugated LC3 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37 °C in a 5% CO<sub>2</sub> incubator.

### 2.4. Histology examination

Isolated livers were fixed with 10% formalin, dehydrated with ethanol, hyalinized with xylene, and embedded in paraffin. Paraffin blocks were cut into 8 µm sections and deparaffinized with xylene, and then rehydrated from alcohol to deionized water for further staining, including H&E and immunofluorescent staining. For H&E staining, the sections were stained with 0.5% (w/v) hematoxylin at 25 °C for 1 min and then 0.5% (w/v) eosin Y at 25 °C for 10 s. For immunofluorescent staining, the sections were incubated with primary antibody at 4 °C for overnight, and then incubated with corresponding secondary antibody at 25 °C for 2 h. The sections were covered by the mounting medium with DAPI. For oil red O staining, the cryostat sections of the livers were fixed with 10% formalin, soaked in 30% sucrose solution overnight, and embedded in optimal cutting temperature compound. Cryostat blocks were cut into 10 µm frozen sections and subjected to oil red O staining. The sections were stained with oil red O (3 mg/mL) at 25 °C for 10 min. After washing, the sections were mounted by the mounting gel.

### 2.5. Western blot analysis

Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 10 µg/mL aprotinin and phosphatase inhibitor cocktail II and III). Aliquots of cell lysates (50 µg protein) were mixed with 5 µL of loading dye (250 mM Tris HCl pH 6.8, 500 mM dithiothreitol, 10% SDS, 50% glycerol, and bromophenol blue). The mixture was then heated at

95 °C for 10 min. Proteins were separated on 8–12% SDS-polyacrylamide gel by electrophoresis, and the gels were transferred onto a polyvinylidene fluoride (PVDF) membrane (Pall, Port Washington, NY, USA), blocked with 5% skim milk for 1 h at 37 °C, and then incubated with primary antibodies overnight, followed by incubation with corresponding secondary antibodies for 2 h. The protein bands were detected using Ultra ECL-HRP Substrate (TU-ECL02, Tools Biotech, Taiwan) and quantified using ImageJ 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

### 2.6. Quantification of intracellular levels of triglycerides

The levels of cellular triglycerides were examined by Oil Red O and Nile red staining. The fluorescence intensity of Nile red staining was measured by fluorometry (Molecular Devices, Sunnyvale, CA, USA) using excitation/emission spectra at 540/590 nm. Photomicrographs were digitally captured under a Leica DMIRB microscope (Leica Biosystems, Deer Park, IL, USA).

### 2.7. Acidic vacuoles detection

Acridine orange staining was used to detect acidic vacuoles, as previously described [24]. Acridine orange emits red fluorescence in acidic environments, including lysosomes and autolysosomes. The fluorescence intensity of acridine orange was used to assess the formation of autophagolysosome.

### 2.8. Small interference RNA transfection

Huh 7 cells were seeded in 3.5 cm dishes overnight and then starved for 6 h in serum-free DMEM. After starvation, control siRNA or AMPK siRNA mixed with Lipofectamine RNAiMAX transfection reagent was added to serum-free DMEM and incubated for 24 h. DMEM was replaced with normal DMEM (10% FBS) and transfected cells were used for experiments.

### 2.9. Statistical analysis

The results are presented as mean ± SEM from five independent experiments. The Mann–Whitney U test was used for comparisons of data between the two groups. For comparisons of data from more than two groups, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons or the Mann–Whitney U test was performed. SPSS software v8.0 (SPSS Inc., Chicago, IL, USA)



was used for all statistical analyses. Differences were considered statistically significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Bromelain attenuates HFD-induced lipid accumulation in the liver and increases the activation of AMPK and autophagy

We first examined whether the activation of AMPK and autophagy are involved in the beneficial effects of bromelain on hepatic lipid accumulation in HFD-fed mice. Oil red O and H&E staining showed that daily treatment of mice with bromelain for 12 weeks decreased lipid accumulation in the liver compared to the vehicle group (Fig. 1A and B). Previous findings suggest that the activation of AMPK and autophagy exerts a protective effect against the development of NAFLD [25–28]. Next, we explored whether autophagy and AMPK are involved in the lipid-lowering effect of bromelain. Compared to vehicle treatment, daily bromelain treatment upregulated the protein expression of ATG5, ATG7, and LC3-II, and downregulated the levels of p62 (Fig. 1C), all of which are standard markers for the activation of autophagy flux [22,29]. Treatment with bromelain also increased the protein expression of LAMP2, the lysosome marker in the livers of HFD-fed mice (Fig. 1C). Additionally, bromelain treatment increased AMPK activation, as evidenced by the elevated AMPK phosphorylation (Fig. 1C). Moreover, immunohistochemistry results demonstrated that the levels of LC3 puncta were increased and the levels of p62 were decreased in the livers of bromelain-treated mice (Fig. 1D and E). These results suggest that the activation of autophagy and AMPK may play crucial roles in the beneficial effects of bromelain against the development of NAFLD.

#### 3.2. Bromelain ameliorates OA-induced lipid accumulation in hepatocytes

We further clarified the causal relationship between autophagy, AMPK, and the beneficial effects of bromelain on hepatic lipid metabolism. We used human hepatoma Huh 7 cells as an *in vitro* model. To mimic the pathology of NAFLD, treatment with OA was used induce lipid accumulation in Huh 7 cells. Oil red O and Nile red staining showed that the intracellular levels of lipids in Huh 7 cells were increased in response to OA challenge (Fig. 2A and B). Furthermore, the OA-induced increase in intracellular lipid accumulation was abolished by bromelain treatment (Fig. 2A and B). These results

suggest that bromelain has a protective effect against OA-induced deregulation of lipid metabolism in hepatocytes.

#### 3.3. Autophagy mediates the lipid-lowering effect of bromelain in hepatocytes

Autophagy plays an important role in orchestrating the metabolism of intracellular lipid droplets [20,21]. We then examined the effect of bromelain on autophagy and found that exposure of OA-treated Huh 7 cells to bromelain induced an increase in the expression of autophagy-related proteins, including ATG5, ATG7, LC3-I, LC3-II, and p62, in a time-dependent manner (Fig. 3A). Moreover, bromelain time-dependently increased the protein expression of LAMP2 in OA-treated Huh 7 cells (Fig. 3A). Furthermore, treatment with bromelain induced the formation of autophagosomes, as evidenced by the appearance of GFP-LC3 puncta 9 h after treatment (Fig. 3B). Moreover, acridine orange staining showed that bromelain increased autophagolysosomal activity, as evidenced by the increase in the intracellular levels of acidic vacuoles within 9 h of treatment (Fig. 3C). We next assessed whether activation of autophagy flux is required for the beneficial effect of bromelain on OA-induced lipid accumulation in hepatocytes. Pretreatment with chloroquine (CQ) or wortmannin (WM), two autophagy inhibitors for blocking the formation of autophagolysosomes, abrogated the protective effect of bromelain on OA-induced lipid accumulation and activation of autophagy flux (Fig. 4A and B). Collectively, these findings suggest that activation of the autophagy pathway is required for the beneficial effect of bromelain on OA-induced degradation of lipids in hepatocytes.

#### 3.4. AMPK phosphorylation is required for bromelain-induced activation of autophagy flux

AMPK is a crucial regulator of autophagy and lipid metabolism [27,28]. Phosphorylation of AMPK at Thr-172 promotes autophagy activation [26,27]. Therefore, we explored the role of AMPK in bromelain-mediated activation of autophagy and protective effect on lipid accumulation. Huh 7 cells were treated with bromelain (2  $\mu\text{g/mL}$ ) for up to 240 min. We found that bromelain induced the phosphorylation of AMPK in a time-dependent manner at Thr-172; phosphorylation increased as early as 15 min after treatment and peaked at 240 min (Fig. 5A). Inhibition of AMPK activation by the pharmacological inhibitors compound C (C.C.) and WZ4003 (WZ) abolished the protective effect of

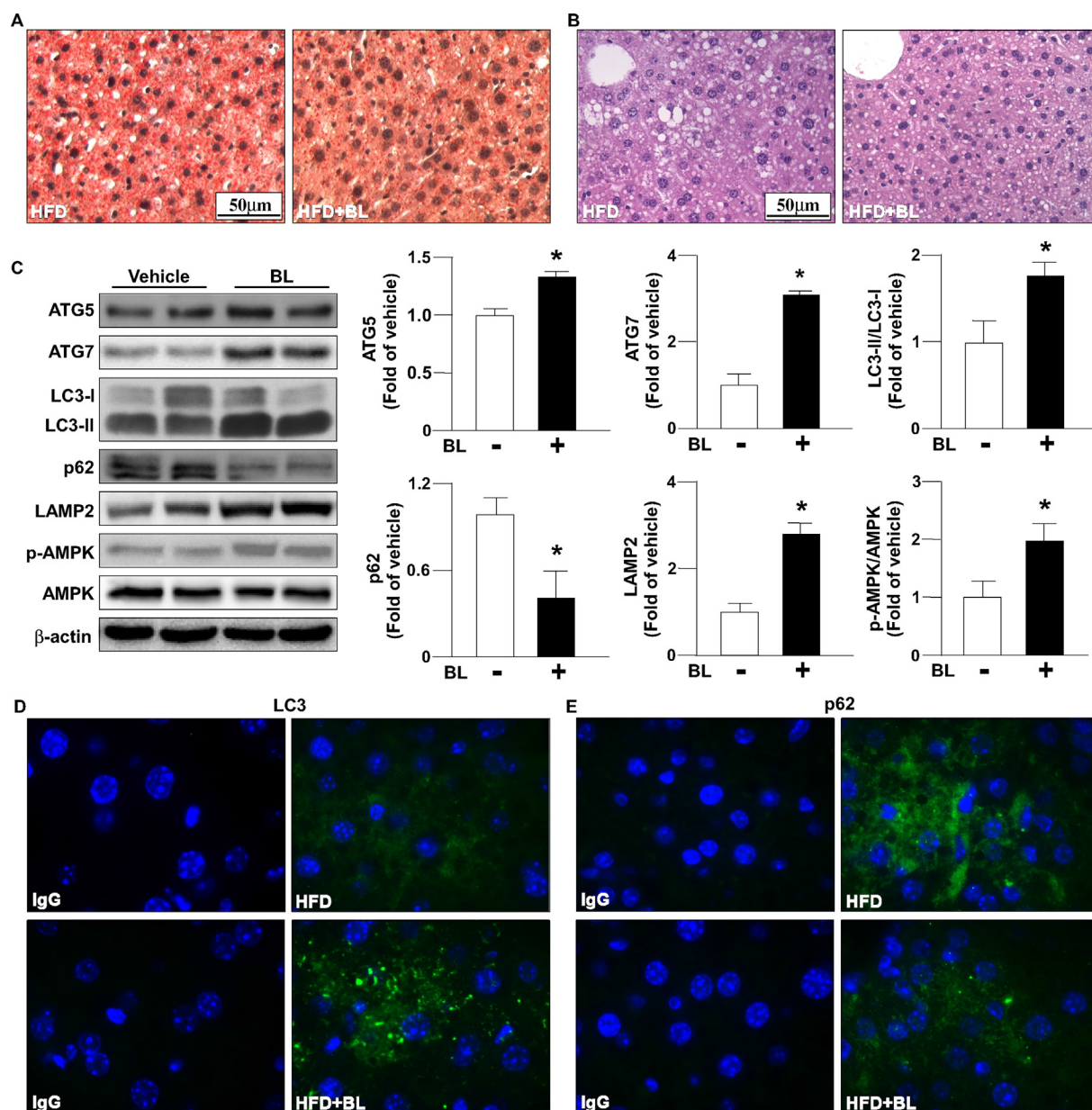


Fig. 1. Bromelain attenuates hepatic lipid levels, and activates AMP-activated protein kinase (AMPK) activity and autophagy in HFD-fed mice. HFD-fed C57BL/6 mice were administered with vehicle (PBS) or bromelain (20 mg/kg/day) for 12 weeks. Representative images of (A) oil red O staining and (B) H&E staining. (C) Western blotting analysis of p-AMPK, AMPK, ATG-5, ATG-7, LC3-I/II, p62, LAMP2 and  $\beta$ -actin. (D and E) Immunofluorescent staining for LC3 and p62. Data are represented as mean  $\pm$  SEM from five mice. \* $p < 0.05$  vs. vehicle group.

bromelain on the accumulation of intracellular lipids (Fig. 5B and C). Moreover, pretreatment with C.C. or WZ prevented the effect of bromelain on the activation of autophagy flux (Fig. 5D). To further confirm the role of AMPK activity in bromelain-induced protection against the deregulation of lipid metabolism and activation of autophagy in hepatocytes, we used siRNA targeting AMPK expression. Our results demonstrated that knockdown of AMPK abrogated the beneficial effects of bromelain on OA-induced lipid accumulation and activation of

autophagy flux (Fig. 6A–C). Collectively, these findings strongly suggest that the AMPK-autophagy pathway is required for the beneficial effects of bromelain on the deregulation of lipid metabolism in hepatocytes.

#### 4. Discussion

The anti-inflammatory and anti-coagulation effects of bromelain have been well-documented [1–4]. However, much remains to be learned about



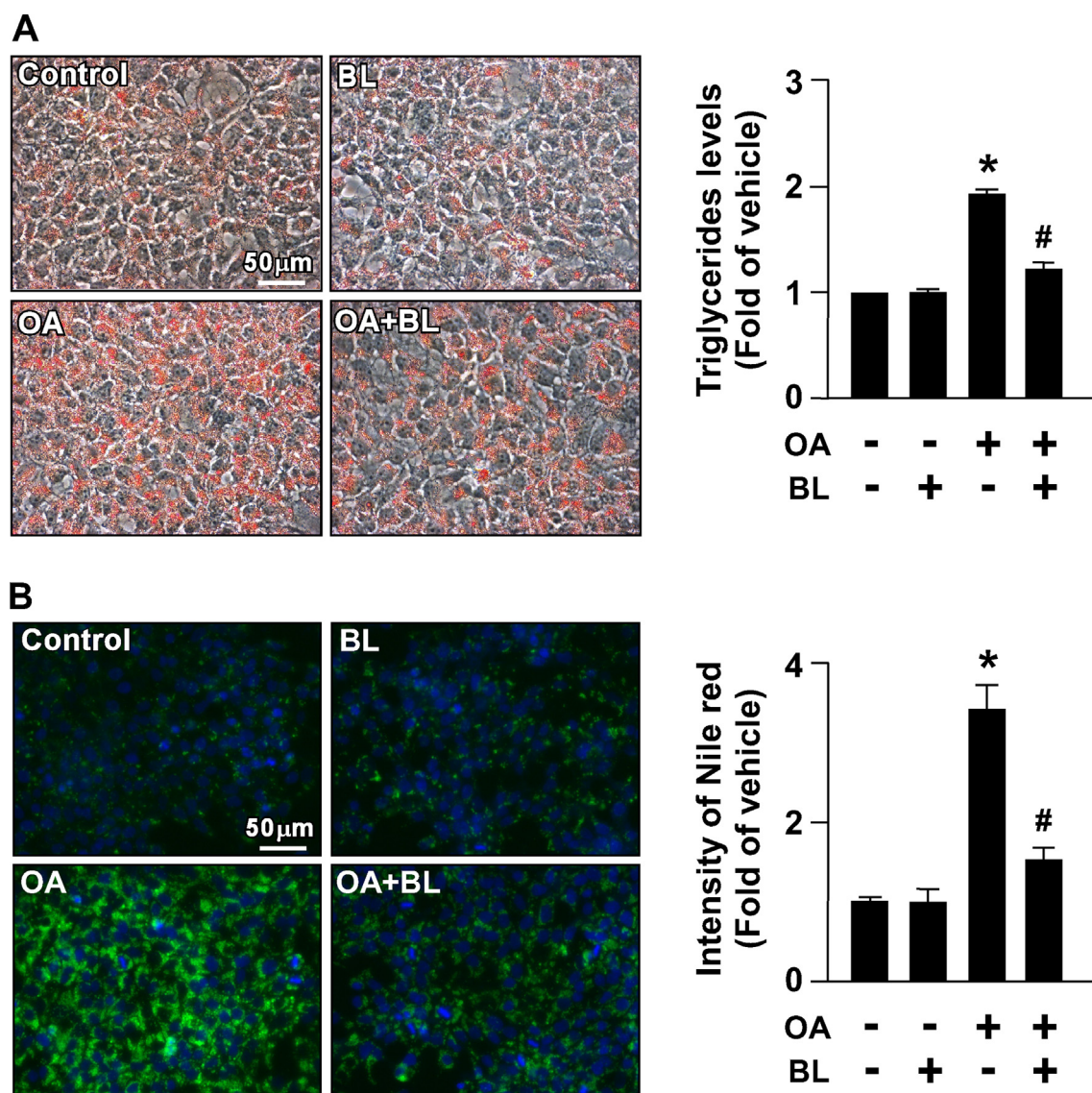


Fig. 2. Bromelain prevents intracellular lipid accumulation in oleic acid (OA)-treated Huh 7 cells. Huh 7 cells were pretreated with OA (125  $\mu\text{g/mL}$ ) for 18 h and then with or without bromelain (2  $\mu\text{g/mL}$ ) for an additional 18 h. (A) Representative images of oil red O staining and the relative intracellular levels of triglycerides. (B) Representative images and the quantitation of the intensity of Nile red staining. Data are the mean  $\pm$  SEM from five independent experiments. \* $p < 0.05$  vs. vehicle-treated group; # $p < 0.05$  vs. OA-treated group.

the effects and molecular mechanisms of bromelain on metabolic diseases. In this study, we provide new evidence for the beneficial effects of bromelain on excess fatty acid-induced lipid accumulation and the underlying molecular mechanisms. We used a HFD-fed mouse model and an OA-induced lipid accumulation cell model to investigate the molecular mechanism underlying the beneficial effect of bromelain on the deregulation of lipid metabolism in hepatocytes. We found that daily treatment with bromelain for 12 weeks alleviated the HFD-induced lipid accumulation in the liver. Bromelain also induced the activation of AMPK and autophagy

flux. Additionally, an *in vitro* study further confirmed the causal relationship between these key events in that bromelain prevented OA-induced lipid accumulation by activating the AMPK-autophagy signaling pathway. The observations of our study suggest that bromelain elicits the activation of AMPK, which in turn initiates the process of autophagy flux and promotes the hydrolysis of lipid droplets, leading to a reduction in intracellular lipid accumulation. Although the role of autophagy in anti-cancer action of bromelain has been reported [3]; the role of autophagy in bromelain-conferred protection from the deregulation of hepatic lipid

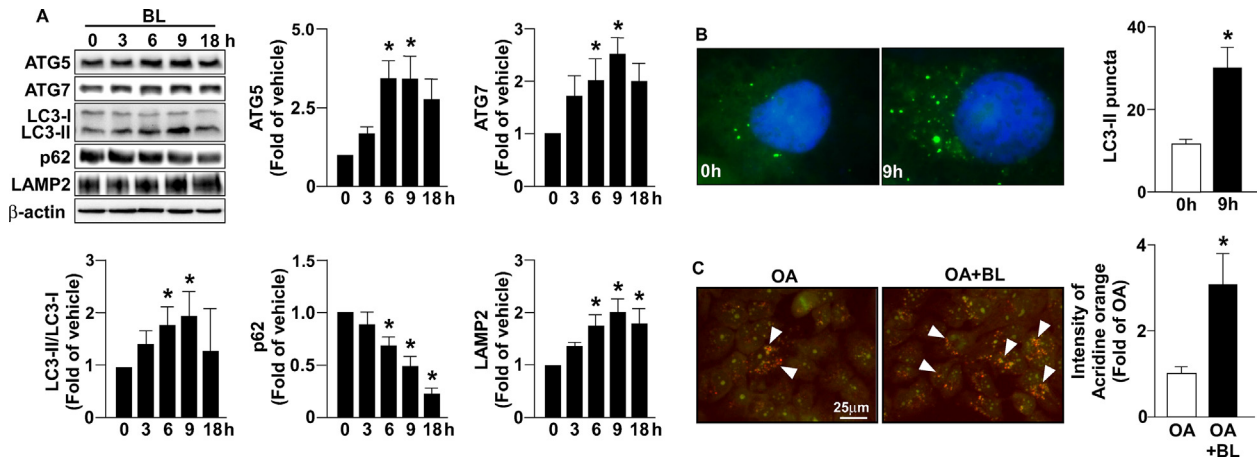


Fig. 3. Bromelain induces activation of autophagy flux in hepatocytes. Huh 7 cells were pretreated with oleic acid (OA) (125  $\mu$ g/mL) for 18 h and then with bromelain (2  $\mu$ g/mL) for the indicated times. (A) Western blotting analysis of autophagy-related proteins, LAMP2 and  $\beta$ -actin. (B) Representative images and quantitation of the intensity of GFP-LC3 puncta in Huh 7.5 cells. (C) Representative images of acidic vacuoles (red fluorescence) and quantitation of the intensity of acridine orange staining. Data are the mean  $\pm$  SEM from five independent experiments. A, \* $p$  < 0.05 vs. vehicle group; B, \* $p$  < 0.05 vs. time zero group; C, \* $p$  < 0.05 vs. OA-treated group.

metabolism is still unclear. Our study is the first to demonstrate that bromelain alleviates the excess fatty acid-induced lipid accumulation in hepatocytes and NAFLD by activating AMPK-autophagy pathway. Collectively, these findings clarify the molecular mechanisms underlying the protective effects of bromelain on the deregulation of hepatic lipid metabolism.

Regarding with the concentration of bromelain used in this study, we have previously reported that treatment with bromelain (20 mg/kg) for 12 weeks effectively attenuate the progression of non-alcoholic fatty liver disease (NAFLD) in C57BL/6 mice [23]. Therefore, we simply adopted this dosage and tried to address the role of AMPK-autophagy pathway in bromelain-conferred protection from

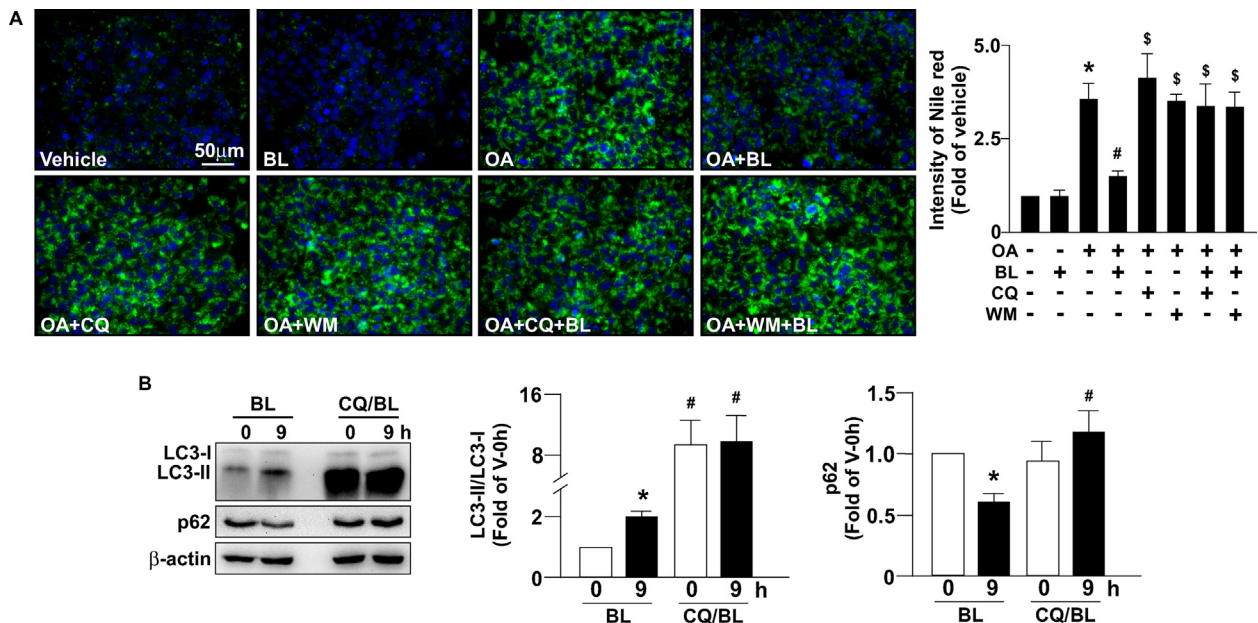


Fig. 4. Inhibition of autophagy abolishes the protective effect of bromelain on intracellular lipid accumulation in hepatocytes. Huh 7 cells were pretreated with oleic acid (OA) (125  $\mu$ g/mL) for 18 h and then chloroquine (CQ, 40  $\mu$ M) and wortmannin (WM, 1  $\mu$ M) for 2 h, followed by bromelain (BL) (2  $\mu$ g/mL) for an additional 18 h. (A) Representative images and quantitation of the intensity of Nile red staining. (B) Western blotting analysis of LC3-I/II, p62, and  $\beta$ -actin. Data are the mean  $\pm$  SEM from five independent experiments. A, \* $p$  < 0.05 vs. vehicle group, # $p$  < 0.05 vs. OA-treated group, \$ $p$  < 0.05 vs. OA + BL-treated group; B, \* $p$  < 0.05 vs. BL-0 h group, # $p$  < 0.05 vs. BL-9h group.



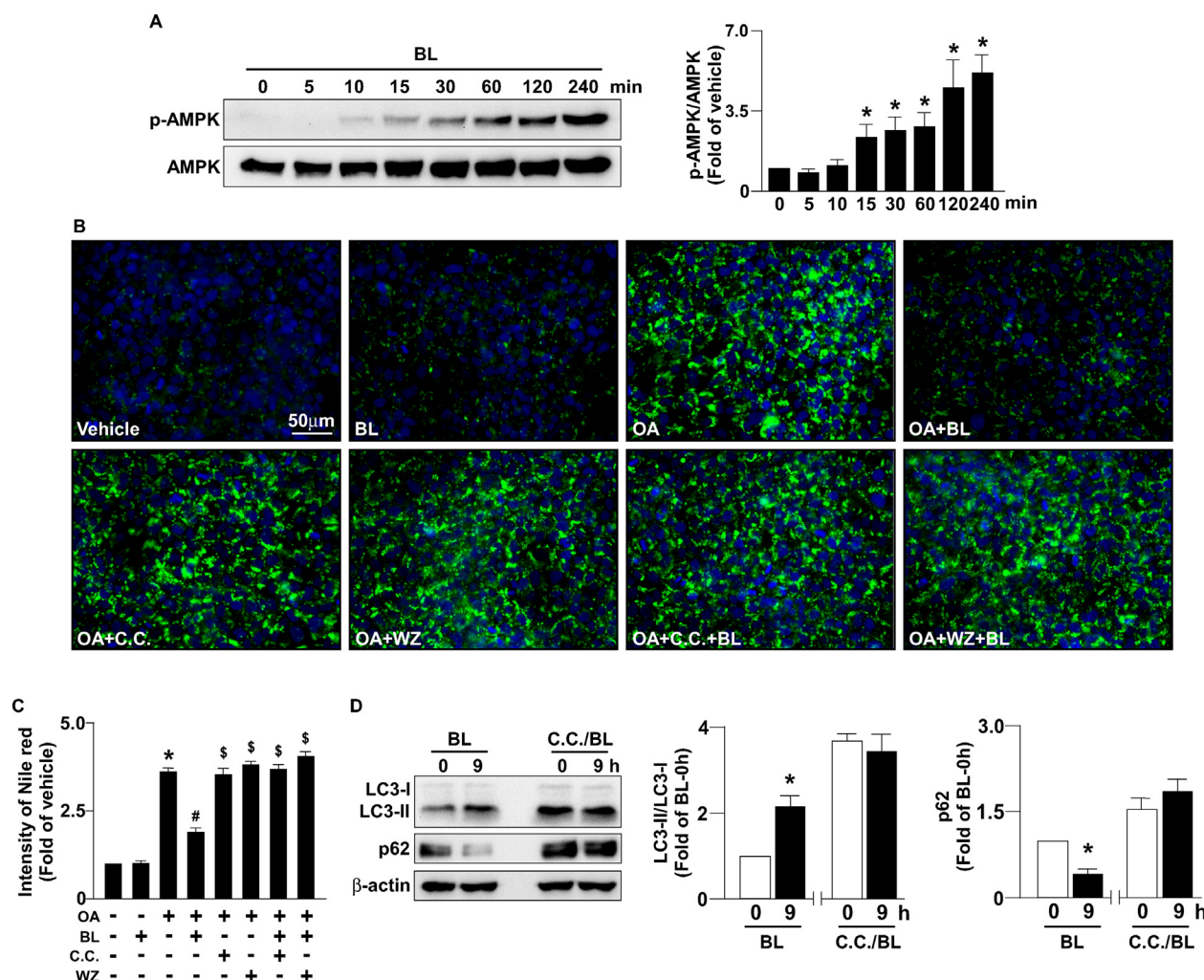


Fig. 5. AMP-activated protein kinase (AMPK) activation is required for the bromelain-mediated beneficial effect on lipid accumulation and autophagy induction in hepatocytes. (A) Huh 7 cells were treated with bromelain (2  $\mu$ g/mL) for the indicated times. Western blot analysis of phosphorylated AMPK (p-AMPK) and AMPK. (B and C) Huh 7 cells were pretreated with oleic acid (OA) (125  $\mu$ g/mL) for 18 h and then with the AMPK inhibitor compound C (CC, 10  $\mu$ M) or WZ4003 (WZ, 5  $\mu$ M) for 2 h, followed by bromelain (BL) (2  $\mu$ g/mL) treatment for an additional 18 h. Representative images and quantitation of the intensity of Nile red staining. (D) Western blotting analysis of LC3-I/II, p62, and  $\beta$ -actin. Data are the mean  $\pm$  SEM from five independent experiments. A and D, \* $p$  < 0.05 vs. time zero group; C, \* $p$  < 0.05 vs. vehicle group, # $p$  < 0.05 vs. OA-treated group, \$ $p$  < 0.05 vs. OA + BL-treated group.

NAFLD in C57BL/6 mice. On the other hand, the doses of bromelain used in our *in vitro* study were determined by MTT assay. Huh 7 cells were treated with various concentrations (0.5, 1, 2, 5, 10  $\mu$ g/mL) of bromelain for 18 h and then subjected to MTT assay. The results showed that treatment with bromelain at the concentrations of 0.5, 1 and 2  $\mu$ g/mL had no cytotoxic effects in Huh 7 cells; however, 5 and 10  $\mu$ g/mL of bromelain showed a decrease in cell viability (data not shown). Thus, we adopted the concentration of 2  $\mu$ g/mL bromelain for further *in vitro* study.

Interestingly, the beneficial effects of bromelain are thought to be attributed to its proteolytic activity [29–31]. For example, bromelain regulates the

leukocyte function by selectively modifying cell surface molecules via the proteolytic activity [29,30]. Very recently, Sagar et al. reported that bromelain inhibits SARS-CoV-2 infection by cleaving the spike protein, transmembrane serine protease 2 and angiotensin-converting enzyme 2, all of which are key molecules for the entry of SARS-CoV-2 into host cells [31]. Thus, we speculate that the proteolytic activity is required for the beneficial effect of bromelain from deregulation of lipid metabolism in hepatocytes. However, the exact target proteins on the surface of hepatocyte to the enzymatic activity of bromelain remain to be investigated.

Autophagy is an important self-degradative process that plays a fundamental role in maintaining

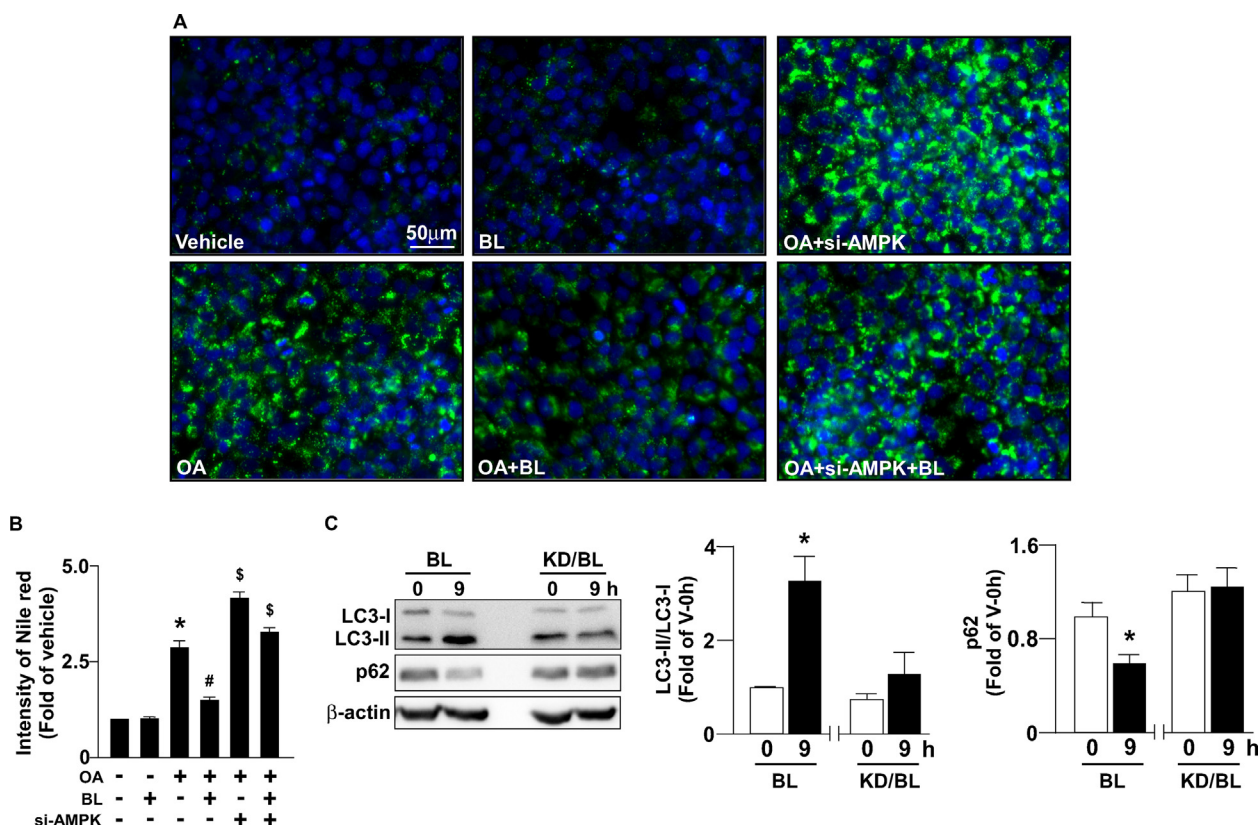


Fig. 6. Knockdown of the expression of AMP-activated protein kinase (AMPK) abrogates the protective effect of bromelain on hepatic lipid accumulation and activation of autophagy flux. (A and B) Huh 7 cell were transfected with AMPK siRNA (20 nM) for 24 h and then were treated with oleic acid (OA) (125  $\mu$ g/mL) in the presence of bromelain (2  $\mu$ g/mL) for an additional 18 h. Representative images and quantitation of the intensity of Nile red staining. (C) Western blotting analysis of LC3-I/II, p62, and  $\beta$ -actin. Data are the mean  $\pm$  SEM from five independent experiments. B, \* $p$  < 0.05 vs. vehicle group, # $p$  < 0.05 vs. OA-treated group, \$ $p$  < 0.05 vs. OA + BL-treated group; C, \* $p$  < 0.05 vs. time zero group.

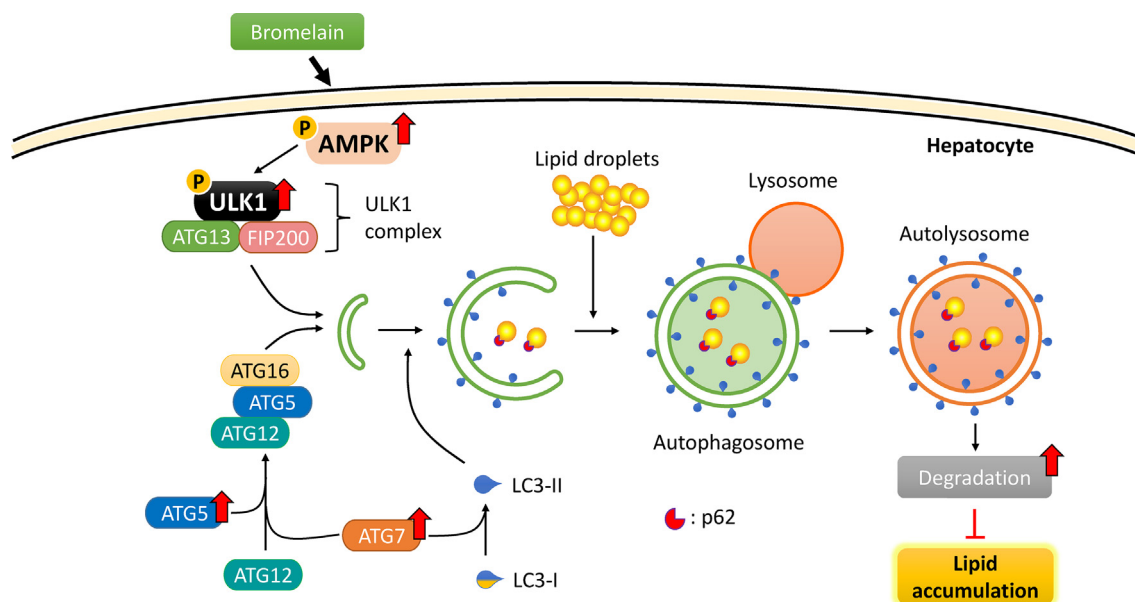


Fig. 7. Schematic representation of the mechanism of bromelain induced AMP-activated protein kinase (AMPK)-autophagy activation in the liver. Schematic illustration of the proposed mechanism by which bromelain alleviates lipid accumulation in hepatocytes. As shown, bromelain induces the phosphorylation of AMPK and ULK1, which initiates the activation of autophagy flux and ultimately decreases hepatic lipid accumulation.

cellular and organismal homeostasis at critical times in response to cellular stress [32]. Recently, increasing evidence suggests that the autophagic machinery also promotes lipid clearance by breaking down intracellular lipid droplets in lysosomes [33,34]. To clarify whether bromelain can activate autophagy to attenuate the lipid accumulation in hepatocytes, we analyzed the protein levels of LC3-II and p62, indicators of the activation of autophagy flux [35,36], in the liver of mice. Our results demonstrated that bromelain could decrease lipid accumulation in the liver by activating autophagy flux. Furthermore, our *in vitro* study supports the notion that treatment with bromelain activates autophagy, as evidenced by increased ATG5, ATG7, LC3-I, LC3-II, autophagy puncta, and lysosomal activity, and decreased p62 and lipid accumulation in Huh 7 cells, suggesting that bromelain promotes the formation of autophagosomes and autophagolysosomes, and leads to the degradation of lipid droplets in the lysosomes of hepatocytes. Moreover, inhibition of autophagy by the pharmacological inhibitors CQ and WM [37–40] abrogated the protective effect of bromelain on lipid accumulation in Huh 7 cells, suggesting the essential role of autophagy in bromelain-conferred protection from deregulation of lipid metabolism. These observations revealed the potential molecular mechanisms underlying bromelain-mediated protection from deregulation of hepatic lipid metabolism by excess fatty acids. However, the molecular mechanism by which bromelain activates autophagy flux in hepatocytes is not understood.

Importantly, AMPK is a key sensor of energy homeostasis in mammalian cells, [18,41]. AMPK can be activated by an increase in the ratio of AMP to ATP and phosphorylate downstream substrate proteins to inhibit energy-consuming biosynthetic and ATP catabolic pathways [19]. Notably, the physiological role of AMPK in the regulation of lipid metabolism has been extensively investigated [41,42]. Clinical therapeutic agents for metabolic syndrome and lipid metabolism-related disorders, such as metformin and statins, exert protective effects against metabolic diseases via the AMPK signaling pathway [42,43]. Moreover, emerging evidence indicates that AMPK signaling is involved in the activation of autophagy flux [26,27]. Our results further confirmed this notion; inhibition of AMPK by C.C. and WZ diminished bromelain-induced autophagy activation and lipid clearance, suggesting that AMPK is an upstream regulator of the activation of autophagy flux by bromelain. Furthermore, previous studies have reported that AMPK directly activates unc-51 like autophagy activating kinase 1 (ULK1) by inducing

phosphorylation at Ser-317, which consequently initiates the activation of autophagy process [44,45]. Additionally, phosphorylated ULK1 (p-ULK1 at Ser-317) activates the class III PI 3-kinase (VPS34) complex to activate autophagy [46]. Interestingly, our data revealed that ULK1 phosphorylation at Ser-317 is also required for bromelain-mediated activation of autophagy and lipid clearance (Fig. S1A). This finding also suggests that ULK1 phosphorylation at Ser-317 is important for the protective effect of bromelain on the deregulation of lipid metabolism. However, the causal relationship between AMPK and ULK1 in bromelain that conferred protection against hepatic lipid metabolism requires further investigation. Collectively, these findings provide new insights into the role of the AMPK-autophagy pathway in bromelain-mediated hepatic lipid clearance under pathological conditions.

Despite the unique pathway discovered in this study, the detailed mechanisms by which bromelain regulates hepatic lipid metabolism merits further study. Overall, our results further support the notion that activation of AMPK-autophagy signaling is crucial in regulating lipid metabolism, which again demonstrates the beneficial effect of bromelain on lipid metabolism. In conclusion, our *in vivo* and *in vitro* findings suggest that activation of the AMPK-autophagy signaling pathway is essential for the bromelain-conferred protection from deregulation of hepatic lipid metabolism and the development of NAFLD (Fig. 7). Our findings reveal a new molecular mechanism underlying the beneficial effects of bromelain on the pathogenesis of NAFLD, which provides supporting evidence for a link between bromelain and the AMPK-autophagy component in the liver. These findings are essential for a better understanding of the molecular mechanism of bromelain and for identifying new therapeutic targets for treating NAFLD and related lipid disorders.

## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

Conceptualization: Hu PA, Kou YR, Lee TS; methodology: Hu PA, Hsu MC, Chen SH, Chen CH; formal analysis: Hu PA; investigation: Hu PA, Hsu MC, Chen SH; data curation: Hu PA and Lee TS; writing—original draft preparation: Hu PA, Kou YR and Lee TS; supervision: Huang JW and Lee TS; funding acquisition: Huang JW and Lee TS. All authors have read and approved the published version of the manuscript.



## Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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## Appendix.

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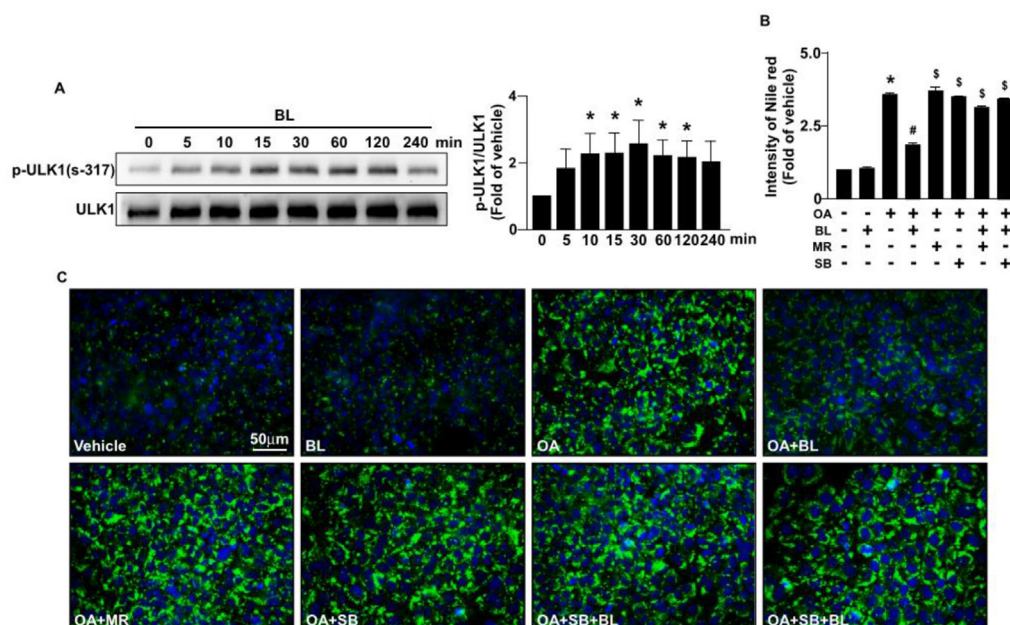


Fig. S1. Inhibition of ULK1 eliminates bromelain-induced hepatic lipid clearance effect. Treatment with bromelain increased the p-ULK1(s-317) to ULK1 ratio in Huh7 cells (A). Nile red staining showing that ULK1 inhibitors blocked the effect of bromelain on lipid clearance in Huh7 cells (B, C). Results are presented by the mean  $\pm$  SEM ( $n=5$ ). (A, B)  $*p < 0.05$  vs. vehicle group. (B)  $^{\#}p < 0.05$  vs. oleic acid (OA) group,  $^{\$}p < 0.05$  vs. OA/bromelain (BL) group. MR, MRT68921; SB, SBI-0206965.

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