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# Autophagy regulates inflammation in adipocytes

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ABSTRACT

Autophagy is an essential process for both the maintenance and the survival of cells, with homeostatic low levels of autophagy being critical for intracellular organelles and proteins. In insulin resistant adipocytes, various dysfunctional/damaged molecules, organelles, proteins, and end-products accumulate. However, the role of autophagy (in particular, whether autophagy is activated or not) is poorly understood. In this study we found that in adipose tissue of insulin resistant mice and hypertrophic 3T3-L1 adipocytes autophagy was suppressed. Also in hypertrophic adipocytes, autophagy-related gene expression, such as LAMP1, LAMP2, and Atg5 was reduced, whereas gene expression in the inflammatory-related genes, such as MCP-1, IL-6, and IL-1β was increased. To find out whether suppressed autophagy was linked to inflammation we used the autophagy inhibitor, 3-methyladenine, to inhibit autophagy. Our results suggest that such inhibition leads to an increase in inflammatory gene expression and causes endoplasmic reticulum (ER) stress (which can be attenuated by treatment with the ER stress inhibitor, Tauroursodeoxycholic Acid). Conversely, the levels of inflammatory gene expression were reduced by the activation of autophagy or by the inhibition of ER stress. The results indicate that the suppression of autophagy increases inflammatory responses via ER stress, and also defines a novel role of autophagy as an important regulator of adipocyte inflammation in systemic insulin resistance.



## 1. Introduction

Metabolic disorders such as obesity, Type 2 diabetes, and vascular disease appear as clusters and constitute a major global insulin resistance-related health problem. Chronic inflammation in insulin target tissues is a key etiologic component causing decreased insulin sensitivity, particularly in obesity [1–4], and it is now well recognized that adipose tissue is an important etiological tissue in insulin resistance and chronic inflammation [5–7]. In insulin resistant hypertrophic adipocytes, dysfunctional mitochondria cause reactive oxygen species and increase oxidative stress, both of which play a critical role in the pathogenesis of various diseases [8,9]. An increase in size of fat droplets in hypertrophic adipocytes force compartments of the adipocyte further from blood vessels, and thus the oxygen supply, which causes the adipocyte to become hypoxic and to activate the hypoxia inducible pathway, hypoxic stress [10]. Furthermore, over-nutrition, an increased demand on synthetic machinery, and an imbalance of endoplasmic reticulum (ER) calcium levels leads to the accumulation of unfolded or misfolded proteins, which could give rise to perturbations in the ER lumen and create stress, called ER stress [4,11]. Hypertrophic insulin resistant adipocytes are therefore prone to numerous stresses, with various dysfunctional/damaged molecules, organelles, proteins, and end-products accumulating.

To mitigate possible damage, accumulated molecules should be removed. There are two major systems (the autophagy lysosome system and the ubiquitin proteasome system) employed for such intracellular clean up. At least three autophagy forms have been identified: (1) chaperone-mediated autophagy; (2) microautophagy; and (3) macroautophagy, each differing in their physiological functions and the mode of cargo delivery. In this study we focused on macroautophagy (referred to in this paper as autophagy).

Autophagy, which can be induced by starvation, is an essential process by which cells break down their own components, however, homeostatic low levels of autophagy is important for the regular maintenance and disposal of intracellular organelles and proteins [12]. Suppression of basal autophagy in neural cells causes neurodegenerative disease [13], a phenomenon which has also been observed in other cell types [14]. Since the role of autophagy in matured adipocytes remains unknown, we hypothesized that autophagy could play a role in regulating the maintenance of accumulated molecules, with subsequent effects on inflammation in hypertrophic adipocytes; and that decreased autophagy would unavoidably slow down the removal of dysfunctional/damaged molecules, organelles, proteins and end-products, the accumulation of which contributes to cellular stresses and leads to insulin resistance.

In this study we demonstrate that autophagy is down-regulated in adipose tissue of high fat diet (HFD) mice. This was recapitulated

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in vitro, as autophagy was suppressed in hypertrophic 3T3-L1 adipocytes. Furthermore, we show that inhibition of autophagy leads to inflammatory responses through ER stress. Our results indicate that autophagy plays a role in the pathogenesis of insulin resistance in adipocytes.

## 2. Materials and methods

### 2.1. Materials

Anti-light chain (LC) 3 antibodies were from Novus Biologicals Inc. (Littleton, CO). The anti-phospho-PERK (Thr980), anti-PERK, anti-eIF2α (Ser51), anti-eIF2α, and anti-CHOP antibodies were from Cell Signaling (Beverly, MA). SP600125, SB203580, PD98059, and Tauroursodeoxycholic Acid (TUDCA) were from Calbiochem (San Diego, CA). Horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Dulbecco's Modified Eagle's medium and fetal calf serum were obtained from Life Technologies, Inc. (Grand Island, NY). All other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Animals

All procedures were performed in accordance with the guidelines of the Research Center for Animal Life Science of Shiga University of Medical Science. GFP-LC3 transgenic (TG) mice [15] were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Male GFP-LC3-TG littermates were fed a normal chow (NC) (13.5% fat) or HFD (60% fat) ad libitum for 16 weeks from 8 weeks of age. Mice were anesthetized after a fast of 36 h, and the epididymal fat pad was removed. Fixed adipose tissue, embedded in paraffin, was sectioned at a thickness of 3 μm.

### 2.3. Cell culture

3T3-L1 adipocytes were provided by Dr. Jerrold M. Olefsky and were cultured and differentiated as previously described [16]. Hypertrophic 3T3-L1 adipocytes, with larger lipid droplets when cultured up to day 21, were also used [17]. Experiments in this study utilized 3T3-L1 adipocytes cultured after completion of the differentiation process as described [18].

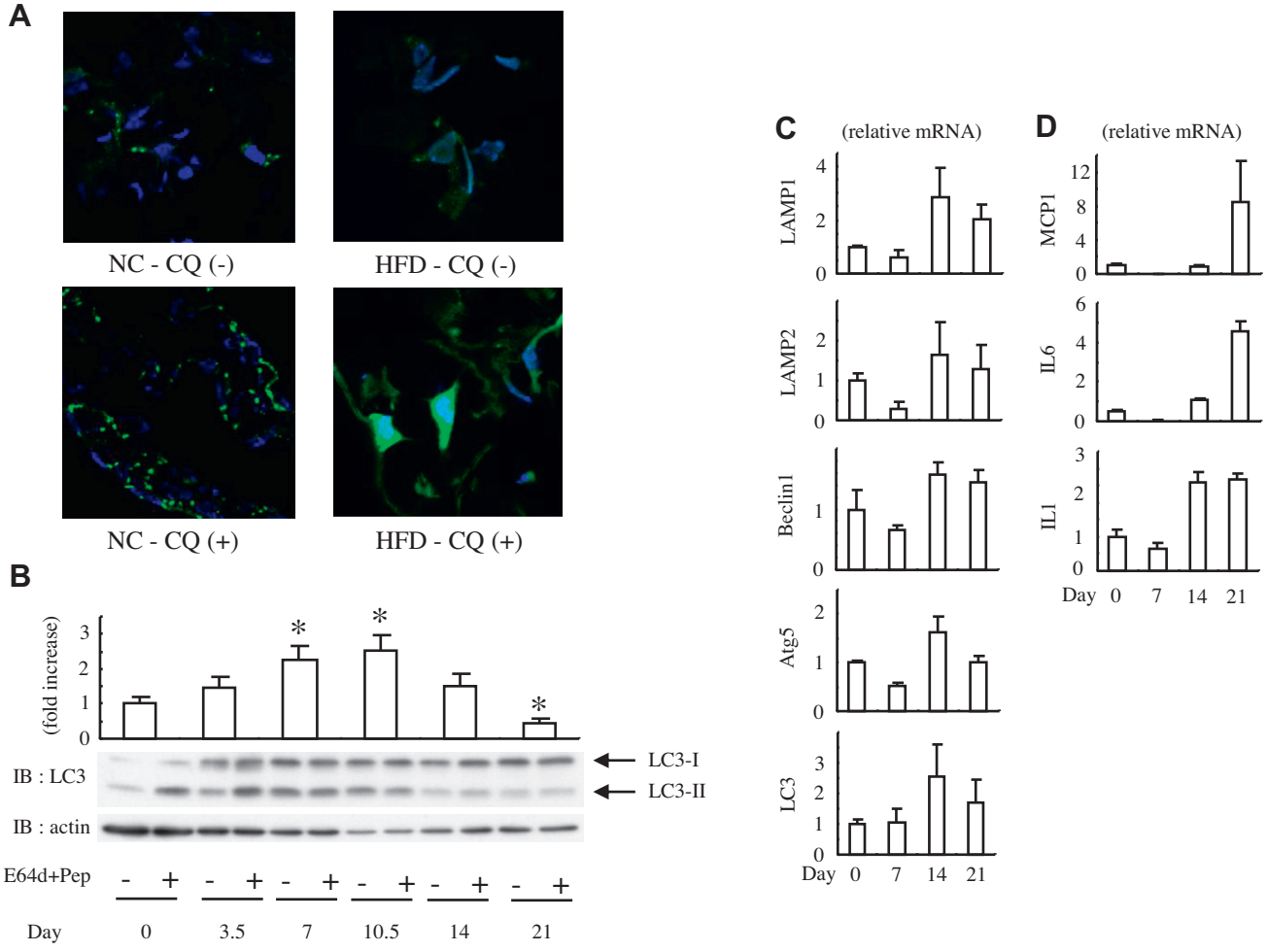


**Fig. 1.** Autophagy is down-regulated in adipose tissue from high fat diet mice and hypertrophic 3T3-L1 adipocytes. (A) GFP-LC3 TG mice were placed on a normal chow diet (NC) or high fat diet (HFD), injected with (+) or without (−) chloroquine (CQ), and the epididymal fat pads were isolated as described under Section 2. Autophagy was directly visualized with confocal microscopy. (B–D) Differentiation of 3T3-L1 fibroblasts started on day 0 and were cultured for 21 days. (B) On each day indicated, cells were pretreated with (+) or without (−) 10 μg/ml pepstatin A (Pep) and 10 μg/ml E64-d for 2 h and lysed, and immunoblotting (IB) was performed with indicated antibodies. The graphs show the mean ± S.E., and the values are expressed as fold increase when compared with those observed in day 0. $^{*}p < 0.05$ day 0 versus day 7, 10.5, and 21. (C and D) On each day indicated, total RNA was purified and quantitative real-time-PCR was performed. Data are presented as the relative expression. Error bars represent the mean ± S.E.

### 2.4. Western blotting

Samples were prepared using the method previously reported [19], and briefly cells were lysed in a solubilizing buffer. The soluble fractions were boiled with Laemmli sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with the previously mentioned antibodies. On phosphorylation and total protein blots, the phosphorylated blotted membrane was stripped and reprobed with an anti-target protein antibody. If the stripped and reblotted membranes did not work adequately, due to different non-specific bands or uneven protein blots (mirror imaging due to insufficient strip), then the same samples were loaded with the same amount of protein per lane on separate gels, and one gel was blotted for the phospho-protein and the other for total protein.

### 2.5. RNA preparation and quantitative RT-PCR

Total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA). Real-time PCR was performed using the 7500 real-time PCR system (ABI Applied Biosystems) and power SYBR Green PCR Master Mix. Gene expression levels were calculated after normalization to the standard housekeeping genes, 18RPS and GAPDH, using the delta/delta Ct methods as described previously [20], and expressed as relative mRNA levels when compared with the internal control. Primer information is available upon request.

### 2.6. Autophagy assays

In vivo, autophagy was visualized using confocal microscopy. LC3-GFP was detected using a 488 nm argon laser and a 515–540 nm band pass filter. In vitro, autophagy was monitored by LC3 blotting. To detect autophagy by Western blot analysis, the cells were treated with E64-d and pepstatin A to inhibit lysosomal enzyme activity [21].

### 2.7. XBP assays

Total RNA was prepared and cDNA was synthesized as described in the RNA preparation section (above). Amplification conditions were: a single cycle at 94 °C for 4 min followed by 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final single cycle of 72 °C extension for 10 min. PCR products were separated by electrophoresis in 2% agarose gel.

### 2.8. Statistics analysis

All values are expressed as mean ± standard error (S.E.). Statistical significance between two groups was determined using the Student's *t* test. A *p*-value of less than 0.05 was considered significant.

## 3. Results

### 3.1. Autophagy is suppressed in adipose tissue from high fat-fed mice

In adipose tissue of starved NC mice, GFP-LC3 signals were detected as punctuated dots. No signals were detected in the adipose tissue of HFD mice (Fig. 1A). For enhancement, chloroquine (an inhibitor of lysosomal activity) was administrated, which increased the punctuated dots in adipose tissue of the NC mice (Fig. 1A). In contrast, in adipocytes from the HFD mice, GFP-LC3 signals were only detected diffusely in the cytoplasm (Fig. 1A), indicating that autophagy was suppressed.

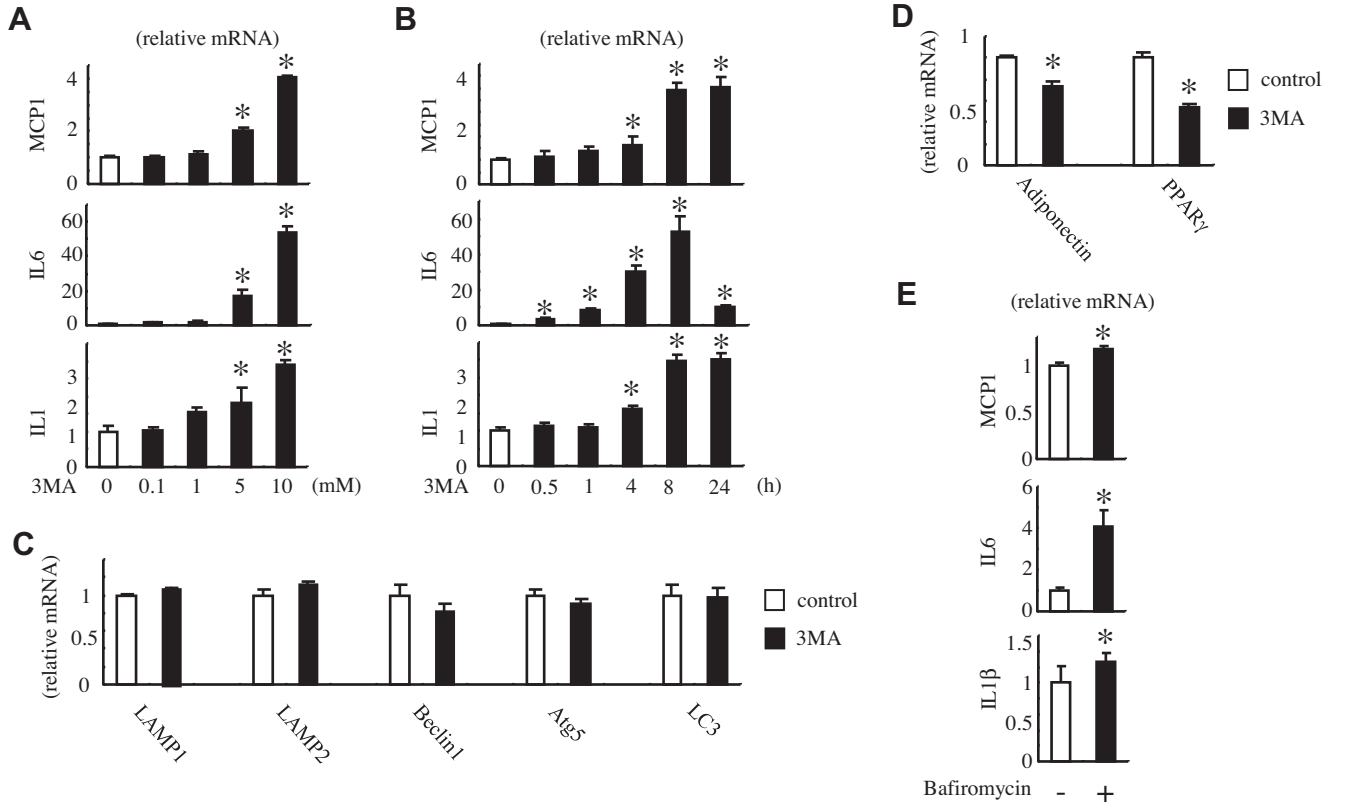


**Fig. 2.** Inhibition of autophagy increases inflammatory responses. The hypertrophic 3T3-L1 adipocytes were pretreated with the concentrations indicated (A) or 10 mM 3-methyladenine (3MA) (B–D) for the indicated time (B), or 8 h (A, C, and D) or 10 nM bafilomycin for 8 h (E). Total RNA was purified and quantitative real-time-PCR performed. Data are presented as the relative expression. Error bars represent the mean ± S.E. $^{*}p < 0.05$ non-treatment versus treatment.

### 3.2. Autophagy is decreased in hypertrophic 3T3-L1 adipocytes

To clarify the in vivo results, we cultured 3T3-L1 adipocytes for 21 days after differentiation and generated hypertrophic 3T3-L1 adipocytes using the method previously reported [17]. These adipocytes displayed insulin resistance, for example, by decreased insulin responsiveness to glucose uptake. Autophagy was measured during the differentiation of 3T3-L1 fibroblasts into hypertrophic adipocytes by LC3 blotting. Autophagy, detected by LC3-II was gradually increased by day 11 following differentiation (Fig. 1B). By day 14, autophagy levels of matured adipocytes were near that of fibroblasts, however, there was a significantly decrease in levels in hypertrophic adipocytes (Fig. 1B).

### 3.3. Expression of inflammatory genes are increased in hypertrophic adipocytes

To further define the hypertrophic adipocytes, we quantified the transcription levels of several autophagy and inflammatory-related genes in hypertrophic 3T3-L1 adipocytes. In matured adipocytes, some autophagy-related genes, such as lysosomal-associated membrane protein 1 (LAMP1), Beclin1, autophagy-related gene 5 (Atg5), and LC3, were increased (Fig. 1C). Compared with matured adipocytes, autophagy-related gene expression in hypertrophic adipocytes showed a decrease but was near the same levels as those of fibroblasts (Fig. 1C). Expansion of adipocytes resulted in enhanced expression of MCP-1, IL-6, and IL-1β (Fig. 1D).

### 3.4. Suppression of autophagy increases inflammatory responses

The findings presented in Fig. 1 raised the question of the potential role of autophagy in inflammatory responses. To assess this we utilized an inhibitor of autophagy, 3-methyladenine (3-MA). Pretreatment with 3-MA clearly increased expression of MCP-1, IL-6, and IL-1β in a dose- and time-dependent manner (Fig. 2A and B, respectively), and without changing autophagy-related gene expression (Fig. 2C). In contrast to pro-inflammatory gene expression, inhibition of autophagy led to a significant decrease in the expression of the anti-inflammatory gene adiponectin and peroxisome proliferator-activated receptor γ (Fig. 2D). Since 3-MA (which is commonly used as a specific inhibitor of autophagy) is an inhibitor of class 3 phosphatidylinositol (PI) 3-kinase, Vps34 [25], we used bafilomycin (which inhibits fusion of autophagosomes with lysosomes [26]) to exclude possible non-specific PI 3-kinase inhibitory effects. Consistent with the effects of 3-MA, pre-treatment with bafilomycin significantly increased expression of MCP-1, IL-6, and IL-1β (Fig. 2E). Together, inhibition of autophagosomal formation, or fusion of autophagosomes with lysosomes, suggests the suppression of autophagy activated inflammatory gene expression in adipocytes.

### 3.5. Inhibition of autophagy increased inflammation via ER stress

Since inflammatory responses are regulated by several signaling pathways, we investigated which pathways were critical for

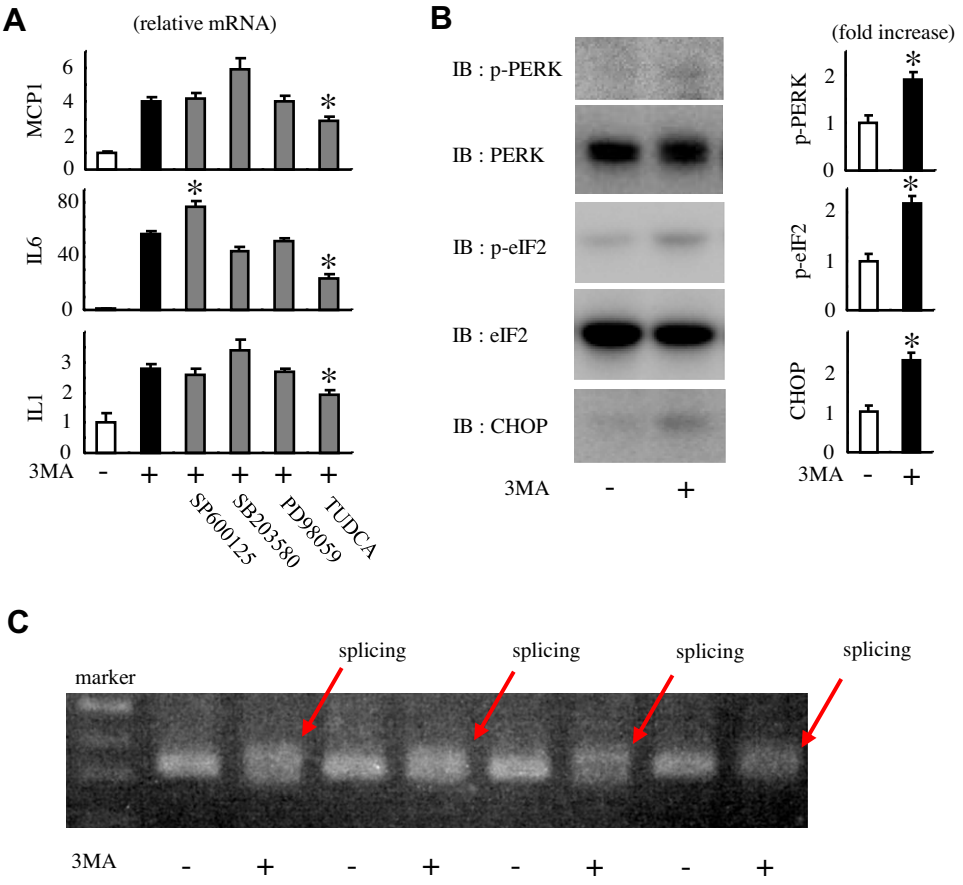


**Fig. 3.** Autophagy inhibition increases inflammatory responses through ER-stress. After pretreatment with 10 μM SP600125, 10 μM SB203580, 50 μM PD98059, or 1 mM Tauroursodeoxycholic Acid (TUDCA) for 1 h, hypertrophic 3T3-L1 adipocytes were stimulated with 10 mM 3-methyladenine (3MA) for 8 h. (A) Total RNA was purified and quantitative real-time-PCR performed. Data are presented as the relative expression. Error bars represent the mean ± S.E. $^{*}p < 0.05$ non-pretreated 3MA-stimulated cells versus treated cells. (B) The cells were lysed and immunoblotting (IB) was performed with the indicated antibodies. The graphs show the mean ± S.E., the values are expressed as fold increase in phosphorylation or expression compared with control cells. $^{*}p < 0.05$ control cells versus 3MA treated cells. (C) Total RNA was purified and XBP assays were performed as described under Section 2.

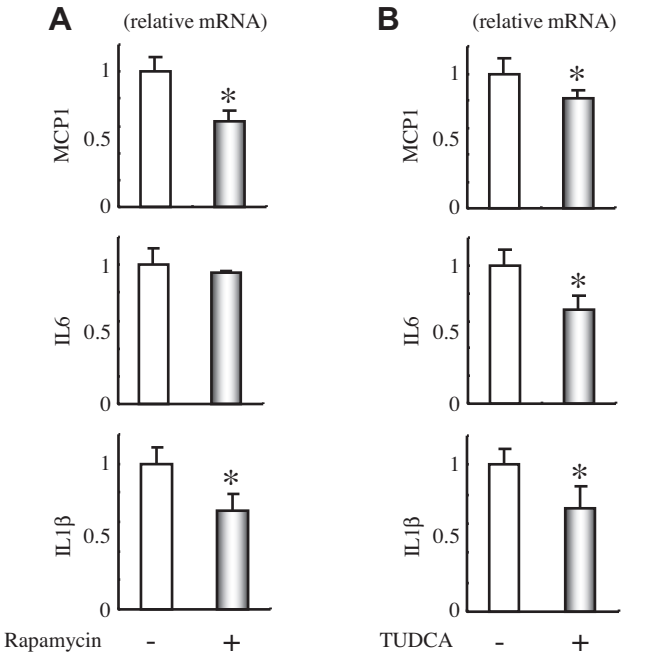


**Fig. 4.** Stimulation of autophagy or inhibition of ER stress decreases inflammatory responses. Hypertrophic 3T3-L1 adipocytes were treated with 100 ng/ml rapamycin (A) or 1 mM Tauroursodeoxycholic Acid (TUDCA) (B) for 8 h. Total RNA was purified and quantitative real-time-PCR performed. Data are presented as the relative expression. Error bars represent the mean ± S.E. $^{*}p < 0.05$ control cells versus treated cells.

regulation of inflammation by autophagy. The inflammatory responses induced by 3-MA were assessed in adipocytes treated with several inhibitors. The treatment of adipocytes with SB203580 (p38 inhibitor) or PD98059 (ERK1/2 inhibitor) did not counteract 3-MA-induced gene expression. SP600125 (JNK inhibitor) showed a slight increase in IL-6 expression, with no change of MCP-1 nor IL-1β (Fig. 3A). Conversely, the ER stress inhibitor TUDCA significantly attenuated the 3-MA-induced MCP-1, IL-6, and IL-1β expressions (Fig. 3A). To further confirm the involvement of ER stress in 3-MA-induced up-regulation of inflammatory responses, we then tested ER stress in adipocytes in which autophagy was suppressed. The ER stress markers [27], phosphorylation of protein kinase-like ER kinase (PERK), eukaryotic translational initiation factor 2α (e-IF2α), and C/EBP homologous protein (CHOP) were determined by Western blot. Treatment with 3-MA clearly increased phosphorylation of PERK and e-IF2α, and expression of CHOP (Fig. 3B). We also measured X-box binding protein (XBP) splicing, which was increased by the unfolded protein response. XBP-1 splicing was significantly increased by treatment with 3-MA (Fig. 3C). These results suggest that inhibition of autophagy induces ER stress and inflammatory responses.

### *3.6. Activation of autophagy or inhibition of ER stress reduced inflammatory gene expression*

To confirm the pro-inflammatory data, we activated autophagy or inhibited ER stress and then tested inflammatory responses. In contrast to treatment with 3-MA, activation of autophagy by rapamycin decreased expression of MCP-1 and IL-1β (Fig. 4A). Treatment with TUDCA alone reduced expression of MCP-1, IL-6, and IL-1β (Fig. 4B).

## 4. Discussion

Autophagy is the only way to degrade large molecules, organelles, proteins, and end-products. Homeostatic autophagy eliminates damaged components and serves an important housekeeping function [12]. Defects in autophagy have been considered as etiology of neurodegeneration, aging, cancer and many other diseases. In insulin resistant adipocytes, various dysfunctional/damaged components are retained and can lead to cellular stresses and inflammation, which cause systemic insulin resistance. Therefore, we hypothesized that autophagy might be down-regulated in cells in an insulin resistant state. However, since insulin inhibits autophagy [28,29], autophagy may be activated by impaired insulin signaling [22]. Whether or not autophagy is suppressed is therefore an important issue to understanding the mechanisms of insulin resistance. In this study we found that autophagy was decreased in adipose tissue from HFD-induced insulin resistant mice as well as in hypertrophic 3T3-L1 adipocytes. It has previously been reported that autophagy is also suppressed in insulin resistant hepatocytes [24,31], a finding that is consistent with our results which suggest that autophagy is down-regulated in insulin resistant adipose tissue and adipocytes.

The results of this study raise the possibility that decreased autophagy leads to the retention of molecules which cause insulin resistance. Adipose tissue specific Atg7 knockout (KO) mice indicate that autophagy is involved in adipogenesis [30,32]. Since the adipose tissue in KO mice has not matured, the possibility of using KO mice to investigate the role of autophagy in mature adipocytes is not suitable, and further studies, such as conditional KO mice studies, should therefore be conducted. Furthermore, KO mice do not display the healthy phenotype, as when they are fed with either NC or HFD, they are at high risk of early death [30]. A defect in autophagy may lead to the accumulation of dysfunctional molecules, which can cause various morbid states and early death. This data supports our hypothesis that the role of autophagy in adipocytes is important for the maintenance of cellular health.

Chronic inflammation plays an important role in the pathogenesis of insulin resistance [33]. Most studies concerned with the connection between autophagy and insulin resistance have focused only on autophagy within insulin target tissues [23,24,30,31]. However, based on the established understanding of inflammation as a cause of insulin resistance, the role of autophagy in regulating inflammation is important. In this regard, we have found that in adipocytes there is a link between defective autophagy and inflammation. Thus, inhibition of autophagy by 3-MA or bafilomycin activated inflammatory responses, which includes increased expression of pro-inflammatory genes and decreased expression of anti-inflammatory genes. The mechanisms of autophagy repressive effects on adipocyte inflammation are of interest. Therefore, on the basis of our inhibitors study, we suggest that ER stress plays a role in 3-MA-induced inflammatory responses. Such a suggestion concurs with a report that highlighted that defective hepatic autophagy promotes ER stress [31]. Additionally, our results suggest that both activation of autophagy by rapamycin and inhibition of ER stress by TUDCA reduce inflammation in 3T3-L1 adipocytes, findings that further confirm that suppression of autophagy increases inflammation. We therefore believe an important role of autophagy is as an anti-inflammatory regulator within adipocytes.

To conclude, the results of this study demonstrate that autophagy is suppressed in insulin resistant adipocytes, and that suppression of autophagy leads to inflammatory responses via ER stress. Although various stresses have been identified as causing insulin resistance, we propose that decreased autophagy in adipocytes,

which we refer to as Autophagic Stress, is one of the important underlying causes of adipocyte inflammation and insulin resistance. Therapeutic strategies to activate autophagy in adipocytes may therefore provide a new approach to prevent insulin resistance and its related diseases.

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