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Autophagosomes accumulate in differentiated and hypertrophic adipocytes in a p53-independent manner

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ABSTRACT

Autophagy is induced by several kinds of stress, including oxidative, genotoxic, endoplasmic reticulum and nutrient stresses. The tumor suppressor p53, which is a stress sensor, plays a critical role in the regulation of autophagy. Although p53 is required for starvation (nutrient deficient stress)-induced autophagy, it is still not clear whether p53 is also required for the autophagy observed in differentiated and hypertrophic adipocytes, which accumulate excessive amounts of nutrients in the form of triglycerides. In this study, we demonstrated that starvation induces autophagy in p53-proficient adipocytes, but not in p53-deficient adipocytes as previously reported. On the other hand, autophagy was equally observed in both p53-deficient and -proficient differentiated and hypertrophic adipocytes. Similar results were obtained by *in vivo* analysis using white adipose tissue of high-fat diet-induced obese mice. Moreover, unexpectedly, the autophagy observed in the differentiated and hypertrophic adipocytes involved increased accumulation of autophagosomes and decreased autophagic flux. Thus, we concluded that in differentiated and hypertrophic adipocytes autophagosomes accumulate in a p53-independent manner, and this accumulation is caused by reduced autophagic flux.

1. Introduction

Autophagy is a major cytosolic catabolic process operating through the lysosomal machinery, and playing an important role in cellular and/or organism homeostasis against diverse pathologies [1–4]. In this process, autophagy is initiated by autophagosome formation, surrounding cytoplasmic components with a double-membrane. Then, the autophagosome fuses with a lysosome to form an autolysosome and subsequently degrades the intramembrane contents. During autophagosome formation, microtubule-associated protein 1–light chain 3 (LC3) is lipidated, converting LC3-I (non-lipidated form) to LC3-II (lipidated form). Autophagy is the key machinery for the turnover of cellular components and/or proteins including p62, which seems to be a selective substrate for autophagy [2–4]. Therefore, the conversion of LC3-I to LC3-II, the aggregation of LC3-II and the degradation of p62 are hallmarks of the autophagic process [2–4].

The tumor suppressor p53 is involved in several cellular stress responses [5]. Recently, it has been reported that p53 plays a critical role in the autophagic process and metabolic process [6–10]. p53 promotes autophagy through inhibition of mammalian target of rapamycin (mTOR) [8]. p53 also transcriptionally promotes the expression of autophagy-regulated genes such as Sestrin2 and DRAM [9,10].

Previously, it has been reported that autophagic vacuoles are frequently found in differentiated and hypertrophic 3T3-L1 adipocytes [11]. Furthermore, targeted deletion of atg5 or atg7, which are necessary for the autophagic process, interferes with normal adipocyte differentiation, suggesting that autophagy regulates adipocyte differentiation and/or lipid accumulation [12–15]. Moreover, inhibition of autophagy increases triglyceride storage in lipid droplets in cultured hepatocytes and mouse liver [16]. Thus, these studies indicate that autophagy is involved in adipogenesis and lipid metabolism.

In the present study, to understand the molecular basis of autophagy observed in differentiated and hypertrophic adipocytes, we investigated whether p53 is required for autophagy in differentiated and hypertrophic adipocytes associated with excessive nutrient accumulation using both *in vitro* and *in vivo* models. Moreover, we evaluated whether the autophagic machinery is fully activated in differentiated and hypertrophic adipocytes *in vitro*.

2. Materials and methods

2.1. Animals

Experiments on mice were conducted in accordance with the provisions of the Ethics Review Committee for Animal

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Experimentation at Tokyo University of Science. p53 heterozygous knockout ($p53^{+/-}$) mice with C57BL/6J background (Accession Number, CDB0001K) were purchased from RIKENBRC (Saitama, Japan). These mice were intercrossed to obtain wild-type ($p53^{+/+}$) and homozygous knockout ($p53^{-/-}$) mice. Male $p53^{+/+}$ and $p53^{-/-}$ mice were weaned four weeks after birth. Mice were housed in a temperature-controlled environment with a 12-h light/dark cycle and free access to water and a normal-fat diet (NFD; NOSAN, Kanagawa, Japan) or high-fat diet (HFD; CREA, Tokyo, Japan) for nine weeks. Mice were sacrificed at 13 weeks of age and the epididymal white adipose tissue (WAT) was collected.

2.2. Cell lines and reagents

The p53-proficient preadipocyte cell line, 3T3-L1, was purchased from RIKENBRC. The p53-deficient preadipocyte cell line, HW, was kindly provided by Dr. Masayuki Saito (Tenshi University, Japan). Primary mouse embryonic fibroblasts (MEFs) derived from wildtype ($p53^{+/+}$) and p53-knockout ($p53^{-/-}$) mice were established as previously described [17].

Etoposide and camptothecin were purchased from WAKO (Osaka, Japan). Rapamycin and bafilomycin A1 were purchased from LC Laboratories (Woburn, MA, USA).

2.3. Vector construction

The pMXs-puro (-U3) –Cul2 and pMXs-puro (-U3) Kpc1 vectors were kindly gifted by Dr. Takumi Kamura (Nagoya University, Japan). To construct the backbone vector, pMXs-puro-mU6 [18], the mouse U6 gene promoter was obtained from the pMXs-puro (-U3) Kpc1 vector by PCR, using the following primers: forward – 5′-GGCAAAACTCGAGTTCGAACGCGTGATCAATTGTTTAAACAAGGCT TTTCTCCAGGGATATTTATAGTA-3′, and reverse – 5′-GTCGAC-CACTGTGCTGGC-3′. The PCR product was digested with NotI/XhoI and subcloned into the NotI/XhoI sites of the pMXs-puro (-U3) –Cul2 vector, yielding the pMXs-puro-mU6 shRNA vector. We designed a mouse p53 shRNA expression vector based on target sequences for effective p53 knockdown as previously reported [19]. The oligonucleotides for shp53 and the shGFP control were chemically synthesized (Operon Biotechnology, Tokyo, Japan) as follows. shp53-1: 5′-GTACGTGTGTAGTAGCTTCttcaagagaGGAGC-TATTACACATGTACttttt-3′ and 5′-cgaaaaaGTACATGTGTAATAGCTCC tctcttgaaGAAGCTACTACACACGTAC-3′; shp53-2: 5′-GGAGTAGGTT GGTAGTTGTTATTCAAGAGATGACAACTATCAACCTATTCCCCttttt-3′ and 5′-cgaaaaaGGGGAATAGGTTGATAGTTGTCATCTCTTGAATA ACAACTACCAACCTACTCC-3′; shGFP: 5′-GGCTATGTCCAGGGGCG-CATCttcaagagaGGTGCGCTCCTGGACGTAGCCttttt-3′ and 5′-cgaaaaa GGCTACGTCCAGGAGCGCACCtctcttgaaGATGCGCCCCTGGACATAGC C-3′ (upper case letters, target sequences against p53 or GFP; lower case letters, BstBI or loop structure sequences). The annealed oligos were directly ligated into a PmeI and BstBI-digested pMXs-puro-mU6 shRNA expression vector.

2.4. Establishment of stable p53-knockdown 3T3-L1 preadipocytes

Stable p53-knockdown cell lines were generated using retroviral infection as previously reported [20]. The produced vectors, termed pMXs-puro-mU6-shp53 or shGFP, were transfected into Plat-E cells with FuGENE®6 (Promega, Madison, WI, USA), according to the manufacturer's protocol. Virus-containing culture supernatants were collected 2 d after the transfection and filtered through 0.22-μm filters (Millipore, Billerica, MA, USA). To obtain stable p53- or GFP-knockdown cell lines (3T3-L1/shp53 or 3T3-L1/shGFP), 3T3-L1 cells were incubated with virus-containing medium for 2 d, followed by selection with 2 μg/mL puromycin for 5 d.

2.5. Cell culture and treatments

Preadipocyte cell lines were maintained in maintenance medium. Preadipocyte cell lines were differentiated as previously described [21]. In brief, cells were seeded to reach confluency after 2 d. At confluence, the maintenance medium was changed to adipocyte differentiation medium (AD medium), and the cells were cultured for another 2 d. For adipocyte maturation, the differentiation-induced cells were grown in adipocyte maturation medium (AM medium), which was changed every other day. The maintenance medium contained 10% FBS (Sigma, Saint Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) in DMEM low glucose (WAKO). AD medium contained 500 μM 3-isobutyl-1-methylxanthine (Sigma) and 1 μM dexamethasone (Sigma) in maintenance medium, and AM medium contained 10 μg/mL insulin (Sigma) and 50 nM tri-iode thyronine (T3; Sigma) in maintenance medium.

To induce adipocyte differentiation in MEFs, MEFs were seeded to reach confluence. At confluence, the maintenance medium was changed to MEF differentiation medium, which contained 500 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/mL insulin and 100 μM troglitazone (WAKO). The medium was changed to fresh MEF differentiation medium every other day.

To induce nutrient starvation, the maintenance medium was changed to DMEM without FBS (serum-free) followed by incubation for 24 h. To induce autophagy via inhibition of mTOR, cells were treated with 500 nM rapamycin for 6 h.

To analyze autophagic flux, adipocytes were differentiated for 3 or 11 d, followed by a medium change with fresh AM medium containing 10 nM bafilomycin A1 (Baf; LC Laboratories) for a 24-h incubation.

2.6. Western blot

Western blot analysis was performed as previously described [17] with the following primary antibodies: LC3 (PM036, MBL, Nagoya, Japan), p62 (PM045, MBL), p53 (PO03, MBL), β-actin (A1978, Sigma) and α-tubulin (T6199, Calbiochem, Darmstadt, Germany). The secondary antibodies used were: horseradish peroxidase-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG or anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA).

2.7. Oil Red O staining

Cells were fixed with 10% neutral-buffered formalin. Fixed cells were washed with 60% isopropanol and stained with 60% isopropanol containing 0.18% Oil Red O for 20 min. Stained cells were washed with 60% isopropanol for 1 min. Images were captured by a BIOREVO BZ9000 fluorescence microscope (KEYENCE, Osaka, Japan).

2.8. Immunocytochemistry and confocal laser microscopy

Cells were seeded on poly-D-lysine-coated coverslips. For LC3 immunocytochemistry, cells were fixed in 4% paraformaldehyde and permeabilized with PBS containing 0.2% Triton-X 100 for 10 min. After washing with PBS containing 0.1% Tween 20 (TPBS), cells were blocked in TPBS containing 2% BSA and 5% goat serum. Following washes with TPBS, cells were incubated with LC3 antibodies in a moist chamber overnight at 4 °C. Next, cells were washed with TPBS and probed with the secondary antibody, Alexa Fluor 488-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), for 30 min at room temperature. Confocal fluorescence images were captured using an LSM5Pascal Exciter laser-scanning microscope (Zeiss, Oberkochen, Germany).

2.9. Transmission electron microscopy analysis

Differentiated adipocytes were fixed with 2.5% gluteraldehyde in 0.2 M cacodylate buffer for 60 min, followed by incubation with 1% osmium tetroxide for 30 min. The samples were embedded in Epon. Selected areas were then sectioned with a Sorvall ultramicrotome MT-2 (Leica Mikrosystem GmbH, Vienna, Austria). Sections were observed under a JEM 1200EX II electron microscope (JEOL LTD, Tokyo, Japan) at 90 kV accelerating voltage.

2.10. Statistical analysis

Statistical analysis was performed by Tukey–Kramer test or Student's *t*-test (comparison of two means). The data are presented as mean ± standard deviation (S.D.). A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. p53 is required for starvation-induced autophagy in 3T3-L1 and HW adipocytes

Treatment with the topoisomerase II inhibitor, etoposide, activated p53 in 3T3-L1 ($p53^{+/+}$) preadipocytes, but not in HW ($p53^{-/-}$) preadipocytes (Fig. 1A). mTOR plays a central role in the regulation of autophagy, and rapamycin induces autophagy via inhibition of mTOR [22]. In both 3T3-L1 and HW cells, 4 d after the induction of differentiation (day 4 adipocytes) rapamycin induced the conversion of LC3-I to LC3-II (Fig. 1B and C). In contrast, nutrient starvation (serum-free) induced the LC3 conversion and p62 degradation in day 4 3T3-L1 adipocytes, but not in day 4 HW adipocytes (Fig. 1D and E). Using confocal fluorescence microscopy analysis we observed that rapamycin treatment markedly increased LC3-positive puncta in both day 4 3T3-L1 and HW adipocytes. In contrast, after nutrient starvation LC3-positive puncta were increased in 3T3-L1 adipocytes but not in HW adipocytes (Fig. 1F). In agreement with previous reports [9,23], these findings suggest that p53 is necessary for starvation-induced autophagy in adipocytes.

3.2. p53 does not influence autophagy observed in differentiated and hypertrophic adipocytes

Oil Red O staining showed that 3T3-L1 and HW adipocytes accumulated TG (triglyceride) during adipocyte differentiation (Fig. 2A). Next, we investigated whether p53 participates in autophagy found at the late stage of adipocyte differentiation. The expression level of LC3-II in day 4 3T3-L1 and HW adipocytes was markedly suppressed compared with 3T3-L1 and HW preadipocytes, probably due to the medium change to insulin and tri-iode thyronine (T3) containing medium. The expression level of LC3-II in both 3T3-L1 and HW cells was similarly and significantly increased at the late stage of adipocyte differentiation (day 12 hypertrophic adipocytes) compared with day 4 adipocytes (Fig. 2B and C). In addition, LC3-positive puncta were found in both day 12 hypertrophic 3T3-L1 and HW adipocytes (Fig. 2D). Using electron microscopy several autophagosomes were observed in both day 12 hypertrophic adipocytes (Fig. 2E). These findings suggest that autophagy might be activated in differentiated and hypertrophic adipocytes in a p53-independent manner.

To confirm that the autophagy found in differentiated and hypertrophic adipocytes is p53-independent, we established stable shp53 3T3-L1 preadipocytes (3T3-L1/shp53). In 3T3-L1/shp53 preadipocytes, the camptothecin-induced p53 activation was markedly suppressed compared with 3T3-L1/shGFP preadipocytes (Fig. 3A). The expression of LC3-II and p62 was similarly increased during adipocyte differentiation in both 3T3-L1/shGFP and 3T3-L1/shp53 adipocytes (Fig. 3B). To further examine whether the autophagy observed in differentiated and hypertrophic adipocytes is p53-independent, primary mouse embryonic fibroblasts (MEFs) derived from wild type ($p53^{+/+}$) and p53-knockout ($p53^{-/-}$) mice were established. Oil Red O staining showed that both MEFs accumulated TG similarly after the induction of adipocyte differentiation for 20 d (Fig. 3C). Furthermore, the conversion of LC3-I to LC3-II was equally enhanced with the increased TG accumulation (Fig. 3D). These findings suggest that p53 does not influence the autophagic machinery observed in differentiated and hypertrophic adipocytes.

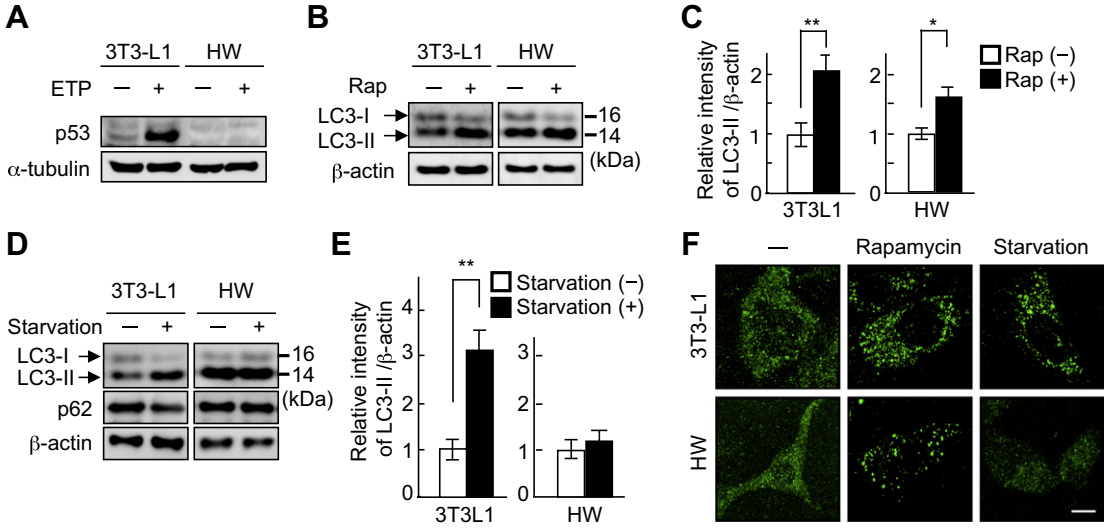

Fig. 1. p53 is required for starvation-induced autophagy. (A) 3T3-L1 and HW preadipocytes were treated with 100 μM etoposide (ETP) for 24 h. Cells were lysed and immunoblot analysis was performed using anti-p53 and anti-α-tubulin antibodies. α-Tubulin was used as a loading control. (B–E) 3T3-L1 and HW adipocytes (day 4 adipocytes) were treated with 500 nM rapamycin (Rap) for 6 h (B, C) or serum-free medium (Starvation) for 24 h (D, E). Cells were lysed and immunoblot analysis was performed with anti-LC3, anti-p62 and anti-β-actin antibodies. β-Actin was used as a loading control. The results are expressed as the relative intensity of LC3-II/β-actin compared with control cells. Values are mean ± S.D. (experiments were performed in duplicate with three independent cultures per experiment). Differences between values were analyzed by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$. (F) Immunofluorescence analysis of LC3 was performed on day 4 adipocytes. Scale bar 20 μm.

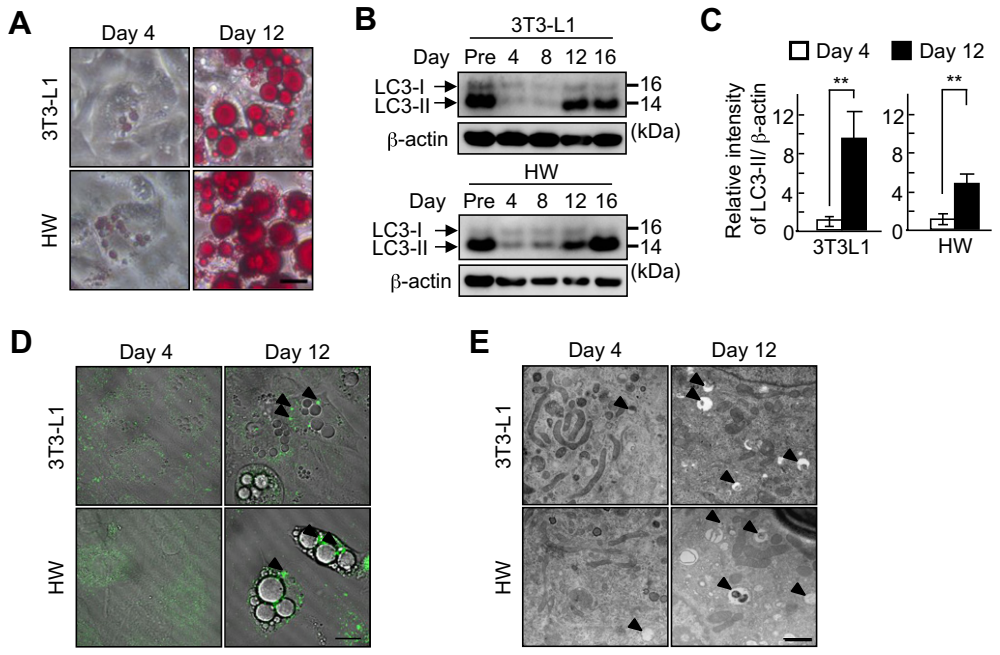

Fig. 2. Autophagy is induced in differentiated and hypertrophic 3T3-L1 and HW adipocytes. (A) Days 4 and 12 3T3-L1 and HW adipocytes were stained with Oil Red O. Scale bar 20 μm. (B) Differentiated adipocytes at the indicated time points were harvested and immunoblot analysis was performed with anti-LC3 and anti-β-actin antibodies. β-Actin was used as a loading control. (C) The results are expressed as the relative intensity of LC3-II/β-actin in day 12 hypertrophic adipocytes compared with day 4 adipocytes. Values are mean ± S.D. (experiments were performed in duplicate with three independent cultures per experiment). Differences between values were analyzed by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$. Immunofluorescence analysis with anti-LC3 antibody (D) and electron microscopy analysis (E) were performed at the indicated time points. The arrowheads point at autophagosomes and/or autolysosomes. Scale bars 20 μm and 1 μm, respectively.

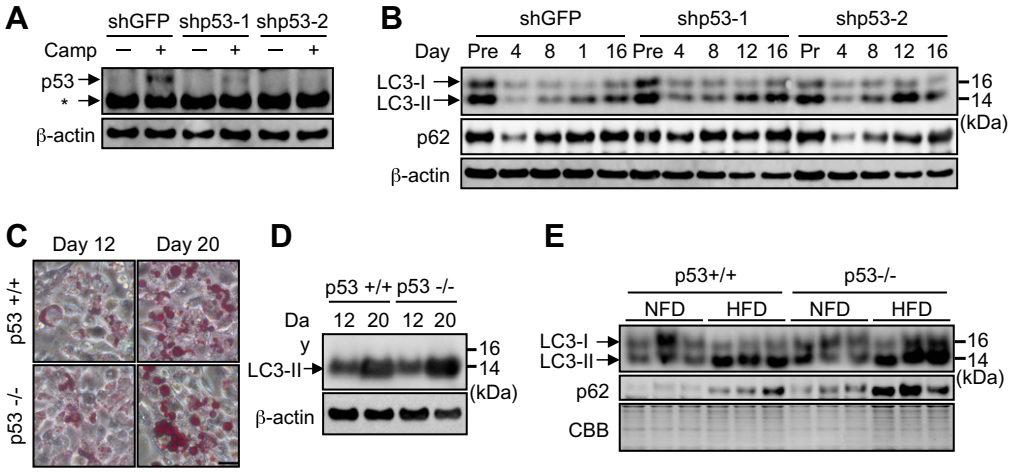

Fig. 3. p53 does not influence autophagy observed in hypertrophic adipocytes and white adipose tissue (WAT) from obese mice. (A) 3T3-L1/shGFP preadipocytes and 3T3-L1/shp53 preadipocytes were treated with 5 μM camptothecin (Camp) for 24 h. Cells were lysed and immunoblot analysis was performed using anti-p53 and anti-β-actin antibodies. β-Actin was used as a loading control. The asterisk indicates nonspecific bands. (B) The differentiated 3T3-L1/shGFP and 3T3-L1/shp53 adipocytes were harvested at the indicated time points and immunoblot analysis was performed with anti-LC3, anti-p62 and anti-β-actin antibodies. β-Actin was used as a loading control. (C, D) Differentiated $p53^{+/+}$ and $p53^{-/-}$ MEFs were stained with Oil Red O staining (C; scale bar 25 μm), or harvested at the indicated time points for immunoblot analysis with anti-LC3 antibody (D). β-Actin was used as a loading control. (E) Representative western blot of LC3. Total protein was extracted from WAT of $p53^{+/+}$ and $p53^{-/-}$ mice fed a normal-fat diet (NFD) or a high-fat diet (HFD) and immunoblot analysis was performed with anti-LC3 and anti-p62 antibodies. CBB stain was used as a loading control. The experiments were performed with 5–6 mice in each group.

To investigate whether p53 influences obesity-induced autophagy *in vivo* as well as *in vitro*, we analyzed the epididymal WAT of wild type ($p53^{+/+}$) and p53-knockout ($p53^{-/-}$) mice, which were fed an NFD or a HFD. HFD increased the body and adipose tissue weight in both mice (data not shown). Previously, it has been reported that obesity increases the expression of p53 in WAT [24,25]. In accordance with these reports, we observed that HFD-induced obesity enhanced p53 expression (data not shown). The conversion of LC3-I to LC3-II and p62 expression were dramatically and equally increased by HFD in both $p53^{+/+}$ and $p53^{-/-}$ mice (Fig. 3E), suggesting that obesity-induced autophagy in mice is regulated in a p53-independent manner as well.

3.3. Autophagic flux is suppressed in differentiated and hypertrophic adipocytes

To clarify whether the autophagic machinery is fully activated in differentiated and hypertrophic adipocytes, we compared

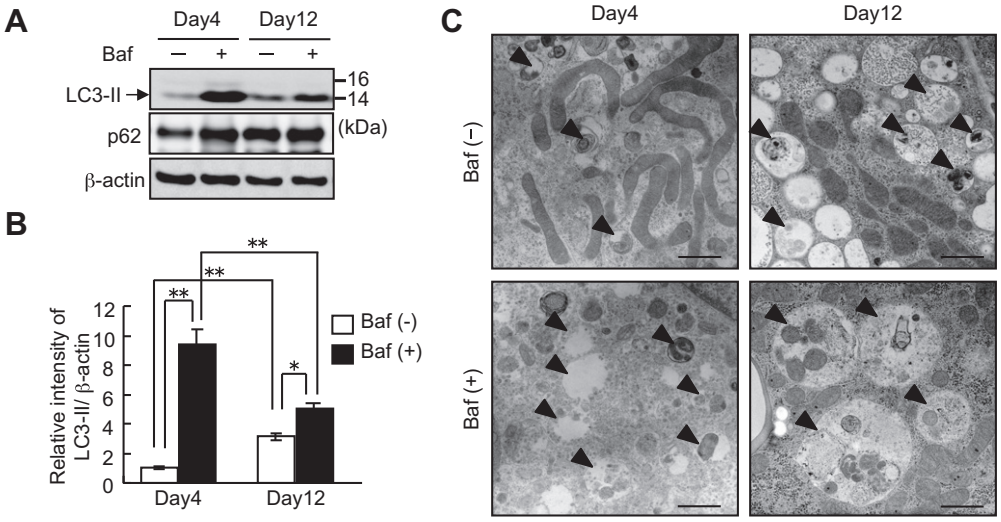

Fig. 4. Autophagic flux is suppressed in differentiated and hypertrophic adipocytes. Day 3 adipocytes and day 11 hypertrophic adipocytes were treated with 10 nM bafilomycin A1 (Baf) for 24 h. (A) Day 4 adipocytes and day 12 hypertrophic adipocytes were harvested and immunoblot analysis was performed with anti-LC3, anti-p62 and anti-β-actin antibodies. β-Actin was used as a loading control. (B) The results are expressed as the relative intensity of LC3-II/β-actin. Values are mean ± S.D. (experiments were performed in duplicate with three independent cultures per experiment). Differences between values were analyzed by Tukey–Kramer test. $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Electron microscopy analysis. The arrowheads point at autophagosomes and/or autolysosomes. Scale bar 1 μm.

autophagic flux of day 12 hypertrophic 3T3-L1 adipocytes with that of day 4 3T3-L1 adipocytes. Treatment with the lysosomal inhibitor, bafilomycin A1 (Baf), significantly increased the expression of LC3-II and p62 in both day 4 adipocytes and day 12 hypertrophic adipocytes (Fig. 4A and B). Unexpectedly, however, the Baf-induced enhancement of the LC3-I to LC3-II conversion and p62 accumulation were lower in day 12 hypertrophic adipocytes than in day 4 adipocytes, suggesting that the autophagic flux was markedly suppressed in day 12 hypertrophic adipocytes compared with day 4 adipocytes (Fig. 4A and B). Using electron microscopy we observed that without Baf treatment day 12 hypertrophic adipocytes contained slightly more autophagosomes than day 4 adipocytes. The autophagosomes' size in day 12 hypertrophic adipocytes was much larger than in day 4 adipocytes (Fig. 4C). After Baf treatment, the autophagosomes' size was markedly increased in both day 4 adipocytes and day 12 hypertrophic adipocytes. Furthermore, regardless of Baf treatment, the autophagosomes containing unspecified structures, which could be undigested contents, were more frequently observed in day 12 hypertrophic adipocytes compared with day 4 adipocytes. Interestingly, unspecified structures were detected in most autophagosomes observed in day 12 hypertrophic adipocytes after Baf treatment (Fig. 4C).

4. Discussion

Previously, it has been reported that p53 is necessary for starvation-induced autophagy in MEFs, HCT116 and Saos-2 cells [7–10]. Herein, we demonstrated that p53 is necessary for starvation-induced autophagy in adipocyte cell lines (3T3-L1 and HW) as well. Electron microscopy analysis revealed that the number of autophagic vacuoles is increased in differentiated and hypertrophic 3T3-L1 adipocytes [11]. However, it has not been clarified yet whether p53 is also required for the autophagy observed in the late stage of adipocyte differentiation. Because differentiated and hypertrophic adipocytes, which accumulate large amounts of TG, may be under stress, we hypothesized that p53 is required for the autophagy observed in differentiated and hypertrophic adipocytes *in vitro* and *in vivo*. Unexpectedly, however, our findings suggest that autophagy observed in differentiated and hypertrophic adipocytes is p53-independent.

Here, we also evaluated the autophagic flux of day 4 adipocytes and day 12 hypertrophic adipocytes, and found that it was markedly suppressed in day 12 hypertrophic adipocytes compared with day 4 adipocytes (Fig. 4A and B). The autophagic machinery consists of three major steps, autophagosome formation, autolysosome formation and degradation of autolysosome contents. During the autolysosome formation, the autophagosome fuses with a lysosome containing many hydrolases. Lieberman et al. [26] hypothesized that lysosomal dysfunction could be seen primarily as autophagy disorder in several types of lysosomal storage diseases. Our electron microscopy analysis of day 12 hypertrophic adipocytes clearly indicated that the autophagosome increases in size and certain structures remain undigested in the autophagosome, and that Baf-treatment enhances the hypertrophy-associated changes. Based on the above-mentioned hypothesis, our data suggest that either autolysosome formation or lysosome function may be impaired in differentiated and hypertrophic adipocytes. Recently, using GFP-LC3 transgenic mice with the lysosome inhibitor, chloroquine, it has been shown that autophagy is suppressed in obese WAT. Moreover, autophagy regulates the inflammatory response in hypertrophic adipocytes [27]. Therefore, we suggest the possibility that autolysosome formation and/or lysosome functions may be impaired in hypertrophic adipocytes *in vitro* and *in vivo*, and the autophagic machinery may be a novel therapeutic target for adipocyte inflammation in obesity and type 2 diabetes.

Acknowledgments

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