Excess Peroxisomes Are Degraded by Autophagic Machinery in Mammals

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Peroxisomes are degraded by autophagic machinery termed “pexophagy” in yeast; however, whether this is essential for peroxisome degradation in mammals remains unknown. Here we have shown that Atg7, an essential gene for autophagy, plays a pivotal role in the degradation of excess peroxisomes in mammals. Following induction of peroxisomes by a 2-week treatment with phthalate esters in control and Atg7-deficient livers, peroxisomal degradation was monitored within 1 week after discontinuation of phthalate esters. Although most of the excess peroxisomes in the control liver were selectively degraded within 1 week, this rapid removal was exclusively impaired in the mutant liver. Furthermore, morphological analysis revealed that surplus peroxisomes, but not mutant hepatocytes, were surrounded by autophagosomes in the control. Our results indicated that the autophagic machinery is essential for the selective clearance of excess peroxisomes in mammals. This is the first direct evidence for the contribution of autophagic machinery in peroxisomal degradation in mammals.

Reorganization of organelles constitutively or suddenly occurs in eukaryotic cells as an adaptation to environmental changes accompanying the cell cycle, development, and differentiation. Indeed, peroxisome proliferators increase the size, number, and enzymes involved in fatty acid metabolism: e.g. peroxisomal thiolase (PT), peroxisomal bifunctional protein (BF), and fatty acid β-oxidation of peroxisomes. However, the mechanistic basis of peroxisome turnover remains poorly understood.

In yeast species, such as Pichia pastoris, Hansenula polymorpha, Candida boidinii, and Saccharomyces cerevisiae, proliferating peroxisomes are degenerated by an autophagy-related process named pexophagy during glucose or ethanol adaptation (10–14). Yeast genetics of pexophagy revealed that most autophagy-related (Atg) genes play dispensable roles in this selective degradation of peroxisomes as well as autophagy (8, 9, 13, 15, 16). In mammals, however, whether or not the autophagic machinery is involved in the degradation of excess peroxisomes biosynthesized in response to drug cues remains a mystery. In particular, there is no direct evidence for the degradation of disused peroxisomes by the autophagic machinery, and it is not clear whether such a degradation process, if any, is selective or non-selective. It has also been reported that selective degradation of mitochondria may occur via autophagy-related mechanism in yeast (17, 18). Therefore, selectivity in the organelle turnover via autophagy is an important issue.

Among the many Atg genes that regulate autophagy, Atg7, which encodes a ubiquitin-activating enzyme (E1)-like enzyme common to two ubiquitilation-like conjugations, the LC3 (Atg8 in yeast) and Atg12 conjugation systems, is a critical gene for autophagosome formation in yeast and mammalian cells (19–26). It has been reported that in yeast, Atg7/Atg8/Gsa7 is essential for pexophagy in addition to autophagy (19, 22, 24). During mammalian autophagy, LC3-I (a cytosolic form of LC3) is lipidated to LC3-II (its autophagosome membrane-bound form) by Atg7 (an E1-like enzyme) and Atg3 (a ubiquitin carrier protein (E2)-like enzyme) (21, 27). Recently, we have established conditional knock-out mice of Atg7 and have shown that Atg7 is indispensable for mammalian autophagy and that the autophagy deficiency in liver leads to marked accumulation of cytoplasmic proteins (20). In the normal liver, LC3 is continuously synthesized to form LC3-I, and LC3-I is subsequently conjugated with phosphatidylethanolamine to form LC3-II during autophagy. LC3-II is then recruited to autophagosomal membranes (21, 28), and the autophagosomal LC3-II is rapidly degraded after fusion of autophagosomes with lysosomes (29). This dynamic flow of LC3 is completely inhibited in Atg7-deficient liver and, as a consequence, more LC3-I accumulates in the mutant liver (20). Considering that deletion of yeast Atg7/Gsa7 gene results in a defect of pexophagy in P. pastoris (24), the liver-specific Atg7-conditional knock-out mice will be an advantageous tool in investigating the degradation of peroxisomes in mammals.

In this study, we analyzed the clearance of surplus peroxisomes using the conditional-knock-out mice of Atg7 (20). The results indicated that autophagy is essential for the degradation of accumulated peroxisomes in the mouse liver.

EXPERIMENTAL PROCEDURES

Reagents—Phthalate esters (diethylhexyl phthalate (DEHP)), corn oil, and leupeptin were purchased from Sigma.

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FIGURE 1. The recovery process of excess peroxisomes induced by DEHP treatment. A, wild-type mice were treated with DEHP for 2 weeks (2 w DEHP) and then chased for 1 week (2 w DEHP + 1 w). Untreated and treated mice were dissected, and liver homogenates were fractionated into MLP, microsomal (Ms), and cytosolic (Cyt) fractions. The protein amount in each fraction was measured. Data are mean ± S.D. values of five mice in each group; *, p < 0.02 and ***, p < 0.001. B, wild-type mice were treated as described in A. The vehicle
Animals and Treatment Regimen—C57Bl6 mice were used as wild-type mice. Male mice received DEHP (1,150 mg/kg/day) or vehicle (corn oil, 5 ml/kg/day) via subcutaneous injection for 2 weeks, and the mice were subsequently fed on a normal diet for 1 week to investigate the changes in proliferated peroxisomes during the recovery process according to the protocol reported previously (7). For detection of autophagosomes by electron microscopy, mice were injected with leupeptin (2 mg/100 g of body weight) after administration of DEHP. All animals were sacrificed by deep anesthesia.

Deletion of Atg7 in Mouse Liver—Atg7 conditional knock-out mice and the heterozygotes were prepared as described previously (20). Briefly, creatine expression in the liver was induced by intraperitoneal injection of polyinosinic acid-polycytidylic acid (pIlC). pIlC was injected three times at a 48-h interval.

Preparation of the Fractions—Livers from Atg7+/+:Mx1 and Atg7−/−: Mx1 mice were treated with DEHP or corn oil for 2 weeks, and at 1 week after treatment, they were dissected. Subfractionation of the livers was accomplished by differential centrifugation according to the method of de Duve et al. (30). Briefly, 20% homogenates were prepared in 0.25 M sucrose, 10 mM HEPES-NaOH, pH 7.4 (homogenizing buffer). The homogenate of the liver was centrifuged at 650 x g for 5 min to remove nuclei and unbroken cells. The pellets were resuspended in the same volume of homogenizing buffer and were then centrifuged. The supernatants from these two centrifugations were combined and used as postnuclear supernatant fractions. Postnuclear supernatant fractions were centrifuged at 10,000 x g for 20 min, and pellets were used as the mitochondrial/lummosomal/peroxisomal (MLP) fractions. The post-MLP supernatants were further centrifuged at 105,000 x g for 60 min to precipitate microsomal fractions in pellet form. All procedures were performed at 4°C.

Immunoblot Analysis—Immunoblotting was performed as described previously (19). The antibody against Mn2+-superoxide dismutase was kindly provided by Prof. Naoyuki Taniguchi (Osaka University, Japan). The antibodies for Atg7 (19), LC3 (20), BF (31), PT (32), and the β-subunit of ATP synthase (33) were prepared as described previously. The antibodies against tubulin and BiP were purchased from Chemicon International, Inc. (Temecula, CA) and Affinity BioReagents, Inc. (Golden, CO), respectively.

Histological Examination—Livers were dissected, fixed in 4% paraformaldehyde, frozen, embedded, and sectioned. For immunohistochemical analysis, the sections were blocked with 5% normal goat serum in phosphate-buffered saline containing 0.2% Triton X-100 and then incubated with anti-PT antibody and Alexa Fluor 488-labeled secondary antibody (Molecular Probes, Eugene, OR). Fluorescence images were obtained using a fluorescence microscope (Q550FV; Leica, Germany) equipped with a cooled charge-coupled device camera (CTR MIC; Leica). Pictures were taken using Leica Qfluoro software (Leica).

Electron Microscopy—Livers were perfusion-fixed with the fixative through the portal vein for 10 min. The fixative consisted of 2% paraformaldehyde, 1% glutaraldehyde, and 0.1 M HEPES-KOH buffer (pH 7.4). To visualize peroxisomes, some liver slices were incubated in alkaline 3,3-diaminobenidine medium consisting of 2 mg/ml 3,3-diaminobenidine, 0.02% hydrogen peroxide, and 0.2 M glycine-NaOH buffer (pH 10.0) for 1 h at room temperature. Then they were postfixed with 1% reduced osmium tetroxide for 1 h. The other tissue slices were postfixed in 1% reduced osmium tetroxide with 3,3-diaminobenidine reaction. All tissue slices were then dehydrated in graded series of ethanol and embedded in Epon. Thin sections were cut with a diamond knife using an ultramicrotome (Reichert, Vienna, Austria). Sections were contrasted with 40 mM lead citrate for 5 min and examined with a Hitachi H7500 electron microscope (Hitachi, Tokyo, Japan).

Quantitative Analysis of Peroxisomes—For each tissue slice, 20 digital electron micrographs were acquired at ×5,000 magnification, enlarged 2.7-fold, and printed by a laser printer. Using the printed figure, we measured the area of peroxisomes and that of the cytoplasmic area of hepatocytes using a SigmaScan scientific measurement system equipped with a computer (Jandel Scientific, San Rafael, CA). The relative total area of peroxisomes was calculated using the following formula: (number of peroxisomes in the average area of peroxisomes/cytoplasmic area) and expressed in μm2/100 μm2 of cytoplasmic area.

Statistical Analysis—The statistical significance of differences between experimental and control groups was determined by the two-tailed Student’s t test. A p value of <0.05 was considered statistically significant.

RESULTS

Selective Degradation of Excess Peroxisomes—Phthalate ester (DEHP) and its active metabolite mono-ethylhexyl phthalate can cause marked increases in both the size and the number of peroxisomes and induce peroxisomal enzymes in the liver (7). Utilizing these phenomena, we first investigated the specific proliferation of peroxisomes and the rapid recovery after removal of the drugs in mice. Wild-type mice were treated with DEHP for 2 weeks and then chased for 1 week as described under “Experimental Procedures.” The mice were dissected at each period, and the liver cell lysates were fractionated into MLP, microsomal, and cytosolic fractions. DEHP administration for 2 weeks was associated with about 2-fold increase in the amount of total protein in MLP, but not in microsomal or cytosolic fractions, as compared with untreated mice, and the amount almost returned to the basal level at 1 week after discontinuation of DEHP (Fig. 1A). These changes were not observed in mice treated with the vehicle (data not shown). Quantitative densitometric analysis of immunoblotting data revealed that PT and BF, marker proteins of peroxisomes, increased significantly after administration of DEHP but not the vehicle, and both diminished significantly to basal levels at 1 week after DEHP discontinuation (Fig. 1, B and C). In comparison, the levels of mitochondrial proteins, the β-subunit of ATP synthase and manganese superoxide dismutase, and the endoplasmic reticulum marker, BiP, remained unchanged during the same manipulations (Fig. 1B). Immunofluorescence analysis using anti-PT antibody revealed that a 2-week administration of DEHP, but not the vehicle, resulted in the appearance of numerous dots representing peroxisomes, and most of these dots disappeared at 1 week after discontinuation of DEHP (Fig. 1D). Considered together, these results indicate that DEHP-induced peroxisomes are selectively degraded following removal of the peroxisome proliferator.

Impairment of Degradation of Proliferated Peroxisomes in Autophagy-deficient Liver—Next, to examine the effects of autophagy deficiency on peroxisome degradation, we took advantage of the conditional knockout-mice, Atg7−/−:Mx1 (mutant mice), and their littermates, Atg7+/+ :Mx1 mice (control mice), the systems of which control mice were treated with corn oil for 2 weeks (2 w vehicle). Untreated and treated mice were sacrificed, and the livers were dissected out and homogenized, and then the postnuclear supernatant fractions were subjected to immunoblotting with anti-PT, BF, β-subunit ATP synthase, Mn2+-superoxide dismutase (SOD), BiP, and tubulin antibodies. Tubulin was used as a control. Data shown are representative of three separate experiments. C, quantitative densitometry of immunoblotting data in A was performed, and the ratios between each of PT, BF, and ATP synthase and tubulin were plotted. ** p < 0.01, *** p < 0.001. D, wild-type mice were treated with DEHP as described in A, and the frozen sections of livers were stained with anti-PT antibody to detect peroxisomes. Magnification, ×400.
were recently established by our group (20). Autophagy is impaired following pIpC injection in Atg7\(^{−/−}\):Mx1 mouse livers. Indeed, we verified that Atg7 protein deletion in Atg7\(^{−/−}\):Mx1 but not Atg7\(^{+/+}\):Mx1 livers was pIpC injection-dependent (Fig. 2A). Furthermore, we also tested the loss of Atg7 activity by investigating the lack of LC3-II (a membrane-bound form of LC3) and accumulation of LC3-I (a cytosolic form of LC3) in the liver. It is generally accepted that LC3-II is a marker protein of autophagosomal membranes (21). Although both forms were detected in the control liver, only LC3-I accumulated in the mutant liver (Fig. 2A), indicating impairment of autophagy in mutant Atg7\(^{−/−}\):Mx1 livers.

**FIGURE 2.** The recovery process of excess peroxisomes is impaired in Atg7-deficient liver. A, Atg7\(^{+/+}\):Mx1 (F/+; Mx1) and Atg7\(^{−/−}\):Mx1 (F/F; Mx1) mice were treated with DEHP for 2 weeks (2 w DEHP) and then chased for 1 week (2 w DEHP + 1 w). Both genotype mice were sacrificed at each time point. The liver was dissected out and homogenized, and then the postnuclear supernatant fractions were subjected to immunoblotting using anti-Atg7, LC3, BF, PT, \(\beta\)-subunit ATP synthase, Mn\(^{2+}\)-superoxide dismutase (SOD), BiP, and tubulin antibodies. Tubulin was used as control. Data shown are representative of three separate experiments. B, quantitative densitometry of Western blotting shown in A was performed, and PT/tubulin, BF/tubulin, \(\beta\)-subunit ATP synthase/tubulin, and Mn\(^{2+}\)-superoxide dismutase/tubulin ratios were plotted; *, \(p < 0.02\); **, \(p < 0.01\); NS, not significant.
mouse liver (20). In the control livers, although LC3-II were induced by the proliferated peroxisomes (Fig. 2A, indicated by 2 w DEHP), it was decreased almost to the basal levels at 1 week after withdrawal of DEHP (Fig. 2A), suggesting that autophagy was induced to remove surplus peroxisomes. After a 2-week treatment with DEHP, the livers were dissected, and total proteins in the lysates of mutant and control livers were separated by SDS-PAGE and subjected to immunoblot analyses. Similar results indicated selective impairment of degradation of excess peroxisomal proteins in autophagy-deficient Atg7F/F:Mx1 liver.

We further confirmed the impairment of peroxisome degradation in autophagy-deficient liver by immunofluorescence analysis using anti-PT antibody (Fig. 3). The PT-positive dots representing peroxisomes were markedly increased following a 2-week DEHP treatment in both genotype livers, as compared with untreated mice (Fig. 3, A and B). Although the dots almost disappeared to the basal levels at 7 days after discontinuation of DEHP in the control (Fig. 3C), most of the peroxisome dots remained visible in mutant liver after the same intervention (Fig. 3F). The data are in agreement with the biochemical results shown in Fig. 2. Based on these results, we concluded that autophagy is essential for selective degradation of excess peroxisomes.

Engulfment of Excess Peroxisomes by Autophagosomal Membranes in Control Hepatocytes—Finally, we used electron microscopy to explore the level of the peroxisomes in Atg7F/F:Mx1 and Atg7F/F:Mx1 livers (Fig. 4). Consistent with the results of immunofluorescent analysis, numerous peroxisomes were detected following a 2-week DEHP treatment in both wild and mutant hepatocytes (Fig. 4, B and E), and most of these structures disappeared after 1 week of discontinuation of DEHP in the control, but not mutant, hepatocytes (Fig. 4, C and F). The relative total area of peroxisomes was determined in each genotype (n = 10). M, mitochondria; G, morphometric analysis of peroxisomes in Atg7F/F:Mx1 and Atg7F/F:Mx1 mice.

Selective Degradation of Excess Peroxisomes

FIGURE 3. Accumulation of excess peroxisomes in Atg7-deficient liver. Immunofluorescent detection of peroxisomes with anti-PT antibody in the Atg7F/F:Mx1 (A–C, F/F:Mx1) and Atg7F/F:Mx1 (D–F, F/F:Mx1) liver is shown. Atg7F/F:Mx1 and Atg7F/F:Mx1 mice were treated with DEHP for 2 weeks (B and E, 2 w DEHP) and then chased for 1 week (C and F, 2 w DEHP + 1 w). Untreated (A and D) and treated mice were sacrificed, and the livers were isolated. The frozen sections of livers were immunostained with anti-PT antibody. Magnification, ×400.

FIGURE 4. Electron microscopic evaluation of livers of Atg7-deficient mice treated with DEHP. A–F, electron micrographs of the liver of representative Atg7F/F:Mx1 mice (F/F:Mx1) and Atg7F/F:Mx1 (F/F:Mx1) mice treated with DEHP for 2 weeks (B and E, 2 w DEHP) and then fed on normal diet for 1 week (C and F, 2 w DEHP + 1 w). The vehicle control mice of each genotype were treated with corn oil for 2 weeks (A and D). The hepatocytes of both genotypes contained a high number of peroxisomes (P) after DEHP treatment (B and E). Note that induced peroxisomes were retained at 1 week after discontinuation of DEHP in Atg7F/F:Mx1 hepatocytes, in contrast to the decreased number in Atg7F/F:Mx1 hepatocytes (C and F). Bars, 1 μm. The total area of peroxisomes relative to the cytoplasmic area was determined in each genotype (n = 10). M, mitochondria; G, morphometric analysis of peroxisomes in Atg7F/F:Mx1 and Atg7F/F:Mx1 mice.
lysosomal cysteine protease inhibitor, into a 2-week DEHP-treated control Atg7F−/−:Mx1 mouse resulted in marked accumulation of autophagosomes, and some peroxisomes were surrounded by a double-membrane structure, autophagosome, in control hepatocytes (Fig. 5). No autophagosome was identified in hepatocytes of Atg7F−/−:Mx1 mice (data not shown). These lines of evidence indicated that the autophagic machinery mediated is essential for selective clearance of excess peroxisomes, as it is so for starvation-induced autophagy in the mouse liver.

DISCUSSION

Most cellular components, if not all, are regulated quantitatively to maintain cell homeostasis. For this regulation, there are growing lines of evidence for the importance of the balance between biosynthesis and degradation. Peroxisomes, a typical cellular component, are dynamic organelles induced and degraded in response to extracellular cues (8). However, little is known about the mechanism for peroxisome degradation in mammals. There are two major concepts for degradation of peroxisomes, i.e. autophagic machinery and autolysis (34, 35). By the analysis of autophagy-deficient livers, we showed the first direct evidence that peroxisomal breakdown is mainly, if not entirely, dependent on autophagic machinery. Based on quantitative densitometry with two peroxisome marker enzymes (Fig. 2, PT and BF) as well as morphometry of the electron micrographs (Fig. 4), ~70–80% of peroxisomes induced by DEHP were degraded via autophagy during 1 week after discontinuation of the drug administration.

Considering peroxisome degradation by autophagic machinery in mammals, it is important to know whether the process occurs via microperoxophagy or macropexophagy. In methylotrophic yeast species, it is well established that the autophagy-related process, termed pexophagy, induces a rapid and selective degradation of excess peroxisomes (13). In P. pastoris cells, following a shift from methanol to ethanol or glucose, unnecessary peroxisomes are degraded by macropexophagy and microperoxophagy, respectively (9). Macropexophagy is the degradation pathway in which autophagosomes selectively surround excess peroxisomes. On the other hand, in microperoxophagy, the excess peroxisomes are not degraded through autophagosome formation. The initial step in microperoxophagy is invagination and seption of a vacuole followed by engulfment of the peroxisomes by the vacuole. In the final stage, the edges of the vacuole fuse with each other followed by vacuolar degradation of the peroxisomal membrane and its contents. Because ATG7 is essential for both macropexophagy and microperoxophagy in P. pastoris cells, it is plausible that excess peroxisomes in mammalian cells are also degraded by both macroautophagy and microperoxophagy. Our data using electron microscopy revealed that autophagosomes preferentially surrounded excess peroxisomes in control hepatocytes (Fig. 5), suggesting that DEHP-induced peroxisomes are degraded mainly through the process of macropexophagy. Thus, we could show the selective role of autophagic machinery in the clearance of surplus peroxisomes after induction of peroxisomes by phthalate esters.

Recent studies provided evidence for the involvement of the autophagic machinery in selective sequestration of proteins in the cell. For example, the precursor form of aminopeptidase I (prApe1) is a selective cargo molecule of autophagy in yeast (36), and cytosolic acetaldehyde dehydrogenase (Ald6p) is preferentially transported to vacuoles via autophagosomes in yeast (37). Consistently, the autophagic machinery could also selectively eliminate pathogenic group A Streptococci invading the cells (38). These reports strongly suggest that autophagosomes sequester the cytosolic protein(s) and invading pathogens in a highly selective manner. We recently reported that Atg7−/− deficient hepatocytes exhibit impaired constitutive autophagy responsible for selective degradation of ubiquitinated proteins (20). Our previous findings together with the present results suggest that the autophagic process eliminates abnormal and/or excess proteins and organelles including peroxisomes in a selective manner even under normal conditions. How the autophagy machinery recognizes these organelles to degrade them awaits further investigation.

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