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Lifespan of Catalase-Deficient Mice Decreases Due to Lysosomal Dysfunction

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Research

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Abstract

Background: Lysosomes are a central hub for cellular metabolism and are involved in the regulation of cell homeostasis through the degradation or recycling of unwanted or dysfunctional organelles through the autophagy pathway. Catalase, a peroxisomal enzyme, plays an important role in cellular antioxidant defense by decomposing hydrogen peroxide into water and oxygen. In accordance with pleiotropic significance, both impaired lysosomes and catalase have been linked to many age-related pathologies with a decline in lifespan. Aging is characterized by progressive accumulation of macromolecular damage and the production of high levels of reactive oxygen species (ROS). Although lysosomes degrade the most long-lived proteins and organelles via the autophagic pathway, the role of lysosomes and their effect on peroxisomes during aging is not known. The present study investigated the role of catalase and lysosomal function in catalase-knockout (KO) mice.

Results: We found that catalase-deficient mice exhibited an aging phenotype faster than wild-type (WT) mice. We also found that aged catalase-KO mice induced leaky lysosomes by progressive accumulation of lysosomal contents, such as cathespin D, into the cytosol. Leaky lysosomes inhibited autophagosome formation and triggered impaired autophagy. The dysregulation of autophagy triggered mTORC1 (mechanistic target of rapamycin complex 1) activation, which plays a pivotal role in modulating aging. However, the antioxidant N-acetyl-L-cysteine (NAC) and mTORC1 inhibitor rapamycin rescued leaky lysosomes and aging phenotypes in catalase-deficient aged mice.

Conclusion: This study unveils the new role of catalase and its role in lysosomal function during aging.

Background

Peroxisomes are highly ubiquitous and dynamic organelles responsible for lipid metabolism, including beta oxidation of very-long-chain fatty acids (VLCFA); alpha oxidation of branched chain FAs; and synthesis of ether lipids, bile acids, and so on [1]. In addition, peroxisomes are also responsible for both anabolism and metabolism of reactive oxygen species (ROS). During β -oxidation, several H₂O₂-producing oxidases are generated in peroxisomes, which are converted to hydroxyl radicals (•OH), making peroxisomal membranes vulnerable to lipid peroxidation [2]. These oxidases are also involved in the production of powerful oxidants that can penetrate the peroxisomal membrane and act as cellular signaling; in contrast, they are also equipped with their accompanying antioxidant catalase to detoxify powerful oxidants [1, 3, 4]. Hence, peroxisomes balance the generation and degradation of ROS to ensure normal cellular function. The inability to maintain a balance between ROS production and scavenging may induce oxidative stress, which is a major contributor to cellular sensecence and pathogenesis of various age-related disorders [1].

Cellular senescence in fibroblasts is characterized by the progressive loss of proliferative capacity with increasing passage number in cells [5]. In early passage, a balance in ROS-generating enzyme (oxidase) and antioxidant exists because ROS generate H_2O_2 and the antioxidant catalase decomposes these toxic

metabolites to water and oxygen. However, as the passage number increases, the defense mechanism of the antioxidant catalase diminishes to counteract the toxic metabolite produced by oxidase and, hence, cells undergo senescence [6]. Similarly, *in vivo*, mice undergo oxidative damage during aging, diminishing the antioxidant enzyme, particularly catalase [7, 8].

Lysosomes are long lived primary degradative organelles that are responsible for the breakdown of proteins, lipids, and polysaccharides for recycling through the autophagic process [9]. During the autophagic process, the double-membrane autophagosome fuses with lysosomes to form autolysosomes; hence, the disposable materials are sequestered in the cytoplasm and undergo degradation through lysosomal hydrolases [10]. Lysosomes contain over 50 hydrolases that are activated at low pH (4.5–5.5) and are maintained by the proton-pumping v-ATPase, chloride channels, and ion transporters in the lysosome membrane [11]. Different stress conditions cause lysosomal deterioration, resulting in the translocation of intralysosomal components, such as cathespins, to the cytosol and can induce leaky lysosomes or lysosomal membrane permeabilization (LMP). Aging is a stress condition that alters the physical and chemical properties of lysosomes, increasing their size and, in some cases, numbers [12]. Although aging prevents lysosomal acidification and worsens autophagic regulation by the accumulation of long-lived proteins, the mechanism by which the peroxisomal enzyme catalase mediates lysosomes and their effects on aging are not known.

The present study investigated the role of catalase and lysosomal function in catalase-knockout (KO) mice. We found that catalase-KO mice exhibited an aging phenotype faster than wild-type (WT) mice. Here, we performed experiments on WT and catalase KO younger (9 weeks) and middle-aged (53 weeks) mice [13–14] and found that KO mice induced an aging phenotype in the middle age mice; however, we did not observe any phenotypic changes in WT mice at that age (53 weeks). We found that ROS generation in catalase-KO mice may be the main player in inducing a senescence-like phenotype; hence, the lifespan decreases rapidly.

Materials And Methods

1. Animal treatments

Homozygous KO mice were kindly provided by Dr. Ye-Shih Ho (Institute of Environmental Health Sciences and Department of Biochemistry and Molecular Biology, Wayne State University, USA). The mice were interbred as previously described [46]. Mice were maintained in accordance with the standard protocol approved by the Animal Care and Use Committee of Gwangju Institute of Science and Technology, Korea. Mice were fed a standard commercial diet (Research Diet, Inc. NJ, USA) and divided into four groups: (I) WT 9W (9-weeks-old or young mice), (II) KO 9W, (III) WT 53W (53-weeks-old or aged mice), and (IV) KO 53W. Mice were fed a normal chow diet (carbohydrate 65%, protein 20%, and fat 5%), with *ad libitum* access to water. All experiments were initiated at approximately 9 or 53 weeks of age. Mouse phenotyping studies were performed with controls, and a minimum of two independent experiments were performed with more than five mice in each group. Animals were sacrificed, and their blood was collected and allowed to clot for 20 min at room temperature (25°C). Serum was separated by centrifugation at 2000 ×g for 20 min at room temperature and stored at -80° C until further analysis. Liver tissue was removed from each mouse, weighed, frozen in liquid nitrogen, and stored at -80° C until further use or fixed with formalin. Tissue sections (8-µm thick) were used for histochemical analysis.

2. Cell culture, reagents, and antibodies

Mouse embryonic fibroblasts (MEFs) from WT and catalase-KO mice were isolated from catalase^{+/+} and catalase^{-/-} mice from E13.5 embryos, as described previously [47]. HepG2 and MEF cell lines were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; #11965092, Gibco) supplemented with 10% fetal bovine serum (FBS; #16000044, Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO₂ incubator. The maximum number of passages for healthy primary MEFs was approximately 5, as cell growth slows beyond passage 5. We worked with MEFs at passages 2 (P2) and 5 (P5) in both WT and KO cells to obtain optimal cellular responses to stimuli [48]. All cell lines were confirmed to be negative for mycoplasma contamination.

Rapamycin (#AY-22989, Selleck Chemicals), NAC (#A7250, Sigma-Aldrich), Lysotracker Red (#L7528, Invitrogen/Thermo Fisher Scientific); MitoSOX Red (#M36008, Invitrogen/Thermo Fisher Scientific), and Leu-Leu methyl ester hydrobromide (LLOMe) (#L7393, Sigma-Aldrich) were added to MEFs and HepG2 cells at the indicated times and concentrations. Indicated antibodies were used as shown in supplementary table 1 (S1).

3. Histological analysis

For histological analysis, tissues were fixed in 10% formalin solution, embedded in paraffin, and sectioned. H&E staining was performed as previously described [46].

For IF staining of tissue and cells (both MEFs and HepG2), snap-frozen tissues were sectioned using a cryostat and then defrosted at room temperature for 1 h. Cells and tissue sections were fixed in 4% paraformaldehyde (HT5014; Sigma-Aldrich) at room temperature for 20 min, washed with PBS, and incubated with 0.2% Triton X-100 for 5 min. The sections were blocked with 5% goat serum and 0.1% Triton X-100, whereas *in vitro*, cells were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. After blocking, the cells were incubated with the target primary antibody (1:200) with their respective blocking solution overnight at 4°C. The next day, tissues and cells were washed with PBS and stained with Alexa Fluor-conjugated secondary antibody in blocking solution at room temperature in the dark for 1 h. After that, both tissue and cells were incubated with 10 μ M DAPI (4',6-diamidino-2-phenylindole) in PBS at room temperature for 10 min and mounted with mounting media. Images were acquired and analyzed using an Olympus FluoView 1000 confocal laser scanning system. Slides were scored in a blinded manner.

4.0il Red O (ORO) staining

Cryosectioned liver samples were fixed with paraformaldehyde for 20 min and washed with distilled water. ORO solution (01391; Sigma-Aldrich) was added to the samples for 1 h at room temperature. Sections were washed with distilled water and counterstained with hematoxylin for 1 min. Images were acquired and analyzed using a light microscope. Slides were scored in a blinded manner.

4. Measurement of hepatic TG

To measure hepatic TG, 50 mg of liver tissue was homogenized, and TG was measured using a triglyceride colorimetric assay kit (#10010303, Cayman Chemical) according to the manufacturer's protocol.

5. SA-β-Gal staining

For SA- β -Gal staining *in vitro* (MEFs and HepG2), cells were washed three times with PBS and stained with senescence β -galactosidase according to the manufacturer's protocol (#9860, Cell Signaling Technology). For *in vivo* experiments, both WT and catalase-KO mice at 9W and 53W were perfused through the heart initially with PBS, followed by 4% paraformaldehyde. Liver tissues were collected, frozen sectioned, and stored at -80° C for future use. Senescence β -galactosidase staining was performed according to the manufacturer's protocol (#9860, Cell Signaling Technology). Images were acquired and analyzed using a light microscope. Slides were scored in a blinded manner. SA- β -Gal staining intensity was determined using the ImageJ software, and the mean SA- β -Gal intensity in the region of interest was measured based on the cell shape. SA- β -Gal positivity was determined as fold-change relative to the control.

6. Lysotracker Red and MitoSOX Staining

Lysotracker red and MitoSOX staining were performed in live cells, according to the manufacturer's instructions. Images were acquired using a fluorescence microscope, with the same fixed exposure time for all samples in each experiment.

7. Western blot analysis

To determine the expression levels of target proteins, western blotting from whole cell analysis using tissue samples and primary adipocytes was performed as described previously [46]. The antibodies used and their sources are listed in Supplementary Table S1.

8. Cell fractionation

Cell fractionation was performed to check cathespin protein (in either the membrane or cytosol) in both cell and liver tissues, as described previously [24]. In brief, treated cells and fresh liver tissues were washed with ice-cold PBS and homogenized with homogenization buffer [10 mM HEPES, 0.25 M sucrose, 10 mM Na₂EDTA, adjusted to pH 7.0 with NaOH, and 1× protease inhibitor cocktail (GenDEPOT, P3100-001; P3200-001)]. After homogenization, both tissues and cells were centrifuged at 1,000 ×g for 10 min, and post-nuclear supernatants were further centrifuged at 100,000 ×g for 1 h to generate supernatant and

pellet fractions. All the procedures were performed at 4°C. After measuring the protein concentration, the supernatant and pellet extracts were boiled at 97°C for 10 min and subjected to western blot analysis.

9. Determination of ROS production

ROS production from tissue lysates and cells was performed as previously described (46).

10. ACOX1 activity

Liver tissues were homogenized in ice-cold PBS. After centrifugation, supernatants were collected, and the levels of acyl-CoA oxidase 1 (ACOX1) and activity were measured using ELISA assay kits (My Biosource).

11. Cathespin D activity

Treated cells and fresh liver tissues were washed with ice-cold PBS and homogenized with lysis buffer provided by a commercially available kit (#ab65302, Abcam). The activity of cathespin D was performed as previously described.

12. Statistical analysis

Data are presented as means ± standard deviation (SD). One-way analysis of variance was used to compare the means of two or more groups. Statistical significance was set at P < 0.05.

Results

1. Catalase-deficient mice induce aging phenotype faster than WT mice

It has been well-documented that increased ROS and diminished antioxidant capacity induce cellular senescence, and catalase enzymes have been used to alleviate senescence through its antioxidant defense mechanism [6, 8, 15]. We investigated whether catalase is used to alleviate ROS and diminish aging in catalase-deficient mice. For this, WT and catalase-KO mouse embryonic fibroblasts (MEFs) were isolated and cultured. Catalase deficiency was confirmed by immunoblot analysis of MEFs (Fig. S1a). KO MEFs displayed flattened and enlarged senescence phenotypic morphology at early passage (P2) and showed increased senescence phenotypic morphology with increasing passage (P4) (Fig. S1a,b). To determine whether the flattened and enlarged MEFs were senescent, β -galactosidase staining was performed. As expected, KO MEFs showed positive staining for senescence-associated β -galactosidase, which significantly increased in P5 but not in WT cells (Fig. 1b and S1c). To illustrate the senescence-induced phenotype, immunoblot analysis was performed for WT and KO MEFs from P2 and P5. Notably, the expression levels of senescence-related proteins p21 and p16 increased in KO P5 MEFs (Fig. 1c). Furthermore, to confirm the aging phenotype, catalase-KO mice with WT littermates at the age of 9 weeks (9W) and 53 weeks (53W) were subjected to the experiment. β -galactosidase staining was performed in the liver of mice, which showed positive staining in KO mice livers at 53W (Fig. 1d). Immunoblot analysis

of liver homogenates from mice showed the induction of senescence-related proteins in KO mice at 53W (Fig. 1e). Together, these data suggest that catalase deficiency induces an aging phenotype faster than WT mice.

2. Catalase-deficient mice induce ROS through mitochondria and peroxisome

As reported earlier, ROS generation is the main cause of aging due to decreased cellular antioxidant capacity [6, 8, 15]. We hypothesized that ROS generation may be increased in catalase-KO mice. As expected, the fluorescence intensity measured by 2'7'-dichlorofluorescein diacetate (DCFH-DA) staining showed an increase in catalase-deficient MEFs at P5 (Fig. 2a). The fluorescent signal of DCFH-DA staining, representing ROS, was quantified and showed that ROS generation was significantly higher in catalase-KO cells at P5 (Fig. 2a). Likewise, we examined the expression of another ROS marker, 4 hydroxynonenal (4-HNE), in MEFs by immunofluorescence staining (IF). Consistent with DCFH-DA staining, the fluorescence intensity of 4-HNE in KO MEFs increased at P5 (Fig. 2b). In addition, to confirm ROS generation *in vivo*, intracellular ROS levels were measured in liver lysates of mice. Total ROS levels increased significantly in KO mice at 53W (Fig. 2c). The endogenous source of ROS contains different cellular locations, with mitochondria and peroxisomes being the major sites [2, 16, 17]. The principal source of ROS produced by mitochondria is the superoxide anion, a byproduct of the electron transport chain responsible for oxidative damage by aerobic energy metabolism [18, 19]. To detect mitochondrial ROS, MitoSOX red, a mitochondrial superoxide indicator, was used in WT and KO MEFs at the P2 and P5 levels (Fig. 2d). Catalase-KO cells at P5 showed increased levels of the red fluorescence signal. In addition, in vivo, the level of ACOX1 (acyl-CoA oxidase 1), a major producer of ROS in the peroxisome and the first and rate-limiting enzyme in fatty acid β-oxidation, increased significantly in KO mice at 53W (Fig. 2e). To confirm the induction of ROS in catalase-KO mice, MEFs were treated with the antioxidant Nacetyl-L-cysteine (NAC). Treatment with NAC, however, inhibited the level of ROS generation in KO MEFs at P5 (Fig. 2a, b, and d). Taken together, these data suggest that catalase-KO mice induce ROS production through both mitochondria and peroxisomes.

We hypothesized that ROS generation in catalase-KO mice may induce cellular senescence, as previously described [6, 8, 15]. Hence, β -galactosidase staining was again performed in WT and catalase-KO MEFs and co-treated with NAC (Fig. 2f). As expected, positive staining of senescence-associated β -galactosidase in KO MEFs was significantly diminished by treatment with NAC. Moreover, NAC treatment also decreased senescence-related protein in KO P5 MEFs (Fig. 2g). Together, these data suggest that catalase deficiency induces an aging phenotype through ROS generation.

3. Catalase-deficient aged mice induce leaky lysosome

Lysosomes are the main catabolic organelles that play an essential role in cellular processes, including responses to nutrient availability, stress resistance, plasma membrane repair and development, and cellular differentiation [20]. In line with catabolic organelles, lysosomal activity is strongly influenced by aging by altering the physical and chemical properties of these organelles and rendering them more

sensitive to stress [12]. Considering this notion, immunoblot analysis of mice liver homogenates was performed to check the lysosomal marker protein LAMP1 (Fig. 3a). Lysosomal protein levels increased in the KO liver. Aging has also been reported to increase lysosomal volume [21]. Hence, to check the volume, the morphology of lysosomes was analyzed using IF. WT and KO MEFs were immunostained with the lysosomal marker LAMP1 (Fig. 3b). Catalase-deficient MEFs at the P5 level showed an enlarged cellular size, which significantly increased the red fluorescence signal toward the cytoplasm of the cell. To confirm acidic lysosomal vesicles, MEFs were immunostained with lysotracker for labeling and tracking of the acidotropic probe for lysosomes (Fig. 3c). The acidic vesicle specifically accumulating in lysosomes decreased, whereas cell size increased in KO MEFs at the P5 level. Although KO MEFs showed increased lysosome size, their acidic vesicles decreased by lysotracker (Fig. 3a-c). Lysosomal activity is highly influenced by hydrolytic enzymes residing in the lumen of the lysosomal membrane, which is highly acidic [24]. As the acidotropic probe for lysosomes decreased in KO MEFs, we assumed that the resident hydrolytic enzymes in the lysosomal lumen were leaked. Hence, leaky lysosomal content may make the hydrolytic enzyme alkaline, causing lysosomes to fuse in the cytosol, which may increase lysosomal size. To check the lysosomal content, we analyzed the protein levels of cathepsin D (cathD) and cathepsin B (cathB), two major lysosomal hydrolases that can serve as molecular reporters for lysosomal functions [25], in WT and KO liver homogenates. Both lysosomal hydrolases were accumulated in the supernatant fraction in KO mice at 53W, whereas both cath D and B were normal in the pellet fraction in the other groups (Fig. 3d). Furthermore, cath D activity was measured in the liver lysates of mice (Fig. 3e). cath D activity decreased in KO mice at 53W, which further confirmed the presence of leaky lysosomes in KO aged mice. Together, these data suggest that catalase deficiency induces lysosomal damage through leaky lysosomes.

4. Leaky lysosome persuades alteration of autophagy through ROS in catalase-deficient aged mice

Damaged lysosomes are selectively sequestered by autophagy [24]. Hence, we hypothesized that damaged lysosomes in KO mice may be recruited by autophagy machinery, which are then engulfed by autophagosomes. To confirm the autophagic process, MEFs were co-immunostained with LC3, an autophagy marker, with lysotracker (Fig. 4a). In contrast to our assumption, the LC3 positive puncta increased but were not fused with lysosomes in KO MEFs due to decreased lysosomal acidic probe, which showed less fusion of autophagosomes with lysosomes. Further, immunoblot analysis was performed in WT and KO MEFs at the P2 and P5 levels. Notably, the expression levels of autophagy substrate marker P62 and autophagosome by LC3II were significantly increased in KO MEFs at P5 (Fig. 4b). To confirm autophagic dysregulation *in vivo*, liver tissues from mice were immunoblotted with an autophagy marker (Fig. 4c). Consistent with MEFs, liver tissues from KO mice at 53W showed dysregulation of basal autophagy in comparison to WT and KO 9W livers (Fig. 4c). ROS mediate leaky lysosomes [25]. Hence, to confirm this, WT and catalase-KO MEFs were treated with or without NAC at both passages (P2 and P5). Treatment with NAC slightly recovered the acidic probe of lysosomes, which fused with autophagosomes in KO MEFs at the P5 level (Fig. 4a). Hence, these data suggest that leaky lysosomes alter autophagy through ROS in catalase-deficient aged mice.

5. Leaky lysosomes induce lipofuscin accumulation through ROS in catalase-deficient aged mice

Although leaky lysosomes are induced in catalase-deficient MEFs, the damaged lysosomes were not degraded through the autophagic process. Instead, basal autophagy was dysregulated in KO MEFs. To find the mechanistic evidence for this, we examined the morphology of the liver by H&E staining in mice. The liver morphology of 53W KO mice showed microvesicular steatosis (i.e., accumulation of small fat droplets) in the cytosol of hepatocytes (arrows), with golden-brown pigment (arrowhead), whereas the livers of WT and KO 9W mice showed normal lobular architecture with hepatocytes arranged in hepatic cords (Fig. 5a). To confirm the accumulation of fat droplets in hepatocytes, triacylglycerol (TG) was measured in the liver lysates of mice. Consistent with H&E staining, liver lysates of 53W KO mice showed a significant increase in liver TG compared to other groups (Fig. 5b). Furthermore, oil red O staining (ORO) also showed the induction of lipid droplets in the hepatocytes of 53W liver sections (Fig. 5c). During aging, the volume and structure of hepatocyte organelles change [26]. Although we observed a significant increase in body weight of 53W old KO mice, but there were no significant changes in liver weight (Fig. S2a-b). We hypothesized that the accumulation of small lipid droplets in the cytoplasm of hepatocytes in KO 53W old mice may be an undigested lipid, lipofuscin, which showed brown pigmentation on hepatocytes (Fig. 5a). Lipofuscin is a highly oxidized insoluble protein that fails to degrade damaged and denatured proteins [27]. Moreover, it is a chemically and morphologically polymorphous waste material that accumulates at the primary site of the lysosome and disturbs lysosomal degradation and causes lysosome leakage [28, 29]. To examine the accumulation of lipofuscin or leaky lysosomes, MEFs were coimmunostained with LGALS1 (galectin-1), a leaky lysosome marker with lysotracker (Fig. 5d). Catalase-KO MEFs at P5 level showed a significant increase in LGALS1 puncta that were loaded on the lysosomes (Fig. 5d, arrow); although acidic vesicles in lysosomes by lysotracker were less, almost all LGALS1 puncta were localized to lysosomes. In contrast, LGALS1 puncta were less or not observed at all in the WT at P2, P5, and KO P2 levels. It is known that enhanced ROS results in the leakage and accumulation of lipofuscin in lysosomes [25, 28]. Hence, to confirm this, WT and catalase-KO MEFs were treated with or without NAC at both passages (P2 and P5). Treatment with NAC slightly rescued the acidic vesicles of lysosomes and decreased the localization of LGALS1 puncta to the lysosomes in KO MEFs at P5 level (Fig. 5d). Hence, these data suggest that leaky lysosomes induce lipofuscin accumulation through ROS in catalase-KO aged mice.

6. Leaky lysosome affects lysosomal pH that activates mTORC1 (mechanistic target of rapamycin complex 1) and leads to cellular senescence

Next, we constructed leaky lysosomes using the well-known lysosomal membrane permeabilization (LMP) marker L-leucyl-L-leucine methyl ester (LLOME) in hepatoma cells and questioned whether leaky lysosomes induce ROS and cellular senescence. For this, we treated HepG2 cells with LMP inducer

LLOME for 24 h and examined the morphology of lysosomes by immunostaining with lysotracker. As expected, the specific accumulation of acidic vesicles in lysosomes decreased in LLOME-treated cells (Fig. 6a). The disruption of acidic hydrolases in lysosomes or leaky lysosomes is induced through extensive ROS [25]. To confirm this, HepG2 cells were stained with DCFH-DA (Fig. 6b). Fluorescence intensity by DCFH-DA staining increased in LLOME-treated cells. Hence, to confirm the leaky lysosomes induced by ROS accumulation, HepG2 cells were co-treated with antioxidant NAC and LLOME. As expected, NAC recovered the acidic vesicles of lysosomes and abolished DCFH-DA fluorescence intensity in HepG2 cells (Fig. 6a–b). LLOME treatment increased the cytosolic release of lysosomal hydrolases [24]. Hence, to check the lysosomal content, the protein levels of cath D and B were immunoblotted in HepG2 cells treated with LLOME (Fig. 6c). As expected, both lysosomal hydrolases (cath D and B) accumulated in the supernatant fraction in LLOME-treated cells, whereas they were normal in the pellet fraction of untreated cells. Further immunoblot analysis of HepG2 cells showed increased protein expression of the ROS marker 4-HNE and peroxisomal oxidase ACOX1 in LLOME-treated cells (Fig. S3a). However, we did not observe any changes in mitochondrial enzymes, including COX1, COX4, voltage-dependent anion channel (VDAC), and antioxidant proteins, including SOD1 and SOD2.

The link between decreased lysosomal function and aging has been well studied [12, 30]. Hence, we investigated whether LMP drug LLOME induces cellular senescence in cells. β-galactosidase staining was performed in HepG2 cells. As expected, cells treated with LLOME showed positive staining for senescence-associated β-galactosidase (Fig. S3b). However, treatment with NAC abolished the positive staining of senescence-associated β-galactosidase. Further immunoblot analysis was performed in HepG2 cells showing increased expression of aging-related proteins, including p16 and p21, in LLOMEtreated cells (Fig. S3c). During lysosomal damage, transcription factor EB (TFEB), a major regulator of autophagy and lysosomal biogenesis, has been shown to rapidly translocate to the nucleus and activate the transcription of its target gene for the activation of lysosomes [31]. Hence, immunostaining was performed for translocation of TFEB to HepG2 cells. As expected, LLOME-treated cells showed translocation of TFEB to the nucleus in LLOME-treated cells (Fig. 6d). Hence, lysosomal rupture induces the biogenesis of lysosomes through autophagy (Fig. S3d), as previously described [24, 31]. Meanwhile, immunoblot analysis also showed that treatment with LLOME increased the expression of phosphorylated S6 (pS6), a marker of downregulation of mTORC1 (Fig. 6e). Further immunostaining with anti-mTORC1was performed in LLOME-treated cells, which showed increased expression of mTORC1 protein (Fig. S3e). Together, these data show that leaky lysosomes affect lysosomes and induce cellular senescence probably through mTORC1 activation.

7. Rapamycin attenuated cellular senescence induced by catalase-deficient cells

mTOR is a key component of cellular metabolism that promotes cell growth and proliferation via nutrient sensing. In addition to cellular growth and proliferation, mTOR has also been associated as a lifespan regulator in mice [32–35]. The lifespan-enhancing effects of mTOR inhibitors have been linked to mTORC1 inhibition [36]. Hence, we treated cells with rapamycin, an mTORC1 inhibitor, to reverse cellular

senescence induced by catalase-KO mice. For this, WT and KO MEFs were treated with rapamycin, and βgalactosidase staining was performed to check the senescence phenotype. KO MEFs displayed flattened and enlarged senescence phenotypic morphology at early passage (P2) and showed increased senescence phenotypic morphology with increasing passage (P5) in addition to positive staining for senescence-associated β-galactosidase, but not in WT MEFs (Fig. 7a). However, treatment with rapamycin inhibited positive staining of senescence-associated β-galactosidase, but KO MEFs still displayed flattened and enlarged senescence phenotypic morphology. Further immunoblot analysis of MEFs showed the induction of senescence-related proteins in KO cells that was suppressed by cotreatment with rapamycin (Fig. 7b). mTORC1 is also known to suppress autophagy, and activation of autophagy by suppression of mTORC1 can slow age by clearing the accumulating old and dysfunctional organelles [36, 37]. Hence, to check the clearance of old and dysfunctional organelles by autophagy, rapamycin was used to treat MEFs, and immunoblot analysis was performed. Remarkably, the increased levels of autophagy substrate marker P62 and autophagosome marker LC3II were significantly decreased by rapamycin in KO MEFs at P5 level (Fig. 7c). Furthermore, phosphorylated S6, downstream of mTORC1 activity was decreased by rapamycin, suggesting that autophagy was initiated (Fig. 7c). In addition, accumulation of lysosomal content, such as cath D, in the supernatant fraction in KO MEFs at level P5 was suppressed by rapamycin (Fig. 7d). Similarly, cath D activity was also recovered in KO MEF following treatment with rapamycin (Fig. 7e). Additionally, we immunostained MEFs with lysotracker to measure the acidic probe of lysosomes by rapamycin. As expected, the decreased acidic puncta of lysosomes were recovered by rapamycin in KO MEFs at P5 (Fig. 7f). Taken together, we showed that mTORC1 depletion by rapamycin slightly attenuated the progression of aging in catalase-KO mice. We also tried to show aging progression by hyperactivation of mTOR by the point mutation S2215Y, identified in the human cancer genome database [38]. We transfected the FLAG-tagged mutated form of mTOR with its WT plasmid in MEFs, followed by immunostaining with lysotracker (Fig. S4). Consistent with the aged phenotype, the mutated form showed a diffuse form of lysotracker, whereas in WT cells, lysotracker puncta were guite distinctive. Together, these data showed that mTOR hyperactivation in catalase-KO mice may aggravate the aging phenotype faster than in WT mice.

Discussion

Aging comprises various probabilities and is usually treated as a discrete variable rather than a continuous variable. Thus, most studies focus on three specific life phases: mature adult, middle age, and old. In mice, the mature adult (3–6 months) group is the reference for any age change, whether it is developmental, maturational, or senescent; middle age (10–14 months) refers to a stage in which senescent changes can be detected in some, but not all, biomarkers of aging; and old age (18–24 months) refers to a stage in which senescent changes can be detected in some, but not all, biomarkers of aging; and old age (18–24 months) refers to a stage in which senescent changes can be detected in almost all biomarkers [13, 14]. In this study, we used two groups of mice, viz.., younger (~2 months or 9W) that were not affected by senescent and middle-aged mice (12 months or 53W) that are on the way to senescence. Although the 53W mice breed normally, they exhibited the senescent phenotype faster, which decreased the lifespan of catalase-KO mice. In addition, during extensive culture passages, normal primary cells undergo malignant

progression through cytostasis and contribute to cell senescence induction (39). Hence, primary MEFs in a recent study showed a senescent-like phenotype only after P5 levels. Thus, we mentioned MEFs for P2 as young cells and P5 as senescent cells.

Senescence refers to a cellular response characterized by a state of stable cell cycle arrest in which proliferating cells become resistant to growth but remain viable and metabolically active after prolonged time in culture [40]. However, senescence is not just a cell cycle arrest. When the cell cycle is arrested, an inappropriate growth-promotion pathway, such as mTOR, converts arrest into senescence and mTOR inhibitor rapamycin decelerates cell proliferation but preserves the re-proliferative potential of inactive cells that are lost during senescence [41, 42]. Based on these findings, we concluded that rapamycin treatment decelerates cell proliferation but inhibits positive staining of senescence-associated β -galactosidase in KO aged MEFs (Fig. 7).

Lipofuscin is comprised of highly oxidized proteins, lipids, and metal elements that accumulate primarily in the lysosomes and reduce its autophagic capacity [43, 44]. These non-degradable lipids and ions or waste accumulation in the form of lipofuscin elevates the lysosomal pH, leading to a snowball effect on lysosomes [45]. Hence, the continuous accumulation of lipofuscin in lysosomes through ROS generation in catalase-KO mice increased the volume of lysosomes. Although lysosomal size increased, lysosomal hydrolytic activity decreased in catalase-KO aged mice (Fig. 3).

Although aging affects many cellular components, our study showed remarkable changes in lysosomes that induce mTORC hyperactivation and, thus, cause an aging phenotype faster in catalase-deficient mice. Our study may unveil the innovative roles of catalase and its relation to lysosomes and its role in aging.

Declarations

Author Contributions

RKD performed conceptualization, analysis, investigations, methodology development, visualization, writing the original draft, and validating the experimental results. **JNL** supervised, investigated, developed the methodology, wrote, reviewed, and edited the manuscript. **YM** performed the analysis and investigation, methodology development, conceptualization, and validation of the experimental results. **CP** contributed to project administration, supervision, resource, and software management. **RKP** contributed to funding acquisition, conceptualization, visualization, and supervision.

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Availability of data and materials:

All data generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

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All procedures were approved by the Animal Care and Use Committee at Gwangju Institute of Science and Technology, Korea.

Consent for publication:

Not applicable.

Competing interests:

The authors have no competing interest to declare

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Catalase-deficient mice induce aging phenotype faster than WT mice (a) Morphology and senescenceassociated β -galactosidase staining of passage WT and catalase KO MEFs at passage 2 (P2) and passage 5 (P5) levels. Percentage of senescent cells at each passage, P2 and P5, in WT and KO MEFs. Positive intensities of β -galactosidase staining were measured using the ImageJ software. Bar graph represents mean ± SD (n = 3 experiments). Scale bar represents 20 µm. *P < 0.05, WT P2 vs. KO P2; WT P5 vs. KO P5. (b) Proteins were extracted from MEFs at P2 and P5 levels. Immunoblot analysis was performed using whole-cell lysates with indicated senescence-associated antibody. (c) Senescenceassociated β -galactosidase staining was performed in the liver section of 9 weeks (9W) and 53 weeks (53W) WT and catalase-KO mice. (d) Immunoblot analysis was performed from the liver sample from WT and catalase-KO mice at the age of 9W and 53W to check the expression of senescence-associated antibody. Scale bar represents 100 µm.



Aging induced in catalase-deficient mice through ROS generation (a) Representative fluorescence images of DCFH-DA staining of WT and catalase-KO MEFs at P2 and P5 levels. Percentage of cells showing green fluorescence (corresponding to DCFH-DA) and fluorescence intensity of MEFs. *P < 0.05 WT P5 vs. KO P5; KO P5 vs. KO P5+NAC. (b) Representative fluorescence images of MEFs fixed and immunostained with anti-4HNE (green). Scale bar represents 20 μ m. (c) Total ROS was measured in liver lysates of WT and catalase-KO mice at 9W and 53W. Values represent mean ± SD (n = 3, 4). *P < 0.05 WT 53W vs. KO 53W. (d) Representative (red) fluorescence image of MitoSOX staining in MEFs, as in (a). (e) ACOX1 levels were measured using the ACOX1 ELISA kit from the liver lysates of mice as in c. (f) MEFs at P2 and P5 were treated with 5 mM NAC overnight, and senescence-associated β-galactosidase staining was analyzed. The percentage of senescent cells was analyzed in WT and KO MEFs treated with NAC. Positive intensities of β-galactosidase staining were measured using the ImageJ software. Bar graph represents mean ± SD (n = 3 experiments). Scale bar represents 20 μ m. *P < 0.05, WT P2 vs. KO P2; KO P2 vs. KO P2+NAC; WT P5 vs. KO P5 vs. KO P5 vs. KO P5+NAC. (g) Proteins were extracted from MEFs as in f. Immunoblot analysis was performed using whole-cell lysates with indicated senescence-associated antibodies.



Catalase-deficient aged mice induce leaky lysosomes (a) Protein was extracted from liver lysates from WT and catalase-KO mice at 9W and 53W. Immunoblot analysis was performed using whole-cell lysates with LAMP1 antibody. (b) Representative fluorescence images of MEFs fixed and immunostained with anti-LAMP1 (red) and DAPI (blue). Scale bar represents 20 μ m. (c) Representative fluorescence images of lysotracker for WT and catalase-KO MEFs at P2 and P5 levels. Scale bar represents 20 μ m. (d) Liver lysates from WT and catalase-KO mice at 9W and 53W were fractioned as described in the Materials and Methods. Equivalent volumes of each fraction were subjected to immunoblotting using the indicated antibodies. (e) Relative cathespin D levels were measured from liver lysates of mice, as indicated in a.



Catalase-deficient aged mice induce alteration of autophagy through ROS (a) MEFs from WT and catalase-KO mice at P2 and P5 levels were treated with 5 mM NAC, fixed, and immunostained with anti-LC3 (green) and Lysotracker (red). Scale bar represents 10 μ m. (b) Proteins were extracted from MEFs as in a. Immunoblot analysis was performed using whole-cell lysates with the indicated antibodies. (c) Protein was extracted from liver lysates from WT and catalase-KO mice at 9W and 53W. Immunoblot analysis was performed using whole-cell lysates with the indicated antibodies.



Catalase-deficient aged mice induce lipofuscin accumulation through ROS (a) Representative hematoxylin and eosin (H&E) staining of livers from WT and catalase-KO mice at the age of 9W and 53W, respectively. The livers of 53W KO mice showed microvesicular steatosis (i.e., accumulation of small fat droplets) in the cytosol of hepatocytes (arrows) with a golden-brown pigment (arrowhead) known as lipofuscin. Scale bar represents 50 μ m. (b) Liver samples from mice as mentioned in a were homogenized, and TG levels were analyzed. Bar graph represents mean \pm SD (n = 3 experiments). *P < 0.05, WT 53W vs. KO 53W. (c) Cryosectioned liver tissues from mice were stained with ORO. Scale bar represents 50 μ m. (d) Representative fluorescence images of MEFs at P2 and P5 treated with 5 mM NAC, fixed, and immunostained with Lysotracker (red), LGALS1 (green), and DAPI (blue). Scale bar represents 5 μ m.



Catalase-deficient aged mice induce mTORC1 activation (a) HepG2 cells were treated with 1 mM LLOMe for 24 h and co-treated with 5 mM NAC overnight. Representative fluorescence images of HepG2 cells immunostained with Lysotracker (red) and DAPI (blue). Scale bar represents 5 µm. (b) Representative fluorescence images of DCFH-DA staining of HepG2 cells treated as in a. Scale bar represents 20 µm. (c) Lysates of HepG2 cells treated as in a were, fractioned as explained in the Materials and Methods section. Equivalent volumes of each fraction were subjected to immunoblotting using the indicated antibodies. (d) Representative fluorescence image of HepG2 cells treated as in a, immunostained with anti-TFEB (red) and DAPI (blue). Scale bar represents 20 µm. (E) Protein was extracted from HepG2 cells as in a. Immunoblot analysis was performed using whole-cell lysates with indicated antibodies.



Rapamycin attenuated cellular senescence induced by catalase-deficient cell (a) MEFs at P2 and P5 were treated with 1 μ M rapamycin (rapa) overnight and senescence-associated β -galactosidase staining was analyzed. The percentage of senescent cells was analyzed in WT and KO MEFs treated with rapa. Positive intensities of β -galactosidase staining were measured using the ImageJ software. Bar graph represents mean \pm SD (n = 3 experiments). Scale bar represents 20 μ m. *P < 0.05, WT P2 vs. KO P2; KO P2 vs. KO P2+rapa; WT P5 vs. KO P5. KO P5 vs. KO P5+rapa. (b-c) Protein was extracted from MEFs as in a. Immunoblot analysis was performed using whole-cell lysates for (b) senescence and (c) autophagy and mTORC1-related antibodies. (d) Lysates of MEFs treated as in a were fractioned as explained in the Materials and Methods section. Equivalent volumes of each fraction were subjected to immunoblotting using cath D antibodies. (e) Relative cath D levels were measured from MEF lysates, treated as in a. Bar graph represents mean \pm SD (n = 3 experiments). *P < 0.05, WT P5 vs. KO P5. (f) Representative fluorescence image of MEFs treated as in a, immunostained with Lysotracker (red) and DAPI (blue). Scale bar represents 20 μ m.

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