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Muscle PGC-1α modulates hepatic mitophagy regulation during aging

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ABSTRACT

Aging has been suggested to be associated with changes in oxidative capacity, autophagy, and mitophagy in the liver, but a simultaneous evaluation of these key cellular processes is lacking. Moreover, skeletal muscle transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α has been reported to mediate inter-organ signaling through myokines with regulatory effects in the liver, but the potential role of muscle PGC-1α on hepatic changes with age remains to be resolved. The aim of the present study was therefore to investigate 1) the effect of aging on mitochondrial autophagy and mitophagy capacity in mouse liver and 2) whether muscle PGC-1α is required for maintaining autophagy and mitophagy capacity in the liver during aging. The liver was obtained from young (Young) and aged (Aged) inducible muscle-specific PGC-1α knockout (iMKO) and floxed littermate control mice (Lox). Aging increased liver p62, Parkin and BCL2/adenovirus E1B 19 kDa protein-interacting protein (BNIP)3 protein with no effect of muscle specific PGC-1α knockout, while liver Microtubule-associated protein (LC3) II/I was unchanged with age, but tended to be lower in iMKO mice than in controls. Markers of liver mitochondrial oxidative capacity and oxidative stress were unchanged with age and iMKO. However, Parkin protein levels in isolated liver mitochondria were 2-fold higher in Aged iMKO mice than in Aged controls. In conclusion, aging had no effect on oxidative capacity and lipid peroxidation in the liver. However, aging was associated with increased levels of autophagy and mitophagy markers. Moreover, muscle PGC-1α appears to regulate hepatic mitochondrial translocation of Parkin in aged mice, suggesting that the metabolic capacity of skeletal muscle can modulate mitophagy regulation in the liver during aging.

1. Introduction

Aging is known to be associated with detrimental changes in many tissues, including sarcopenia with loss of muscle mass and reduced muscle strength, as well as decreased hepatic volume and hepatic steatosis (Frith et al., 2009; Gan et al., 2011; Lauretani et al., 2003; Ogrodnik et al., 2017). The average life expectancy has increased over the last century and it is expected that within the next 10 years the fraction of people above 65 years will increase by 236 million (He et al., 2015). It is therefore also anticipated that the frequency of age-associated diseases will increase, highlighting the importance of understanding the molecular mechanisms of aging (Hung et al., 2011; Martinez, 2017).

The liver is one of the essential organs of the body with multiple functions affecting whole body metabolism. The liver is responsible for production and release of triglycerides, cholesterol, proteins and bile salts as well as detoxification by breaking down lipophilic toxins and drugs (Liu et al., 2017; Thomas et al., 2002). Moreover, the liver is a key player in maintaining glucose homeostasis by controlling various pathways of glucose metabolism. Plasma (Xiong et al., 2014) and hepatic (Kristensen et al., 2017; Xiong et al., 2014) triglyceride concentrations have been reported to increase with aging in mice. This indicates that aging is associated with an imbalance in fatty acid uptake and synthesis versus fatty acid utilization in the liver, resulting in hepatic steatosis.

Several hepatic metabolic processes depend on ATP, and it is thus important that the liver maintains functional metabolic pathways for ATP production (Haase et al., 2011; Yoon et al., 2001). Previous studies have reported lower Cyt c and COXIV protein in the liver of aged than young rodents (Houtkooper et al., 2011; Navarro et al., 2004), and this was shown to be accompanied by higher hepatic protein carbonylation in aged than Young rodents (Houtkooper et al., 2011; Navarro and Boveris, 2004; Navarro et al., 2004). Furthermore, hepatic superoxide dismutase (SOD)2 protein and Catalase protein have been shown to
decrease with aging (Houtkooper et al., 2011; Navarro and Boveris, 2004; Navarro et al., 2004), and increased mitochondrial DNA damage has been reported with aging in rats. Taken together, this suggests that aging is associated with decreased mitochondrial capacity and enhanced oxidative stress in the liver, potentially affecting metabolic function. An increased need for removal of dysfunctional mitochondria is therefore likely required with aging.

Removal of damaged mitochondria is achieved by autophagy and mitophagy, which is important for maintaining cell and mitochondrial function, through lysosomal degradation of cellular components, including mitochondria. Autophagy involves formation of autophagosomes and lipidosome of cytosolic LC3II to LC3I. Previous studies have reported lower content of autophagy markers (Beclin 1 and LC3 I protein) in the liver of aging rats (24 months) than in Young rats (6 months) (Wohlgemuth et al., 2007) and lower LC3II and LC3I protein in 12 and 24 months old mice than in 2 months old mice (Uddin et al., 2012). These results suggest that hepatic autophagy capacity is reduced with aging, which may be a contributing factor to the age-associated impairment of hepatocyte function. However, age-associated regulation of autophagy capacity in the liver requires further investigation.

Dysfunctional mitochondria are removed through mitophagy, which is a selective form of autophagy involving PINK1-Parkin and BNIP3/NIX pathways that target damaged mitochondria to autophagosomal degradation through LC3II linkage. BNIP3 and NIX directly interact with LC3II inducing autophagic degradation of the mitochondria, whereas PINK1 accumulates on the outer mitochondrial membrane in response to mitochondrial damage, recruiting cytosolic Parkin to the damaged mitochondria. Parkin then recruits p62 through poly-ubiquitination resulting in translocation of damaged mitochondria to the autophagosome membrane through p62 linkage to LC3II, with concomitant degradation. Mitochondrial localization of Parkin is thus determined by the level of mitochondrial damage, and reduced Parkin protein content reflects lower capacity for Parkin mediated mitophagy (Yid et al., 2016; Gao et al., 2004; Guo et al., 2008). A single study has examined the regulation of hepatic mitophagy with aging, reporting that hepatic Parkin protein was increased in 12 and 24 months old mice relative to 2 months old mice (Santos-Alves et al., 2015). This indicates increased capacity for clearance of damaged mitochondria in the liver with aging supporting that aging may increase hepatic mitophagy. However, the impact of aging on the hepatic BNIP3/NIX pathway in the liver remains to be determined, and the hepatic PINK1/Parkin pathway requires further investigation. Furthermore, the effect of aging on mitochondrial localization of mitophagy proteins is unresolved.

The transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α is a well described key regulator of mitochondrial biogenesis and energy metabolism (Brandt et al., 2018; Geng et al., 2010; Leick et al., 2008; Lin et al., 2002; Lin et al., 2004). Muscle PGC-1α has also been suggested to mediate organ cross-talk with regulatory effects in the liver. Thus, a previous study reported that muscle specific PGC-1α overexpression was associated with elevated plasma β-aminoisobutyric acid (BAIBA) as well as increased hepatocyte oxygen consumption rate and hepatic β-oxidation through a PPARG-dependent mechanism (Roberts et al., 2014). Furthermore muscle PGC-1α has also been reported to mediate an exercise-induced upregulation of Irisin secretion into blood with a concomitant induction of brown adipose tissue genes in white adipose tissue improving several metabolic parameters, through an increased energy expenditure and glucose tolerance (Boström et al., 2012). However, whether muscle PGC-1α is required for maintenance of oxidative capacity as well as autophagic and mitophagy capacity in the liver with aging remains to be determined.

Therefore, the overall aim of the present study was to investigate 1) the effect of aging on mitochondrial autophagy and mitophagy capacity in mouse liver and 2) whether muscle PGC-1α is required for maintaining autophagy and mitophagy capacity in the liver during aging.

2. Methods
2.1. Mice

Inducible muscle-specific PGC-1α knockout mice were generated by crossing PGC-1α exon 3–5 floxed C57BL/6N mice as previously described (Lin et al., 2004) with mice expressing a tamoxifen inducible Cre recombinase under the control of an upstream human α-skeletal actin promoter (HAS-MerCreMer) (McCarthy et al., 2012). Due to severe testicular abnormalities observed in male mice upon tamoxifen injections, only female mice were used for all experiments. All animal experiments were carried out in accordance with the EU directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Experimental protocol

Eight weeks old (Young) and 15 months old (Aged) PGC-1α floxed mice, either +/- (iMKO) or +/- (Lox) for the HSA-MerCreMer construct, were injected with tamoxifen (40 mg/kg body weight) for 3 consecutive days, to induce the muscle specific knockout of PGC-1α. The mice were housed in groups of 6–8 mice with ad libitum access to chow diet (Altromin 1314F, Brogaarden, Lynge, Denmark) and water. Eight weeks after the tamoxifen injections, mice were euthanized by cervical dislocation, and tissues were either snap-frozen in liquid nitrogen or prepared for analyses as described below. It should be noted that another group of Aged mice were exercise trained for 7 weeks described in the original experimental protocol (Halling et al., 2019), but these mice are not included in the current experimental setup as only aging is in focus.

2.3. Plasma glucose assay

The principal behind the assay for determining plasma glucose is the same as for the glycogen assay described below. A dilution series of Glucose Standard solution was prepared to obtain a standard curve for the glucose concentrations, used for converting the emission to a glucose concentration.

2.4. Plasma FFA

Plasma nonesterified fatty acid (NEFA) concentrations were measured colorimetrically using a NEFA-HR 2 kit, according to the manufacturer’s guidelines (WAKO Diagnostics). The absorbance was measured in a Multiskan (Thermo Scientific, Rockford IL, USA).

2.5. Hepatic triglycerides

Triglycerides were extracted from ~20 mg crushed liver samples by saponification, using ethanolic KOH to produce glycerol. Free glycerol was determined using Free Glycerol Reagent (Sigma Aldrich, Denmark) where the production of the fluorescent dye quinoneimine was measured spectrophotometrically at 540 nm using a Multiscan (Thermo Scientific, Rockford IL, USA). A standard curve was constructed from a dilution series of Glycerol Standard, and used to calculate the free glycerol concentration of each sample. Finally, the free glycerol concentration was converted to triglyceride content.

2.6. Hepatic glycogen

Hepatic glycogen was determined using acid hydrolysis as previously described (Lowry, 1972). In short, hepatic tissue samples of ~10-15 mg were boiled for 2 h in 1 M HCl to hydrolyze glycogen to glycosyl units and subsequently neutralized with NaOH, and measured fluorometrically. A standard curve was constructed using a Glucose Standard Solution (Sigma G-6918) for conversion.
2.7. CS activity

Liver homogenate was prepared from approx. 10 mg liver tissue in a 0.3 M phosphate buffer with BSA added using a Tissue Lyser II (Qiagen, Hilden, Germany). Maximal citrate synthase (CS) activity was determined using the Sigma-Aldrich Citrate Synthase assay kit (Sigma-Aldrich, MO, USA), measuring the absorbance in a Multiskan (Thermo scientific, Rockford, IL, USA).

2.8. RNA isolation, reverse transcription and real-time PCR

Crushed liver samples (~10–15 mg) were homogenized using a Tissue lyser II (Qiagen, Germany) and total RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi (1987) as previously described (Pilegaard et al., 2000). The RNA concentration and purity of the samples were determined by spectrophotometric analysis using a NanoDrop spectrophotometer (Thermo Scientific, De, USA). Reverse transcription of mRNA to cDNA was performed on 3 μg total RNA using Superscript II and Oligo dt (Invitrogen, Carlsbad, CA) as previously described (Pilegaard et al., 2000).

Real-time PCR was performed to quantify the amount of PGC-1α mRNA using forward and reverse primers and Taqman probes designed using Primer Express 3.0 software (Applied Biosystems, Waltham, MA, USA). The cycle threshold for each sample was converted into a relative mRNA, using a standard curve (Applied Biosystems, Waltham, MA, USA). The real-time PCR was run in triplicates with a total reaction volume of 10 μl using Mastercycler II (Applied Biosystems, Waltham, MA, USA). The cycle threshold for each sample was converted into a relative mRNA, using a standard curve generated from a dilution series of a pooled sample made from the cDNA samples. Total single stranded (ss) DNA in each sample was determined using OliGreen reagent (Molecular Probes, Leiden, The Netherlands) as previously described in (Lundby et al., 2005).

2.9. Liver lysates and protein determination

Crushed liver tissue (~25–30 mg) was homogenized in ice-cold buffer (10 % glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1 % NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na3VO4, 3 mM benzamidine) as previously described (Birk and Wojtaszewski, 2006) using a TissueLyser II (Qiagen, Hilden, Germany) at 30 s−1 for 2 min. The protein concentration of the lysate samples was determined using the bicinchoninic acid method (Pierce Biotechnology Inc., Rockford, IL, USA), and lysates were prepared in sample buffer containing sodium dodecy1 sulfate (SDS) at a concentration of 2 μg/ml protein. Samples were boiled for 3 min at 96 °C before SDS-PAGE and western blotting except for OXPHOS.

2.10. SDS-PAGE and western blotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (immobilon-P Transfer Membranes; Millipore, Denmark) by semi-dry blotting. After blocking in 3 % fish gel for 1 h membranes were incubated in primary antibodies against OXPHOS (Abcam ab110413), SOD2 (Millipore NP_006262), Catalase (Santa Cruz sc-50508), 4-Hydroxyxynonenal (HNE) (Abcam ab46545), LC3 (NovusBio NB100-2220), p62 (Abcam ab56416), Parkin (Cell Signaling #4211) and BNIP3 (Cell signaling #3769). The following day the membrane was incubated with Horseradish Peroxide (HRP)-conjugated secondary antibody diluted in TBST with 3 % fish gel and the bands were detected by incubating the membrane with an enhanced chemiluminescence (ECL) reagent Luminata™ Classic (Millipore, Denmark) using the image analyzer ImageQuant LAS 4000. The band intensities were quantified using the ImageQuant TL software (GE Healthcare). The specific protein content is given in arbitrary units (AU) relative to the average of standards, a pool of all samples, loaded on each side of the gel.

2.11. Isolation of mitochondria

Mitochondria were isolated from approximately 0.5–1 gram liver using a mitochondrial isolation buffer (MIM), containing sucrose, HEPES and EGTA (ethylen glycol-bis (β-aminoethy1 ether)-N,N,N’,N’-tetraacetic acid), and a MIM + Bovine Serum Albumin (BSA) buffer. The tissue samples were homogenized in MIM + BSA buffer using a Glass Tissue Grinder (Thomas Scientific, Swedensboro, USA), gently by hand. Homogenates were transferred to 50 ml oakridge tubes (Thermo Scientific™ Nalgene™) and centrifuged at 800g for 10 min at 4 °C twice. The supernatant was transferred to new oakridge tubes and centrifuged at 12,000g for 15 min at 4 °C, precipitating the mitochondria. The pellet was resuspended in 200-400 μl MIM, depending on initial weight, by very gentle pipetting, and cytosolic supernatant was kept as control. The samples were kept on ice in all steps. The mitochondrial isolations were confirmed by western blotting using adenine nucleotide translocase (ANT)2 antibody as a mitochondrial marker and fatty acid synthase (FAS) as a cytosolic marker, with isolated mitochondria and their cytosolic counterparts loaded in pairs.

2.12. Statistics

Data are presented as mean ± SE. Statistical analyses were performed using a two-way analysis of variance (ANOVA) to test for differences between groups and genotypes. When a main effect was identified the Student-Newman-Keuls multiple comparisons test was performed to localize the differences. Differences were considered statistically significant at p < 0.05 and a tendency is reported at 0.05 ≤ p < 0.1. Statistical calculations were performed using SigmaPlot 13.0 software (SYSTAT Software, USA). Figures were made using Graphpad Prism 9 (Graphpad Software, CA, USA).

3. Results

3.1. Plasma glucose and FFA

There was an overall tendency for an interaction between groups and genotype for plasma glucose levels (p = 0.056), but no significant differences were evident in plasma glucose levels between Young and Aged in either Lox or iMKO mice or between Lox and iMKO mice within Young or Aged (Table 1).

| Table 1 | Plasma glucose (mmol/l), plasma free fatty acids (FFA; mmol/l), hepatic tri-glycerides (mmol/g) and hepatic glycogen content (μmol/g) in Young and Aged liver and inducible muscle-specific peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α knockout (iMKO) mice. Values are means ± SE; n = 9–12.* Significantly different from Young within same genotype, p < 0.05. ($) Tends to be significantly different from Young within same genotype, 0.05 ≤ p < 0.1. ($) indicates a tendency for an overall interaction. |

<table>
<thead>
<tr>
<th>Young</th>
<th>Aged</th>
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<tbody>
<tr>
<td></td>
<td>Lox</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>Plasma FFA (mmol/l)</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Hepatic triglycerides (mmol/g)</td>
<td>20.2 ± 3.1</td>
</tr>
<tr>
<td>Hepatic glycogen (μmol/g)</td>
<td>449.3 ± 27.1</td>
</tr>
</tbody>
</table>
There were no differences in plasma FFA between Young and Aged, either within Lox or iMKO mice or between Lox and iMKO mice within Young or Aged (Table 1).

3.2. Hepatic metabolic parameters

Hepatic triglyceride content was approximately 1.8 fold higher \((p < 0.05)\) in Aged than Young Lox and tended to be 1.5 fold higher \((p = 0.081)\) in Aged than Young iMKO mice (Table 1). There were no differences in hepatic triglyceride content between Lox and iMKO within Young or Aged (Table 1).

Hepatic glycogen content tended to be \(\sim 10\%\) lower \((p = 0.098)\) in Aged than Young Lox and was approximately 20\% lower \((p < 0.05)\) in Aged than Young iMKO mice (Table 1). There were no differences in hepatic glycogen content between Lox and iMKO within Young or Aged (Table 1).

3.3. Oxidation and mitochondrial activity

Hepatic PGC-1\(\alpha\) mRNA content (Fig. 1A), total OXPHOS protein (Fig. 1B) and hepatic CS activity (Fig. 1C) were not different between Young and Aged either in Lox and iMKO mice and not different between Lox and iMKO within either Young or Aged. On the other hand, muscle PGC-1\(\alpha\) was reduced by 70–80\% in iMKO mice relative to controls, confirming the muscle specific PGC1-\(\alpha\) knockout model (Halling et al., 2019).

3.4. Antioxidant defence

Hepatic SOD2 protein tended to be 15\% lower \((p = 0.056)\) in Aged than Young Lox mice (Fig. 2A), with no differences in hepatic SOD2 protein between Aged and Young iMKO mice. No differences were observed in hepatic SOD2 protein between Lox and iMKO within Young or Aged (Fig. 2A). Furthermore, no differences were observed in either hepatic Catalase protein (Fig. 2B) or 4-HNE modified proteins (Fig. 2C) between Young and Aged within either Lox or iMKO, and there was no difference in hepatic Catalase or 4-HNE protein between Lox and iMKO mice in either Young or Aged.

3.5. Autophagy and mitophagy pathways

There was no difference in hepatic LC3II/LC3I protein ratio between Young and Aged within Lox or iMKO, however there was an overall tendency for lower \((p = 0.084)\) hepatic LC3II/ LC3I protein ratio in iMKO than Lox (Fig. 3A).

Hepatic p62 protein was approximately 1.4 fold higher \((p < 0.05)\) in Aged than Young Lox mice and tended to be 1.3 fold higher \((p = 0.067)\) in Aged than Young iMKO mice. However, there was no difference in hepatic p62 protein content between Lox and iMKO within Young or Aged (Fig. 3B).

Hepatic Parkin protein was approximately 7 fold higher \((p < 0.05)\) in Aged than Young Lox mice and 3 fold higher \((p < 0.05)\) in Aged than Young iMKO mice (Fig. 3C). On the other hand, no differences were observed in hepatic Parkin protein between Lox and iMKO within Aged
Hepatic BNIP3 protein was 1.4–1.5 fold higher (p < 0.05) in Aged than Young Lox mice and 1.5 fold higher (p < 0.05) in Aged iMKO mice with no difference between Lox and iMKO within Young or Aged (Fig. 3D).

Mitochondria were isolated from the liver to further investigate potential changes in localization of Parkin and BNIP3. Hepatic mitochondrial Parkin protein was approximately 4 fold higher (p < 0.05) in Aged than Young iMKO mice with no difference between Lox and iMKO within Young or Aged (Fig. 4A). Furthermore, hepatic mitochondrial Parkin protein was not significantly different between Lox and iMKO within Young or Aged (Fig. 4A).

There were no differences in hepatic mitochondrial BNIP3 protein between Young and Aged, either within Lox or iMKO mice and there were no difference in mitochondrial BNIP3 protein between Lox and iMKO mice within Young or Aged (Fig. 4B).

4. Discussion

The main findings of the present study are that hepatic total p62, Parkin and BNIP3 protein were higher in Aged than Young mice, indicating a higher capacity for mitophagy with aging through the PINK1/Parkin pathway. Moreover, simultaneously hepatic mitochondrial Parkin protein was also higher in Aged iMKO mice than Aged Lox mice.

The present finding that total hepatic Parkin protein was higher in Aged than Young mice is corroborated by a single study reporting increased Parkin protein in 12- and 24-months old mice relative to 2 months old mice (Santos-Alves et al., 2015). Furthermore, the higher total hepatic BNIP3 protein in Aged than Young mice in the present study, is a novel observation, but in accordance with skeletal muscle studies in rodents reporting increased BNIP3 protein with aging (Carter et al., 2018; O’Leary et al., 2013; Sebastian et al., 2016), and suggests enhanced capacity for mitophagy through the BNIP3/NIX pathway with aging as well.
The present observation that hepatic mitochondrial Parkin protein was higher in iMKO than Lox within the Aged mice, is also novel, and may indicate that hepatic mitochondria have a higher requirement for Parkin, which may suggest a higher degree of hepatic mitochondrial damage when muscle PGC-1α is lacking. It may therefore be speculated that muscle PGC-1α is required for proper mitochondrial function in the liver of Aged mice. This is supported by studies on skeletal muscle that demonstrated impaired mitochondrial function and altered mitochondrial network structure in whole-body PGC-1α KO and inducible muscle-specific PGC-1α KO mice (Adhihetty et al., 2009; Halling et al., 2017; Halling et al., 2019).

The effects of muscle PGC-1α on mitochondrial Parkin protein in the liver may be mediated through myokine release, as previous studies have reported that myokines can deliver muscle PGC-1α mediated
signals to other organs, with implications in mitochondrial biogenesis, and hepatic β-oxidation (Bostrom et al., 2012; Roberts et al., 2014). A potential regulator may be BAIBA, a myokine inducing browning of WAT and increasing hepatic β-oxidation (Roberts et al., 2014). Another potential regulator is the myokine IL-15, which has been reported to regulate lipid oxidation and probably thermogenesis in BAT, processes mediated by UCPs (Almendro et al., 2008). As BAIBA and IL-15 seem to regulate mitochondrial biogenesis as well as β-oxidation, a potential effect of such factors on the function of mitochondria and translocation of Parkin in the liver is likely.

The present observation that hepatic LC3II/I protein ratio was unchanged in Aged compared to Young mice is in accordance with previous studies reporting unchanged LC3II content and LC3II/I ratio in the liver of 15- and 24-months old mice relative to young (Kristensen et al., 2017; Uddin et al., 2012). This indicates that autophagosome number is unaffected by aging in the liver, reflecting that the requirement for removal of damaged cellular components in mouse liver is not affected with aging. However, the observed increase in hepatic p62 protein in Aged relative to Young mice in the present study indicates lower degree of autolysosomal activity or that the capacity for cargo delivery is unchanged in Aged compared to Young mice is in accordance with previous mouse studies on skeletal muscle, in which CS activity remained unchanged in 13 months old mice (Leick et al., 2010) relative to young mice in the respective studies. Taken together, this indicates that the hepatic mitochondrial content was unchanged, and the increase in Parkin and BNIP3 protein content with aging may therefore suggest the need for an enhanced mitophagy capacity in the liver due to mitochondrial damage.

The current observation that hepatic SOD2 protein tended to decrease with aging indicates a slightly lower antioxidant defence which potentially may be associated with increased oxidative stress. However, the observed unchanged 4-HNE modified proteins in Aged relative to Young mice in the present study indicates that lipid oxidation was unchanged and thus oxidative damage in the liver was unaffected with aging. In previous studies protein carbonylation has been shown to increase in the liver of 19/24 months old mice and 23 months old rats (Houtkooper et al., 2011; Navarro and Boveris, 2004; Navarro et al., 2004) or stay unchanged in 15 months old mice (Kristensen et al., 2017). Increased oxidative stress can therefore not be precluded, as decreased SOD2 may lead to other oxidative modifications than lipid peroxidation. However, the unchanged protein carbonylation in Kristensen et al. indicates that oxidative damage may occur later than 15 months in mouse liver explaining the lack of change in lipid oxidation in the present study. In addition, it may be speculated that the elevated mitochondrial Parkin protein levels observed indicate that removal of damaged mitochondria is high, explaining the low level of protein carbonylation.

In conclusion, the present study demonstrates a modulation of autophagy and mitophagy markers in mouse liver during aging, without mitochondrial function being impaired with aging (Buch et al., 2020). The novel observation that hepatic CS activity remained unchanged with aging in the present study is in line with the unchanged OXPHOS protein in the liver with aging and also indicates unchanged mitochondrial content with aging. This observation is in part in accordance with previous mouse studies on skeletal muscle, in which CS activity remained unchanged in 15 months old mice (Halling et al., 2017) while CS activity decreased in 13 months old mice (Leick et al., 2010) relative to young mice in the respective studies. Taken together, this indicates that the hepatic mitochondrial content was unchanged, and the increase in Parkin and BNIP3 protein content with aging may therefore suggest the need for an enhanced mitophagy capacity in the liver due to mitochondrial damage.

Fig. 4. Hepatic Mitochondrial parkin protein content (A) and mitochondrial BCL2/adenovirus E1B 19 kDa protein-interacting protein (BNIP)/3 protein content (B) from Young and Aged lox and inducible muscle-specific peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α knockout (iMKO) mice. Values are means ± SE; n = 2–3. In addition, individual data points are shown. *Significantly different from Young within same genotype, p < 0.05. #Significantly different from iMKO within same age group, p < 0.05.
changes in hepatic oxidative capacity and redox stress markers. These observations were independent of muscle PGC-1α. However, mitochondrial Parkin protein was higher in livers from Aged muscle-specific PGC-1α knockout mice than control mice, suggesting that muscle PGC-1α is in part required for hepatic mitochondrial translocation of Parkin potentially assisting in maintaining functional liver mitochondria at old age.

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**CRediT authorship contribution statement**

Natascha Masselkhi Christensen: Investigation, Analysis, Writing – original draft, Stine Ringholm: Analysis, Writing – Original draft, Bjørn Buch: Investigation, Writing – Review & editing, Anders Gudiksen: Investigation, Writing – Review & editing, Jens Frey Halling: Investigation, Writing – Review & editing, Henriette Pilegaard: Conceptualization, Methodology, Funding acquisition, Supervision, Investigation, Writing – Review & editing.

**Declaration of competing interest**

No conflicts of interest, financial or otherwise, are declared by the authors.

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