Short and long-term effect of reproduction on mitochondrial dynamics and autophagy in rats

Hailey A. Parry\textsuperscript{a,1}, Ryleigh B. Randall\textsuperscript{b,1}, Hayden W. Hyatt\textsuperscript{b,c}, Wendy R. Hood\textsuperscript{d}, Andreas N. Kavazis\textsuperscript{*}

\textsuperscript{a} School of Kinesiology, Auburn University, Auburn, AL, USA
\textsuperscript{b} Department of Chemistry and Biochemistry, Auburn University, Auburn, AL, USA
\textsuperscript{c} Department of Applied Physiology and Kinesiology, University of Florida, Gainesville, FL, USA
\textsuperscript{d} Department of Biological Sciences, Auburn University, Auburn, AL, USA

ARTICLE INFO

Keywords:
Gestation
Lactation
Pregnancy

ABSTRACT

We evaluated mitochondrial dynamics and autophagy by investigating the acute and long-term changes in the liver and skeletal muscle of rats in multiple reproductive stages. A total of 48 rats were used. Rats were randomly assigned to three groups (\(n = 16\) per group): nonreproductive females; females that became pregnant, gave birth, but had their pups removed at birth, and thus, did not lactate; and females that experienced pregnancy, gave birth, and were allowed to lactate. Each group was further divided into two-time subgroups (\(n = 8\) per subgroup) and data were collected at a time-point corresponding to 1) peak lactation (day 14 of lactation) in the lactating animals (4 months of age) and 2) 15 weeks after parturition (12 weeks post-weaning in lactating animals; 7 months of age). Levels of several proteins involved in mitochondrial dynamics and the autophagy system were measured in the liver and skeletal muscle. Beclin1 protein levels in the liver were higher in non-lactating rats two weeks after parturition, while Beclin1 protein levels were highest in 7-month-old animals that had previously experienced a standard reproductive event that included pregnancy and a full 3 week of lactation. These animals also exhibited higher protein levels of the mitochondrial fusion marker Mfn2 in the liver. In skeletal muscle, we also observed increased protein levels of the mitochondrial fission marker DRP1 in non-lactating animals compared to animals that lactated. In summary, our data provide insightful information on the mechanisms that influence liver and skeletal muscle remodeling in response to the metabolic challenges of reproduction, and lactation in particular. Autophagy remodeling and mitochondrial fusion seem to coincide with liver mass size during the lactation stage of reproduction. Our findings highlight the complex changes that occur in the liver and skeletal muscle during reproduction, and highlights the remarkable plasticity required during this demanding metabolic feat.

1. Introduction

Reproduction increases the metabolic demand of a female mammal, resulting in cellular remodeling (Wade and Schneider, 1992). Although reports of mitochondrial function have previously been published in pregnant and lactating animals (Hyatt et al., 2017, 2019), no research has investigated how the morphology of the mitochondria change with lactation. This appears to be paramount since evidence shows a strong link between mitochondrial morphology and function (Bleck et al., 2018; Glancy, 2020; Glancy et al., 2017).

Mitochondria are important cellular organelles that undergo adjustments based on the energy needs of the body and maintaining a healthy pool of mitochondria is critical during cellular remodeling. Two processes that facilitate these changes are mitochondrial fission and fusion. Mitochondrial fission involves the splitting of one mitochondrion into two or more organelles and two of the proteins involved in this pathway include dynamin related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1). Mitochondrial fusion involves the formation of one mitochondrion from other independent mitochondrial structures and requires the use of mitofusion proteins 1/2 (Mfn1/2) and optic atrophy...
type 1 protein (OPA1) (Lee and Yoon, 2018; Scott and Youle, 2010). Importantly it is the balance of these processes that results in healthy functional mitochondria in cells (Meyer et al., 2017; Scott and Youle, 2010).

In addition to mitochondrial fission and fusion, autophagy involves a cellular cascade of signaling molecules which is responsible for cellular turnover (He and Klionsky, 2009). Importantly, autophagy occurs within organisms (Eskelinen, 2019) and is a vital process in maintaining a healthy pool of mitochondria (Oami et al., 2018). Autophagy requires the coordination of a plethora of proteins (e.g., autophagy related (ATG)) in order to facilitate protein turnover. Important markers of autophagy include Beclin1, conjugate marker ATG5-ATG12, ATG16L, and the LC3BII/LC3BI ratio (He and Klionsky, 2009). Beclin1 is the marker for the activation of autophagy. The conjugate marker ATG5-ATG12 and ATG16L are both markers of phagophore formation which represents the start of autophagosome formation. Lastly, when LC3BII is converted into LC3BII on the phagophore membrane, this concludes the formation of the autophagosome before formation with the lysosome. Readers are guided to other thorough reviews for more details of this process (Eskelinen, 2019; He and Klionsky, 2009; Zarzyska and Motyl, 2008). Under times of increased energy demand (e.g., pregnancy and lactation), where high cellular turnover is important for maintaining healthy tissue, autophagy could play a critical role. However, few studies have investigated how reproduction alters autophagy in either the liver or skeletal muscle of female mammals.

Heat shock proteins are a family of chaperone proteins that, in response to chemical or physical stress, prevent excess damage to cells and aid in cellular recovery (Beere, 2004). The 70 kD heat shock protein (HSP70) plays an important role in normal development and an individual’s response to stress (Evans et al., 2010). Under normal conditions, HSP70 is involved in protein folding, transport, and degradation. In response to cellular stressors, however, HSP70 binds and stabilizes substrates in order to prevent their degradation or damage inside the cell (Evans et al., 2010). HSP70 appears to play an important role in protection against metabolic disease and insulin resistance (Chichester et al., 2015) and cellular adaptations to HSP70 during reproduction can be critical to meeting the energy demands of the cell.

In the current study we investigated the acute and long-term changes in mitochondrial dynamics, the autophagy system, and heat shock proteins in multiple reproductive stages in the liver and skeletal muscle of rats. We hypothesized the changes in mitochondrial function previously observed with lactation (Hyatt et al., 2017, 2019) occurred in part by an increase in mitochondrial fusion. Furthermore, we hypothesized that the large changes in liver mass size previously reported by our group and others that occurs during the different stages of reproduction may be mechanistically explained through the autophagy process. Lastly, we hypothesized lactation to increase HSP70, as this protein would contribute against metabolic diseases. The liver and skeletal muscle were chosen for the current investigation since they are two of the most metabolically active tissues within an organism, and both are responsive to the demands of reproduction.

2. Materials & methods

2.1. Animals and experimental design

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to and all experimental procedures were approved by Auburn University’s Institutional Animal Care and Use Committee (PRN, 2014–2591) and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Ten-week-old Sprague-Dawley rats were acclimated with their diet and facility for ten days prior to experimental start. Rats were housed under standard laboratory conditions (46 × 20 cm boxes, 12/12 light/dark cycle, 22 °C, 50% relative humidity) and given ad libitum access to food and water. Liver and skeletal muscle samples used for this study were residual from a study describe in two prior publications (Hyatt et al., 2017, 2019). These samples were flash frozen at the time of collection and were not thawed prior to this analysis.

Specifically, liver and skeletal muscle from six groups were used (n = 8 per group). Groups 1–3 were aged matched and sacrificed at approximately four months of age, and groups 4–6 were aged matched and sacrificed at approximately seven months of age. The groups were: (1) NR-4 month = non-reproductive adult females, (2) P-4 month = adult females that became pregnant, gave birth, but had their pups removed within 12 h of birth, and thus did not lactate; these animals were sacrificed two weeks post-parturition, (3) L-4 month = adult females that became pregnant, gave birth, and suckled their young (litter size adjusted to eight on day of parturition) to peak lactation (i.e., 14 days), (4) NR-7 month = non-reproductive adult females, (5) P-7 month = adult females that became pregnant, gave birth, but had their pups removed within 12 h of birth, and thus did not lactate; these animals were sacrificed fifteen weeks post-parturition (P-7 month), (6) L-7 month = adult females that became pregnant, gave birth (litter size adjusted to eight on day of parturition), and suckled their young for three weeks; pups were removed and the mothers were sacrificed fifteen weeks post-parturition. Figure 1 depicts the animal groups.

2.2. Western blotting

Approximately 60–70 mg of liver or skeletal muscle samples were homogenized via micropestle manipulation in cell lysis buffer (5 mM Tris HCl, 5mM EDTA) containing protease inhibitors (2.5 mM pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate; IB, Road Dubuque, IA). Insoluble proteins from homogenates were removed with centrifugation at 1500g for 5 min. Homogenates were then stored at -80 °C. Protein determination on cell lysis homogenates was performed via Bradford Assay (Bradford, 1976). Homogenates were prepared for Western blotting at 2 μg/μL. Subsequently, 15 μl of prepped sample were separated by polyacrylamide gel electrophoresis via 4–15% or 12% precast polyacrylamide gels (Bio-Rad, Hercules, CA) containing 0.1% sodium dodecyl sulfate for ~1 h at 200 V.

After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Amresco, Radnor, PA) via the Bio-Rad system for 2 h at 200 mA. Nonspecific sites were blocked for 1 h at room temperature in TBS solution containing 0.05% Tween and 5% nonfat milk. Membranes were then incubated for 1 h with primary antibodies directed against the proteins of interest. The primary antibodies used were mitofusin 2 (Mfn2; #3882-100; BioVision, Milpitas, CA), dynamin related protein 1 (Drp1, #NB110-552888S; Novus Biologicals USA, Littleton, CO), microtubule-associated protein light chain 3 (LC3; #NB100-2220; Novus Biologicals USA, Littleton, CO), beclin-1 (Beclin1; #NB110-87318; Novus Biologicals USA, Littleton, CO), autophagy related 12-5 conjugate (ATG12-5; #NB110-53818; Novus Biologicals USA, Littleton, CO), heat shock protein 70 (HSP70; #ab79852, abcam, Cambridge, MA), and heat shock protein 90 (HSP90; #ab13495; abcam, Cambridge, MA). Antibodies used in the current study have previously been validated (An et al., 2018; Challa et al., 2019; Wang et al., 2019; Essick et al., 2013; Kim et al., 2018; Pfötsch et al., 2020; Wang et al., 2019; Xu et al., 2016). Following incubation with primary antibodies, membranes were washed extensively with TBS–TWEEN and then incubated with conjugated horse-radish peroxidase secondary antibodies. Membranes were then developed using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA), and band densitometry was performed using a UVP Imager and associated densitometry software (UVP, LLC, Upland, CA). Ponceau staining was used as the normalizing control.
2.3. Statistical analysis

All data are presented as mean ± standard deviation and figures contain individual data points for each animal. Statistics were performed using Prism (GraphPad Software, San Diego, CA, USA). A two-way analysis of variance (ANOVA) was performed on all variables to determine differences and a Fisher’s least significant difference post-hoc test was performed if a significant interaction effect was found. Statistical significance was determined at \( p < 0.05 \).

3. Results

3.1. Autophagy markers in liver

Beclin1 was differentially expressed (interaction: \( p < 0.001 \), group: \( p = 0.001 \), time: \( p = 0.183 \); Figure 2A). In the 4-month-old rats, the P group had the highest Beclin1, whereas in the 7-month-old rats the L group had the highest Beclin1. Also, the P and L groups had higher levels of Beclin1 than NR. ATG5-ATG12 was differentially regulated (interaction: \( p = 0.033 \), group: \( p = 0.983 \), time: \( p = 0.001 \); Figure 2B) where 7-month-old rats expressed higher levels of ATG5-ATG12 compared to 4-month-old rats. Also, ATG5-ATG12 conjugate was lower in the 4-month-old L group compared to the 7-month-old L group. ATG16L was highest in the NR rats compared to the other two groups (interaction: \( p = 0.525 \), group: \( p = 0.005 \), time: \( p = 0.725 \); Figure 2C). LC3II/LC3BI ratio was differentially regulated (interaction: \( p = 0.024 \), group: \( p = 0.145 \), time: \( p = 0.975 \); Figure 2D) where the 4-month-old P group had the lowest level.

3.2. Autophagy markers in skeletal muscle

Beclin1 was differentially expressed (interaction: \( p = 0.139 \), group: \( p = 0.048 \), time: \( p = 0.091 \); Figure 3A) and ATG12-ATG5 marker was higher in NR compared to L and 4-month-old rats had higher levels compared to 7-month-old rats (interaction: \( p = 0.201 \), group: \( p = 0.006 \), time: \( p < 0.001 \); Figure 3B). No significant changes were observed for the ATG16L (interaction: \( p = 0.204 \), group: \( p = 0.877 \), time: \( p = 0.574 \); Figure 3C) or LC3II/LC3BI ratio (interaction: \( p = 0.798 \), group: \( p = 0.839 \), time: \( p = 0.079 \); Figure 3D).

3.3. Mitochondrial dynamics markers in liver

Mfn2 protein was differentially expressed (interaction: \( p = 0.001 \), group: \( p = 0.163 \), time: \( p = 0.091 \); Figure 4A). In the 4-month-old rats, the P group had the highest Mfn2, whereas in the 7-month-old rats the L group had the highest Mfn2. No significant differences were observed in Drp1 levels (interaction: \( p = 0.135 \), group: \( p = 0.545 \), time: \( p = 0.097 \); Figure 4B).

3.4. Mitochondrial dynamics markers in skeletal muscle

No significant changes were observed in Mfn2 levels (interaction: \( p = 0.721 \), group: \( p = 0.201 \), time: \( p = 0.720 \); Figure 4C). Protein levels of Drp1 were higher in P group compared to L group, and 4-month-old rats had higher levels compared to 7-month-old rats (interaction: \( p = 0.377 \), group: \( p = 0.021 \), time: \( p = 0.004 \); Figure 4D).

3.5. Heat shock proteins in liver

No significant changes were found for HSP70 (interaction: \( p = 0.093 \), group: \( p = 0.286 \), time: \( p = 0.080 \); Figure 5A) or HSP90 (interaction: \( p = 0.424 \), group: \( p = 0.987 \), time: \( p = 0.962 \); Figure 5B).

3.6. Heat shock proteins in skeletal muscle

HSP70 was higher in 7-month-old rats compared to 4-month-old rats (interaction: \( p = 0.098 \), time: \( p < 0.001 \), group: \( p = 0.578 \); Figure 5C), but HSP90 was not significantly different (interaction: \( p = 0.084 \), time: \( p = 0.072 \), group: \( p = 0.070 \); Figure 5D).

Figure 1. Schematic of experimental design. A total of 48 rats were used for the current study. Rats were split into three groups (n = 16 per group): nonreproductive (NR); females that became pregnant, gave birth, but had their pups removed and did not lactate (P); and females that became pregnant, gave birth, and were allowed to lactate (L). Each group was divided into another two subgroups (n = 8 per subgroup) and data were collected when rats were 4 and 7 months old.
4. Discussion

We have previously reported that mitochondria function (e.g., respiration and electron transport chain complex activities) are differentially affected in the liver and skeletal muscle during different reproductive states (Hyatt et al., 2017, 2019). In the current study, we addressed a possible connection between mitochondrial dynamics and autophagy that may explain these reported changes. Our results from the current study provide important new information of how these cellular pathways respond and details are discussed below.

In the liver, we observed higher expression of an autophagy activation marker (i.e., Beclin1) in the 4-month-old non-lactating rats, while Beclin1 protein expression was highest in the 7-month-old lactating group. In addition, these two groups also had higher protein expression of the mitochondrial fusion marker Mfn2 in liver tissue. The increased marker of mitochondrial fusion at these time points would suggest an increase in mitochondrial network. Generally, an increase in mitochondrial fusion has been reported with higher metabolic function in liver cells (Marycz et al., 2018; Mollica et al., 2017). Since autophagy is known to be an energetically demanding process (Pickles et al., 2018), we propose that increased connectivity within the mitochondrial network may have occurred to support increased autophagy initiation. A possible explanation for the increase in autophagy initiation and mitochondrial fusion could involve liver size. We previously reported the liver mass of the 7-month-old lactating group to be smaller compared to the animals that did not lactate (Hyatt et al., 2017). However, in the 4-month-old rats,
the non-lactating rats had the smallest liver mass and the lactating rats had the largest liver mass (Hyatt et al., 2019). This is not surprising as the liver undergoes drastic changes during gestation and lactation (Campbell and Kosterlitz, 1949; Coope and Mottram, 1914), and therefore will respond differently depending on the stage of reproduction and lactation. The current study reports an increase in autophagy induction and mitochondrial fusion in 4-month non-lactating and 7-month lactating animals. These results, in conjunction with the liver masses previously reported, suggest that an increase in autophagy induction may, in part, explain this size difference, as autophagy and cell growth are known to be inversely related (Blommaart et al., 1995; Neufeld, 2012). In summary, animals that do not lactate following parturition lack the need to maintain an enlarge liver. Animals that did lactate will maintain the size of the liver until there is no need to further maintain a large liver. Therefore, autophagy induction at the respective time points would mediate remodeling of the liver as the demand of lactation is no longer needed.

Further, in another energetically demanding tissue, skeletal muscle, Beclin1 protein levels were the lowest in the lactating groups and this

Figure 3. Autophagy markers in skeletal muscle. (A) Beclin1, (B) ATG5-ATG12, (C) ATG16L, (D) LC3BII/LC3BI Ratio, and (E) representative images of the Western blots. Two-way analyses of variance results are shown on top of each graph. Individual data points for each animal are also shown. The uncropped versions of figures are presented in Figure S3A.
pattern was also observed for the marker ATG5-ATG12. These results indicate that autophagy initiation and phagosome formation in the skeletal muscle is decreased in animals that lactated, and the response is seen at both the 4- and 7-month time points. Little research has been done regarding autophagy in skeletal muscle with lactation in rodents. However, there are reports in lactating cows. One study reported that the LC3BII/LC3BI ratio was higher during post calving (lactation), suggesting higher autophagosome formation with lactation in dairy cows (Mann et al., 2016). Additionally, these researchers reported higher amounts of proteasome activity and relative ubiquitin content, suggestive of higher breakdown of cellular material in the skeletal muscle during lactation. Interestingly, these results observed at 4- and 21-days post-calving are opposite of what we observed in rats. This is most likely due to physiological differences between species. Rat’s peak lactation occurs at 14 days post-parturition, whereas dairy cow’s peak lactation time is about 70 days post-parturition (Lean et al., 1989). Therefore, the data collected in cows does not represent data collected near peak lactation. The changes in levels of autophagy may vary in rats depending on where in the lactation cycle an animal is. Lastly, cows are known to have large negative energy balance at the beginning of lactation (de Vries and Veerkamp, 2000; Winkler et al., 2015). Since autophagy is known to be active during periods of starvation (Zarzynska and Motyl, 2008), it is not surprising that dairy cows at 4- and 21-days lactation had high markers of autophagy in skeletal muscle. Although rats are also known to have a high metabolic

Figure 4. Mitochondrial dynamics markers in liver and skeletal muscle. (A) Marker of the mitochondrial fusion: Mitochondrial fusion 2 (Mfn2) in the liver, (B) marker of mitochondrial fission: Dynamin-1-like protein (Drp1) in the liver, (C) Mfn2 in skeletal muscle, (D) Drp1 in skeletal muscle, and (E) representative images of the Western blots. Two-way analyses of variance results are shown on top of each graph and differences (p < 0.05) between groups are denoted by: * NR4 vs. P4, + P4 vs L4, # NR4 vs L7, |L4 vs L7. Individual data points for each animal are also shown. The uncropped versions of figures are presented in Figure S4A.
rate during lactation, their energy intake also increases to maintain energy balance (Kunkele and Kenagy, 1997; Speakman, 2008). Further, in skeletal muscle, HSP70 protein content increased in the 7-month-old rats regardless of reproduction or lactation intervention. Increases in HSP70 in skeletal muscle have been associated with enhanced insulin sensitivity measures in aging animals. Insulin resistance is associated with comorbidities as individuals age and decreases in insulin sensitivity is associated with morbidity and mortality (Chichester et al., 2015; Rincon et al., 2005). Studies have demonstrated that low levels of HSP70 leads to a higher likelihood of developing insulin resistance with age (Chichester et al., 2015). Insulin resistance is associated with increased instances of diabetes and metabolic disease, suggesting that an increase in HSP70 may play a role in preventing the development of metabolic diseases later in life.

How the morphology of the mitochondrial network can influence cellular processes in skeletal muscle requires additional investigation. We currently report an increase in the skeletal muscle protein expression of the mitochondrial fission marker DRP1 in the non-lactating group.
compared to the lactating group. It is well known that mitophagy and mitochondrial fission occur concurrently (Gomes et al., 2011; Rambold et al., 2011; Twigg et al., 2008). Although we did not measure markers of mitophagy, we would anticipate an increase in mitophagy to occur in skeletal muscle. Other important markers of mitochondrial regulation have been measured during lactation. Previous investigations have reported a significant decrease in mRNA content for PPARα, PGC-1α, and PGC-1β in animals that were allowed to lactate (Gutgesell et al., 2009), suggesting a decrease in mitochondrial biogenesis transcription factors. These results may imply that lactation could result in better functioning of mitochondria, as previously observed by an increase in respiratory control ratio (Hyatt et al., 2017) and therefore a decrease need for mitochondrial biogenesis. However, it is important to note that transcriptional changes do not always reflect translational changes, and therefore results should be interpreted cautiously.

We also note that protein expression markers were evaluated in this study and not phosphorylated proteins. This possess the limitation that we may be missing any post translational modifications which may be occurring throughout the autophagy and mitochondrial dynamic pathways resulting in activation or deactivation of pathways. Furthermore, during the investigation of mitochondrial dynamics, we were only able to obtain useable data for the outer mitochondrial membrane fusion marker Mfn2. This constraints our ability to understand what may be occurring at the inner mitochondrial membrane. Additionally, limitations of the LC3BII/LC3BI ratio have been reported (Klionsky et al., 2016; Mizushima and Yoshimori, 2007). Briefly, the main concerns of this marker are as follows: multiple LC3 isoforms, antibody affinity, and the blot product of LC3 immunoblots. The suggestion has been made that the marker of LC3BII/LC3BI be used to determine autophagic structures, however other methods should be used to determine autophagic flux (Klionsky et al., 2016). Further, the results of autophagy cannot be extrapolated to suggest changes in mitophagy. Nonetheless, our collective findings are supportive of increased autophagy and altered mitochondrial function with lactation. Future studies should continue to investigate the effect of lactation on mitophagy and how it may be contributing to mitochondrial function.

5. Conclusion

These findings provide insightful information on the mechanisms that dictate tissue remodeling in response to the metabolic challenges of reproduction. Our findings of altered mitochondrial dynamics highlights the integrated processes required to meet energy demands. Moreover, the increase in autophagy activation suggests a possible mechanism for how the size of the liver changes with lactation status. Collectively, our findings further our understanding of the complex changes that occur with reproduction and lactation, and highlights the remarkable plasticity required during this demanding metabolic feat.

Declarations

Author contribution statement

Hailey A. Parry, Ryleigh B. Randall, Hayden W. Hyatt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Wendy R. Hood, Andreas N. Kavazis: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by US National Institutes of Health grant (RO3 HD083654-01).

Data availability statement

Data included in article supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e08070.

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