Developmental regulation of mitochondrial biogenesis and function in the mouse mammary gland during a prolonged lactation cycle

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Submitted 8 July 2010; accepted in final form 23 December 2010

Hadsell DL, Olea W, Wei J, Fiorotto ML, Matsunami RK, Engler DA, Collier RJ. Developmental regulation of mitochondrial biogenesis and function in the mouse mammary gland during a prolonged lactation cycle. Physiol Genomics 43: 271–285, 2011. First published December 28, 2010; doi:10.1152/physiolgenomics.00133.2010.—The regulation of mitochondrial biogenesis and function in the lactating mammary cell is poorly understood. The goal of this study was to use proteomics to relate temporal changes in mammary cell mitochondrial function during lactation to changes in the proteins that make up this organelle. The hypothesis tested was that changes in mammary cell mitochondrial biogenesis and function during lactation would be accounted for by coordinated changes in the proteins of the electron transport chain and that some of these proteins might be linked by their expression patterns to PPARGC1α and AMP kinase. The mitochondrial proteome was studied along with markers of mitochondrial biogenesis and function in mammary tissue collected from mice over the course of a single prolonged lactation cycle. Mammary tissue concentrations of AMP and ADP were increased ($P < 0.05$) during early lactation and then declined with prolonged lactation. Similar changes were also observed for mitochondrial ATP synthesis activity, mitochondrial mass and DNA copy number. Analysis of the mammary cell mitochondrial proteome identified 244 unique proteins. Of these, only two proteins of the electron transport chain were found to increase during early lactation. In contrast, coordinated changes in numerous electron transport chain proteins were observed both during mid- and late lactation. There were six proteins that could be directly linked to mitochondrial ATP synthesis activity during early lactation results from changes in only a limited number proteins. In addition, decreases in a handful of proteins linked to lipid oxidation could be temporally linked to decreases in PPARGC1α and phospho-AMP kinase suggesting differential roles for these proteins in coordinating mammary gland metabolism during early lactation.

mitochondria; proteome; adenosine 5’-triphosphate

THE MITOCHONDRIUM CONTRIBUTES to as much as 90% of cellular ATP synthesis. Beyond this role, however, the mitochondrion also functions in Ca2+ homeostasis, apoptosis, heme biosynthesis, and the production of reactive oxygen species (45, 56–58, 67). In addition, tissue-specific roles have been described for the mitochondria including urea synthesis, gluconeogenesis, thermogenesis, synthesis of steroid hormones, ketone body metabolism, and lactate utilization (37). Although these tissue-specific functions have been understood for many years, their presence has recently become more visible with the application of proteomic approaches to mitochondrial fractions from different tissues (43, 47). The result of these intensive efforts has been the near complete cataloging of the nearly 700–1,000 proteins that encompass the mitochondrial proteome, along with the idea that there exists both ubiquitous and tissue specific mitochondrial proteins. The mammary gland is a tissue that has thus far been missed in these mitochondrial proteome studies.

In the lactating mammary gland, previous work has highlighted the importance of developmental changes in mitochondrial respiration with the onset of lactation and with mammary involution (17, 44). In addition to this, mammary mitochondria have been suggested to play an important role in the provision of citrate for milk lipid biosynthesis (15, 54, 70). However, much remains to be learned about the regulation of mammary cell mitochondria and their contribution to the process of milk biosynthesis during lactation.

In most species the functional activity of the mammary gland during lactation follows a biphasic developmental pattern (24). This pattern starts with the rapid increase in milk output that occurs during secretory activation and continues with a more gradual increase until the point of peak lactation is reached. Following this gain-of-function phase, the ability of the gland to produce milk decreases. This decrease occurs even if the lactation is prolonged by the presence of continued suckling stimulus and complete milk removal. Only a handful of studies have specifically focused on mitochondrial biogenesis in the mammary secretory cell during a normal lactation cycle (29, 31, 52, 53). Analysis of mammary tissue by electron microscopy demonstrated that during pregnancy there is a notable increase in the number of mitochondria per secretory cell and that during the early postpartum period both size and density of secretory cell mitochondria increase (29, 53). In conjunction with these cytological observations, notable increases have also been reported in the activities of a number of mitochondrial enzymes during pregnancy, but most markedly during the early postpartum period (31, 52, 53). Despite these well-documented and dramatic changes, almost nothing is known about the regulation of mitochondrial biogenesis in mammary secretory cells during a normal lactation. In addition, there have been no studies on mitochondrial function and biogenesis in the mammary gland during prolonged lactation.

Previous work in our laboratory has made use of a litter cross-fostering model in the lactating mouse to study the cellular and biochemical changes that are associated with the...
known temporal variations that occur during a normal lactation and with prolonged lactation (26). The results of that study suggested that alterations in mitochondrial oxidative damage might be linked to temporal changes in milk production during the lactation cycle. The goals of the present study were to conduct a detailed analysis of mitochondrial biogenesis and function during normal and prolonged lactation in the mouse and to relate these changes to changes in the mitochondrial proteome, and to changes in expression of peroxisome proliferator-activated receptor gamma, coactivator 1 (PPARGC1α), a transcriptional coactivator known to regulate mitochondrial function in other tissues, and to changes in the phosphorylation and/or abundance of adenosine monophosphate-activated protein kinase (AMPK), a known sensor of cellular energy status and regulator of PPARGC1α. The hypothesis to be tested was that changes in mammary cell mitochondrial biogenesis and function during the lactation cycle could be directly accounted for by coordinated changes in many, if not most, of the proteins that make up the oxidative phosphorylation (OXPHOS) pathway and that some of these proteins might also be linked by their temporal expression patterns to PPARGC1α and AMP kinase.

MATERIALS AND METHODS

Experimental animals. All animals were studied in accordance with procedures outlined in the NIH Guide to Care and Use of Experimental Animals. The Baylor College of Medicine Animal Care and Use Committee approved these experiments. Two populations of CD-1 lactating mice were utilized in these studies. Secretory activation samples were obtained from mice (n = 5–8/time point) at -1, 1, 2, 3, 4, and 5 days postpartum. Samples over early mid- and prolonged lactation were obtained (n = 5–8/time point) at 2, 8, 14, and 21, 28, and 35 days postpartum, respectively. For the analysis of milk, there were also samples from a second experiment collected on days 2, 8, 14, 16, 20, 22, and 28. The animals were analyzed in their first lactation, and none were concurrently pregnant during the course of these studies. To maintain constant suckling stimulus, the litters were initially normalized to 10 pups/litter, and a set of 8–day-old pups was cross-fostered onto the dams every week as previously described (26).

At the time of harvesting, wet weights were obtained for the mammary glands. They were then either flash-frozen in liquid nitrogen and stored at -80°C for further analysis or processed for the analysis of mitochondrial function. For some animals a small piece of the number three mammary gland was fixed in half-strength Karnovsky’s buffer, to be sent for electron microscopy.

Western blotting. Total tissue protein extracts of mammary tissue were prepared from 50 mg of tissue. PAGE was carried out using 20 μg protein per lane. Blots were prepared using PROTRAN nitrocellulose membrane. Detection was based on enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, IL), horseradish peroxidase-conjugated donkey anti-rabbit, sheep anti-mouse secondary antibodies (GE Healthcare Biosciences Pittsburgh, PA), or anti-sheep secondary antibody at a dilution of 1:2,000 and Kodak Biomax MR-1 film (Sigma Chemical). Cytb-C was detected using sheep polyclonal antibody (ABCAM Cambridge, MA) at 1:5,000 dilution. PPARGC1α was measured using rabbit polyclonal antibody (US Biologicals, Swampscott, MA) at 1:500 dilution. F1-ATP synthase-β was measured using rabbit polyclonal antibody (Santa Cruz Biotechnology Santa Cruz, CA) at 1:500 dilution. Mitochondrial DNA copy number. Total mammary tissue DNA was extracted using Qiagen DNeasy Kit (Invitrogen, Carlsbad, CA). Cytochrome B primers and probe were designed by ABI Primer Express software and had the following sequences; CytB-Fwd 5'-GCC ACC TTT ACC CGA TTC T-3', CytB-Rev 5'-TGT CTA GGG CGG CGA TAA TA-3', CytB-probe 5' 6FAM-CGCG TTT CCA CTT CAT TC-MGBNFQ-3'. The β-actin primers and probe were purchased as a predeveloped Taqman Assay (20× Mouse ActB catalog #4352933E). Each sample was tested for cytochrome B and β-actin in triplicate in an ABI 96-well plate. Samples for β-actin were run on the same plate using 25 μl of 2× Taqman master mix, 2.5 μl of 20× β-Actin Taqman Assay, 12.5 μl H2O, and 100 ng mitochondrial DNA. The plates were run in a 7900 HT Fast-Real Time PCR system (Applied Biosystems, Foster City, CA), and cycle thresholds (Ct) for each sample were calculated. Data were expressed as the ratio (2-ΔΔCT) of cytochrome B-β-actin.

Morphotmetric analysis of electron micrographs. Tissue for electron microscopy was fixed in half strength Karnovsky’s buffer for 2 h at 4°C, then transferred to cacodylate buffer until further processing. Upon processing, the tissue was rinsed three times for 10 min at 4°C in cacodylate buffer, then incubated in 2% osmium tetroxide for 1 h at 4°C, dehydrated in a series of ethanol concentrations, and embedded in Spurr’s plastic.

Thin sections for transmission electron microscopy (TEM) were cut with a diamond knife into ~0.08 μm sections and stained with uranyl acetate (20 min) and lead citrate (3 min). Sections were viewed using a CM 12 Phillips Transmission Electron Microscope (University of Arizona Electron Microscopy Core). Electron micrographs of day 8 and day 35 mammary glands were morphometrically analyzed with the Image Pro software (Media Cybernetics, Bethesda, MD). The number and area of the mitochondria were calculated from the data collected.

Purification of mitochondria and measurement of ATP synthesis. Mitochondria were purified from freshly isolated lactating mouse mammary tissue as previously described (25). ATP synthesis activity was measured using a luciferase-coupled assay as previously described (25). For a subset of these samples, the tissue was minced with scissors in ice-cold Hanks’ balanced salt solution (HBSS; Sigma Chemical, St. Louis, MO) and then digested for 30 min at 30°C in HBSS containing 0.2% wt/vol type III collagenase (130 units/mg; Worthington Biochemicals, Lakewood, NJ), 0.5% heat inactivated fetal bovine serum (Sigma Chemical), and 0.4% wt/vol bovine serum albumin (Sigma Chemical). The epithelium-enriched pellet was then recovered after centrifugation at 1,500 g for 10 min, washed twice with HBSS, and then used to prepare mitochondria.

Staining for succinate dehydrogenase and AMP kinase. Frozen sections from five CD-1 female mice at 2, 8, 14, 21, 28, 35 days of lactation were used for this staining procedure. They were incubated in a solution 4 mg/ml nitro blue tetrazolium chloride (Alpha Aesar, Ward Hill, MA) 0.2 M TRIS, 0.05 M MgCl2, and 0.83 M sodium succinate (Sigma-Aldrich) for 1 h at 37°C. The sections were then transferred to 15% formol saline containing 0.9% wt/vol NaCl and 15% wt/vol paraformaldehyde and incubated for 15 min at room temperature. Sections were washed in distilled water for 3 min, dehydrated in 93% alcohol, 95% alcohol, and 100% alcohol for 3 min each. The sections were rinsed in xylene and mounted with 2 drops of DPX mounting medium and coverslipped. The images were captured with a spot camera using a ×100 oil objective. Adobe Photoshop CS2 (Adobe Systems San Jose, CA) was used to mark the speckles and epithelial area, which were later counted and analyzed using Image Pro plus software (Media Cybernetics). Staining for phospho-AMP kinase was done on frozen sections using an antibody that detects phosphorylation of threonine 173 (Cell Signaling Technology, Danvers, MA). Total AMP kinase was also detected by staining with an antibody (Cell Signaling Technology). Stained sections were imaged using an Olympus FV300 Laser Scanning Confocal Microscope (Olympus America, Centerville, PA). Digital images were then analyzed using Image Pro Plus version 5.1 to obtain relative quantitative measurements of fluorescence intensity.
Measurement of tissue adenine nucleotides. Mammary tissue samples were homogenized in perchloric acid and nucleotides were measured by HPLC (41).

Proteomic experimental design. Mitochondrial fractions were prepared from mice at days 2, 8, 14, and 21 postpartum using an epithelium-enriched fraction of collagenase-digested tissue from the #4 mammary gland of each mouse. The mitochondria were prepared as described above. These fractions were then analyzed by two-dimensional differential in-gel electrophoresis (2D-DIGE) so that estimates of biological variation could be obtained. There were three mice per time point.

Sample preparation for DIGE. Frozen mitochondria pellets isolated from each individual animal’s mammary gland were suspended in a urea-containing extraction buffer [DIGE buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Tris (pH 8.8), 5 mM magnesium acetate] compatible with Cy-dye labeling and 2D-DIGE so that the final protein concentration of each solubilized mitochondrial extract was ~2 mg/ml. Samples were incubated at room temp (~22°C) for 30 min with occasional mixing by vortex to facilitate sample resuspension and solubilization. Samples were then subjected to sonication for 3 pulses of 30 s each at 45 W power with a 30 s interval between power bursts in a Misonix 3,000 cup horn sonicator chilled to 4°C. Samples were centrifuged at 12,000 g (4°C), and the resulting supernatant recovered to a fresh microcentrifuge tube. Any material remaining in the pellet form was re-extracted with DIGE buffer for 15 min, subjected to sonication and recentrifugation as above, and the resulting supernatant pooled with the first extraction’s supernatant material. Protein concentration was determined for all isolated protein extracts using the Bio-Rad Protein Assay.

Cy-dye labeling. Samples were independently labeled with either Cy-3 or Cy-5 dyes, such that at least one aliquot from each individual time point sample was labeled with one of the alternate dyes. A pooled internal reference protein sample was also prepared that comprised an equal amount of protein (by weight) from all samples in the experiment, and this pooled reference sample was labeled with Cy-2. All protein samples were labeled with Cy dyes as described by the manufacturer (19). In brief, 400 pmol of Cy dye was added to 50 μg of protein, incubated on ice and in the dark for 30 min. Following the addition of 1 μl of 10 mM lysine the sample was incubated for an additional 15 min on ice.

2D-electrophoresis. Prior to gel electrophoresis, Cy dye-labeled protein samples were pooled together such that each 2D-gel contained three separate protein samples, allowing each 2D gel to be run in a fluorescent multiplexed fashion. Fluorescent multiplexing allowed the spot pattern from each of the individually labeled protein samples applied to each gel to be distinguished by one of the three spectrally resolvable Cy dyes after gel imaging, while simultaneously minimizing the necessary number of gels run and any potential gel-gel spot-pattern artifacts due to electrophoretic variations between gels. Therefore, each fluorescent multiplexed gel contained two samples from one of the respective individual time-point replicates, one labeled with Cy-5 and one labeled with Cy-3, and a third sample from the common Cy-2 labeled pooled internal reference sample, such that the pooled internal reference sample was represented on every gel in the six-gel series. Electrophoresis was carried out according to (21). First dimension isoelectric focusing was carried out on pH 3–11 nonlinear 24 cm IPG strips (GE Healthcare) using an IPGphor II unit (GE Healthcare) under the following conditions per strip: 1 h at 0 volts, 8 h at 50 volts, 1 h at 500 volts, 1 h at 1,000 volts, and 80,000 volt h at 8,000 volts. Following first dimension focusing, the IPG strips were equilibrated with SDS after reduction and alkylation of cysteine residues, then placed on top of 20 cm × 24 cm × 1 mm, 8–18% gradient polyacrylamide gels and electrophoresed in a DALT 6 apparatus (GE Healthcare) at 12 mA/gel, until the bromphenol blue dye front ran off the bottom of the gel.

Gel image acquisition and spot-volume processing. Following electrophoresis, gels were sequentially scanned at the optimal excitation/emission wavelength settings for each of the three Cy dyes present in the gels on a Typhoon 9400 laser scanner (GE Healthcare). All three scans were done in succession at 100 μm resolution. Gel image data were analyzed using DeCyder v. 6.5 software from GE Healthcare. Intragal spot detection and spot-volume determination was carried out on every gel using the differential in-gel analysis module of the software, while intergel spot matching and spot-volume normalization was carried out using the biological variation analysis module of the software. Automated spot matching across all six gels was performed and manually validated. Statistical analysis calculations performed within the DeCyder software were used to determine which spots were significantly altered in their protein expression levels between experimental samples and were the basis for spot selection for further spot processing and protein identification.

Mass spectrometry and protein identification. Protein spots identified as being differentially expressed between individual time point samples were identified by DeCyder image analysis as described above and delineated for automatic spot picking and processing for protein identification via mass spectrometry (MS). Protein spots of interest were isolated from preparative 2D-gels run under conditions similar to the analytical 2D-DIGE gels as described above, starting with a 500 μg protein load per gel. Preparative gels were stained with colloidal Coomassie blue stain postelectrophoresis, and after image acquisition and matching to the analytical Cy dye images using DeCyder, spots of interest were delineated for further processing. Spot picking, in-gel trypsin digestion, and resulting isolated peptide spotting onto matrix-assisted laser desorption/ionization (MALDI) target plates was done in an automated fashion using the robotic Spot Handling Work Station (GE Healthcare Biosciences, Piscataway, NJ). Proteins were digested with trypsin at a ratio of 200 ng of trypsin per protein spot picked. One-sixth of the resulting tryptic peptides eluted from each gel spot was spotted onto MALDI target plates. Alphacyano-4-hydroxycinnamic acid at 5 mg/ml in 50% acetonitrile, 50% water, 10 mM ammonium citrate was used as matrix and mixed with the dried peptide on the MALDI target plate. MS was performed on an Applied Biosystems 4800 MALDI time-of-flight (tof/tof) mass spectrometer set in an automated mode to collect 1,000 laser shots per sample in MS mode and 2,000 shots/speak in MSMS mode, or less, if a threshold number of ions reached the detector in fewer shots. GPS Explorer software (v3.6 A/B) was used in conjunction with Mascot search engine for searching the Swissprot (v5.0.0) Mus musculus database, containing 12,710 sequences for protein identification. A confidence interval of ≥95% was required in both the peptide mass fingerprinting mode (MS mode), as well as in the MS/MS verification mode before resulting spectra were used to confirm a protein identification.

Real-time PCR analysis of gene expression. Real-time PCR was used to measure the mRNA levels for select proteins identified in the proteome experiment 2. The genes in this analysis included long chain acyl-CoA dehydrogenase (Acad1), acetyl-CoA acetyl transferase 1 (Acat1), Enol-CoA hydratase 1 (Ech1), idicotate dehydrogenase 3a (Idh3a), electron transferring flavoprotein-B (Etfb), malate dehydrogenase 2 (Mdh2), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 3 (Ndufa3), succinate dehydrogenase complex, subunit A, flavoprotein (SdhA), succinate dehydrogenase complex, subunit B, flavoprotein (SdhB), and TNF receptor-associated protein 1 (Trap1). For the primer sequences see table S. 7. Total RNA from the mammary tissue collected from lactating mice at days 2, 8, 14, and 21 was isolated utilizing TRIzol reagent (Invitrogen) as per manufacturer’s instruction. Reverse transcription PCR was performed using High-capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer’s manual. In brief, 5 μg total RNA was used for each 50 μl reaction. The reverse transcription was incubated at 25°C for 10 min, followed by 37°C for 120 min and 85°C for 5 s. Quantitative (q) PCR was performed in the 7900HT Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 25 μl reaction contained 25 ng of DNA template, 3 mM
of MgCl₂, 0.2 mM of dNTP, 100 nM of each primer, 0.4 × Sybr Green 1 (Invitrogen), and 0.2 U of Platinum Taq DNA polymerase with 1× PCR buffer (Invitrogen). cDNA samples were denatured at 95°C for 5 min, followed by a 40 cycle-PCR of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Upon completion of 40 cycles the samples were held at 72°C for 5 min and followed by a dissociation analysis to determine the amplicon size. Samples were prepared and run in duplicate. Relative expression levels were normalized to the level of the housekeeping gene Gapdh.

Data analysis. Analysis of the data for Figs. 1–6 was conducted using SPSS version 15 (http://www.spss.com). Differences among time points were considered significantly different at P value <0.05. Analysis of the proteomic data was conducted using a combination of software packages. To simplify the analysis, data from proteins with multiple isoforms were analyzed as the sum of the isoforms for any particular protein. To determine the degree to which this normalized sum ratio represented the changes in individual isoform, Pearson’s correlation was used. Detection of statistically significant (P < 0.05) changes across the different time points for each of the proteins measured in the experiment was accomplished using the one-way ANOVA procedure in SPSS version 15. The data were also split into three time intervals, early, mid-, and late lactation. The early lactation interval compared day 2 with day 8 postpartum, the midlactation time interval compared day 8 with day 14 postpartum, and the late lactation interval compared day 14 with day 21 postpartum. An F-statistic was used to evaluate the changes in each protein independent of all other proteins in the dataset. Ratios with a value of <1 were expressed as their negative inverse to provide an indication of directionality to any changes. Details of the analysis including mean squares, the value of the F-statistic, and its corresponding P value are presented in Supplemental Table S3.1 An overall ontology analysis was conducted on the list using Mouse Genome Informatics Database Gene Ontology Term Finder. Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) was used to conduct ontology on proteins within the dataset that exhibited statistically significant changes (P < 0.05). Ontology term enrichment was consider significant at a P value of <0.05. The Benjamini-Hochberg correction for multiple comparisons was used. Detection of networks was accomplished using the IPA. Heat maps and dendrograms were generated using the Heatplus package implemented in R (49).

RESULTS

Secretion of 8-hydroxy 2′-deoxyguanine in milk. Our own previous studies have demonstrated that changes in lactation capacity and mammary development during a single prolonged lactation cycle were correlated with significant changes in oxidative damage both to mitochondrial DNA and protein (24, 26). To determine if evidence of oxidative stress could be obtained through analysis of milk, the concentrations of 8-hydroxy 2′-deoxyguanine (8DG) were measured in milk samples collected at different days of lactation during 2 independent experiments (A). Mammary tissue collected at different days postpartum was assayed for the concentrations of ATP (black bars), ADP (gray bars), and AMP (white bars) measured in neutralized perchloric acid extracts by HPLC (B). Energy charge (C) was calculated from the formula \(\frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}\). ATP synthesis activity (D) was measured in isolated mammary mitochondria using a luciferase-coupled assay. For A and B each bar represents the mean ± SE for 3–5 mice. For C each bar represents the mean ± SE for 5 mice. *Differences (P < 0.05) from day 2 postpartum.

Fig. 1. Changes in oxidative stress, mammary tissue adenine nucleotide concentrations, and mammary mitochondrial ATP synthesis activity are developmentally regulated during the lactation cycle. Concentrations of 8-hydroxy 2′-deoxyguanine (8DG) were measured by ELISA in milk samples collected at different days of lactation during 2 independent experiments (A). Mammary tissue collected at different days postpartum was assayed for the concentrations of ATP (black bars), ADP (gray bars), and AMP (white bars) measured in neutralized perchloric acid extracts by HPLC (B). Energy charge (C) was calculated from the formula \(\frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}\). ATP synthesis activity (D) was measured in isolated mammary mitochondria using a luciferase-coupled assay. For A and B each bar represents the mean ± SE for 3–5 mice. For C each bar represents the mean ± SE for 5 mice. *Differences (P < 0.05) from day 2 postpartum.

capacity, tissue adenine nucleotide concentrations and mitochondrial ATP synthesis activity were measured over the course of a prolonged lactation cycle (Fig. 1B). Comparison of tissue concentrations of AMP, ADP, and ATP demonstrated (P < 0.05) increases in the tissue concentration of AMP during early lactation followed by a decrease in later lactation (Fig. 1B). The calculation of energy charge based on these tissue concentrations suggested that the relative abundance of high energy ATP and ADP was decreased during peak lactation and then increased again as lactation capacity declined (Fig. 1C).

To determine how mitochondrial function changed over the course of a prolonged lactation cycle, a firefly luciferase-coupled assay was used to measure the ATP synthesis capacity of mitochondria freshly isolated from whole tissue homogenates prepared from the mammary tissue of lactating mice during the prolonged lactation cycle (Fig. 1D). This assay demonstrated that mitochondrial ATP synthesis activity increased by twofold between day 2 and 8 postpartum, remained

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1 The online version of this article contains supplemental material.
elevated through day 14 postpartum, and then decreased on day 21 postpartum. To confirm that this increase in mitochondrial function was specific to mammary epithelial cells (MEC) and not due to potential changes in other cell types within the gland, ATP synthesis activity was also measured in mitochondria prepared from MEC-enriched collagenase digests of mammary tissue taken from lactating mice on day 2 or 8 postpartum. This analysis also demonstrated a significant ($P < 0.05$) increase in mitochondrial ATP synthesis activity (2.67 ± 0.1 and 10.06 ± 1.8 units/mg mitochondrial protein for day 2 and 8, respectively) between day 2 and 8 postpartum. These results support the conclusion that MEC mitochondrial ATP synthesis activity is temporally linked to milk production in lactating mice, which reaches a maximum on day 8 postpartum and then declines after day 14 postpartum.

**MEC mitochondrial number.** To determine the extent to which changes in mitochondrial number were temporally correlated with changes in lactation capacity during the prolonged lactation cycle, histochemical staining was conducted for succinate dehydrogenase activity (SDH). Staining of mammary tissue sections with nitro-blue tetrazolium in the presence of sodium succinate demonstrated a speckled pattern of staining that was predominantly visible in epithelial cells (Fig. 2A). As a negative control, the assay was conducted in specimens in which the succinate was excluded from the reaction (Fig. 2B). Quantitation of the number of speckles over epithelial structures within the tissue demonstrated that the activity of this enzyme is increased by fivefold during the gain-of-function phase of the cycle (Fig. 2C). During the loss-of-function phase of the cycle, the number of SDH-positive speckles remained similar to that observed at peak lactation. In a separate analysis (Fig. 3), Uranyl-acetate-stained tissue sections prepared from mammary tissue collected on either day 8 (Fig. 3A) or day 35 (Fig. 3B) postpartum were imaged by TEM. Morphometric analysis of these images demonstrated that neither the number of mitochondria per epithelial cell (C) nor the average area per epithelial mitochondrion (D) were different between the two time points. This analysis supports the conclusion that although the early phase of lactation is associated

![Fig. 2](image-url)  
**Fig. 2.** Changes in mammary epithelial cell mitochondrial number over a prolonged lactation cycle. Succinate dehydrogenase activity (A) was detected in mammary tissue sections by using the in situ oxidation of nitro-blue tetrazolium in the presence of sodium succinate. Mitochondrial number within the mammary epithelial compartment was enumerated by counting speckles within epithelial cells identified and outlined in digital images. Incubation of sections in reaction mixture lacking the succinate substrate was used as a negative control for specificity (B). Relative changes in epithelial mitochondrial number was expressed per mm$^2$ of epithelial cell area (C). Each bar in C represents the mean ± SE for 5 samples. *Differences ($P < 0.05$) from day 2 postpartum.

![Fig. 3](image-url)  
**Fig. 3.** Quantitation of changes in mammary epithelial cell mitochondrial number and area between day 8 and 35 postpartum using electron microscopy. Electron micrographs were prepared from samples of mammary tissue collected from lactating mice on day 8 (A) postpartum and on day 35 (B) postpartum. The total number of mitochondria per epithelial cell (C) and mitochondrial area (D) was measured using image analysis tools within the software package Image-Pro Plus. Each bar represents the mean ± SE for 10 mice. Arrows depict mitochondria.
with an increased mitochondrial number, loss of mitochondrial function during prolonged lactation may not be linked to a loss of MEC mitochondria per se.

Mitochondrial electron transport chain proteins and DNA copy number. To estimate the relative contribution of mitochondrial proliferation or maturation to these changes in mitochondrial number and activity we measured mitochondrial DNA copy number and the total tissue abundance of the electron transport chain (ETC) proteins, cytochrome c and F1 ATP synthase-β (Fig. 4). Analysis of total tissue DNA for the cytochrome B gene by real-time PCR (Fig. 4A) demonstrated that during the first 8 days of lactation mitochondrial DNA copy number increased by ~50%, reached a peak on day 8 postpartum, and then decreased by day 21 postpartum to a level that was similar to that observed on day 2. Western blotting (Fig. 4B), followed by densitometry revealed that the abundance of cytochrome c and F1 ATP synthase-β within whole mammary extracts increased during early lactation, reached a maximum on day 8, and then declined during the remainder of the lactation (Fig. 4C). Taken together, these data suggest that changes in both mitochondrial biogenic processes and ETC activity play a role in the temporal changes that occur in milk production over the course of a lactation cycle.

Analysis of the mammary mitochondrial proteome. A total of 2,194 spots could be detected by 2D-DIGE on mitochondria prepared from the epithelium-enriched fraction of collagenase-digested mammary tissue (Supplemental Fig. S1, Supplemental Table S1). Of these, a total of 585 spots were identified by MALDI-tof/TOF analysis of spots picked from the 2D gels (Supplemental Table S1). Ontology analysis using the Mouse Genome Informatics Database demonstrated that 53% of the proteins found within the extracts from this experiment were mitochondrial (data not shown). Also within this fraction, 8% were endoplasmic reticulum, 7% were plasma membrane, 6% cytosolic, 5% were nuclear, 3% were cytoskeletal, 2% were Golgi, 2% were ribosomal, 2% were peroxisomal, 3% were extracellular space, 1% was endosomal, and 7% were unannotated. In addition, 35 known subunits of the OXPHOS pathway were also detected.

Of the accesses identified within the mammary mitochondrial proteome 121 corresponded to unique proteins represented by a single isoform and 123 proteins that were represented by 2 or more isoforms (Supplemental Table S2). To simplify the quantitative analysis for these proteins with multiple isoforms an overall normalized sum ratio was calculated from the sums of the fluorescence intensity for the individual isoforms (Supplemental Table S2). Analysis of the resulting data revealed that of the 244 proteins present in the data set, 166 demonstrated statistically significant changes ($P < 0.05$) for at least one of the three time intervals analyzed (Supplemental Table S3). For the early lactation interval, the magnitude of changes (55 proteins) were from 10% to 3.5-fold with an average of $1.5 \pm 0.4$-fold. For the midlactation interval, the magnitude of changes (81 proteins) ranged from $-6\%$ to 2.5-fold with an average of $1.7 \pm 0.3$-fold. For the late lactation interval the magnitude of changes (105 proteins) ranged from $-17\%$ to 4.2-fold with an average of $1.8 \pm 0.5$-fold. The resulting dataset can be visualized by the heat map in Supplemental Fig. S2.

To ensure that the analysis of ratios derived from sums did not mask potential changes in isoform abundance indicative of posttranslational modifications, normalized ratios for the individual isoforms of each protein were compared with the normalized sum ratio for that protein. For 87 of the 123 proteins with multiple isoforms, 100% of the isoforms had a significant positive Pearson’s correlation ($P < 0.05$, $r > 0.5$) to the normalized sum ratio (Supplemental Table S4). For the remaining proteins, the percentage of isoforms that were correlated to the normalized sum ratio varied from 33 to 89%, and several had at least one isoform that was negatively correlated (Supplemental Table S4, Supplemental Fig. S3). Although these negative or noncorrelated isoforms generally had a low relative abundance, the results suggest that there were at least 36 proteins in the dataset for which posttranslational modifications might have influenced abundance or activity at different times throughout the lactation cycle.

To obtain a broader understanding of how observed changes within the proteome might impact mitochondrial function, ontology analysis using fold-changes and $P$ values calculated for the comparison of early, mid-, and late lactation time intervals (Supplemental Table S3) was conducted with the IPA package. The top 10 biological function terms enriched for in this analysis were energy production, nucleic acid metabolism, small molecule biochemistry, DNA replication, recombination.
and repair, free radical scavenging, cellular function and maintenance, lipid metabolism, cell death, and tumor morphology (Fig. 5A). Comparison of the number of proteins for which changes ($P < 0.05$) were observed among the three time intervals revealed that for the top four categories, the largest changes occurred during mid- or late lactation (Fig. 5A, Supplemental Table S5). Importantly, a significant number of these proteins were subunits of the electron transport chain and contributed to the enrichment of mitochondrial dysfunction and OXPHOS pathways within the dataset (Fig. 6A, Supplemental Tables S5 and S6). However, before spending further time on specific pathways, enrichment in proteins linked to the free radical scavenging and cell death deserve mention (Fig. 5A).

With regard to free radical scavenging, there were a total of 15 proteins that changed ($P < 0.05$) during one or more of the time intervals studied (Fig. 5B). Hierarchical clustering of these revealed the presence of three major sets. The first set contained proteins that were generally high at day 2 postpartum and decreased during the early lactation interval. The second set contained five proteins that were all generally low on days 2 and 8 postpartum and increased on day 14. In this set, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (Uqcrfs1), and voltage-dependent anion channel 1 (Vdac1) had minor-abundance isoforms that were not correlated to changes their respective normalized sum ratio (Supplemental Fig S3). The third set consisted of three proteins that were low on day 2 postpartum and did not increase until day 21 postpartum. These included clusterin (Clu), superoxide dismutase 1 (Sod1), and cathepsin D (Ctsd). Of the two isoforms identified for Ctsd, only the most abundant was correlated to the normalized sum ratio (Supplemental Fig S3). Overall, the patterns observed in this group of proteins suggested that the majority of free radical scavenger proteins increase with mid- or late lactation.

With regard to cell death there were 54 proteins identified that change at one or more of the intervals studied (Supplemental Table S5). Hierarchical clustering of these proteins revealed five sets (Fig. 5C). The largest of these accounted for 33% of the cell death associated proteins and was generally low during early and mild lactation but increased on day 21 (Fig. 5C and Supplemental Table S5). The largest functional grouping within this set were heat shock proteins (Hsp). The protein Clu was also in this set, as was another cell death regulating protein, programmed cell death 6 interacting protein (Pdcd6ip). The second largest group of proteins within the cell death category accounted for 27% of the set and were generally increased on day 14 postpartum and decreased on day 21 postpartum. About half of these proteins were also components of the OXPHOS pathway, which will be discussed later. There were four proteins in this ontology group that had multiple isoforms for which at least one was not correlated to the normalized sum ratio. However, As with the free radical scavenging proteins, the majority of cell death proteins were increased either during mid or late lactation.

Analysis of the data set for canonical pathway enrichment identified mitochondrial dysfunction and OXPHOS, as the most highly enriched (Fig. 6A). These two pathways, along with ubiquinone biosynthesis, demonstrated the greatest enrichment during the mid- and late lactation intervals. Enrichment for the ubiquinone biosynthesis pathway was primarily driven by the presence of NADH ubiquinone dehydrogenase subunits, while the mitochondrial dysfunction and OXPHOS pathways contained these as well as subunits from other ETC complexes (Supplemental Table S6). Hierarchical clustering and heat map analysis of proteins in the OXPHOS pathway demonstrated that essentially all of these proteins increased during mild lactation and then decreased at late lactation (Fig. 6B). Only two proteins, ubiquinol-cytochrome c reductase binding protein (Uqcrb) and NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 3 (Ndufaf3), increased ($P < 0.05$) during the early lactation. In this set there were also six proteins that had one or more low abundance isoforms that were not correlated to the normalized sum ratio (Supplemental Fig S3). The most noteworthy of these was Atp5a1. Of the 19 isoforms detected for this protein there were three that were not correlated with the remaining high abundance isoforms and one that was negatively correlated ($r = -0.68, P = 0.01$). These results suggest that while increased ATP synthesis capacity of mammary cell mitochondria during

![Image](http://physiolgenomics.physiology.org/ by 10.220.32.247 on November 6, 2017)
early lactation requires changes in only one or a few proteins, loss of ATP synthesis activity during late lactation is linked with an orchestrated event involving numerous subunits of the OXPHOS pathway.

The citrate cycle was the third most enriched pathway within the mammary mitochondrial proteome (Fig. 6A). Most of the changes to protein abundance within this pathway occurred during the early lactation phase. Hierarchical clustering and heat map analysis (Fig. 6D) for proteins in the citrate cycle revealed two major subgroups based on changes in protein abundance. The first consisted of five proteins that were high on day 2 postpartum and decreased (P < 0.05) with progression to day 8 postpartum (Supplemental Table S7). The second group consisted of five proteins that were generally low at day 2 postpartum and demonstrated an increase on day 14 postpartum (Supplemental Table S7). The two proteins, succinyl-CoA ligase γ (Suc1g) and ATP citrate lyase (Acly), were distinct from other two groups in that their relative abundance increased during early lactation. This increase was most dramatic for Acly being ~67% (P = 0.006) The remaining four proteins in the data set did not change significantly. In addition to these changes however, there were also six multi-isoform proteins in this set with at least one uncorrelated isoform (Supplemental Fig. S3). These results suggest that during early lactation there is a coordinated decrease in citrate cycle enzymes that are downstream of citrate synthase and responsible for the oxidative steps of the cycle. The results further suggests that the oxidative activity of the cycle increases again with progression into late lactation and that there may also be changes in the activities of these enzymes as a result of posttranslational modifications.

Among the remaining of the highest ranking pathways, butanoate metabolism had the greatest number changes during the early and late stages of lactation, while pathways related to amino acid and fatty acid metabolism and propanoate and pyruvate metabolism underwent the largest number of changes during early lactation (Fig. 6A, Supplemental Table S7). There were 24 proteins within this set of pathways that changed at one or more of the intervals studied. Over half of these (58%) fell into the class of enzymes know as dehydrogenases. Hierarchical clustering of these proteins along with heat map analysis demonstrated the presence of two major subgroups based on expression (Fig. 6C). The first of these contained 11 proteins that were generally most highly expressed on day 2 postpartum, decreased on days 8 and 14, and then increased on day 21. Of these, six were directly involved in beta-oxidation of fatty acids. The second major group within this set of metabolic proteins also contain 11 proteins that were generally most highly expressed on day 2 postpartum, decreased on days 8 and 14, and then increased on day 21. Of these, six were directly involved in beta-oxidation of fatty acids. The second major group within this set of metabolic proteins also contain 11 proteins that were generally most highly expressed on day 2 postpartum, decreased on days 8 and 14, and then increased on day 21. Of these, six were directly involved in beta-oxidation of fatty acids. The second major group within this set of metabolic proteins also contain 11 proteins that were generally most highly expressed on day 2 postpartum, decreased on days 8 and 14, and then increased on day 21. Of these, six were directly involved in beta-oxidation of fatty acids.
For Me1, only the increase on day 8 was significant ($P < 0.05$). Overall the data on these pathways suggest that mammary cell long chain fatty acid oxidation is decreased with progression from early to midlactation. In addition, increased Me1 abundance is consistent with the concept that the mammary cell alters citrate cycle function during lactation to provide substrates for milk lipid synthesis.

A secondary goal in the proteome analysis reported here was to identify interacting networks that could potentially regulate changes to mammary mitochondrial biogenesis and function during lactation. To identify these networks we applied the networks analysis algorithm of the ingenuity pathway analysis software to identify connectivity networks based on direct interactions. For this comparison, a total of 33 proteins were significantly altered. Proteins in the network (A) are colored coded based on whether they increased (red) or decreased (green) during the time interval. Proteins colored gray were present in the data set but did not reach the significance threshold ($P < 0.05$). Proteins colored white were not present in the data set but were still potentially part of the network based on high connectivity. Values presented near each protein represent the fold change and the corresponding $P$ value for the change.

Real-time RT-PCR was used to measure mRNAs for select proteins to determine if their expression at the mRNA and protein levels were correlated (Pearson’s $r > 0.5$) to each other (B) or not (C). Each data point represents the average of 3 biological replicates per time point.
also, though not present in the dataset were four highly connected molecules with potential to play a regulatory role. These were huntington (Htt), Ppargc1a, amyloid-β (A4) precursor protein (App), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ (YWHAZ, also known as 14–3-3ζ). Because both Htt and Ppargc1a are both transcriptional regulators, real-time qRT-PCR was conducted for several of the focus molecules that were connected to these to determine if there was a correlation between protein and mRNA levels. For the 10 focus molecules within this network that were analyzed by real-time RT-PCR, four displayed RNA levels that were correlated to the corresponding proteins levels (Fig. 7B), while the other six did not (Fig. 7C). Of five focus molecules within this network that were linked to Ppargc1a, two could be demonstrated to have correlations (Pearson’s R >0.5) between the DIGE data and real-time PCR data: Acat1 and Idh3a. For Htt there was one of seven focus molecules analyzed by real-time RT-PCR revealed a correlation between the mRNA abundance and the DIGE data. These data suggest that a transcriptional mechanism mediated by Ppargc1a and Htt may play a role in regulating select mammary mitochondrial proteins linked to the citrate cycle and fatty acid oxidation during early lactation.

The top five networks identified from the analysis of proteins that changed during the midlactation interval (days 8-14) had P values that were all <10^{-25} and consisted of at least 15 focus molecules, respectively. The most highly ranked (P = 10^{-37}) network in this analysis contained 18 focus molecules and was enriched for proteins linked to the biofunctions of energy production and small molecule biochemistry (Fig. 8). Of these 18 molecules, 10 were increased (P < 0.05) and eight were decreased (P < 0.05). The most striking aspect of the network was the presence of a highly interconnected module made up of protein subunits that compose NADH dehydrogenase, or complex I of the OXPHOS pathway. In this module there were a total of 15 proteins of which six were increased (P < 0.05). The increases ranged from 26% for NADH dehydrogenase (ubiquinone) Fe-S protein 8 (Ndufs8) to 56% for NADH dehydrogenase (ubiquinone) flavoprotein 1(Ndufv1). Similar modules for OXPHOS pathway complexes III, IV, and V were detected in two of the other most highly ranked networks for this time interval (Supplemental Fig. S4). Select proteins within these modules were linked to the transcriptional regulators Ppargc1a, nuclear respiratory factor I (Nrf1), and myelocytomatosis viral oncogene homolog (Myc). These results support the conclusion that with midlactation there is a coordinated increase in many of the proteins that make up the electron transport chain.

Analysis of the late lactation interval for interacting networks detected six networks. The most highly ranked network in this set had 26 focus molecules and a P value of 10^{-56} (Fig. 9). Within this network there were nine proteins that decreased and 17 that increased. Of the proteins that decreased, six contributed to a module comprising OXPHOS pathway complex V, the ATP synthase. As with the increases in ETC modules for complexes I, III, and IV that were observed between days 8 and 14 postpartum, there were decreases in these same modules between days 14 and 21. (Supplemental
Among all of the above networks, potential transcriptional regulators included PPARGC1α, Myc, and hepatic nuclear factor 4A (Hnf4a). These results support the conclusion that during late lactation there is a coordinated decrease in many of the proteins which make up the electron transport chain.

Abundance of PPARGC1α and phospho-AMPK in mammary tissue. Activation of AMP activated protein kinase (AMPK) induces the expression of the transcriptional coactivator PPARGC1α, which is known to stimulate mitochondrial biogenesis (20). To assess the involvement of these proteins in mammary mitochondrial biogenesis we measured their abundance in the mammary gland during the different phases of lactation (Figs. 10 and 11). Densitometric analysis of Western blots (Fig. 10, A and C) for PPARGC1α demonstrated that this protein is induced within 24 h of parturition, remains high from days 2 through 4, and then is low throughout the remainder of both early and prolonged lactation (Fig. 10, B and D). Immunofluorescent staining of frozen mammary tissue sections for phospho-AMPK (Fig. 11A) demonstrated the protein to be highly abundant in MEC. Quantitative analysis of images captured from mammary tissue sections stained for either phospho- or total AMPK demonstrated that phosphorylation of Thr172 was high on day 2, decreased \( (P < 0.05) \) with progression into early lactation, then increased again at the end of lactation (Fig. 11C). The amount of total AMPK as measured by immunofluorescence did not change with time postpartum (Fig. 11C). These results suggest that changes in AMPK phosphorylation and the abundance of PPARGC1A during early lactation may be linked to mitochondrial biogenesis and function as lactation progresses.

DISCUSSION

The results of these studies highlight the importance of mitochondrial biogenesis and ATP synthesis activity to MEC milk production during normal lactation and provide the first description of the mammary gland mitochondrial proteome during lactation. Because the mammary gland has both epithelial and stromal elements and the proportion of these elements are known to change over the course of a developmental cycle (40, 69), a combination of histochemical and biochemical approaches was used to provide corroborating data to support the conclusion of this study. In addition, the analysis of ATP synthesis from selected time points and of the mitochondrial proteome was done using both whole tissue homogenates and epithelial fractions prepared from collagenase-digested mammary tissue.

Previous work in our laboratory has demonstrated that the 8DG content of mammary mitochondrial DNA increased with early lactation and then declined with prolonged lactation (26). Oxidative damage to genomic DNA during aging is known to be repaired very efficiently, with resulting metabolites such as 8DG being excreted into the urine (59, 65). Because metabolic intermediates in the lactating mammary cell are often secreted into milk (4), the milk 8DG concentrations described in the...
present study could be interpreted as an indicator of oxidative damage to the MEC. To follow up on these results, independent methods were used to determine the relationship of mammary mitochondrial biogenesis and function to oxidative damage and to identify potential regulatory mechanisms. Adenine nucleotide concentrations were initially chosen as an indicator of mammary energy status because they have been suggested to serve as a useful marker of cellular mitochondrial function during aging and disease in other tissues (32, 39, 63, 74), however, it became apparent that with lactating mammary tissue, adenine nucleotides may not have been the best indicator of cellular energy status during lactation as the relationship of this endpoint to temporal changes in milk production is variable (13, 30, 41, 66, 68). Some have observed that the pattern of increase in mammary tissue nucleotides with lactation may be more related to increases in RNA synthesis than to changes in metabolic activities (68). Therefore a direct measurement of ATP synthesis activity in isolated mitochondria was also used.

The changes in mammary mitochondrial ATP synthesis activity that were observed in this study were consistent with previous observations on mammary mitochondrial respiration in lactating guinea pigs (44). In addition, the decrease of mammary mitochondrial ATP synthesis on day 21 postpartum correlates with previously reported decreases in milk production, as assessed by litter gain and with increases in mammary apoptosis (26). These results are consistent with the idea that the ATP synthesis activity of mitochondria isolated from the mammary tissue of lactating mice is temporally correlated to milk production during the lactation. In addition, the changes in mitochondrial number, mass, and DNA content further suggest that there are coordinated changes within the MEC of not only mitochondrial ATP synthesis activity, but also in the processes that regulate the replication of the mitochondrial genome and the turnover of mitochondria. These results suggest that there may exist important regulatory mechanisms within the lactating mammary cell to control mitochondrial biogenesis and function. Part of these may involve oxidative damage.

Several earlier studies have collected proteomic data from the mammary tissue of lactating animals (1, 6, 14, 16, 18, 48, 50, 60). None have studied mammary mitochondria. With all proteomic studies, there are several issues that warrant consideration. First, in isolated organelles, the extent to which the fraction of interest is contaminated with proteins from other organelles is important (42). The presence of milk proteins within the mitochondrial fraction described in the study illustrates this challenge. However, it is important to note that the largest category within cellular component gene ontology was

Fig. 10. Expression of PPARGC-1a protein in the mammary gland increases transiently during early lactation and then remains low through prolonged lactation. Western blotting was conducted for PPARGC-1a proteins on mammary tissue extracts prepared from mice on days 1 through 5 postpartum (A and B) or on days 2, 8, 14, and 21 of the lactation cycle (C and D). Tubulin was used to control for loading total protein loading (A and C) Densitometry and statistical analysis (B and D) demonstrated significant changes (P < 0.05) in PPARGC-1a abundance during the immediate periparturient period. Each bar represents the mean ± SE for 8 mice. *Statistically significant differences (P < 0.05).

Fig. 11. Phospho-AMPK in mammary epithelial cells is highest during early lactation, decreases with midlactation, and increases again with prolonged lactation. Immunofluorescent staining of mammary tissue frozen section was conducted using antibodies against the phospho-AMPK (Thr172) (staining shown in green) (A) and total AMPK (not shown). Specificity of the staining was demonstrated by omitting the 1st antibody step from the staining procedure (B). All sections were also counterstained with the nuclear dye TOPRO3 (red). Tissue sections obtained from mice at days 2, 8, 14, 21, 28, and 35 days postpartum were stained for phospho- (black bars) and total (gray bars) AMPK and imaged by confocal microscopy to generate digital images, which were then used to measure staining intensity (C). Each bar represents the mean ± SE for 8 mice. *Statistically significant differences from day 2 postpartum (P < 0.05).
“mitochondrion,” which contained sevenfold more accessions than any other category in the analysis. Comparison of this dataset with the recently described Mitobase database also found about a 50% overlap (47). In contrast, an analysis of 218 accessions described in a whole tissue proteome from the lactating bovine mammary gland consisted of ~15% mitochondrial proteins (6). This suggests that the proteins in this dataset were enriched for mitochondria by a factor of 3.5.

Secondly, there were a number relatively small changes detected in the dataset. In DIGE, the use of the internal standard has been reported to result in a precision of 8–10% (2). Therefore it was not surprising that a number of the changes observed in this study were of this magnitude. However, it is important to note that most of the observed changes were larger than this and likely to have had a significant impact on mitochondrial function.

Lastly, the issue of using a normalized sum ratio for the quantitative analysis of proteins with multiple isoforms needs some consideration. This approach proved valuable because it simplified the analysis and facilitated follow-up using Pearson’s correlation to produce a short list of proteins with the potential for regulation by posttranslational modification. (Supplemental Fig S3). Some of these proteins have already been demonstrated to undergo phosphorylation or acetylation (8, 11). In addition, changes in phosphorylation are known to regulate the activity of several of these proteins including Pdh1, Acly, and Vdac1 (34–36, 73).

Based on the mitochondrial ATP synthesis result, we predicted that there would have been measurable increases in components of the OXPHOS pathway between day 2 and 8 postpartum. Surprisingly, most of the OXPHOS proteins increased on day 14 postpartum after ATP synthesis activity had already reached a maximum. These results suggest that although coordinated changes occur in numerous OXPHOS proteins during mid- to late lactation, changes in only one or two during early lactation are involved with the increased mitochondrial ATP synthesis activity that was observed. Of the two OXPHOS proteins that did increase during early lactation NDUFAF3 was the most notable since it displayed a 3.5-fold increase. Although this subunit of the OXPHOS pathway has only recently been described, it appears to play an important role in the activity of complex I (55, 72). Mutations in the gene encoding this protein have been linked to a fatal mitochondrial disease in human infants. These observations suggest that NDUFAF3 may be a rate limiting factor in mammary mitochondrial ATP synthesis activity during early lactation.

With regard to the other ontology terms and pathways that were enriched in this data set, the results are largely consistent with expectations. For example, the enrichment for proteins linked to free-radical scavenging and cell death during late lactation supports the idea that oxidative damage increases in the gland with progression through lactation and is consistent with the fact that mammary cell apoptosis also increases (26, 27). In fact proteins such as Clu, Hspa8, and Anxa5 have previously been demonstrated to increase with mammary involution (51, 61, 64). The function of these proteins in this process, however, remains unclear.

The observed changes in citrate cycle proteins are also significant in that previous work has shown that certain cataplerotic reactions in lactating mammary tissue function to divert metabolic substrates such as citrate out into the cytoplasm for use in milk lipid synthesis (5, 38). Although Me 1 and Acly have classically been referred to as cytosolic enzymes, they have also been identified in mitochondrial preparations from other tissues and can be found in the Mitobase database of proteins with known localization to the mitochondria (47). The increase observed for these two proteins in the present dataset is consistent with previous observations demonstrating that these enzymes increase in mammary tissue during early lactation (12, 23, 62). The other interesting aspect of these coordinated changes is in the fact that many of the enzymes involved with fatty acid oxidation were actually decreased in the same time frame. Although these observations clearly point to the role of mammary mitochondria in lipid biosynthesis during lactation the question remains as to what the nature of the cellular mechanism are that regulate mammary mitochondrial biogenesis during lactation.

The principle value of the networks identification algorithm of IPA is in its ability to identify interacting modules. Figs. 7–9 illustrate how these modules are interconnected and high-light potential regulator nodes. The highest scoring networks in our data set clearly illustrated the fact that there were coordinated changes in modules comprising all five subunits of the OXPHOS pathway. The presence of transcriptional regulators as regulatory nodes in these networks, though not surprising, provides motivation for future studies. That protein and mRNA levels were positively correlated for only four out of the 10 protein-mRNA pairs examined does not rule out a role for these transcriptional regulators in determining mammary mitochondrial function. Previous work comparing proteomic measurements with mRNA measurements has demonstrated that high correlation between protein and mRNA levels are more the exception than the rule (3, 9, 10, 22).

PPARGC1A is a master regulator of genes involved with metabolism, mitochondrial biogenesis and the activity of the OXPHOS pathway in other tissues (28). Previous analysis of mammary tissue microarray data and genetic marker data in lactating dairy cows has suggested that PPARGC1α may be important to milk production and/or milk fat synthesis (7, 33, 71). In the present study, a connection to seven proteins in the early lactation mitochondrial proteome suggests PPARGC1A may play a role in the regulation of mammary cell metabolism and mitochondrial function during early lactation. In this regard, it is interesting to note that most of the proteins connected to PPARGC1A, decreased during early lactation as did PPARGC1A itself. In addition most of these were linked to the citrate cycle and lipid metabolism as opposed to the OXPHOS pathway. The decrease in phosphorylation of mammary AMPK during this time period is also very interesting since in other systems, AMPK activates PPARGC1α (46). Lastly, although PPARGC1A was also linked to two proteins during midlactation that were a part of the OXPHOS pathway, the majority of proteins within this pathway showed no connection to this protein. In contrast, other transcriptional regulators such as MYC, NRF1, and HNF4A were connected to these networks. These may also be worth considering further with regard to the regulation of mammary cell mitochondria during lactation.

In conclusion, these studies demonstrate the presence of biphasic changes in mitochondrial biogenesis and function that directly correlate to the known changes in milk production.
capacity that occur over a prolonged lactation cycle. They further demonstrate that although the gain in milk production during early lactation is linked with dramatic increase in mammary mitochondrial number. The loss of function that occurs during prolonged lactation may not necessarily be linked to a decreased in MEC mitochondrial number. The studies highlight the importance of changes in the abundance of metabolic enzymes linked to the provision of substrate for milk lipid synthesis and suggest that PPARGC-1α may be one of several transcription factors acting to regulate the metabolic activity of mammary mitochondria during lactation. More definitive studies on the function of this and other candidate proteins described in this paper are necessary.

ACKNOWLEDGMENTS

The authors thank Jessy George, Daniel Torres, and Su Pan for technical assistance with this project and Dr. Leo Nijtmans for the antibody to NDUFAF3. The authors also thank Dr. Monique Rijnkels and Louise Hadsell for critically reading the manuscript and providing editorial suggestions.

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REFERENCES

10. DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


33. Khattib H, Zaitoun I, Wiebelhaus-Finger J, Chang YM, Rosa GJ. The association of bovine PPARGC1A and OPN genes with milk composition