Gestational Diabetes Is Characterized by Reduced Mitochondrial Protein Expression and Altered Calcium Signaling Proteins in Skeletal Muscle

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1371/journal.pone.0106872

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12987328

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Gestational Diabetes Is Characterized by Reduced Mitochondrial Protein Expression and Altered Calcium Signaling Proteins in Skeletal Muscle

Kristen E. Boyle¹, Hyonson Hwang⁴, Rachel C. Janssen¹, James M. DeVente⁵, Linda A. Barbour²,³, Teri L. Hernandez², Lawrence J. Mandarino⁶,⁷, Martha Lappas⁸,⁹, Jacob E. Friedman¹

¹Department of Pediatrics, University of Colorado Denver School of Medicine, Aurora, Colorado, United States of America, ²Department of Medicine, University of Colorado Denver School of Medicine, Aurora, Colorado, United States of America, ³Department of Obstetrics and Gynecology, University of Colorado Denver School of Medicine, Aurora, Colorado, United States of America, ⁴Boston Children’s Hospital/Harvard Medical School, Boston, Massachusetts, United States of America, ⁵Department of Obstetrics & Gynecology, Brody School of Medicine, East Carolina University, Greenville, North Carolina, United States of America, ⁶Department of Medicine, Mayo Clinic Arizona, Tempe, Arizona, United States of America, ⁷Department of Medicine, University of Melbourne, Melbourne, Victoria, Australia, ⁸Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Victoria, Australia, ⁹Mercy Perinatal Research Centre, Mercy Hospital for Women, Heidelberg, Victoria, Australia

Abstract

The rising prevalence of gestational diabetes mellitus (GDM) affects up to 18% of pregnant women with immediate and long-term metabolic consequences for both mother and infant. Abnormal glucose uptake and lipid oxidation are hallmark features of GDM prompting us to use an exploratory proteomics approach to investigate the cellular mechanisms underlying differences in skeletal muscle metabolism between obese pregnant women with GDM (OGDM) and obese pregnant women with normal glucose tolerance (ONGT). Functional validation was performed in a second cohort of obese OGDM and ONGT pregnant women. Quantitative proteomic analysis in rectus abdominus skeletal muscle tissue collected at delivery revealed reduced protein content of mitochondrial complex I (C-I) subunits (NDUFS3, NDUFV2) and altered content of proteins involved in calcium homeostasis/signaling (calcineurin A, α1-syntrophin, annexin A4) in OGDM (n = 6) vs. ONGT (n = 6). Follow-up analyses showed reduced enzymatic activity of mitochondrial complexes C-I, C-III, and C-IV (~60–75%) in the OGDM (n = 8) compared with ONGT (n = 10) subjects, though no differences were observed for mitochondrial complex protein content. Upstream regulators of mitochondrial biogenesis and oxidative phosphorylation were not different between groups. However, AMPK phosphorylation was dramatically reduced by 75% in the OGDM women. These data suggest that GDM is associated with reduced skeletal muscle oxidative phosphorylation and disordered calcium homeostasis. These relationships deserve further attention as they may represent novel risk factors for development of GDM and may have implications on the effectiveness of physical activity interventions on both treatment strategies for GDM and for prevention of type 2 diabetes postpartum.


Editor: Mauro Salvi, University of Padova, Italy

Received May 8, 2014; Accepted August 4, 2014; Published September 12, 2014

Copyright: © 2014 Boyle et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This research was supported by grants from the National Institutes of Health SR01 DK062155 (J.E.F.), Colorado Nutrition Obesity Research Center, P30 DK048520 (J.E.F.), and R01 DK078645 (L.A.B.) [http://www.nih.gov], and from the Medical Research Foundation for Women and Babies; (M.L.) [http://www.mrfwb.org.au] and the Diabetes Australia Research Trust Trust; (M.L.) [http://www.diabetesaustralia.com.au]. M.L. was supported by a Career Development Fellowship from the National Health and Medical Research Council, grant no. 1047025 [https://www.nhmrc.gov.au]. K.E.B. was supported by the National Institutes of Health F32 DK089743 and K12 HD057022 [http://www.nih.gov]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: kristen.boyle@ucdenver.edu

Introduction

Gestational diabetes mellitus (GDM) is a rapidly growing public health concern. Adoption of new diagnostic criteria recommended by the American Diabetes Association (ADA) [1,2] estimates a global prevalence of nearly one in five women (~18%) who are considered at risk for GDM. Obesity occurs in ~one in three women of child-bearing age [1,3] and is a driving force accelerating the prevalence of GDM. GDM not only complicates pregnancy by increasing risk of pre-eclampsia and cesarean delivery, but is an independent risk factor for excess fetal growth and childhood obesity [3–7], and a consequence of even greater insulin resistance and nutrient availability than associated with maternal obesity alone [4–8]. In addition, GDM diagnosis identifies a population of women at markedly increased risk for future diabetes [8,9], in part due to abnormal skeletal muscle signaling. Up to fifty percent of women diagnosed with GDM will go on to develop type 2 diabetes (T2DM) [9–11], and physical activity and dietary interventions to prevent this progression have been disappointing due to compliance difficulties [10–13]. Thus, understanding the pathogenesis of GDM is extremely important.
from a public health perspective for both maternal and child health.

Late in gestation, due to the demands of the placental-fetal unit and rapid depletion of glycogen stores, all women exhibit a shift in metabolism to increase reliance on lipid for metabolic substrate, a term called accelerated starvation. This metabolic shift is accompanied by a large decrease in skeletal muscle insulin sensitivity (~50%), both of which serve to allow for increased glycolytic supply to the growing fetus [12–14]. Women with GDM, however, demonstrate lower rates of whole body lipid oxidation both during early and late gestation [14,15], with a less robust shift from glucose to lipid metabolism in late pregnancy compared with their normal glucose tolerant (NGT) counterparts [15–17]. Skeletal muscle, by virtue of its mass, is the principle site of glucose and lipid oxidation [3,16–18], and therefore plays an important role in whether fuels are utilized by maternal tissues or shunted across the placenta to the developing fetus. The pathways underlying insulin resistance in GDM are well studied and multifactorial. However, little is known about the cellular mechanisms for altered skeletal muscle lipid or glucose metabolism, which is likely to significantly alter nutrient availability, thus increasing risk for increased fetal fat accretion and an increased risk of childhood obesity in offspring of women with GDM [3,18,19].

Therefore, the purpose of this study was to employ a discovery proteomic approach to identify candidate proteins that may underlie differences in skeletal muscle metabolism in obese, GDM pregnant women. The advantage of proteomic analysis, as opposed to a transcriptomic approach, is that we are able to measure content of global functional molecules involved in skeletal muscle metabolism rather than expression of genes that may or may not be related to protein content. We used an established quantitative proteomic analysis [19,20] to compare skeletal muscle of obese pregnant women with GDM (OGDM) with obese pregnant women with NGT (ONGT). We then carried out a functional validation in a second, larger cohort of obese pregnant women with and without GDM. We are the first to demonstrate that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport system (ETS) are downregulated in the OGDM women, a factor that several proteins of the mitochondrial electron transport}
search the decoy randomized database. The search parameters used were: 10 ppm mass tolerance for precursor ion masses and 0.5 Da for production masses; digestion with trypsin; a maximum of two missed tryptic cleavages; fixed modification of carboxymethylation; variable modifications of oxidation of methionine and phosphorylation of serine, threonine and tyrosine. Probability assessment of peptide assignments and protein identifications were made through use of Scaffold (version Scaffold_2_00_06, Proteome Software Inc., Portland, OR). Only peptides with ≥ 95% probability were considered. Proteins that contained identical peptides and could not be differentiated based on MS/MS analysis alone were grouped.

Protein and gene ontology annotation were performed as described, as were extraction of tandem mass spectra and
verification of charge states and monoisotopic peak assignments [19]. To quantify protein abundance, normalized spectral abundance factors (NSAFs) were used [19]. Briefly, MS/MS spectra assigned to a protein were normalized to the number of amino acids for that protein, resulting in a spectral abundance factor (SAF). Each SAF was normalized against the sum of all SAFs in one sample, resulting in the NSAF value. This calculation is represented by the following equation, where \( N \) is equal to the number of proteins detected in a sample:

\[
NSAF_i = SAF_i / \sum_{i=1}^{N} SAF_i
\]

Thus, NSAF values allow for direct comparison of a protein’s abundance between individual runs in a fashion similar to microarray data analysis. The reproducibility and linearity of this method are described previously [19,23].

### Study 2: Metabolic Analysis

**Ethics Statement.** Approval for this study was obtained from the Mercy Hospital for Women’s Research and Ethics Committee and written informed consent was obtained from all participants prior to cesarean delivery.

**Patients and Sample Collection.** To extend our observations from Study 1, a larger cohort of ONGT and OGDM subjects were studied. Pregnant women over 21 years of age were screened for GDM at 24–28 weeks gestation and were diagnosed according to the criteria set by the Australasian Diabetes in Pregnancy Society (ADIPS), by either a fasting venous plasma glucose level of 5.5 (compared to 5.3 using CC criteria) and/or greater than 8.0 mmol/L (8.6 using CC criteria) glucose 2 h after a 75 g oral glucose tolerance test. Blood samples were immediately centrifuged at 1,500 g for 10 min and the plasma aliquoted into microfuge tubes and samples were immediately stored at −80°C until assayed for glucose and insulin. Blood glucose determination was performed by the hospital pathology department using an automated glucose oxidase/oxygen-rate method. Standard ELISA assay kit for insulin (Diagnostic Systems Laboratories, Webster, TX; limit of detection 0.26 IU/mL) was purchased and used according to the manufacturer’s instructions. Insulin resistance at time of diagnosis was calculated using the homeostasis model assessment for insulin resistance (HOMA-IR) method where HOMA-IR = fasting plasma glucose (mmol/l) times fasting plasma insulin (µU/mL) divided by 22.5 [26,27].

**Mitochondrial Enzyme Activity Assays.** Mitochondrial-enriched supernatants (post 600 g) were prepared from frozen skeletal muscle samples, as described [27]. Supernatants were used to assay activity of respiratory chain enzyme complexes I, II, III, and IV (C-I through C-IV, respectively); and citrate synthase (CS), spectrophotometrically on a Synergy H1 microplate reader (Biotek, Winooski, VT). Enzyme assays for respiratory chain complexes and CS were performed as described [27,28] with minor modifications for microplate reading. For C-I and C-II, enzyme activities were calculated as initial rates (nmol/min). For complexes III and IV, enzyme activities were calculated as the first-order rate constants derived within 2–3 min of reaction initiation. All assays were performed in duplicate. The protein content of each sample was determined using a BCA assay. All activities were normalized to the total protein content and to CS activity (calculated as initial rate of reaction as nmol/min) of each sample and expressed relative to the mean for NGT women.

**Western Blot.** Protein levels of MitoProfile total OXPHOS antibody cocktail, AMPK, phosphoAMPK, PGC-1α, and PPARα were determined in the muscle biopsy samples with calnexin as loading control as previously described [28]. OXPHOS antibody cocktail measured specific subunits of C-I, C-II, C-III, and C-IV (NDUFB8, SDHB, UQCRCl2, and MT-CO1, respectively). All antibody dilutions were 1:1,000 for primary antibodies and 1:10,000 for secondary antibodies, unless otherwise stated. All results were expressed relative to the mean for NGT women.

---

**Table 1. Clinical characteristics of the subjects for Study 1.**

<table>
<thead>
<tr>
<th></th>
<th>ONGT (n = 6)</th>
<th>OGDM (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery BMI (kg/m²)</td>
<td>34.2±2.3</td>
<td>37.5±2.1</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/L)</td>
<td>4.2±0.2</td>
<td>5.3±0.3*</td>
</tr>
<tr>
<td>Insulin (µU/L)</td>
<td>11.3±3.2</td>
<td>19.5±4.8*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1±0.7</td>
<td>4.6±1.0*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>161.0±22.6</td>
<td>265.5±40.3</td>
</tr>
<tr>
<td>Gravida</td>
<td>2.0±0.0</td>
<td>2.5±2.1</td>
</tr>
<tr>
<td>Parity</td>
<td>1.0±0.0</td>
<td>1.0±1.4</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. OGTT, oral glucose tolerance test. Independent t-test *P<0.05. doi:10.1371/journal.pone.0106872.t001
OXPHOS antibody cocktail (host: mouse), calnexin (host: rabbit), PGC-1α (host: rabbit), and PPARα (host: rabbit) antibodies were purchased from Abcam (Cambridge, MA). AMPK (host: rabbit) and phospho-AMPK (Thr172) (host: rabbit) antibodies were from Cell Signaling Technology (Danvers, MA). Secondary antibodies were purchased from Bio-Rad (Hercules, CA).

Mitochondrial DNA Copy Number. Approximately 15 mg of skeletal muscle was homogenized and DNA was isolated by phenol/chloroform extraction with ethanol precipitation. Mitochondrial DNA (mtDNA) copy number was then measured as previously described [28].

### Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics, Version 22 (IBM Corp., Armonk, NY). For the Study 1 proteomic analysis, a large number of proteins were assigned in at least one of 12 subjects studied (979 proteins; Table S1). A series of filters were used to narrow the number of proteins that were used in comparisons between groups (Figure 1). First only those proteins with representation in at least three subjects from each group were chosen (415 proteins). Of these, only those proteins with greater than 1.5-fold difference between groups (22 proteins) were examined for statistically significant differences between groups. First, data were tested for normality using the Shapiro-Wilk test, which were performed for ONGT and OGDM separately.

#### Table 2. Proteins with 1.5-fold difference between ONGT and OGDM, Study 1.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Protein Function</th>
<th>Fold Difference</th>
<th>t-test P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYOM1</td>
<td>Myomesin 1</td>
<td>5.21</td>
<td>0.07</td>
</tr>
<tr>
<td>HSPA1A/1B</td>
<td>Heat shock 70 kDa protein 1A/1B</td>
<td>2.98</td>
<td>0.09</td>
</tr>
<tr>
<td>ME1</td>
<td>NADP-dependent malic enzyme</td>
<td>2.39</td>
<td>0.09</td>
</tr>
<tr>
<td>CMBL</td>
<td>Carboxymethylenebutenolidase homolog</td>
<td>2.30</td>
<td>0.01*</td>
</tr>
<tr>
<td>PPP3CB</td>
<td>Calmodulin-dependent calcineurin A β</td>
<td>2.22</td>
<td>0.02*</td>
</tr>
<tr>
<td>CAMK2A</td>
<td>Calcium/calmodulin-dependent protein kinase 2 α</td>
<td>2.17</td>
<td>0.15</td>
</tr>
<tr>
<td>FKBP3</td>
<td>Peptidyl-prolyl cis-trans isomerase FKBP3</td>
<td>1.86</td>
<td>0.38</td>
</tr>
<tr>
<td>ERP29</td>
<td>Endoplasmic reticulum resident protein 29</td>
<td>1.75</td>
<td>0.40</td>
</tr>
<tr>
<td>CD36</td>
<td>Platelet glycoprotein 4</td>
<td>1.73</td>
<td>0.05*</td>
</tr>
<tr>
<td>LRRC47</td>
<td>Leucine-rich repeat-containing protein 47</td>
<td>N/A</td>
<td>1.71</td>
</tr>
<tr>
<td>TUBA8</td>
<td>Tubulin alpha-8 chain</td>
<td>−1.54</td>
<td>0.10</td>
</tr>
<tr>
<td>CAPZA2</td>
<td>F-actin-capping protein subunit alpha-2</td>
<td>−1.58</td>
<td>0.12</td>
</tr>
<tr>
<td>NDUFV2</td>
<td>NADH dehydrogenase flavoprotein 2</td>
<td>−1.59</td>
<td>0.04*</td>
</tr>
<tr>
<td>NDUFS3</td>
<td>NADH dehydrogenase iron-sulfur protein 3</td>
<td>−1.60</td>
<td>0.03*</td>
</tr>
<tr>
<td>ANXA4</td>
<td>Annexin A4</td>
<td>−1.65</td>
<td>0.05*</td>
</tr>
<tr>
<td>NDUFS8</td>
<td>NADH dehydrogenase iron-sulfur protein 8</td>
<td>−1.74</td>
<td>0.07</td>
</tr>
<tr>
<td>PSMA2</td>
<td>Proteasome subunit alpha type-1</td>
<td>−1.97</td>
<td>0.04*</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
<td>−1.97</td>
<td>0.10</td>
</tr>
<tr>
<td>SERPINC1</td>
<td>Antithrombin-III</td>
<td>−2.03</td>
<td>0.12</td>
</tr>
<tr>
<td>AHSG</td>
<td>Alpha-2-H5-glycoprotein</td>
<td>−2.07</td>
<td>0.07</td>
</tr>
<tr>
<td>CP</td>
<td>Ceruloplasmin</td>
<td>−2.07</td>
<td>0.19</td>
</tr>
<tr>
<td>SNTA1</td>
<td>Alpha1-syntrophin</td>
<td>−2.93</td>
<td>0.03*</td>
</tr>
</tbody>
</table>
Differences between groups were tested using non-parametric Mann Whitney U tests where data were not normally distributed, and remaining proteins were subjected to independent t-tests. Study 2 was powered off our previous analysis of skeletal muscle enzyme activity in pregnant women [28–30], which showed a calculated effect size of 1.32 (averaged for C-I, C-II, and C-III) for differences between NGT and GDM women. We calculated 80% power with n = 10 subjects per group where \( \alpha = 0.05 \) for two-sided independent t-tests. Data were tested for normality using the Shapiro-Wilk test, which were performed for ONGT and OGDM separately. Independent t-tests were performed on raw or log-transformed data where data were not normally distributed. Statistical difference is indicated by \( P \leq 0.05 \). Data are expressed as the mean ± SEM.

Results

Study 1: Proteomic Analysis

Patient Characteristics. Patient characteristics for the women in Study 1 are shown in Table 1. Women in the ONGT (n = 6) and OGDM (n = 6) groups were of similar BMI. As expected, OGDM women had higher fasting blood glucose and insulin values, which translated to higher HOMA-IR scores (\( P < 0.05 \)).

Mitochondrial and Calcium Signaling Proteins are Altered in Women with GDM. Of the 979 unique proteins identified by tandem MS/MS mass spectrometry and NSAF analysis (Table S1), 22 met our criteria for statistical comparison between the ONGT and OGDM participants (protein detectable in at least three subjects per group and greater than 1.5-fold difference between groups; Table 2). Of these, eight proteins were statistically different between the groups (\( P \leq 0.05 \)). Two of these proteins were subunits of ETS Complex I (C-I; NDUFV2 and NDUFS3), which were reduced by 60% in women with OGDM (\( P < 0.05 \)), while a third subunit of C-I (NDUFS8) tended to be reduced by 75% in the women with OGDM, compared with ONGT women (\( P = 0.07 \)). Fatty acid transporter CD36 was increased by almost 75% in women with OGDM (\( P = 0.05 \)). In addition, proteasome subunit alpha-1, a protein involved in protein degradation, was reduced by nearly 2-fold in women with OGDM (\( P < 0.05 \)). Surprisingly, several proteins involved in calcium homeostasis and calcium-dependent cellular signaling were also altered in skeletal muscle of women with OGDM, including calmodulin-dependent calcineurin A B (PPP3CB), which was increased by over 2-fold in women with OGDM compared with ONGT women (\( P < 0.05 \)). Similarly, calcium/calmodulin-dependent protein kinase type II, subunit A (CaMKIIa), tended to be higher in women with OGDM, though this was not significantly different between groups (\( +2\)-fold in GDM, \( P = 0.15 \)). Furthermore, annexin A4, a calcium binding protein, was decreased by 65% in the women with OGDM (\( P = 0.05 \)) and \( \alpha \)-syntrophin, a cellular localization protein involved in ion transport and cellular signaling, including calcium ion flux, was decreased by nearly 3-fold in women with OGDM (\( P < 0.05 \)).

Study 2: Metabolic Analysis

Patient Characteristics. For Study 2, we followed up the results from Study 1 in a larger, separate cohort of OGDM and ONGT women (Table 3). The ONGT (n = 10) tended to be more obese than the OGDM women (n = 8; \( P = 0.06 \)), though fasting blood glucose and 1 and 2 hour OGTT glucose values were higher in OGDM than ONGT women (\( P < 0.05 \)).

Mitochondrial Enzyme Activity is Reduced in Women with GDM. Because we observed reduced protein content of mitochondrial oxidative phosphorylation subunits (Study 1, Table 2), we measured two common markers of mitochondrial content (mitochondrial DNA copy number [mtDNA] and CS activity) in Study 2. However, mtDNA tended to be 30% lower in OGDM compared to ONGT women (\( P = 0.10 \); Figure 2A). CS activity was increased by 30% higher in the women with OGDM (\( P = 0.06 \); Figure 2B).

Next, given the reduced content of several C-I subunits (NDUFV2, NDUFS3) in skeletal muscle of OGDM compared to ONGT women (Study 1), we determined whether the activity of key enzymes involved in mitochondrial oxidative phosphorylation was also different between groups in Study 2. We found that women OGDM women exhibit reduced activity of C-I and C-III, (by 45% and 45%, and 30%, respectively; Figure 2C & E; \( P < 0.05 \)) and a trend for reduced C-IV activity (by 30%; Figure 2F; \( P = 0.06 \)) compared to ONGT women. However, OXPHOS protein content was not similarly reduced (Figure 3A, C, & D). There were no differences in C-II activity or protein content (Figure 2D & 3B).

To examine whether transcriptional regulators of mitochondrial biogenesis were affected, we measured PPAR\( \gamma \) and PGC-1\( \alpha \) protein content, though these were not different between ONGT and OGDM women (Figure 4A & B; ns). Lastly, because AMPK is a calcium-dependent protein involved in cellular metabolism

---

**Table 3. Clinical characteristics of the subjects for Study 2.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ONGT (n = 10)</th>
<th>OGDM (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>33.4±1.8</td>
<td>34.1±1.1</td>
</tr>
<tr>
<td>12-week BMI (kg/m²)</td>
<td>38.4±1.7</td>
<td>33.8±1.5</td>
</tr>
<tr>
<td>Delivery BMI (kg/m²)</td>
<td>41.2±2.2</td>
<td>36.8±1.6</td>
</tr>
<tr>
<td>Glucose: 0 h OGTT (mmol/L)</td>
<td>4.7±0.1</td>
<td>5.3±0.2*</td>
</tr>
<tr>
<td>Glucose: 1 h OGTT (mmol/L)</td>
<td>7.4±0.6</td>
<td>9.9±0.6*</td>
</tr>
<tr>
<td>Glucose: 2 h OGTT (mmol/L)</td>
<td>5.3±0.3</td>
<td>7.7±0.7*</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>38.7±0.2</td>
<td>38.2±0.4</td>
</tr>
<tr>
<td>Parity</td>
<td>2.4±0.4</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>Neonate birth weight (g)</td>
<td>3554.5±107.1</td>
<td>3616.1±183.0</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. OGTT, oral glucose tolerance test. Independent t-test *\( P < 0.05 \).

doi:10.1371/journal.pone.0106872.t003
and mitochondrial biogenesis [29–33], we then measured AMPK phosphorylation and found that Thr172 phosphorylation of AMPK was markedly downregulated by 75% in OGDM compared to ONGT women (Figure 4).

**Discussion**

The present study is the first proteomic analysis of skeletal muscle tissue from obese, pregnant women with GDM compared with their obese, NGT counterparts. In this hypothesis-generating analysis we found that several proteins of the mitochondrial ETS were downregulated in the women with GDM, a factor that was corroborated by reduced activity of several enzyme complexes of the respiratory chain in the skeletal muscle of OGDM women. Quantitative bar graphs of mitochondrial DNA copy number (A) and enzyme activity of complex I (C), complex II (D), complex III (E), and complex IV (F) of the respiratory chain in pyramidalis muscle collected during scheduled cesarean section. Data are mean ± SEM. *P<0.05 vs. NGT.

doi:10.1371/journal.pone.0106872.g002
the ETS in a similar, larger cohort of both OGDM and ONGT women. Proteomic analysis also revealed disrupted calcium signaling/homeostasis proteins in skeletal muscle of OGDM women. Subsequent measures also showed a pronounced reduction in the phosphorylation of AMPK in skeletal muscle in OGDM compared to ONGT women, which has been linked to both calcium homeostasis and reduced oxidative metabolism.

Obese, insulin resistant humans often exhibit reduced skeletal muscle mitochondrial function when compared with normal weight, insulin sensitive individuals, generally in the form of reduced electron transport flux or ATP turnover [31–34]. We

Figure 3. Mitochondrial respiratory chain complex proteins are not different between ONGT and OGDM women. Complex I (A), complex II (B), complex III (C), complex IV (D). Representative Western blots where calnexin is used as loading control (E). doi:10.1371/journal.pone.0106872.g003
Figure 4. AMPK phosphorylation is reduced in OGDM women, though regulators of mitochondrial biogenesis are not. Quantitative bar graphs of PPARα (A) and PGC-1α (B), total AMPK (C), and phospho-AMPK (D) protein content in pyramidalis muscle collected during scheduled cesarean section. Representative Western blots where calnexin is used as loading control (E). Data are mean ± SEM. *P<0.05 vs. NGT. doi:10.1371/journal.pone.0106872.g004
found that obese women diagnosed with GDM showed significantly lower skeletal muscle mitochondrial C-I subunit protein content (Study 1) and enzyme activity (Study 2) compared to obese, NGT women. Even though there was a trend for reduced mtDNA copy number in the OGDM women, the trend for increased CS activity in these same women, makes it difficult to determine whether mitochondrial content was reduced in the women with GDM. However, given that enzyme activity data were normalized to CS activity in Study 2, the lower enzyme activities in the OGDM women do not appear to be the result of lower mitochondrial content and these discrepancies in mitochondrial content simply highlight the fact that these data are markers of mitochondrial content, not direct measures. For this reason we also measured upstream regulators of oxidative metabolism and mitochondrial biogenesis (PGC-1α and PPARα) and found that they were not different between the OGDM and ONGT women. These data indicate that mitochondrial functional capacity may be linked to specific subunit protein content rather than global mitochondrial deficits in the skeletal muscle of women with GDM. Thus, in this cohort where the principal differences between the OGDM and ONGT women is impaired glucose tolerance, these results suggest that reduced mitochondrial enzyme capacity may be an important determinant of excessive glucose intolerance during pregnancy, perhaps contributing to postpartum diabetes risk. However, the association between reduced skeletal muscle mitochondrial capacity and T2DM is not universally reported. For example, Asian Indians with T2DM show greater skeletal muscle mitochondrial activity than their non-diabetic counterparts [34,35], and Boushel et al. [35–37] report similar mitochondrial respiration rates in permeabilized muscle fibers from obese, type 2 diabetics compared with non-diabetic, overweight counterparts, suggesting that the relationship between T2DM and reduced mitochondrial capacity is not causal. Gestational diabetes, like T2DM, is characterized by both insulin resistance and pancreatic beta cell dysfunction [8,36,37]. Therefore, in addition to factors such as skeletal muscle mitochondrial dysfunction and skeletal muscle insulin signaling [8,38–40], which can exacerbate insulin resistance, factors determining beta cell function likely play a prominent role in the development of GDM and subsequent T2DM postpartum.

Calcium homeostasis and calcium-dependent signaling play important roles in many skeletal muscle processes in addition to muscle contraction, such as insulin-mediated glucose uptake, AMPK signaling and mitochondrial biogenesis [38–41]. Our proteomic analysis revealed decreased α1-syntrophin and annexin A4, proteins involved in intracellular calcium signaling/homeostasis. In addition, we observed large increases in both calcineurin A and CaMKIIα in OGDM compared with ONGT women, though differences in CaMKIIα did not reach statistical significance. Such data suggest a disruption in calcium handling in skeletal muscle of women with GDM that warrants further investigation into its role in insulin signaling and mitochondrial capacity.

Alpha 1-syntrophin is a member of the syntrophin family of scaffold proteins that interacts with many signaling proteins, linking them with various cell surface receptors, ion channels, and downstream effectors [41–43]. In particular, α1-syntrophin, interacts with dystrophin and related proteins [38,41–44] and disruption of the dystrophin-syntrophin complex has been suggested to promote the excessive calcium influx and altered MAPK and GTPase signaling observed in muscular dystrophy [38,41–44]. In this context, silencing of α1-syntrophin in myotube cultures induces cellular calcium influx via store-operated calcium channels [39,40,45,46]. Thus, the observed reduction in α1-syntrophin in the skeletal muscle of OGDM women in our study may affect myocellular calcium levels. Contrary to transient increases in intramyocellular calcium that induce AMPK phosphorylation, such as occurs with muscle contraction, sustained elevations of intramyocellular calcium can reduce AICAR-induced AMPK phosphorylation in myotube cultures, which appears to be regulated by elevated CaMKII [29,39,40]. Thus, sustained elevations in cellular calcium may induce CaMKII to inhibit AMPK phosphorylation. As a potent activator of lipid oxidation and mitochondrial biogenesis [10,11,29], the reduced AMPK phosphorylation observed in Study 2 could explain their reduced mitochondrial enzyme capacity via calcium regulatory mechanisms. Though we did not observe differences in overall mitochondrial content in Study 2, as estimated by mtDNA copy number and citrate synthase activity, it is possible that there was a deficit in content of specific subunit proteins, which contributed to reduced enzyme activity. For example, the C-I molecule contains 45 subunit proteins [47]. Our proteomic analysis only identified 24 C-I subunit proteins assigned in at least 1 individual (Table S1), of which only two were statistically different between groups. This suggests that, while overall subunit content of C-I is not different in skeletal muscle of the OGDM women, content of specific, important C-I subunit proteins is reduced, resulting in reduced mitochondrial enzyme activity. Indeed, gene silencing of NDUF53 and mutations in the NDUFV2 gene have been shown to lead to mitochondrial dysfunction, hypertrophic cardiomyopathy, and Leigh’s Syndrome [48,49]. Thus, calcium regulatory control of AMPK phosphorylation and mitochondrial enzyme capacity may have clinical implications that may partially explain why physical activity interventions in women with GDM, for the control glycemia and prevention of its progression to T2DM, are disappointing and often associated with a high non-compliance rate [10,11,50]. While this is the first report of reduced α1-syntrophin in human insulin resistant skeletal muscle, similar observations have been made in diabetic Goto-Kakizaki rats, where skeletal muscle dystrophin and α1-syntrophin content was markedly reduced and thought to be a contributor to the excessive insulin resistance observed in these animals [50,51].

Annexin A4 is a lipid-binding protein involved in exocytosis and ion transport in a calcium-dependent manner [51,52]. Among its many functions, annexin A4 interacts with the p50 subunit of NF-kB, reducing is transcriptional activity [8,52,53]. Thus, the inflammatory cascade may be enhanced in skeletal muscle where annexin A4 protein is reduced. This may be one mechanism whereby inflammation is elevated in skeletal muscle of women with GDM, as we have shown previously [8,53]. As a result, serine phosphorylation of JNK and/or p70S6 kinase may link inflammation with disrupted insulin signaling in skeletal muscle of women with GDM [8,53,54]. While this is the first report of reduced annexin A4 in skeletal muscle of GDM patients, members of our group have recently reported reduced content of other annexin proteins (A2, A5, A6) in placental tissue from obese GDM patients [54,55], suggesting a universal disruption of annexin protein content in pregnant women with GDM.

Calcineurin A was reduced in skeletal muscle of OGDM women. Calcineurin A is the catalytic subunit of calcineurin that forms a heterodimer with the calcineurin B regulatory subunit [55,56]. Once activated by calcium/calmodulin, calcineurin stimulates insulin sensitizing pathways and has also been shown to induce lipid oxidation and mitochondrial biogenesis pathways, including increased expression of CD36 and all of the ETS complexes [56,57], which may explain increased CD36 protein in OGDM women in the proteomic analysis. Increased calcineurin A may also account for the slight, yet non-significant increases in
electron transport complex proteins measured in the OGDM women (Study 2). However, studies show that calcineurin expression is induced in response to mitochondrial stress and mtDNA damage [57,58]. For example, in early stage diabetic cardiomyopathy, mitochondrial stress is commonly observed, along with disrupted calcium homeostasis, elevated calcineurin, and insulin resistance [28,58]. While these observations may seem paradoxical given calcineurin action, they seem to suggest a compensatory function of calcineurin, with regard to mitochondrial biogenesis and insulin sensitivity, which may be precluded in cardiac muscle of diabetic cardiomyopathy patients. Perhaps a similar compensatory increase in calcium is observed in skeletal muscle of GDM patients in response to mitochondrial stress.

We have previously reported that obese women with mild, diet-treated GDM have similar skeletal muscle mitochondrial enzyme activity to obese NGT pregnant women [28,59,60]. Here, we have found that those with more severe GDM (insulin-treated) exhibit lower mitochondrial enzyme activity for C-I and C-III than obese NGT pregnant women, despite the tendency for lower BMI, suggesting that the extent of glucose intolerance may play a more important role in skeletal muscle mitochondrial enzyme capacity than BMI in these women. This is further corroborated by the fact that the women with insulin treated GDM presented here had similar BMIs as the women with diet-treated GDM reported previously [28]. While it is possible that exogenous insulin administration may have induced the observed proteomic or enzymatic differences in the OGDM women, it is equally plausible that, due to skeletal muscle insulin resistance of the OGDM women, exogenous insulin simply equalized the effect of insulin on skeletal muscle tissues to those observed in the non-insulin treated women. Nevertheless, reduced mitochondrial enzyme activity in the OGDM women does not appear to be the result of differences in total mitochondrial content, but rather may be the result of reduced subunit protein content. Even though we discovered reduced content of two C-I subunits, and a trend for reduced content of a third C-I subunit in our proteomic analysis, we did not observe differences in total OXPHOS content, which is likely due to the fact that the OXPHOS antibody cocktail only measures the NDUF8 C-I subunit. Nevertheless, we must recognize that these observations can only be extended to obese women diagnosed with GDM, and may differ for those women with less severe GDM (diet-treated).

GDM women and their offspring are a population which globally and significantly contributes to the escalation of T2DM and childhood obesity and our proteomic analysis of skeletal muscle from obese pregnant women revealed that proteins involved in mitochondrial oxidative phosphorylation are reduced in OGDM compared to their NGT counterparts. These observations were corroborated by reduced skeletal muscle mitochondrial enzyme activity in a larger cohort of obese pregnant women. We further demonstrated that several proteins involved in calcium homeostasis/signaling were altered in obese women with GDM, several of which may be linked to disrupted insulin signaling and reduced mitochondrial activity. Such disturbances in calcium homeostasis proteins could contribute to the inflammation, insulin resistance, and altered substrate metabolism observed in skeletal muscle of OGDM patients, consequently shunting maternal nutrients to the growing fetus. Furthermore, disruption of calcium homeostasis could help explain the high failure rate of exercise intervention studies designed to treat women with GDM during pregnancy and/or to prevent the development of T2DM postpartum [59,60].

In conclusion, this exploratory analysis identifies several calcium regulatory proteins that could represent novel risk factors for both the development of GDM and the progression to T2DM. Larger, more targeted studies are required to confirm these exploratory analyses. Further examination of calcium regulatory proteins could reveal an important link between insulin resistance and reduced mitochondrial activity in obese women with GDM that could potentially be targeted for strategies geared toward prevention and treatment of T2DM in this rapidly growing cohort of at risk women.

Supporting Information

Table S1 Spectral counts and NSAF values for 979 proteins identified in at least 1 subject.

Acknowledgments

The clinical research midwives Genevieve Christophers, Debra Jinks, Rachel Murdoch and Gabrielle Fleming, are gratefully acknowledged for assistance with sample collection. The Obstetrics and Midwifery staff of the Mercy Hospital for Women is also thanked for their co-operation.

Author Contributions

Conceived and designed the experiments: KEB JEF JMD TLH LAB. Performed the experiments: HH KEB RCJ JMD TLH LAB ML. Analyzed the data: HH KEB RCJ. Contributed reagents/materials/analysis tools: JEF LJM LAB ML. Contributed to the writing of the manuscript: KEB JEF.

References


