Skeletal Muscle MnSOD, Mitochondrial Complex II, and SIRT3 Enzyme Activities Are Decreased in Maternal Obesity During Human Pregnancy and Gestational Diabetes Mellitus

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Context: Insulin resistance and systemic oxidative stress are prominent features of pregnancies complicated by maternal obesity or gestational diabetes mellitus (GDM). The role of skeletal muscle oxidative stress or mitochondrial capacity in obese pregnant women or obese women with GDM is unknown.

Objective: We investigated whether obese pregnant women, compared with normal weight (NW) pregnant women, demonstrate decreased skeletal muscle mitochondrial enzyme activity and elevated markers of oxidative stress, and if these differences are more severe in obese women diagnosed with GDM.

Design: We measured mitochondrial enzyme activity and markers of oxidative stress in skeletal muscle tissue from NW pregnant women (n = 10), obese pregnant women with normal glucose tolerance (NGT; n = 10), and obese pregnant women with GDM (n = 8), undergoing cesarean delivery (~37 wk gestation).

Results: Electron transport complex-II and manganese superoxide dismutase (MnSOD) enzyme activities were decreased in obese-NGT and obese-GDM, compared with NW women. The glutathione redox ratio (GSH:GSSG) was decreased in obese-NGT and obese-GDM, indicative of increased oxidative stress. Mitochondrial sirtuin (SIRT)3 mRNA content and enzyme activity were lower in skeletal muscle of obese-NGT and obese-GDM women. Importantly, acetylation of MnSOD, a SIRT3 target, was increased in obese-NGT and obese-GDM vs NW women and was inversely correlated with SIRT3 activity ($r = -0.603$), suggesting a mechanism for reduced MnSOD activity.

Conclusions: These data show that obese pregnant women demonstrate decreased skeletal muscle mitochondrial respiratory chain enzyme activity and decreased mitochondrial antioxidant defense. Furthermore, reduced skeletal muscle SIRT3 activity may play a role in the increased oxidative stress associated with pregnancies complicated by obesity. **(*J Clin Endocrinol Metab* 98: E1601–E1609, 2013)**

Nearly 25% of women entering pregnancy are obese (1), which places them at a 3-fold greater risk for developing gestational diabetes mellitus (GDM) (2). Up to 60% of women diagnosed with GDM will go on to develop type 2 diabetes within 10 years postpartum (3), suggesting that the metabolic and physiologic stress of pregnancy may present an "unmasking" of future disease risk. Although skeletal muscle insulin resistance is a universal

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Abbreviations: BMI, body mass index; CS, citrate synthase; GDM, gestational diabetes mellitus; GSH, total glutathione; GSSG, oxidized glutathione; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; NGT, normal glucose tolerance; NW, normal weight; OGTT, oral glucose tolerance test; PGC-1α, proliferator-activated receptor γ coactivator-1α; SDHa, succinate dehydrogenase subunit A; SIRT, sirtuin.

finding of human pregnancy, the severity of insulin resistance tends to be increased in obese compared with normal weight (NW) pregnant women and is even more severe in obese women diagnosed with GDM (obese-GDM) (4–6). Given that skeletal muscle is responsible for up to 90% of insulin-stimulated glucose uptake in healthy adults and accounts for the vast majority of whole body decrements in glucose tolerance under conditions of insulin resistance (7), skeletal muscle is an important determinant of whole body glucose metabolism and overall metabolic health. Increasingly, skeletal muscle insulin resistance is associated with decreased mitochondrial metabolism and increased skeletal muscle oxidative stress in nonpregnant cohorts (8–10). However, little is known about skeletal muscle metabolism or oxidative stress in human pregnancy, particularly in pregnancies complicated by obesity or obese-GDM.

Accelerated reactive oxygen species production is triggered when energy substrate supply substantially exceeds mitochondrial energetic demands, as occurs with human obesity (8). If this is not counteracted with antioxidant defenses, the result is increased oxidative stress, which has been linked to insulin resistance in obese humans and rodents (8). Pregnant women also exhibit increases in circulating oxidative stress markers (11–13). However, whether increased oxidative stress or decreased mitochondrial metabolism is evident in skeletal muscle of obese pregnant women or obese-GDM has not been previously investigated. Accordingly, we sought to determine whether markers of oxidative stress are increased and/or mitochondrial complex enzyme activities are lower in skeletal muscle of obese compared with NW pregnant women, and whether these differences may be more severe in obese-GDM.

We also explored mechanisms that may underlie potential differences in skeletal muscle mitochondrial enzyme activity and oxidative stress, including activity of sirtuin (SIRT)3, the primary mitochondrial deacetylase (14). Acetylation is a reversible form of posttranslational modification that has recently emerged as an important mechanism for controlling the activity of a broad array of enzymes involved in mitochondrial metabolism, including the citric acid cycle, fatty acid oxidation, and the electron transport system, as well as important antioxidant defense enzymes (15–17). Indeed, SIRT3 expression and activity are reduced in animal models of obesity (18, 19) and may contribute to the obesity-associated decrease in oxidative metabolism and antioxidant defense. Nevertheless, very little is known about the possible role of SIRT3 in the regulation of mitochondrial metabolism and redox balance in human skeletal muscle.

Herein, we show for the first time that, compared with NW pregnant women, skeletal muscle of obese pregnant women, with or without GDM, exhibits decreased mitochondrial enzyme activity and antioxidant capacity coupled with increased oxidative stress markers. We also report that SIRT3 enzyme activity is lower in obese and obese-GDM women and was inversely correlated with hyperacetylation of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD). Together, these results suggest that decreased skeletal muscle mitochondrial enzyme activity and antioxidant defense may contribute to increased oxidative stress in obese pregnant women, independent of GDM diagnosis.

Research Design and Methods

Patients and sample collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and informed consent was obtained from all participants prior to cesarean delivery. Women were screened for GDM at 24 to 28 weeks' gestation and were diagnosed according to the criteria set by the Australasian Diabetes in Pregnancy Society, by either a fasting venous plasma glucose level of 5.5 or greater than 8.0 mmol/L glucose 2 hours after a 75 g oral glucose tolerance test (OGTT). Patients whose GDM was treated with insulin, glyburide, or metformin were excluded. Women with polycystic ovarian syndrome, pre-eclampsia, and macrovascular complications were excluded. Body mass index (BMI) was calculated based on measurements from patients' first antenatal visit (~12 wk gestation). NW pregnant women had a BMI <25 kg/m^2 and obese patients had a BMI >30 kg/m^2.

Between 300 and 500 mg of pyramidalis skeletal muscle was obtained from a total of 28 pregnant women undergoing elective caesarean section (term ~37 wk gestation). The pyramidalis muscle is located anterior to the rectus abdominus and is "mixed" in fiber type (20). Dissections of skeletal muscle were obtained within 10 minutes of delivery and snap-frozen in liquid nitrogen and stored at −80°C until further analysis. Tissues were also imbedded for histology to verify that they were free from adipose or connective tissue contamination by hemotoxylin and eosin staining as previously described (21). mRNA content of perilipin 1 was also measured to assure lack of adipose tissue contamination (data not shown).

For all deliveries, spinal anesthesia and/or epidural was used. All muscle samples were taken at the time of cesarean delivery (between 8:30 AM and 3:00 PM). Women delivering in the morning hours were fasted overnight, and women delivering after 12:00 noon were fasted from 7:30 AM. Women diagnosed with GDM were counseled by a nutritionist to follow the recommended Standard of Care diet for controlling blood glucose (40% carbohydrate, 15% protein, and 45% fat).

Mitochondrial enzyme activity assays

Mitochondrial-enriched supernatants (post 600 g) were prepared from frozen skeletal muscle samples as described (22). Supernatants were used to assay activity of respiratory chain enzyme complexes I, II, II+III, III, and IV; citrate synthase (CS), aconitase, MnSOD, and catalase spectrophotometrically on a Synergy H1 microplate reader (Biotek). Enzyme assays for re-

spiratory chain complexes and CS were performed as described with minor modifications for microplate reading (22). For complexes I, II, and II+III, and CS, 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 to 3 minutes of reaction initiation.

For aconitase, MnSOD, and catalase assays, mitochondrial supernatants were incubated with appropriate reaction buffers for 2 minutes at 30°C, after which specific activators were added and reactions were followed for 5 minutes at specified wavelengths. Aconitase activity was measured as described by Xu et al (23) with modifications for microplate reading. Activity of MnSOD and catalase were measured by the protocol of Boden et al (24) with modifications for microplate reading. SIRT3 enzymatic activity was assayed using a fluorometric kit (Enzo Life Sciences Inc) following the manufacturer's instructions with modifications as described (19). All assays were performed in duplicate. The protein content of each sample was determined using a bicinchoninic acid assay. All enzyme activities were normalized to the total protein content of each sample and results are expressed relative to the mean for NW women.

Western blot and immunoprecipitation

Protein levels of MitoProfile total OXPHOS antibody cocktail, MnSOD, succinate dehydrogenase subunit A (SDHa), proliferator-activated receptor γ coactivator-1α (PGC-1α), and SIRT3 were determined in the muscle biopsy samples as previously described (19), with calnexin as loading control. For detection of acetylated lysine, 200 μg of total protein was rotated at 4°C for 4 hours with 2 μg anti-acetyl-lysine antibody. Western blot was performed for SDHa and MnSOD and normalized to total SDHa or MnSOD protein, respectively. All results were expressed relative to the mean for NW women. Antibodies to PGC-1α, SIRT3, and acetylated lysine were purchased from Cell Signaling Technology; MnSOD was purchased from Enzo Life Sciences Inc, and OXPHOS antibody cocktail, SDHa, and calnexin were from Abcam.

Glutathione redox state

Overall oxidative stress was measured by total and oxidized glutathione (GSH and GSSG, respectively) using a commercially available kit (Caymen Chemical).

Mitochondrial DNA (mtDNA) and quantitative PCR

Approximately 15 mg of skeletal muscle was homogenized and DNA was isolated by phenol/chloroform extraction with ethanol precipitation. mtDNA copy number was then measured as previously described (25) with modifications for use with iQ SYBR Supermix (Bio-Rad Laboratories).

Quantitative PCR was performed using primer sets for genes of interest and RPL13 and ubiquitin C as reference genes and iQ SYBR Supermix (Bio-Rad) following the manufacturer's protocol. Reactions were run in duplicate on an iQ5 Real-Time PCR Detection System (Bio-Rad) along with a no-template control per gene. RNA expression data were normalized to reference genes using the comparative threshold cycle method. To demonstrate that efficiencies of target and reference genes were approximately equal, validation experiments were performed using standard curves for genes of interest and reference genes.

Statistical analysis

Statistical analyses were performed using PASW Statistics (SPSS, IBM Corp). Based on our hypothesis of increasing severity of metabolic perturbation from NW, to obese-normal glucose tolerance (NGT), to obese-GDM groups, we used a planned contrast ANOVA, which included two orthogonal contrasts chosen a priori to individually compare the effects of obesity and GDM. Contrast 1 tested the effect of obesity (NW vs obese-NGT + obese-GDM), whereas contrast 2 tested the effect of GDM (obese-NGT vs obese-GDM). Using this model, we were able to reduce the type I error rate by only testing comparisons that were of scientific interest. Correlation analyses for relevant related parameters were performed using the linear regression model. Statistical difference is indicated at $P < .05$. Data are expressed as the mean ± SEM.

Results

Participants

Participant characteristics are summarized in Table 1. By design, maternal BMI at 12 weeks' gestation and at delivery was greater in both obese-NGT and obese-GDM women compared with NW women (contrast 1; $P < .05$).

Table 1. Clinical Characteristics of the Subjects

	NW (n = 10)	Obese-NGT (n = 10)	Obese-GDM (n = 8)
Maternal age, y	32.2 ± 1.3	33.4 ± 1.8	36.0 ± 2.5
12-wk BMI, kg/m^2	21.6 ± 0.7	38.4 ± 1.7[a]	32.4 ± 2.0[a,b]
Delivery BMI, kg/m^2	25.7 ± 1.9	41.2 ± 2.2[a]	33.4 ± 3.2[a,b]
Glucose: 0 h OGTT, mmol/L	4.3 ± 0.7	4.7 ± 0.1	5.0 ± 0.3
Glucose: 1 h OGTT, mmol/L	6.3 ± 0.5	7.4 ± 0.6[a]	10.0 ± 0.4[a,b]
Glucose: 2 h OGTT, mmol/L	5.9 ± 0.4	5.3 ± 0.3	7.9 ± 0.6[b]
Gestational age, wk	38.8 ± 0.1	38.7 ± 0.2	39.3 ± 0.3[b]
Gravida	3.2 ± 0.4	3.5 ± 0.6	2.8 ± 0.4
Parity	2.3 ± 0.2	2.4 ± 0.4	2.4 ± 0.3
Neonate birth weight, g	3516.0 ± 131.2	3554.5 ± 107.1	3432.5 ± 180.0

Data are mean ± SEM.

[a] $P < .05$ for NW vs obese-NGT + obese-GDM.

[b] $P < .05$ for obese-NGT vs obese-GDM.

Notably, BMI was slightly yet significantly greater in obese-NGT compared with obese-GDM women (contrast 2; $P < .05$). Fasting glucose values were not different between the groups, although 1 hour OGTT glucose values were elevated in obese-NGT and obese-GDM compared with NW women (contrast 1; $P < .05$). Two-hour OGTT glucose values were elevated in obese-GDM compared with obese-NGT women (contrast 2; $P < .05$).

Decreased mitochondrial respiratory enzyme activity in obese pregnant women

We found no differences in CS activity among the groups (Figure 1A). Similarly, other estimates of mitochondrial content, including mtDNA copy number (900 ± 89, 1379 ± 154, and 1159 ± 169 mtDNA copy number per diploid nuclear genome in NW, obese-NGT, and obese-GDM, respectively) and C-IV protein content (Figure 2D), were not different among the groups. Nevertheless, as is customary, all mitochondrial enzyme activity data were normalized to CS activity to control for individual differences in mitochondrial content (all respiratory complexes, MnSOD, SIRT3, and aconitase) (22). Enzyme activity of C-II and coupled C-II+III were decreased in skeletal muscle from obese-NGT and obese-GDM compared with NW pregnant women (contrast 1; $P < 0.05$; Figure 1, C and E). Enzyme activity of all other complexes of the respiratory chain was not different among groups (Figure 1, B, D, and F). Despite these differences in mitochondrial enzyme activity at C-II and C-II+III (Figure 1), there was no difference in mitochondrial respiratory complex content in skeletal muscle of obese-NGT or obese-GDM women compared with NW control women (Figure 2, A–E), indicating that differences in mitochondrial enzyme activity are not due to differences in mitochondrial protein content.

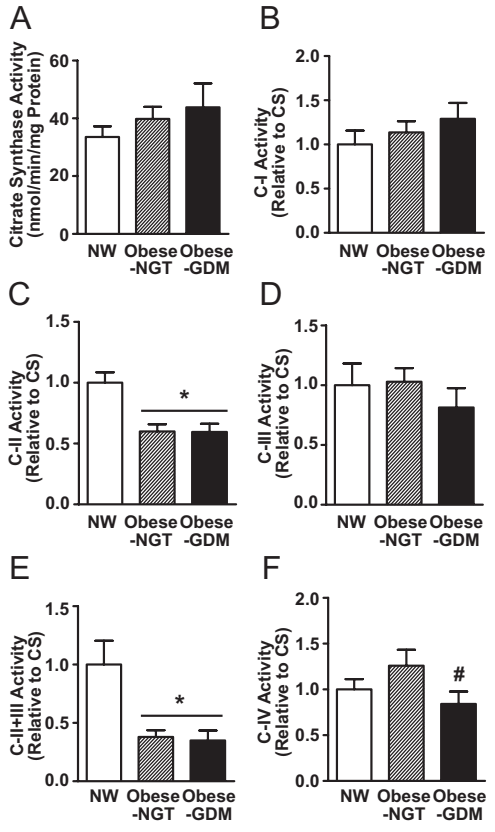

Figure 1. C-II and C-II+III mitochondrial enzyme activity is reduced in skeletal muscle of obese-NGT and obese-GDM compared with NW women. Quantitative bar graphs of enzyme activity of CS (A) and complex I (B), complex II (C), complex III (D), collective complex II+III (E), and complex IV (F) in skeletal muscle collected during scheduled cesarean section. Data are mean ± SEM. *, $P < .05$ vs NW. #, $P > .05$ vs Obese-NGT. Unless otherwise stated, n or n − 1 subjects were included for each group.

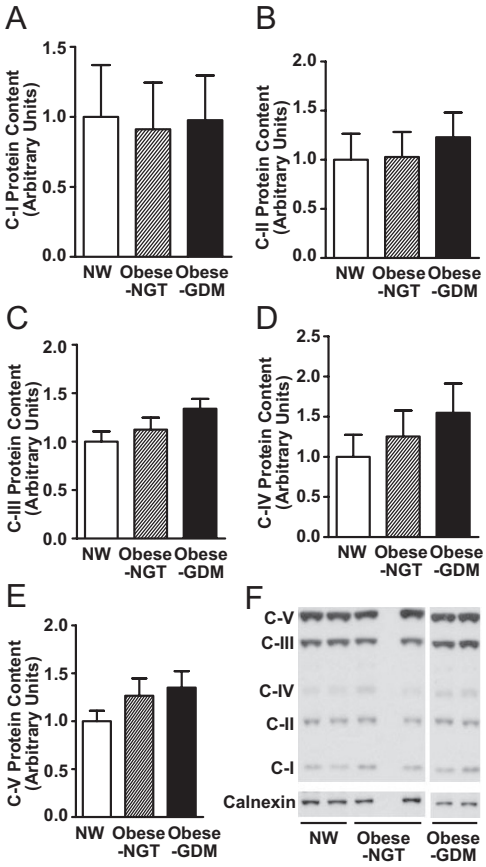

Figure 2. Mitochondrial respiratory chain complex proteins were not different in skeletal muscle of NW, obese-NGT, and obese-GDM pregnant women. A quantitative bar graph of complex I (A), complex II (B), complex III (C), complex IV (D), and complex V (E). Representative Western blots where calnexin is used as loading control (F). Data are mean ± SEM. Unless otherwise stated, n or n − 1 subjects were included for each group.

Decreased antioxidant defense and increased oxidative stress in skeletal muscle of obese pregnant women

The ratio of skeletal muscle GSH:GSSG was decreased by 15% in obese-NGT and obese-GDM women (contrast 1; $P < .05$; Figure 3A), indicating increased oxidative

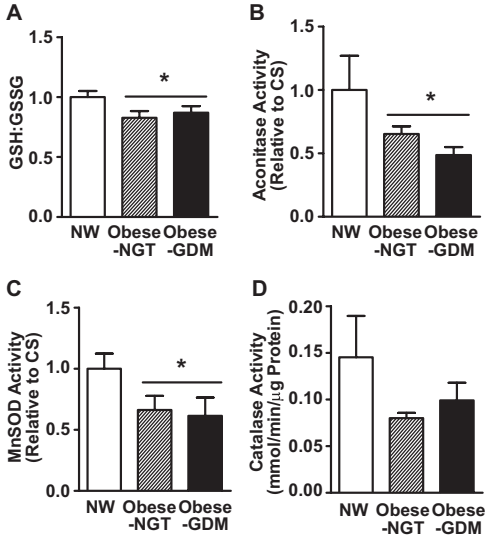

Figure 3. Reduced antioxidant defense and increased oxidant burden in skeletal muscle of obese pregnant women. Enzyme activity for antioxidants MnSOD (A) and catalase (B) was reduced in obese-NGT and obese-GDM compared with NW women (n = 6). The relative oxidant burden was also increased in obese-NGT (n = 8) and obese-GDM compared with the NW women (n = 7) (GSH:GSSG; C). Aconitase activity (D), which is sensitive to oxidative damage, was reduced in obese-NGT (n = 5) and obese-GDM (n = 4) compared with NW women (n = 3). Data are mean ± SEM. *, $P < .05$ vs NW. Unless otherwise stated, n or n − 1 subjects were included for each group.

stress. Furthermore, activity of the citric acid cycle enzyme aconitase, which is highly susceptible to oxidative damage and is another indicator of oxidative stress (26), was decreased in the obese-NGT and obese-GDM compared with NW women (contrast 1; $P < .05$; Figure 3B). Importantly, no significant differences were found for contrast 2 (obese-NGT vs obese-GDM) for either GSH:GSSG or aconitase activity. Because increased oxidative stress represents increased oxidant burden and/or decreased antioxidant defense, we then measured activity of antioxidant enzymes. As shown in Figure 3C, MnSOD enzyme activity was lower in obese-NGT and obese-GDM compared with NW women (contrast 1; $P > .05$), with no difference in MnSOD protein among groups (Figure 5E). Similarly, catalase activity was decreased by almost 45% in obese-NGT and obese-GDM, although this did not reach statistical significance due to a high degree of variability among the NW women (contrast 1; $P = .27$; Figure 3D).

Decreased SIRT3 activity is associated with MnSOD protein hyperacetylation in skeletal muscle of obese pregnant women

SIRT3 mRNA was decreased in obese-NGT and obese-GDM compared with the NW women (contrast 1; $P < .05$; Figure 4A), although the more modest decrease in SIRT3 protein content did not reach statistical significance (Figure 4B). These data were in agreement with lower transcript levels and a trend for decreased protein content of PGC-1α in obese-NGT and obese GDM compared with NW women (contrast 1; $P > .05$ and $P = .09$, respectively; Figure 4, D and E), as PGC-1α is known to regulate SIRT3 gene expression (27, 28). Perhaps most importantly, SIRT3 enzyme activity was decreased in obese-NGT and obese-GDM compared with NW women (contrast 1; $P < .05$; Figure 4C). In agreement with our in vitro measure of SIRT3 activity, acetylation of MnSOD, a known target of SIRT3 deacetylase activity, was increased in obese-NGT and obese-GDM compared with NW women (contrast 1; $P < .05$; Figure 5F). Furthermore, MnSOD acetylation was inversely correlated with SIRT3 activity among all women ($r = -0.603$, $P < .05$; Figure 5G), and SIRT3 activity was inversely correlated with BMI ($r = -0.454$, $P < .05$; Figure 5H). Conversely, acetylation of SDHa, another known SIRT3 target, was not different among groups (Figure 5C).

Discussion

Both obesity and insulin resistance have been associated with lower skeletal muscle oxidative capacity and increased oxidative stress (8–10); however, little is known about skeletal muscle oxidative stress and mitochondrial complex activity in pregnant obese women or obese-GDM. In the present study obese-NGT women had lower activity of key skeletal muscle mitochondrial complexes and antioxidant enzymes compared to NW subjects. However, somewhat counter to our expectations, obese-GDM did not exhibit a further reduction in mitochondrial complex activity or a greater increase in oxidative stress markers compared with obese-NGT women. This may be due to several factors. First, the obese-NGT women were more obese than the women with GDM in our study, which may have limited our ability to detect any additive effects of GDM in the presence of obesity. Second, we investigated young women with a comparatively mild form of glucose intolerance. GDM patients with more severe glucose intolerance were excluded from our study because they require insulin or metformin throughout pregnancy to control their blood glucose. Third, the obese-GDM patients studied here were well controlled as a result of diet therapy and therefore may have improved their insulin resistance in the time between diagnosis and tissue collection. All of these factors may be important for why we did not observe differences in between obese-NGT and obese-GDM women, and adding substantially more subjects would have had very little effect on the results given the small effect size noted in this population of relatively well-controlled, mild GDM patients. Nevertheless, our findings tend to support the notion that increased oxida-

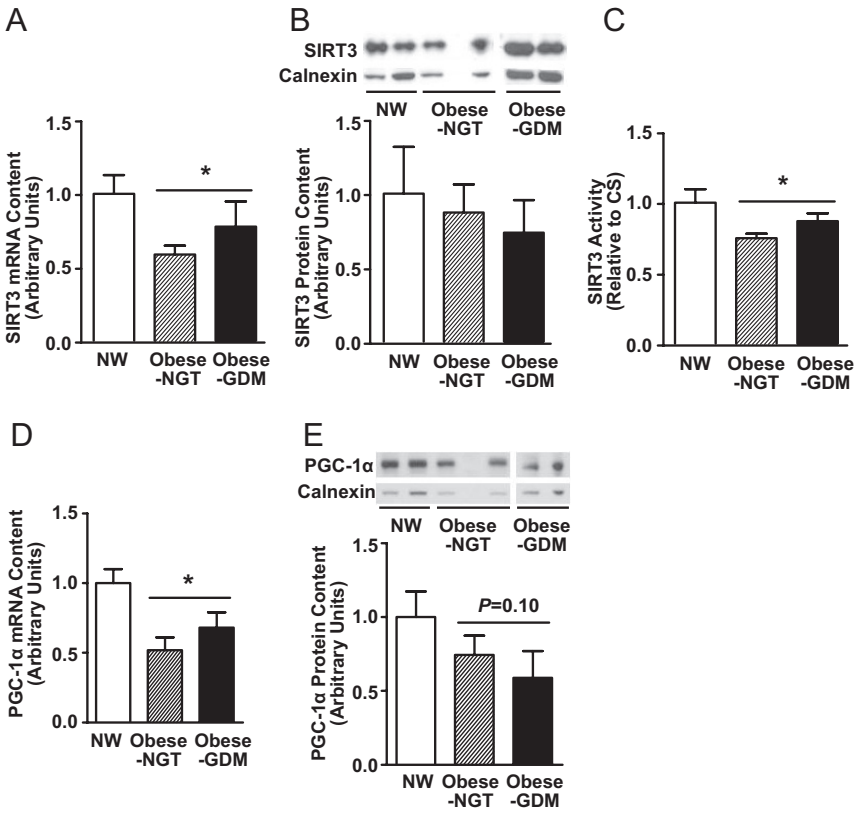

Figure 4. Reduced SIRT3 enzyme activity contributes to hyperacetylation of MnSOD, but not SDHa, in skeletal muscle of obese-NGT and obese-GDM. SIRT3 mRNA content (A) and enzyme activity (C), but not protein content (B), were reduced in obese-NGT and obese-GDM compared with NW women. PGC-1α mRNA content (D) was reduced in the obese-NGT and obese-GDM compared with NW women (n = 7), and there was a trend for reduced PGC-1α protein content (E). Representative Western blots are shown where calnexin is used as loading control. Data are mean ± SEM. *, $P < .05$ vs NW. Unless otherwise stated, n or n − 1 subjects were included for each group.

tive stress and decreased mitochondrial capacity in late pregnancy may be driven more by obesity than by factors underlying or resulting from glucose intolerance (and/or β-cell dysfunction) per se. Thus, it is within the context of obesity-related differences that we discuss our results.

Obese pregnant women exhibited greater skeletal muscle oxidative stress compared with NW women, as measured by decreased GSH:GSSG ratio and decreased aconitase activity. These measures are sensitive markers of an oxidized redox balance and oxidative damage, respectively (8, 26). Similarly, C-II activity was considerably lower in the obese-NGT and obese-GDM, compared with NW women. These results may be expected given that increased oxidative stress and mitochondrial metabolism are common features of nonpregnant obese and insulin-resistant humans and animals (8, 10). Nevertheless, this is the first report of lower mitochondrial enzyme capacity in skeletal muscle of obese pregnant women. C-II enzyme activity is particularly sensitive to free radical damage observed in situations of chronic oxidative stress, resulting in part from the loss of iron from its iron-sulfur enzyme cubane center (26). This, and likely other mechanisms of oxidative damage, may be responsible for C-II loss of function in the obese pregnant women in our study. Moreover, oxidative damage to the flavoprotein subunit of C-II (SDHa) may result in excess reactive oxygen species production due to reverse electron flow at C-II (29, 30). Indeed, muscle fibers from obese, insulin-resistant humans not only have increased oxidative stress markers compared to lean, insulin-sensitive counterparts, but also exhibit excess H_2O_2 emission during state 4 respiration of C-II (8). Together, these findings support the notion that there is increased oxidant burden and decreased C-II enzyme activity in skeletal muscle of obese pregnant women. These factors may contribute to the increases in systemic oxidative stress observed in obese-NGT and obese-GDM (11, 12).

Despite evidence of increased oxidative damage in skeletal muscle from obese-NGT and obese-GDM women, these women also demonstrated impaired antioxidant defense, as indicated by lower MnSOD activity when compared with NW women. MnSOD activity is normally enhanced when oxidative stress is increased, via both induction of its gene expression and deacetylation by SIRT3 (31). SIRT3 is a mitochondrial-localized sirtuin that removes acetyl groups from target proteins, including MnSOD and SDHa, thereby increasing their enzymatic activity (15–17). Although short-term high-fat feeding is known to induce SIRT3 gene expression, chronic high-fat feeding/obesity decreases SIRT3 expression (18), suggesting that prolonged metabolic stress leads to altered SIRT3 regulation. Because SIRT3 knockout mice exhibit metabolic changes commonly associated with obesity, such as increased oxidative stress/lipid peroxidation, disrupted skeletal muscle insulin signaling, and lower hepatic mitochondrial respiration compared with wild-type mice (32–34), it is tempting to speculate that decreased SIRT3 activity might account for the decreased C-II and MnSOD enzyme activity in our obese pregnant women. In our study, we did not detect differences in SDHa acetylation among groups. However, SDHa has many acetylation

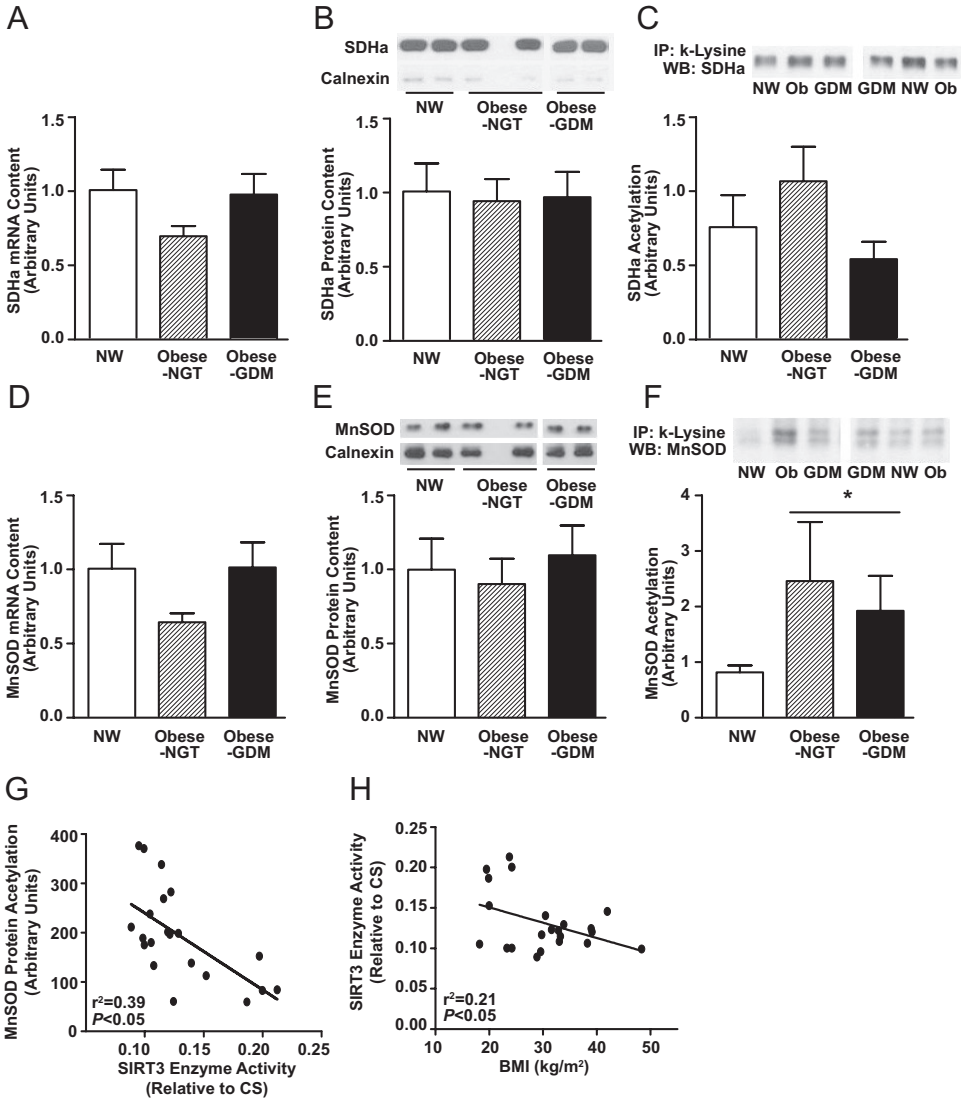

Figure 5. MnSOD acetylation is increased in obese-NGT (n = 8) and obese-GDM compared with NW women (n = 7) and was correlated with SIRT3 activity. SDHa mRNA content (A), protein content (B), and acetylation status (C) were not different (n = 8 for NW). MnSOD mRNA content (D) and protein content (E) were not different (n = 8 for NW), but MnSOD acetylation (F) was increased in skeletal muscle of obese-NGT and obese-GDM. MnSOD hyperacetylation is inversely correlated with decreases in SIRT3 activity (G). Although SIRT3 enzyme activity was inversely correlated with BMI (I), MnSOD acetylation was not (H). Data are mean ± SEM. *, $P < .05$ vs NW. Unless otherwise stated, n or n − 1 subjects were included for each group.

sites, which may not all be regulated by SIRT3 (35). In addition, immunoprecipitation/ immunoblot techniques are likely not sensitive enough to detect site-specific differences in acetylation. However, in contrast, MnSOD acetylation was increased in obese compared with NW pregnant women in our study and was negatively correlated with SIRT3 activity. Given the nature of our SIRT3 activity assay, we must acknowledge that the activity of other mitochondrial deacetylases could have contributed to our measure of SIRT3 activity in our mitochondrial-enriched preparations. Nevertheless, SIRT3 is the primary known mitochondrial deacetylase and, perhaps more importantly, is known to interact with several key functional residues on MnSOD (16, 17, 36). These facts, coupled with the strong correlation we observed between MnSOD acetylation and SIRT3 activity, support the notion that SIRT3 does play a role in governing MnSOD activity in skeletal muscle of obese pregnant women.

In addition to lower SIRT3 enzyme activity in the obese pregnant women, we also observed a severe decrease in SIRT3 mRNA content. Recent studies have demonstrated that PGC-1α induces SIRT3 gene expression (27, 28). Thus, our observations of decreased PGC-1α mRNA content and a tendency for decreased PGC-1α protein content in the obese, compared with NW women, suggest that PGC-1α may contribute to reductions in SIRT3 activity, even though decreased SIRT3 protein in the obese-NGT and obese-GDM women did not reach statistical signifi-

cance. However, other direct regulators of SIRT3 activity are also likely involved. For example, SIRT3 activity is NAD^+-dependent and, therefore, sensitive to the cellular energy state. We and others have previously reported decreased NAD^+ content and NAD^+/NADH ratio in livers of obese and high fat-fed rodents (19, 37). Likewise, peroxidized lipids are commonly associated with increased cellular and mitochondrial oxidative stress. The lipid peroxidation byproduct 4-hydroxynonenal was recently shown to inhibit SIRT3 enzyme activity via protein carbonylation (38). Although we did not measure NAD^+ content or lipid peroxides in this study, it is possible that these factors may also play a role in decreased SIRT3 activity observed in our obese pregnant women.

In summary, the present study is the first to show that obese pregnant women, regardless of GDM diagnosis, exhibit increased skeletal muscle mitochondrial stress, including: 1) mitochondrial respiratory chain deficiency via lower C-II and C-II+III enzyme capacity; 2) decreased antioxidant function via lower enzyme capacity of MnSOD, coupled with evidence of increased oxidative stress; and 3) decreased SIRT3 activity, which inversely correlated with MnSOD hyperacetylation. Together, our results suggest that substrate excess during pregnancy (ie, obesity) is associated with a cyclical relationship between increased oxidative stress and decreased mitochondrial enzyme capacity, in which SIRT3 may play an important role via regulation of MnSOD. It is important to recognize that the "initiator" of this cycle is not known and, perhaps, cannot be attributed to one particular factor, but rather to a host of small changes that propagate until they manifest as more severe metabolic dysregulation in skeletal muscle tissue. It is not known whether the relationships reported here are due to obesity per se or if they only become evident during pregnancy due to the physiological stress of pregnancy-associated insulin resistance and inflammation. Nevertheless, the altered substrate metabolism and increased oxidative stress of the obese pregnancy have been linked to adverse fetal programming events that confer obesity and type 2 diabetes risk to the offspring (39, 40). The mechanisms underlying how maternal oxidative stress and skeletal muscle mitochondrial capacity may impart this disease risk to the offspring are not known, but these questions are certainly deserving of further investigation.

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