Deoxyribonucleic Acid Methylation and Gene Expression of *PPARGC1A* in Human Muscle Is Influenced by High-Fat Overfeeding in a Birth-Weight-Dependent Manner

Charlotte Brøns,* Stine Jacobsen,* Emma Nilsson, Tina Rönn, Christine B. Jensen, Heidi Storgaard, Pernille Poulsen, Leif Groop, Charlotte Ling, Arne Astrup, and Allan Vaag

Steno Diabetes Center (C.B., S.J., E.N., C.B.J., H.S., P.P., A.V.), Type 2 Diabetes Pathophysiology, 2820 Gentofte, Denmark; Department of Human Nutrition (C.B., A.A.), Faculty of Life Sciences, University of Copenhagen, 1958 Frederiksberg, Denmark; and Department of Clinical Sciences (T.R., L.G., C.L., A.V.), Diabetes and Endocrinology Research Unit, Lund University, Malmö University Hospital, S-205 02 Malmö, Sweden

Context: Low birth weight (LBW) and unhealthy diets are risk factors of metabolic disease including type 2 diabetes (T2D). Genetic, nongenetic, and epigenetic data propose a role of the key metabolic regulator peroxisome proliferator-activated receptor γ, coactivator 1α (*PPARGC1A*) in the development of T2D.

Objective: Our objective was to investigate gene expression and DNA methylation of *PPARGC1A* and coregulated oxidative phosphorylation (OXPHOS) genes in LBW and normal birth weight (NBW) subjects during control and high-fat diets.

Design, Subjects, and Main Outcome Measures: Twenty young healthy men with LBW and 26 matched NBW controls were studied after 5 d high-fat overfeeding (+50% calories) and after a control diet in a randomized manner. Hyperinsulinemic-euglycemic clamps were performed and skeletal muscle biopsies excised. DNA methylation and gene expression were measured using bisulfite sequencing and quantitative real-time PCR, respectively.

Results: When challenged with high-fat overfeeding, LBW subjects developed peripheral insulin resistance and reduced *PPARGC1A* and OXPHOS ($P < 0.05$) gene expression. *PPARGC1A* methylation was significantly higher in LBW subjects ($P = 0.0002$) during the control diet. However, *PPARGC1A* methylation increased in only NBW subjects after overfeeding in a reversible manner. DNA methylation of *PPARGC1A* did not correlate with mRNA expression.

Conclusions: LBW subjects developed peripheral insulin resistance and decreased gene expression of *PPARGC1A* and OXPHOS genes when challenged with fat overfeeding. The extent to which our finding of a constitutively increased DNA methylation in the *PPARGC1A* promoter in LBW subjects may contribute needs to be determined. We provide the first experimental support in humans that DNA methylation induced by overfeeding is reversible. **(*J Clin Endocrinol Metab* 95: 3048–3056, 2010)**

Individuals born with low birth weight (LBW) are at increased risk of developing insulin resistance, type 2 diabetes (T2D), and the metabolic syndrome later in life (1). We have previously identified numerous metabolic abnormalities relevant to the pathophysiology of T2D in healthy, young LBW men including impaired insulin-stim-

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* C.B. and S.J. contributed equally to this work.

Abbreviations: BMI, Body mass index; FFA, free fatty acid; LBW, low birth weight; NBW, normal birth weight; OXPHOS, oxidative phosphorylation; T2D, type 2 diabetes.

ulated glucose uptake in the forearm muscle (2), decreased whole-body insulin-stimulated glycolytic flux (3), hepatic insulin resistance (3), and changes in muscle and fat insulin-signaling proteins (4, 5). The increased disease susceptibility in LBW subjects has long been speculated to involve epigenetic programming *in utero*, and recent studies have provided preliminary evidence of a connection between maternal nutrition and health on one side and of DNA methylation in the offspring on the other (6–8).

One of the best characterized epigenetic mechanisms is DNA methylation, which may affect gene expression (9) and genome stability (10). DNA methylations occur throughout the genome but are mostly studied and thought to be important at CpG dinucleotides, which are found in high densities and often unmethylated in gene promoters, referred to as CpG islands (11). DNA methylations are influenced by environmental factors as evident by diverging epigenetic patterns with increasing age in monozygotic twin pairs (12), higher DNA methylation of cytochrome-c-oxidase subunit VIIa polypeptide 1 (*COX7A1*) (13), and NADH dehydrogenase-1β subcomplex, 6 (*NDUFB6*) in old compared with young subjects (14) and by a generally higher DNA methylation with older age of CpG islands across multiple tissues (15).

Decreased expression of peroxisome proliferator-activated receptor-γ, coactivator-1α (*PPARGC1A*) may cause insulin resistance by interfering with multiple key cellular functions including mitochondrial function (16), lipid oxidation (17), angiogenesis, and microvascular flow (18) as well as oxidative stress (19). *PPARGC1A* may also play a role in regulation of pancreatic insulin secretion (20). Decreased expression of *PPARGC1A* and oxidative phosphorylation (OXPHOS) genes in skeletal muscle has been observed in some (16, 21, 22), but not all (23), studies of T2D patients. Recent studies have shown increased promoter DNA methylation and decreased gene expression of *PPARGC1A* in both pancreatic islets (20) and skeletal muscle biopsies (24) from patients with T2D. Importantly, we have found that decreased expression of *PPARGC1A* in skeletal muscle is associated with LBW in elderly, but not young, twins (25). Another recent study showed that maternal obesity is associated with DNA methylation of *PPARGC1A* in cord blood from the newborn offspring pointing toward a potential key role of *PPARGC1A* methylation in programming of obesity, T2D, and the metabolic syndrome (6).

Recent studies have suggested that epigenetic traits are influenced by different dietary conditions and interventions including calorie restriction in humans (26). Studies in rodents have shown that high-fat diets increase DNA methylation of the leptin promoter (27) and prolong the presence of DNA methylation in liver and lung tissue after treatment with a carcinogenic agent (28, 29). A study of human skeletal muscle cell cultures showed a higher degree of methylation of *PPARGC1A* when incubated with free fatty acids (FFA) (24). Interestingly, sustained cellular exposure to saturated fatty acids (30) as well as 3 d intake of a high-fat diet resulted in reduced expression of *PPARGC1A* in young men (31).

We hypothesized that young and healthy LBW men exhibit altered DNA methylation in the promoter region of *PPARGC1A* in skeletal muscle as a result of fetal programming resembling that seen in patients with overt T2D. To unmask metabolic abnormalities potentially conferring susceptibility to T2D in LBW subjects, LBW as well as normal birth weight (NBW) subjects were studied both during a control diet as well as after exposure to a diet high in fat and calories for a 5-d period.

Subjects and Methods

Subjects

Twenty-six healthy, young, lean male volunteers with LBW (<10th percentile) and 20 subjects with NBW (50–90th percentile) were recruited as previously described (2–4, 32). All subjects were born at term, and the groups were matched according to age and body mass index (BMI). The subjects had no family history of diabetes in two generations, and subjects with a BMI higher than 30 kg/m^2 or a high physical activity level (>10 h/wk) were excluded. Control *in vivo* data including muscle OXPHOS gene expression and metabolic responses to high-fat overfeeding in NBW subjects (not in LBW subjects) were published previously (3, 33). The protocol conformed to the Declaration of Helsinki and was approved by the ethics committee for Copenhagen County, and all subjects signed an informed consent form.

Experimental protocol

The study was a randomized crossover study, with a washout period of 6–8 wk, as previously reported for the NBW control subjects (33). In brief, subjects were examined twice with a hyperinsulinemic-euglycemic clamp after intake of a 3-d control diet including 30% fat (mean 11.9 ± 1.1 MJ) and after a 5-d high-fat overfeeding diet (mean 17.6 ± 1.4 MJ) containing 50% extra calories and 60% fat.

Hyperinsulinemic-euglycemic clamp

The clamp was performed and analyzed as previously described (3). A primed continuous infusion of tritiated [3-^{3}H]glucose was initiated at 0 h, and an insulin infusion of 80 mU/m^2 · min was used throughout the 180-min clamp to maintain euglycemia. Glucose and fat oxidation rates were measured by indirect calorimetry (34), and basal and insulin-stimulated biopsies were excised from musculus vastus lateralis in 35 of the 46 subjects using a Bergström needle. The tissue was immediately frozen in liquid nitrogen and stored at −80 C. Body composition was assessed by dual-energy x-ray absorptiometry scanning (Lunar Radiation, Madison, WI).

Quantitative real-time-PCR

Total RNA was extracted from the muscle biopsies using TRI reagent (Sigma-Aldrich, St. Louis, MO). cDNA was synthesized using QuantiTect reverse transcription kit (QIAGEN, Valencia, CA). mRNA levels were detected with the ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA) using gene-specific probe/primer pairs for *NDUFB6* (Hs00159583_m1), ubiquinol-cytochrome c reductase binding protein (*UQCRB*) (Hs00559884_m1), *COX7A1* (Hs00156989_m1), ATP synthase, H^+ transporting, mitochondrial F1 complex, O subunit (*ATP5O*) (Hs00426889_m1), and *PPARGC1A* (Hs00173304_m1) (Applied Biosystems). All samples were run in duplicate and the standard curve approach used for quantification. The transcript quantity was normalized to mRNA levels of cyclophilin A (*PPIA*) (4326316E; Applied Biosystems).

DNA methylation

Genomic DNA was extracted collectively with RNA from muscle biopsies and partitioned into a separate fraction for further processing. Bisulfite conversion was completed using the EZ DNA methylation kit (Zymo Research, Orange, CA) and amplified using forward and reverse primers designed by MethPrimer (35). The *PPARGC1A* promoter sequence contains four CpG sites and is identical to that studied by Ling *et al.* (20) in β-cells from T2D patients and was located 624–867 bp upstream from the transcription start and amplified using forward primer 5′-TATTTTA-AGGTAGTTAGGGAGGAAA-3′ and reverse primer 5′-CCCATAACAATAAAAAATACCAACTC-3′ (Fig. 1A). We included *NDUFB6* as a control gene because it is not directly involved in transcriptional activities, is reduced in T2D, and is possibly regulated by epigenetic factors (14). The *NDUFB6* sequence was located 392–611 bp upstream from the transcription start and amplified with forward primer 5′-GTTGTTTTTT-GATTGTTGTATTATAATTTA-3′ and reverse primer 5′-AAAATACCCTAAACAACTATCTCAT-3′. The *NDUFB6* sequence contains three CpG sites and one polymorphism creating a possible methylation site (rs629566, A/G). The amplicons were visualized after electrophoresis through a 2% ethidium bromide-stained agarose gel. Small fragments were removed by ExoSAP-IT treatment (USB Corp., Cleveland, OH). DNA was precipitated with ethanol and sequencing PCR performed using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). The samples were sequenced with the ABI 3130xl genetic analyzer (Applied Biosystems). Trace files were subject to quality control and analysis using the ESME software (Epigenomics, Berlin, Germany) (Fig. 1B) (36).

Statistical analysis

Statistical analyses were performed with SAS Statistical Analysis Package (SAS Institute, Cary, NC; version 8.2). Normality was evaluated by Kolmogorov-Smirnov's test and normality plots. Data are presented as mean ± SD or SEM, whenever appropriate. Basal and insulin-stimulated gene expression was combined because no effect of insulin on gene expression was observed. Two-sided Student's *t* test was used to identify significant differences between NBW and LBW subjects (unpaired) and between diets (paired). $P \leq 0.05$ was considered significant. Correlations were calculated using Pearson's correlation coefficient.

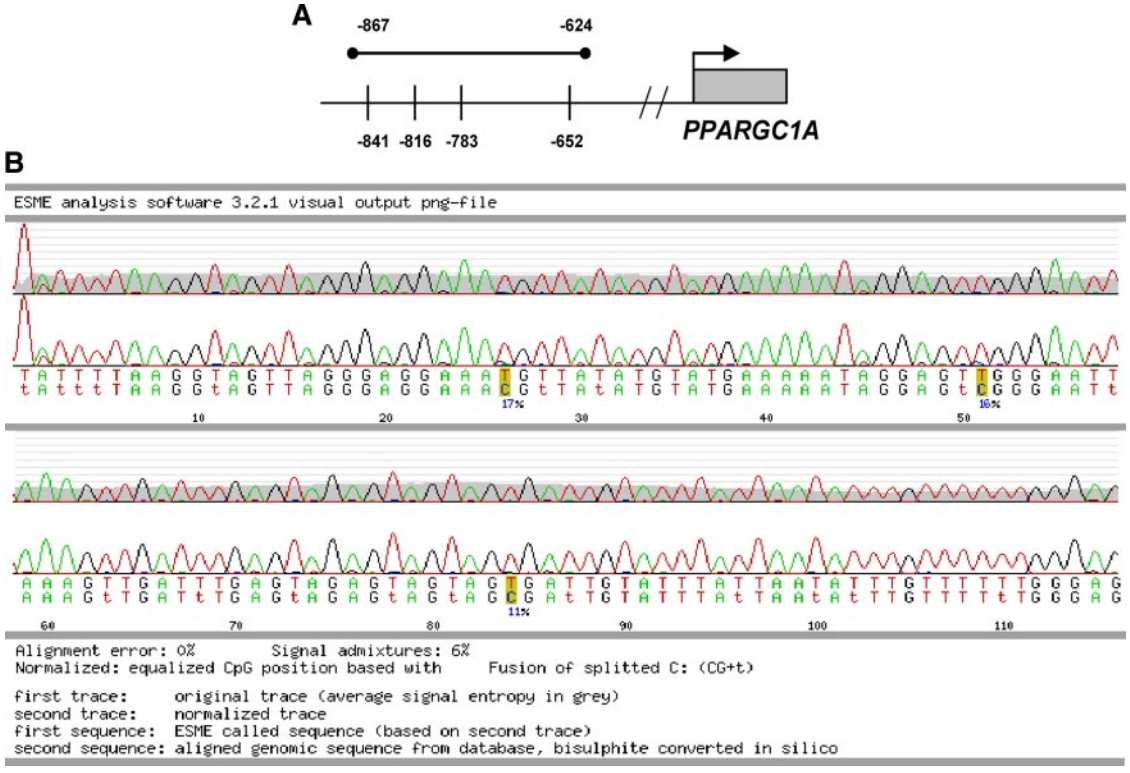

FIG. 1. Sequencing of a subset of the *PPARGC1A* promoter. A, Visualization of the specific section of the promoter region of *PPARGC1A*, which was sequenced, stretching from −624 to −867 upstream of the transcription start (*arrow*). The four CpG sites investigated are marked with a *perpendicular line*. B, ESME output file of methylation sites and degree of methylation (site −841, 17%; site −816, 16%; and site −783, 11%, respectively) in the *PPARGC1A* promoter. The sample sequence is aligned to a genomic database reference sequence. Methylation sites are identified by unconverted cytosine (C) bases in the sample sequence.

TABLE 1. Subject characteristics of the NBW and LBW groups at baseline (control diet)

	NBW	LBW
Birth weight (g)	3893 ± 207	2688 ± 269[c]
Age (yr)	24.6 ± 1.0	24.2 ± 0.5
Height (m)	1.83 ± 0.07	1.77 ± 0.05[b]
Weight (kg)	78.3 ± 9.1	77.7 ± 10.9
BMI (kg/m^2)	23.4 ± 2.4	24.8 ± 3.7
Trunk fat mass/total fat mass (g)	0.50 ± 0.04	0.53 ± 0.04[a]
Leg fat mass/total fat mass (g)	0.37 ± 0.04	0.34 ± 0.03[a]
Trunk fat mass/total fat mass (%)	1.09 ± 0.18	1.23 ± 0.17[a]

Data are mean ± SD.

[a-c] NBW (n = 26) *vs. LBW (n = 20):* [a] $P \leq 0.05$; [b] $P \leq 0.01$; [c] $P \leq 0.001$.

Interaction of birth weight and diet was investigated by a normal mixed model. ESME analysis software version 3.2.1 (Epigenomics, Berlin, Germany) was employed in quality control and analysis of the methylation data. In brief, ESME align the bisulfite converted sequence to a reference sequence and provides an average degree of methylation at each CpG site according to the fluorescent signal detected from methylated and unmethylated cytosines. The mean methylations obtained for the same CpG site using forward and reverse primers were used to express the CpG site-specific degree of methylation.

Results

Subjects characteristics

The LBW subjects were smaller at birth, had a significantly lower final height, and were more abdominally obese compared with NBW subjects (Table 1), which is in accordance with previous findings (3). Weight and body composition were not affected by overfeeding (data not shown).

In vivo metabolism

The LBW subjects had elevated fasting glucose and insulin levels compared with NBW subjects on the control diet (Table 2). In response to high-fat overfeeding, both groups had significantly increased fasting blood glucose and C-peptide levels (NBW only) and decreased FFA levels, whereas there were no changes in serum insulin. As previously published, LBW and NBW subjects had a similar degree of peripheral insulin action (M-value) after the control diet (3). Likewise, basal and insulin-stimulated rates of whole-body fat and glucose oxidation (Table 2) and hepatic glucose production were similar in the two groups on the control diet (3). Nevertheless, in contrast to the NBW subjects (33), the LBW subjects developed a significant deterioration of peripheral insulin action (Table 2), when exposed to 5 days of overfeeding. Moreover, the LBW subjects showed an increased rate of insulin-stimulated fat oxidation after overfeeding, which was also higher than the NBW subjects during both the control and overfeeding diet. In other words, the high-fat overfeeding

TABLE 2. *In vivo* data from the hyperinsulinemic-euglycemic clamp examination including glucose, insulin, and C-peptide concentrations and metabolic flux data after the control and overfeeding diet

Diet	NBW	LBW
Fasting (baseline)		
Blood glucose (mmol/liter)		
Control	4.59 ± 0.46	4.96 ± 0.46[a]
Overfeeding	5.05 ± 0.40[c]	5.18 ± 0.33[b]
Serum insulin (pmol/liter)		
Control	30.9 ± 14.1	40.7 ± 14.4[a]
Overfeeding	43.4 ± 29.2	42.9 ± 18.8
Serum C-peptide (pmol/liter)		
Control	408 ± 146	483 ± 118
Overfeeding	521 ± 269[b]	530 ± 174
Plasma FFA (mmol/liter)		
Control	334 ± 136	401 ± 190
Overfeeding	205 ± 82[c]	188 ± 91[c]
Glucose oxidation (mg/kg FFM · min)		
Control	2.41 ± 0.83	1.96 ± 0.74
Overfeeding	2.43 ± 0.90	2.20 ± 0.56
Fat oxidation (mg/kg FFM · min)		
Control	0.98 ± 0.39	1.12 ± 0.51
Overfeeding	0.94 ± 0.39	1.17 ± 0.33[a]
Insulin stimulated (clamp)		
Blood glucose (mmol/liter)		
Control	5.11 ± 0.31	5.06 ± 0.30
Overfeeding	5.17 ± 0.30	5.07 ± 0.30
Serum insulin (pmol/liter)		
Control	870 ± 232	816 ± 114
Overfeeding	867 ± 181	842 ± 125
Serum C-peptide (pmol/liter)		
Control	406 ± 279	366 ± 120
Overfeeding	453 ± 195	390 ± 143
Plasma FFA (mmol/liter)		
Control	9.3 ± 4.4	9.6 ± 4.8
Overfeeding	12.4 ± 6.4	14.4 ± 7.8[b]
M-value (mg/kg FFM · min)		
Control	13.73 ± 2.32	13.43 ± 2.98
Overfeeding	13.29 ± 3.32	11.89 ± 3.57[b]
Glucose oxidation (mg/kg FFM · min)		
Control	5.18 ± 0.80	4.89 ± 0.93
Overfeeding	4.83 ± 1.34	4.74 ± 0.82
Fat oxidation (mg/kg FFM · min)		
Control	0.02 ± 0.25	0.14 ± 0.44
Overfeeding	0.15 ± 0.29	0.37 ± 0.34[a,b]

Data are mean ± SD and are shown for NBW (n = 26) *vs.* LBW (n = 20) subjects. FFM, Fat-free mass.

[a] Significant differences between NBW and LBW subjects at $P \leq 0.05$.

[b,c] Paired comparisons between control and overfeeding include n = 25 (NBW) and n = 18 (LBW) subjects. Significant differences between the control and overfeeding diet are indicated as follows: [b] $P \leq 0.05$; [c] $P \leq 0.001$.

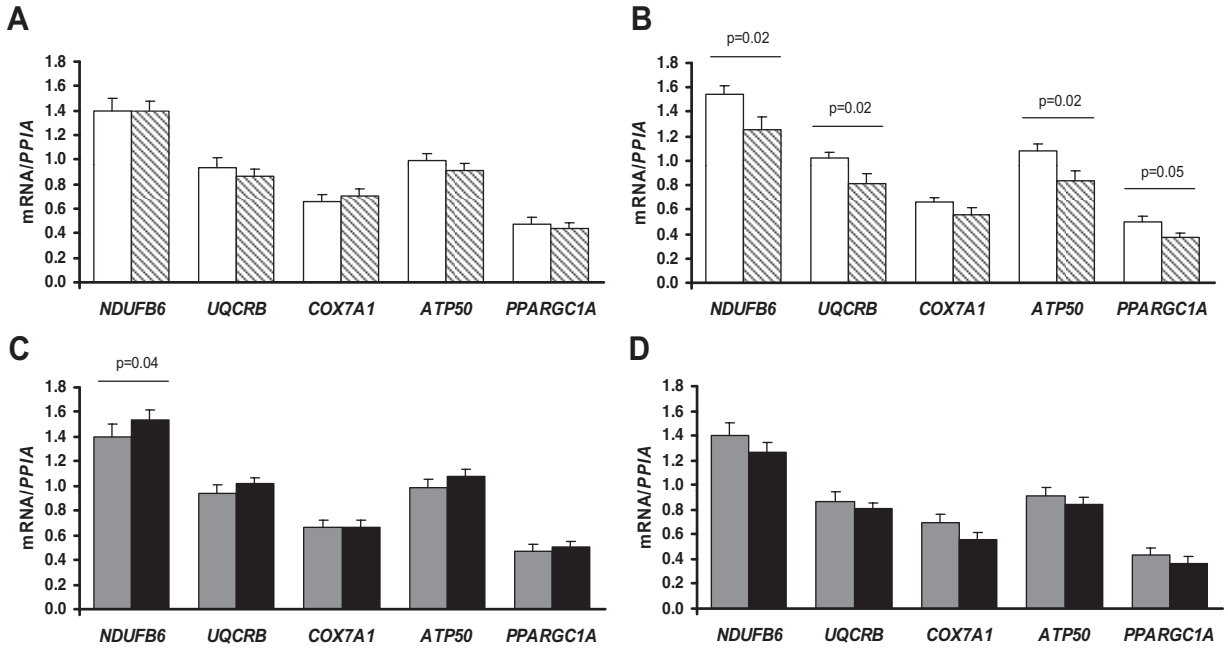

FIG. 2. *PPARGC1A* and OXPHOS gene expression normalized to *PPIA* in NBW (*white*) and LBW (*hatched*) subjects during control (*grey*) and overfeeding (*black*) diets. A, Gene expression in NBW (n = 21) and LBW (n = 17) subjects during the control diet. B, Gene expression in NBW (n = 21) and LBW (n = 13) subjects during overfeeding. C and D, Effect of overfeeding on gene expression within the NBW (C) (n = 20) and the LBW (D) (n = 13) subjects. Data are mean ± SEM.

challenge unmasked a selective risk of developing insulin resistance and elevated fat oxidation in only the LBW subjects.

Gene expression

The NBW and LBW subjects had similar gene expression on the control diet (Fig. 2A). When exposed to high-fat overfeeding, mRNA levels of *NDUFB6*, *UQCRB*, and *ATP5O* (all $P = 0.02$) as well as of *PPARGC1A* ($P = 0.05$) were significantly lower by approximately 25% in skeletal muscle from LBW compared with NBW subjects (Fig. 2B). A similar trend was observed for *COX7A1* ($P = 0.12$) (Fig. 2B). The difference arose as the NBW subjects showed a trend toward higher expression after the overfeeding diet, being statistically significant only for *NDUFB6* ($P = 0.04$) (Fig. 2C), whereas the LBW subjects remained unchanged with a tendency toward decreased expression (Fig. 2D). The dietary intervention was performed in a randomized crossover manner, enabling us to document reversibility of changes of gene expression induced by the high-fat diet. We were, however, unable to identify any reversibility of gene expression induced by the overfeeding diet of either *PPARCG1A* or OXPHOS genes (data not shown). OXPHOS genes are considered to be tightly regulated by *PPARGC1A*, which was supported by significant positive correlations between *PPARGC1A* and the OXPHOS genes during both the control (r = 0.56–0.83, $P < 0.01$) and overfeeding (r = 0.50–0.63; $P < 0.01$) diets. As previously published (3), skeletal muscle *PPARGC1A* and OXPHOS mRNA expression was similar in the two groups during the control diet (Fig. 2A). The gene expression level of *PPARGC1A* as well as of the OXPHOS gene did not correlate with whole-body insulin action (M-value) or with the rates of glucose or fat oxidation, as assessed during either the basal or the insulin-stimulated state.

DNA methylation

DNA methylation was investigated in the promoter region of *PPARGC1A* at four CpG sites (Fig. 1A). The mean DNA methylation was significantly higher in the LBW than NBW subjects after the control diet ($P = 0.0002$). The differences between NBW and LBW subjects were significant at three CpG sites during the control diet (Fig. 3A) (site −841: 32%, $P = 0.05$; site −816: 59%, $P = 0.001$; site −783: 59%, $P = 0.008$; site −652: 41%, $P = 0.42$). Measures of the fourth CpG site (−652) were obtained for only half of the subjects, resulting in a less powerful analysis. When exposed to overfeeding, methylation of *PPARGC1A* increased in the NBW only, and the differences between the groups were subsequently attenuated (Fig. 3B). The change in methylation during overfeeding in NBW subjects reached statistical significance for two CpG sites (site −841: 30%, $P = 0.01$; site −816: 31%, $P = 0.10$; site −783: 43%, $P = 0.008$; site −652: 6%, $P = 0.93$) (Fig. 3C). The LBW subjects showed similar *PPARGC1A* methylation during the two diets (Fig. 3D). When investigating the reversibility of the DNA methyl-

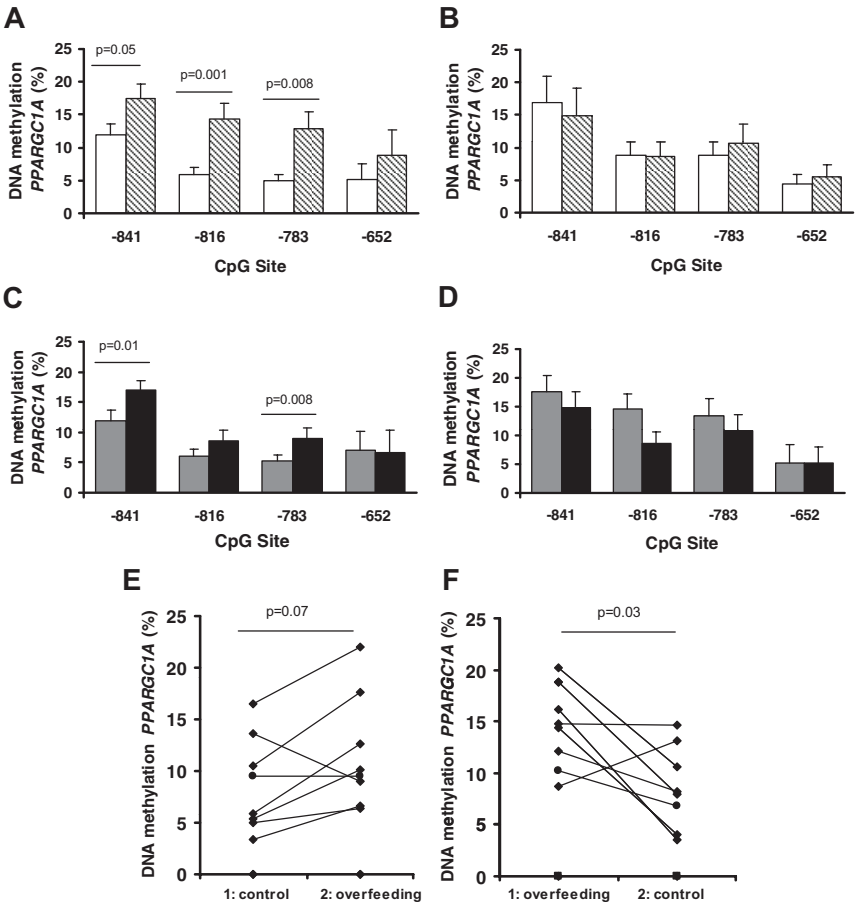

FIG. 3. DNA methylation of the *PPARGC1A* promoter region in NBW (*white*) and LBW (*hatched*) subjects during control (*grey*) and overfeeding (*black*) diet. A, DNA methylation during the control diet in NBW (n = 22) and LBW (n = 17) subjects. B, DNA methylation in NBW (n = 21) and LBW (n = 14) subjects during overfeeding. C and D, Effect of overfeeding on DNA methylation within the NBW (C) (n = 20) and the LBW (D) (n = 14) subjects. Data are mean ± SEM. E and F show the reversibility of methylation in the NBW subjects: E, DNA methylation induced by overfeeding in NBW (n = 10) subjects that received control diet first followed by the high-fat overfeeding diet; F, reversibility of methylation in the NBW (n = 10) subjects that received the high-fat overfeeding first followed by the control diet. DNA methylation is integrated over the three sites (-841, -816, -783) CpG sites in E and F.

ation induced by the high-fat diet, we were able to document a significant reduction of methylation in NBW subjects that were first studied after overfeeding and subsequently shifted to the control diet ($P = 0.03$) (Fig. 3, E and F). Furthermore, a highly statistical significant interaction of birth weight group by dietary intervention affected DNA methylation ($P = 0.009$), strongly supporting the idea that the difference in methylation responses to the two diets was dependent on birth weight. Interestingly, *NDUFB6* was methylated to a much higher degree (81 ± 11%) than *PPARGC1A* (10 ± 2%), and no methylation changes were observed for *NDUFB6* in relation to the birth weight or diet intervention (Fig. 4, A–D). Limited data were obtained for the CpG site (−418) in *NDUFB6*, containing the polymorphism rs629566, that indicate a low occurrence of G at the A/G polymorphism site. We observed no significant correlations between DNA methylation and gene expression of *PPARGC1A* or *NDUFB6*. Furthermore, no statistical significant correlations between the level of DNA methylation of *PPARGC1A* or of *NDUFB6* with basal or insulin-stimulated *in vivo* metabolism including the M-value was observed in any of the study groups before or after overfeeding (data not shown).

Discussion

Several findings of potential importance for understanding the mechanisms by which both the prenatal (LBW) as well as postnatal (high-fat overfeeding) environments interact to cause insulin resistance and eventually T2D were observed in this study. First, young and otherwise healthy LBW subjects but not NBW controls developed peripheral insulin resistance and a disproportionately elevated lipid oxidation rate after exposure to 5 d of high-fat feeding. Second, the LBW subjects had elevated DNA methylation of the *PPARGC1A* promoter at baseline, and the mRNA expression of *PPARGC1A* and coregulated OXPHOS genes was reduced in LBW subjects when challenged by the high-fat overfeeding diet. Finally, we found that short-term high-fat feeding increased DNA methylation of *PPARGC1A* in a reversible manner in the NBW subjects only. The extent to which the constitutively elevated *PPARGC1A* promotor methylation may be involved in, or responsible for, the differential development of insulin resistance as well as decreased expression of *PPARGC1A* and OXPHOS genes in the LBW subjects during overfeeding remains to be determined.

Recently, increased DNA methylation of *PPARGC1A* in pancreatic β-cells as well as in muscle biopsies from T2D patients was reported (20, 24), which was speculated to be involved in the reduced tissue gene expression of *PPARGC1A* and subsequently in the development of impaired insulin secretion and action in T2D patients. Epigenetic alterations observed in LBW subjects at increased risk of developing T2D could therefore reflect DNA methylation remnants established during fetal life, possibly affecting tissue development in organs relevant to T2D pathophysiology during phases with active cell di-

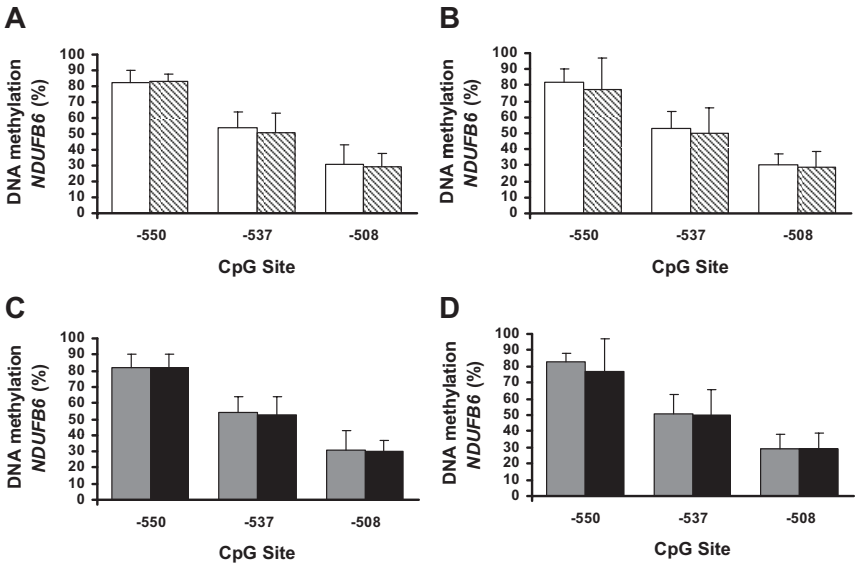

FIG. 4. DNA Methylation of the *NDUFB6* promoter region in NBW (*white*) and LBW (*hatched*) subjects during control (*gray*) and overfeeding (*black*) diet. A, DNA methylation during the control diet in NBW (n = 21) and LBW (n = 17) subjects. B, Methylation in NBW (n = 19) and LBW (n = 14) subjects during the overfeeding diet. C and D, Effect of overfeeding on DNA methylation in the NBW (C) (n = 19) subjects and the LBW (D) (n = 14) subjects. Data are mean ± SEM.

visions. Importantly, this study shows that the exact same *PPARGC1A* CpG sites reported to be more highly methylated in pancreatic β-cells from T2D subjects (20) are methylated to a higher extent in young and lean LBW compared with NBW subjects when studied during an isocaloric control diet.

Our finding that 5 d of high-fat overfeeding caused an increased DNA methylation of *PPARGC1A* in muscle tissue of NBW subjects is in accordance with the recent report of increased DNA methylation of *PPARGC1A* after 48 h fatty acid exposure in cultured muscle cells (24). In light of these findings, the coordinated changes in DNA methylation across all CpG sites after the overfeeding diet may reflect a more widespread genome-wide response, at least in promoter regions that are responsive to methylation changes. We cannot exclude the possibility that short-term diet-induced methylation, in contrast to a long-term and more constitutive methylation, may exert some beneficial cellular effects as a part of a normal physiological response involved in the day-to-day regulation of mechanisms influenced by the diet. Short-term methylation (and potentially corresponding gene expression) changes of *PPARGC1A* could for example relate to protection of the cell against futile OXPHOS and ATP synthesis and, with that, the generation of free radicals and oxidative stress (19). It remains to be determined whether diet-induced DNA methylation becomes more permanent with longer exposures to a high-fat diet. If so, this could contribute to the notion of a general increase of DNA methylation with age (12) and/or reduced longevity associated with chronic high-energy diet intake (37). The observed reversibility of the DNA methylation induced by 5 d of high-fat overfeeding in the NBW subjects is another novel finding, which theoretically could be essential for biological signals to function and thereby in a dynamic fashion respond to changing physiological cues (38). In contrast, the constitutively increased DNA methylation of *PPARGC1A* in LBW subjects indicates a lower sensitivity to environmental challenges such as high-fat feeding. The extent to which such metabolic inflexibility in terms of acute regulation of DNA methylation may contribute to an increased risk of T2D in LBW subjects remains uncertain.

We determined mRNA expression of *PPARGC1A* and coregulated OXPHOS genes to establish whether a functional significance of LBW, high-fat overfeeding and/or of DNA methylation *per se* could be identified. Although DNA methylation of the *PPARGC1A* promoter was elevated in LBW subjects already during the control diet, we found no difference in *PPARGC1A* or OXPHOS gene expression between the two groups, and no correlation between DNA methylation and gene expression was observed. However, when challenged with high-fat overfeeding, the expression of *PPARGC1A* and OXPHOS genes was decreased in LBW compared with NBW subjects. In other words, the high-fat diet unmasked a disproportionately reduced expression of *PPARGC1A* as well as a panel of OXPHOS genes that previously were reported to be down-regulated in skeletal muscle obtained from patients with overt T2D in some studies (16, 21, 22). Thus, metabolic challenges such as high-fat diets and/or exercise experiments (39) may be required to unmask significant down-regulation of genes including *PPARGC1A* relevant to metabolic control and insulin action. Following this line of thinking, it may help understanding and accepting that the degree of DNA methylation may not necessarily correlate with the level of gene expression during nonchallenged resting states. Sparks *et al.* (31) reported down-regulation of *PPARGC1A* and OXPHOS genes after a 3-d high-fat diet in healthy young men. However, they did not stratify for birth weight, and the subjects may have consumed high-fat diets for even longer periods of time.

Another novel finding in this study was the differential development of peripheral insulin resistance and a disproportionately elevated fat oxidation rate in LBW but not in NBW subjects when exposed to a high-fat diet. This pro-

vides further support of LBW representing a significant prediabetic state. However, the extent to which differential DNA methylation and/or reduced expression of *PPARGC1A* and OXPHOS genes are related in a potential causal manner to the development of peripheral insulin resistance in the LBW subjects after high-fat overfeeding remains to be elucidated. Indeed, and in contrast to previous studies of *PPARGC1A* methylation in patients with overt T2D (20, 24), we were unable to demonstrate significant correlations between skeletal muscle *PPARGC1A* methylation on one side and *PPARGC1A* mRNA expression on the other side. This may be explained by a too short duration of the excess methylation in the NBW subjects to exhibit any functional impact as well as the notion that some regions of the gene promoter could be more or less accessible to the binding of transcription factors (*e.g.* due to histone acetylation). Another explanation for the lack of correlation between DNA methylation and gene expression may be that DNA methylation of genes such as *PPARGC1A* influence gene expression and organ development during fetal life primarily or exclusively and that little functional impact of altered DNA methylation, at least for some genes, may be operating in the fully developed adult subjects. Poor correlations between degree of DNA methylation and gene expression for most genes was also reported in the Human Epigenome Project (40) as well as in a recently published study on calorie restriction (26), supporting the lack of correlations in the current study.

The constitutively elevated DNA methylation of *PPARGC1A*, but not of *NDUFB6*, in the LBW subjects demonstrates some degree of gene specificity of epigenetic programming in LBW subjects. Recent observations showed that offspring of mothers periconceptionally exposed to famine had lower methylation of several genes including *IGF2* in their adult life than their unexposed, same-sex siblings, supporting our current finding that early-life conditions may indeed cause epigenetic changes persisting throughout life (7, 8).

In conclusion, our data demonstrate increased DNA methylation in the promoter region of *PPARGC1A* in skeletal muscle of young and otherwise healthy LBW subjects when studied on a control diet. Furthermore, we provide the first experimental support in humans that DNA methylations are induced by high-fat overfeeding in a reversible manner only in NBW control subjects. The extent to which a constitutively elevated DNA methylation of *PPARGC1A* contributes to the novel finding of a differential development of peripheral insulin resistance and decreased gene expression of *PPARGC1A* and co-regulated OXPHOS genes in LBW subjects during high-fat overfeeding, as well as to the later risk of metabolic disease including T2D in LBW subjects, remains to be determined.

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Address all correspondence and requests for reprints to: Charlotte Brøns, Steno Diabetes Center, Niels Steensensvej 1, 2820 Gentofte, Denmark. E-mail: chbe@steno.dk.

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C.B., S.J., A.V., A.A., C.L., L.G., and P.P. designed and/or supervised the experiments. C.B., S.J., and A.V. wrote the manuscript. C.B., C.B.J., and H.S. performed hyperinsulinemic-euglycemic clamps, collected *in vivo* data, and muscle biopsies. S.J., E.N., and T.R. performed gene expression and DNA methylation analyses and supervised laboratory tasks. All authors discussed the results and commented on the manuscript.

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