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Insulin and Glucose Alter Death-Associated Protein Kinase 3 (DAPK3) DNA Methylation in Human Skeletal Muscle

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DNA methylation is altered by environmental factors. We hypothesized that DNA methylation is altered in skeletal muscle in response to either insulin or glucose exposure. We performed a genome-wide DNA methylation analysis in muscle from healthy men before and after insulin exposure. DNA methylation of selected genes was determined in muscle from healthy men and men with type 2 diabetes before and after a glucose tolerance test. Insulin altered DNA methylation in the 3' untranslated region of the calcium pump *ATP2A3* gene. Insulin increased DNA methylation in the gene body of *DAPK3*, a gene involved in cell proliferation, apoptosis, and autophagy. *DAPK3* methylation was reduced in patients with type 2 diabetes. Carbohydrate ingestion reduced *DAPK3* DNA methylation in healthy men and men with type 2 diabetes, suggesting glucose may play a role. Supporting this, *DAPK3* DNA methylation was inversely correlated with the 2-h glucose concentration. Whereas glucose incorporation to glycogen was unaltered by small interfering RNA against *DAPK3*, palmitate oxidation was increased. In conclusion, insulin and glucose exposure acutely alter the DNA methylation profile of skeletal muscle, indicating that DNA methylation constitutes a rapidly adaptive epigenetic mark. Furthermore, insulin and glucose modulate *DAPK3* DNA methylation in a reciprocal manner, suggesting a feedback loop in the control of the epigenome.

Type 2 diabetes is a life-threatening metabolic disease reaching epidemic-like proportions. The pathophysiology

of type 2 diabetes is incompletely understood, but both genetic and environmental factors contribute to disease development (1). People with type 2 diabetes are characterized by skeletal muscle insulin resistance arising from impaired insulin signaling and reduced glucose transport activity (2,3). Many of these same steps are impaired in skeletal muscle from relatives of patients with type 2 diabetes who are glucose intolerant (4,5). Thus, skeletal muscle insulin resistance is a characteristic feature of type 2 diabetes that can be noted at all stages of disease development.

Skeletal muscle insulin resistance may arise in part from a dysfunctional metabolic program, either of an epigenetic or genetic origin (6). This notion is partly based on the observation that impairments in glucose and lipid metabolism persist in skeletal muscle cultures derived from people with type 2 diabetes (7–10), as well as in those from relatives of patients with type 2 diabetes who are insulin resistant but do not have diabetes (11,12). Strikingly, these metabolic derangements observed in cultured cells mirror the clinical presentation of type 2 diabetes in the donors, indicating that insulin resistance is an underlying intrinsic defect. However, skeletal muscle insulin resistance can also be acquired by exposure to high levels of insulin, glucose, free fatty acids, or cytokines (7,13–16), systemic factors associated with type 2 diabetes. Although acquired insulin resistance has largely been attributed to defective signal transduction and enzymatic modifications, epigenetic modifications may also play a role (1).

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Epigenetic modifications including DNA methylation provide a potential molecular basis for the interaction between genetic and environmental factors on glucose homeostasis. Recent evidence suggests that systematic factors associated with obesity or insulin resistance can modify DNA methylation in somatic tissues (9,13,17–19), indicating that the regulation of the epigenome is a more dynamic process than previously appreciated. Using whole-genome promoter methylation analysis of DNA from skeletal muscle from healthy participants and participants with type 2 diabetes, we screened for differentially methylated genes and identified hypermethylation of promoters of genes involved in glucose metabolism and mitochondrial function (13). We also found that elevated levels of cytokines and free fatty acids altered DNA methylation of genes important for lipid metabolism in primary human muscle cultures (13), suggesting that systemic factors associated with insulin resistance may play a role. Changes in DNA methylation in skeletal muscle are also triggered by exercise (20–22), dietary factors (19,23,24), and gastric bypass–induced weight loss (17). Whether DNA methylation of genes involved in metabolic regulation is altered in response to dynamic shifts in metabolic demand experienced during an acute insulin stimulation or a glucose challenge is unknown.

We have shown that changes in skeletal muscle DNA methylation in type 2 diabetes are inversely correlated with mRNA expression of multiple genes involved in the regulation

of insulin sensitivity (13,17), indicating that an epigenetic fingerprint emerges in response to metabolic dysregulation. Insulin treatment acutely alters the gene expression profile of skeletal muscle biopsies of healthy individuals and patients with type 2 diabetes (25). Thus, we hypothesized that insulin directly mediates changes in DNA methylation of skeletal muscle. We found that insulin treatment reduced DNA methylation in the calcium pump *ATP2A3* gene and increased DNA methylation in the gene body of death-associated protein kinase 3 (*DAPK3*), a gene involved in cell proliferation, apoptosis, and autophagy. Short-term fasting/refeeding is also associated with changes in skeletal muscle gene expression profiles concomitant with shifts in substrate utilization between carbohydrates and fats as main fuel sources (26). Thus, we determined whether a glucose challenge alters DNA methylation of *ATP2A3* and *DAPK3* in skeletal muscle. Establishing the epigenomic response to these physiological challenges in metabolically healthy people with normal glucose tolerance will build profiles to better predict the development of skeletal muscle insulin resistance.

RESEARCH DESIGN AND METHODS

Clinical Cohorts

Two separate cohorts were studied. Ten healthy sedentary men with normal glucose tolerance were recruited for the open muscle biopsy study. Clinical characteristics of these participants are presented in Table 1A. Twenty-four men

Table 1—Anthropometric measurements and metabolic parameters of the men enrolled in the skeletal muscle biopsy studies

A		B	
	NGT		Type 2 diabetes
<i>n</i>	10	<i>n</i>	12
Age, years	53 ± 2	Age, years	60 ± 3
Height, cm	181 ± 2	Height, cm	178 ± 2
Weight, kg	82.4 ± 2.7	Weight, kg	80.8 ± 2.3
BMI, kg/m ²	25.2 ± 0.6	BMI, kg/m ²	25.4 ± 0.5
Waist-to-hip ratio	0.89 ± 0.02	Waist-to-hip ratio	0.92 ± 0.01
SBP, mmHg	125 ± 5	SBP, mmHg	133 ± 5
DBP, mmHg	81 ± 3	DBP, mmHg	81 ± 3
FPG, mmol/L	5.3 ± 0.1	FPG, mmol/L	5.3 ± 0.1
		2-h PG, mmol/L	5.9 ± 0.3
Fasting insulin, pmol/L	36.9 ± 8.0	Fasting insulin, pmol/L	51.2 ± 6.4
		2-h insulin, pmol/L	293.6 ± 56.2
HbA _{1c} , %	5.4 ± 0.1	HbA _{1c} , %	5.5 ± 0.1
HbA _{1c} , mmol/mol	35.9 ± 0.6	HbA _{1c} , mmol/mol	37.1 ± 1.0
		HOMA-IR	1.6 ± 0.2
HDL cholesterol, mmol/L	1.4 ± 0.1	HDL cholesterol, mmol/L	1.3 ± 0.1
LDL cholesterol, mmol/L	3.7 ± 0.2	LDL cholesterol, mmol/L	3.4 ± 0.1
Triglycerides, mmol/L	0.85 ± 0.14	Triglycerides, mmol/L	0.98 ± 0.16
Total cholesterol, mmol/L	5.3 ± 0.3	Total cholesterol, mmol/L	5.1 ± 0.2

Data are mean ± SEM unless otherwise indicated. A: Biopsies were obtained after an overnight fast. B: Biopsies were taken after an overnight fast before and during an oral glucose tolerance test. 2-h PG, plasma glucose 2 h after oral glucose ingestion; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HOMA-IR, HOMA of insulin resistance; NGT, normal glucose tolerance; SBP, systolic blood pressure. **P* < 0.05 calculated using Student *t* test.

with either normal glucose tolerance ($n = 12$) or type 2 diabetes ($n = 12$) were recruited for the glucose tolerance study. Clinical characteristics of these participants are presented in Table 1B. Study protocols were approved by the local ethics committee in Stockholm and performed according to the Declaration of Helsinki. Informed written consent was obtained from all participants. All subjects included were instructed to not participate in any physical exercise 2 days prior to the visit to the clinic. Subjects reported to the clinic at 0800 h after an overnight fast; they had been asked not to eat or drink anything but water after 2000 h the night before.

Open Muscle Biopsy and In Vitro Incubation

Open muscle biopsies were performed as previously described (27). A skeletal muscle biopsy (~ 2 g) was excised and placed in oxygenated Krebs-Henseleit buffer (KHB) containing 5 mmol/L HEPES and 0.1% BSA (RIA Grade; Sigma-Aldrich, St. Louis, MO). Smaller muscle strips were dissected from the biopsy specimen and mounted on Plexiglas clamps; placed in oxygenated KHB containing 5 mmol/L HEPES 0.1% BSA, 15 mmol/L mannitol, and 5 mmol/L glucose; and transported to the laboratory. Thereafter, muscle strips were incubated in the absence (basal) or presence of insulin (120 nmol/L) for 60 min in individual glass vials containing oxygenated KHB plus the addition of 5 mmol/L HEPES 0.1% BSA, 15 mmol/L mannitol, and 5 mmol/L glucose. Muscle strips were frozen in liquid nitrogen for subsequent methylation analysis.

Oral Glucose Tolerance Test and Skeletal Muscle Biopsy

Fasting blood samples were taken for clinical chemistry (Table 1B), and a skeletal muscle biopsy (40–100 mg) was obtained from the vastus lateralis with a Weil-Blakesley conchotome tong instrument (Agnthos, Sweden) under local anesthesia (mepivacaine hydrochloride 10 mg/mL (AstraZeneca). Biopsies were frozen and stored in liquid nitrogen. Thereafter, the subjects ingested a standardized solution containing 75 g of glucose. Two hours after the glucose ingestion, a blood sample and skeletal muscle biopsy were collected as described above.

Cell Isolation and Culture

Skeletal muscle biopsies were obtained from healthy male volunteers as previously described (28). Cell subculture was performed as described (28), with the following adaptations. Primary myoblasts were grown in DMEM/F-12 with 20% FBS, 1% penicillin-streptomycin (10,000 units/mL), and 1% Fungizone on uncoated culture flasks. At 80% confluence, the medium was changed to a fusion medium of 76% DMEM with 25 mmol/L glucose, 20% M199 (5.5 mmol/L), 2% HEPES, 1% penicillin-streptomycin (10,000 units/mL), and 1% Fungizone (all from Thermo Fisher Scientific, Waltham, MA, except the FBS from Sigma-Aldrich) with 0.03 μ g/mL zinc sulfate, 1.4 mg/mL vitamin B12 (both from Sigma-Aldrich), 100 μ g/mL

apo-transferrin (from BBI solutions, Cardiff, U.K.), and 0.286 IU/mL insulin (Novo Nordisk, Bagsværd, Denmark) for 4–5 days. Following fusion, the medium was switched to the same medium but without apo-transferrin and insulin for an additional 4–5 days (postfusion media) with 2% FBS, except for the insulin-treatment study, where no FBS was used. In the For the low/high glucose experiment, DMEM with 5.5 mmol/L glucose was used in the postfusion medium. The medium was changed every 2–3 days and prior to each experiment. Myotubes were incubated for 1 h in the absence or presence of insulin (120 nmol/L) and either harvested immediately or washed with fresh postfusion media and harvested after an additional 4 h incubation in the absence of insulin.

ATP2A3 or DAPK3 Gene Silencing in Primary Human Skeletal Muscle Cells

Myotubes were transfected (Lipofectamine RNAiMAX Transfection Reagent; Thermo Fisher Scientific) with 10 nmol/L of a nontargeting negative control small interfering RNA (siRNA) (*Silencer* Select Negative Control No. 2, catalog no. 4390847; Thermo Fisher Scientific) or siRNA directed against ATP2A3 or DAPK3 (validated *Silencer* Select siRNA s1749 or s557, respectively; Thermo Fisher Scientific). Cells were transfected twice, separated by a period of 48 h, and the final transfection was initiated 48 h prior to further experiments.

Lipid oxidation and glucose incorporation into glycogen was determined as described (28). For lipid oxidation, myotubes were exposed to serum-free postdifferentiation media containing 0.025 mmol/L palmitate and incubated in the absence or presence of 2 mmol/L 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; Toronto Research Chemicals, Toronto, CA) for 6 h. For glycogen synthesis, cells were incubated for 4 h in serum-free postfusion media prior to a 2-h incubation in fresh serum-free postfusion media in the presence or absence of 120 nmol/L insulin.

To assess signal transduction, cells were incubated in the absence or presence of 120 nmol/L insulin or 2 mmol/L AICAR for 1 h. Cells were rinsed twice in ice-cold PBS and then frozen at -20°C . mRNA analysis and description of primer oligonucleotides are detailed in Supplementary Table 1. Western blot analysis and description of antibodies are detailed in Supplementary Table 2.

DNA and mRNA Extraction

Genomic DNA and mRNA was extracted from skeletal muscle and cultured myotubes using AllPrep DNA/RNA Protein Kit or DNeasy and RNeasy kits (Qiagen, Hilden, Germany). DNA and RNA concentration was measured with NanoDrop 1000 (Thermo Fisher Scientific). Genomic DNA was bisulfite treated using EpiTect Fast Bisulfite Conversion Kit (Qiagen).

Methylation Array

Genome-wide methylation was run on the Infinium HumanMethylation450 BeadChip arrays (Illumina, San Diego, CA). Analysis was performed at the Bioinformatics

and Expression Analysis core facility at Karolinska Institutet. Beta-values were converted into M-values in R, using Bioconductor packages lumi and methylumi (29,30). Paired data were analyzed as Beta-values and then converted to M-values. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using WebGestalt as updated on 30 January 2013 (31,32). DNA pyrosequencing analysis and primer oligonucleotides are detailed in Supplementary Table 4.

mRNA Analysis

RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using SYBR Green-based primers. *HPRT1* was used as the reference gene for skeletal muscle biopsies. The geometric mean of reference genes (*B2M*, *GAPDH*, *HPRT*, and *RPLP0*) was used to compare results of experiments performed using cultured cells, except for gene-silencing experiments, where the geometric mean of *PP1B* and *TBP* were used. Primer oligonucleotides for the quantitative PCR analysis is shown in Supplementary Table 1.

Statistical Analysis

Parametric tests or nonparametric tests were used as appropriate followed by either Bonferroni post hoc correction or Benjamini-Hochberg correction (KEGG pathway analysis). Correlation analysis was determined using Spearman correlation coefficient. Analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA) or R base version 3.3.1 and open-source packages. Comparisons were considered to be statistically significant at $P < 0.05$.

RESULTS

Insulin Alters DNA Methylation in Isolated Skeletal Muscle

Genome-wide methylation was determined in isolated skeletal muscle strips incubated in the absence (basal) or presence of insulin using an Illumina 450k array. The methylation array data are deposited in the Gene Expression Omnibus (GEO accession: GSE87655). Genes showing differentially methylated CpGs were selected ($P < 0.05$). Genes showing CpGs with differences in Beta-values and M-values above 0.03 between basal and insulin-stimulated muscle were considered further (Supplementary Table 3). Insulin treatment altered methylation of over 1,600 genes on at least one CpG site. The list was further interrogated using WebGestalt (31,32). If several CpGs relating to same gene were identified, the list was curated, such that any gene associated with a significant change in CpG methylation between basal and insulin-stimulated skeletal muscle occurred only once in the list. KEGG pathway analysis revealed that insulin stimulation significantly altered DNA methylation of genes related to over 15 pathways, including genes involved in insulin signaling, metabolic pathways, and type 2 diabetes (Fig. 1).

Pyrosequencing Validation of the 450k Methylation Array

To validate the results from the 450k array, we studied four specific regions/genes of interest: *DAPK3* (cg18748062), *ATP2A3* (cg08498987), *HK1* (cg17575314), and *HDAC5* (cg16217242) (Table 2). We purchased or designed primers for pyrosequencing analysis (Supplementary Table 4). *HK1* was selected as part of the insulin signaling pathway as

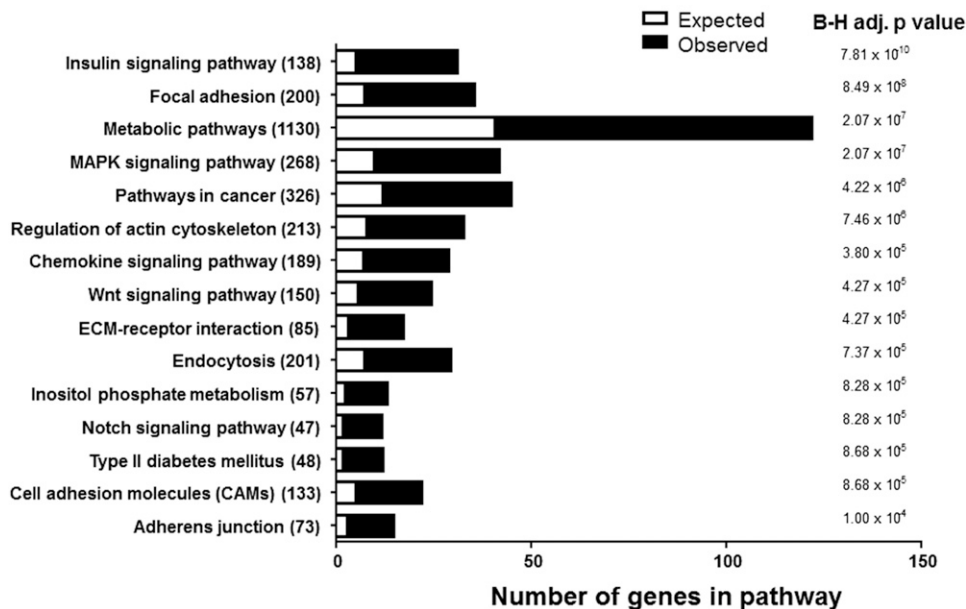


Figure 1—Genome-wide methylation analysis reveals insulin-induced DNA methylation in isolated human skeletal muscle. Genome-wide analysis showing the 15 most significantly enriched KEGG pathways (Benjamini-Hochberg adjusted [B-H adj.] P value) of genes with significant changes in DNA methylation between basal and insulin-stimulated skeletal muscle from three healthy subjects. ECM, extracellular matrix; MAPK, mitogen-activated protein kinase.

Table 2—Insulin-induced percent and fold change in DNA methylation in skeletal muscle

Gene	Target ID	Gene region	Percent change	Fold change	P value
<i>HK1</i>	cg17575314	Body	5%	1.07	0.034
<i>HDAC5</i>	cg16217242	TSS 1500	2%	1.64	0.001
<i>DAPK3</i>	cg18748062	Body	18%	1.25	0.006
<i>ATP2A3</i>	cg08498987	3' UTR	−21%	0.68	0.014

Targets selected for validation from the Illumina 450k array. Values for *HK1* and *HDAC5* are from the M-values analysis, and values for *DAPK3* and *ATP2A3* are from the Beta-values analysis. Percent and fold change in methylation is reported from the Illumina 450k array for basal versus insulin-stimulated human skeletal muscle. $n = 3$ subjects. TSS 1500, 200–1,500 bases upstream of the transcription start site. UTR, untranslated region.

revealed by the KEGG analysis. The other three sites were selected based on either the high M-value or Beta-value difference, as well as the role of each gene in metabolism. Pyrosequencing of DNA extracted from isolated skeletal muscle strips incubated in the absence or presence of insulin was performed. Consistent with results from the 450k array, methylation of one CpG site (cg08498987) in the gene body of *DAPK3* was increased in response to insulin. As pyrosequencing allows for the sequencing of regions over 100 base pairs in length, we investigated the adjacent CpGs and found that methylation differed between CpGs and that insulin impacted methylation in the whole region (Fig. 2A). We next analyzed five different CpGs in the 3' untranslated region of the *ATP2A3* gene. CpG5 corresponded to the probe on the array (cg08498987). Whereas methylation was unchanged in CpG5 in response to insulin, methylation of CpG2 and CpG3 was altered (Fig. 2B). Moreover, the methylation pattern was unaltered over five different CpGs in *HK1* in response to insulin (Fig. 2C). The level of methylation in the *HDAC5* region probed was close to the unmethylated negative control (<1% of the CpGs methylated) and therefore considered below background (data not shown). Given the change in methylation of *DAPK3* and *ATP2A3*, we next determined mRNA expression of each respective gene. Despite the changes in DNA methylation, mRNA of *DAPK3* or *ATP2A3* was unaltered in response to insulin (Fig. 2D).

To determine whether an insulin-induced methylation change occurred specifically at the studied CpG site or whether it was a more general effect over a stretch of the chromosome, we interrogated the methylation array by focusing on the genes directly adjacent to *DAPK3* (Supplementary Table 5).

DNA Methylation and mRNA in Cultured Human Skeletal Muscle Cells

DNA methylation of *DAPK3* and *ATP2A3* was determined in myotubes incubated for 1 h in the absence or presence of insulin (120 nmol/L) (Fig. 3). Myotubes were studied immediately after insulin exposure or allowed to recover in standard media for an additional 4 h. Insulin treatment did not significantly increase *DAPK3* DNA methylation in myotubes acutely ($P < 0.09$; Fig. 3A). Although *ATP2A3* methylation was consistently lower after insulin stimulation, no significant change was noted between time points (Fig. 3B). The level of methylation in basal and insulin-

stimulated myotubes was variable between donors (Fig. 3C), whereas the insulin-stimulated change in percent DNA copies methylated was more stable (Fig. 3D). mRNA of *DAPK3* and *ATP2A3* was unaltered between basal and insulin-stimulated conditions (data not shown).

Glucose Tolerance Alters DNA Methylation in Skeletal Muscle

We next measured DNA methylation of *DAPK3* and *ATP2A3* in skeletal muscle obtained from a separate cohort of men with normal glucose tolerance or type 2 diabetes before and 2 h after a glucose challenge. *ATP2A3* methylation was not altered between patients with type 2 diabetes and healthy men (data not shown). Moreover, a glucose challenge did not alter *ATP2A3* methylation (data not shown). We assessed methylation of *DAPK3* at two CpG sites (CpG1; Fig. 4A and CpG2; Fig. 4B). Methylation of *DAPK3* at CpG1 but not CpG2 was lower in men with type 2 diabetes compared with healthy control men. Moreover, a glucose challenge was associated with lower CpG1 and CpG2 methylation in *DAPK3* in both men with type 2 diabetes and healthy men. To determine whether glucose altered DNA methylation in vitro, differentiated human myotubes were incubated in the presence of 25 mmol/L glucose for 2 h and CpG1 and CpG2 methylation in *DAPK3* was assessed. Whereas methylation at the CpG1 site (Fig. 4C) was unaltered, a trend for reduced methylation was observed at the CpG2 site (Fig. 4D).

As plasma glucose was significantly different between the groups before and after the glucose tolerance test, as well as between men with type 2 diabetes and healthy men (Table 1B), we correlated percentage of *DAPK3* methylation with glucose concentration. *DAPK3* methylation at CpG1 and CpG2 was inversely correlated with the 2-h glucose concentration. However, the relationship between fasting glucose concentration and *DAPK3* methylation did not reach statistical significance (Fig. 5). BMI did not associate with *DAPK3* methylation, while waist-to-hip ratio correlated with *DAPK3* CpG1 methylation in skeletal muscle obtained 2 h after a glucose challenge ($P = 0.004$) (data not shown).

Effects of Silencing *ATP2A3* or *DAPK3* on Insulin Signaling and Metabolism in Primary Human Skeletal Muscle Cells

siRNA was used to knock down the expression of *ATP2A3* or *DAPK3* in human skeletal muscle. Subsequently, glucose

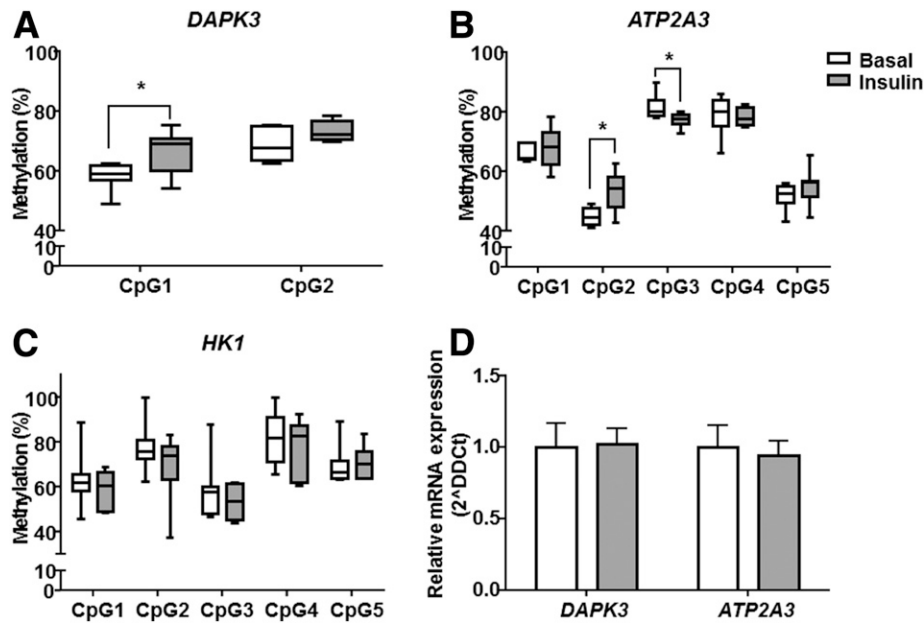


Figure 2—Effects of insulin on DNA methylation and mRNA expression in isolated human skeletal muscle. DNA methylation of *DAPK3* (A), *ATP2A3* (B), and *HK1* (C) in isolated skeletal muscle incubated for 1 h in the absence (Basal; open boxes) or presence (Insulin; filled boxes) of 120 nmol/L insulin. Whiskers in box plots indicate maximal and minimal values. D: mRNA expression of *DAPK3* and *ATP2A3* in skeletal muscle incubated for 1 h in the absence (Basal; open bars) or presence (Insulin; gray bars) of 120 nmol/L insulin. Data in panel D are mean \pm SEM. *Indicates significant pairwise comparison.

incorporation into glycogen, palmitate oxidation, and signal transduction was assessed. *ATP2A3* and *DAPK3* mRNA was reduced after gene silencing in differentiated primary human skeletal muscle cells (Fig. 6A and B). Silencing of

ATP2A3 (Fig. 6C) or *DAPK3* (Fig. 6D) did not alter basal or insulin-stimulated glucose incorporation into glycogen. Silencing *ATP2A3* (Fig. 6E) did not alter basal or AICAR-stimulated palmitate oxidation. Conversely, *DAPK3* silencing

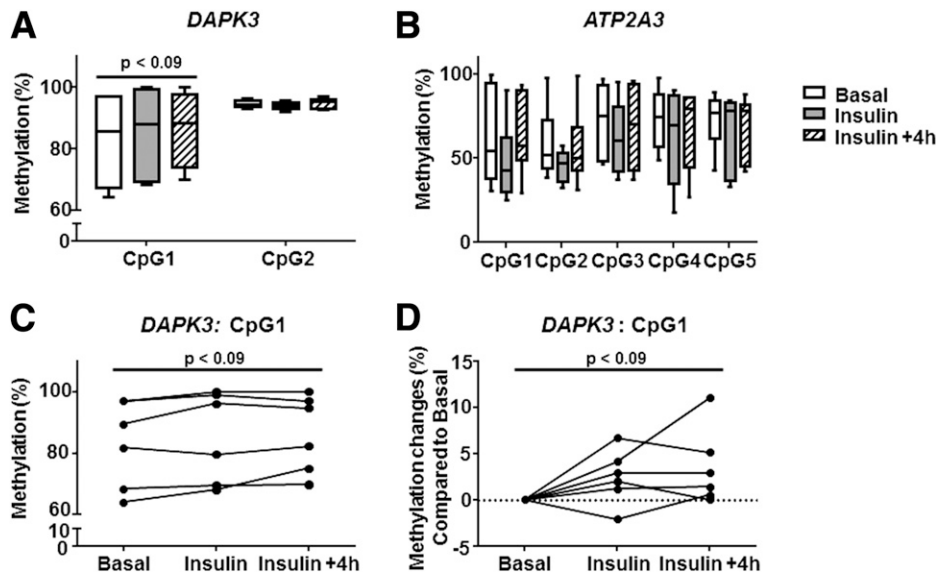


Figure 3—Effects of insulin on DNA methylation in cultured human skeletal muscle cells. Primary human skeletal muscle cultures from healthy donors were incubated for 1 h in the absence (Basal; open boxes) or presence (Insulin; gray boxes) of 120 nmol/L insulin and either harvested immediately or washed with fresh postfusion media and harvested after an additional 4 h incubation in the absence of insulin (Insulin +4h; hatched boxes). Whiskers in box plots indicate maximal and minimal values. A: Effect of insulin on *DAPK3* DNA methylation ($P < 0.09$). B: *ATP2A3* DNA methylation was unaltered between basal and insulin-stimulated conditions. Individual changes in DNA methylation of *DAPK3* at CpG1 at each time point in percentage of DNA copies methylated (C) or relative to the basal level (D) is shown. Each dot represents a subject at a specific time point.

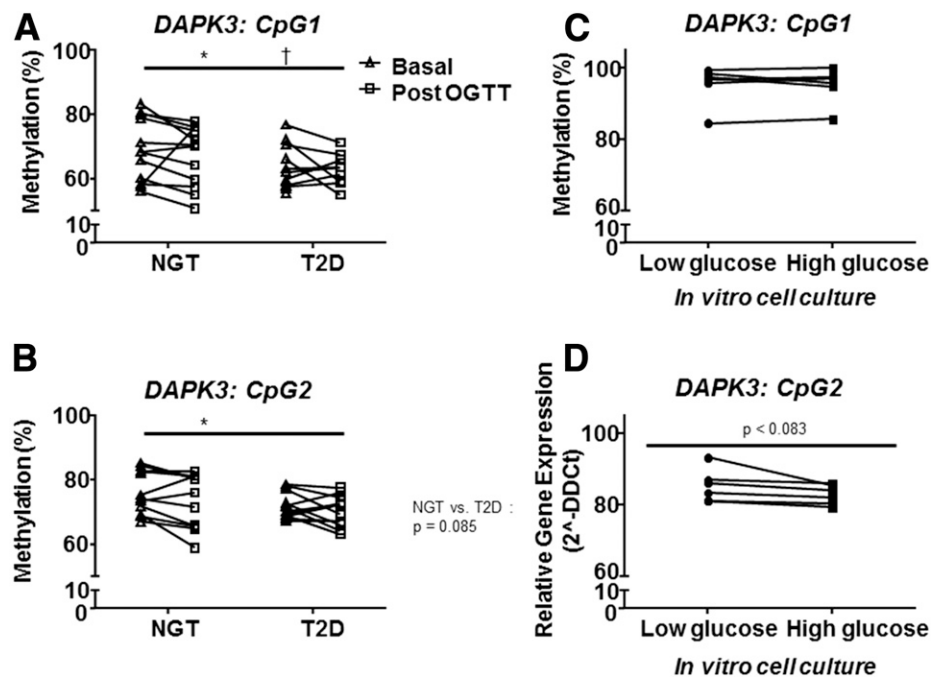


Figure 4—Effect of a glucose challenge on *DAPK3* DNA methylation in skeletal muscle from people with normal glucose tolerance or type 2 diabetes (A and B) or cultured human myotubes in vitro (C and D). Skeletal muscle biopsies were obtained from people with normal glucose tolerance (NGT) or type 2 diabetes (T2D) before (Basal; open triangles) and 2 h after (Post OGTT; open boxes) an oral glucose tolerance test (OGTT). A: *DAPK3* CpG1 methylation was reduced after the OGTT and in T2D versus NGT. B: *DAPK3* CpG2 methylation was also reduced after the OGTT, but there was no significant difference between T2D and NGT ($P < 0.09$). Glucose challenge was associated with lower *DAPK3* CpG1 methylation (A) and *DAPK3* CpG2 methylation (B) in skeletal muscle from people with NGT or T2D. Differentiated human myotubes were exposed to low (5 mmol/L) or high (25 mmol/L) glucose for 2 h. *DAPK3* CpG1 methylation was unaltered by high glucose in human myotubes (C); *DAPK3* CpG2 was unaltered by high glucose in human myotubes (D). *Indicates a main effect due to the OGTT. †Indicates a main effect of T2D.

increased basal and AICAR-stimulated palmitate oxidation (Fig. 6F).

To further explore the effects of *ATP2A3* or *DAPK3* on myotube function, we determined insulin signaling and autophagic signaling after silencing these genes. Silencing *ATP2A3* or *DAPK3* did not impact phosphorylation of protein kinase B (PKB; also known as Akt) or TBC1 domain family member 4 (TBC1D4; also known as Akt substrate of 160 kDa, AS160) in basal or insulin-stimulated myotubes (Fig. 7A–D). As *DAPK3* is involved in autophagy, we measured p62 and LC3 protein abundance. *DAPK3* silencing did not alter the protein abundance of p62 and LC3 in cultured myotubes (Fig. 7E–H). GAPDH protein abundance was not altered by gene silencing (data not shown).

DISCUSSION

Epigenetic modifications of the genome, including DNA methylation, provide a molecular basis for the interaction between genetic and environmental factors on glucose homeostasis (1). While dynamic changes in DNA methylation have been mainly studied during the narrow window of early development, evidence is emerging that diabetes, obesity, and weight loss have a dynamic effect on the epigenome (1). Changes in skeletal muscle DNA methylation in type 2 diabetes are inversely correlated

with mRNA expression of multiple genes involved in the regulation of insulin sensitivity (13,17), indicating that an epigenetic fingerprint emerges in response to metabolic dysregulation. Careful mapping of epigenetic signatures in healthy people and patients with type 2 diabetes at rest and in response to various metabolic challenges may uncover novel biomarkers and targets that can be leveraged to predict and treat insulin resistance. Here we show that insulin stimulation or oral glucose ingestion rapidly alters the DNA methylation in human skeletal muscle, supporting the notion that systemic factors can modify the epigenome in type 2 diabetes.

Type 2 diabetes and obesity are associated with genome-wide changes in DNA methylation in skeletal muscle (13,17). Interestingly, therapeutic interventions to improve insulin sensitivity including weight loss (17) and exercise training (20–22) remodel DNA methylation patterns in skeletal muscle. Moreover, exposure of cultured muscle cells to systemic factors that induce insulin resistance such as elevated lipids or cytokines or to agents that mimic the effects of exercise on glucose and lipid metabolism alter DNA methylation of metabolic genes (9,33). Thus, alterations in the extracellular milieu, including hyperglycemia, hyperinsulinemia, and elevated free fatty acids and cytokines may reprogram skeletal muscle insulin sensitivity through changes in

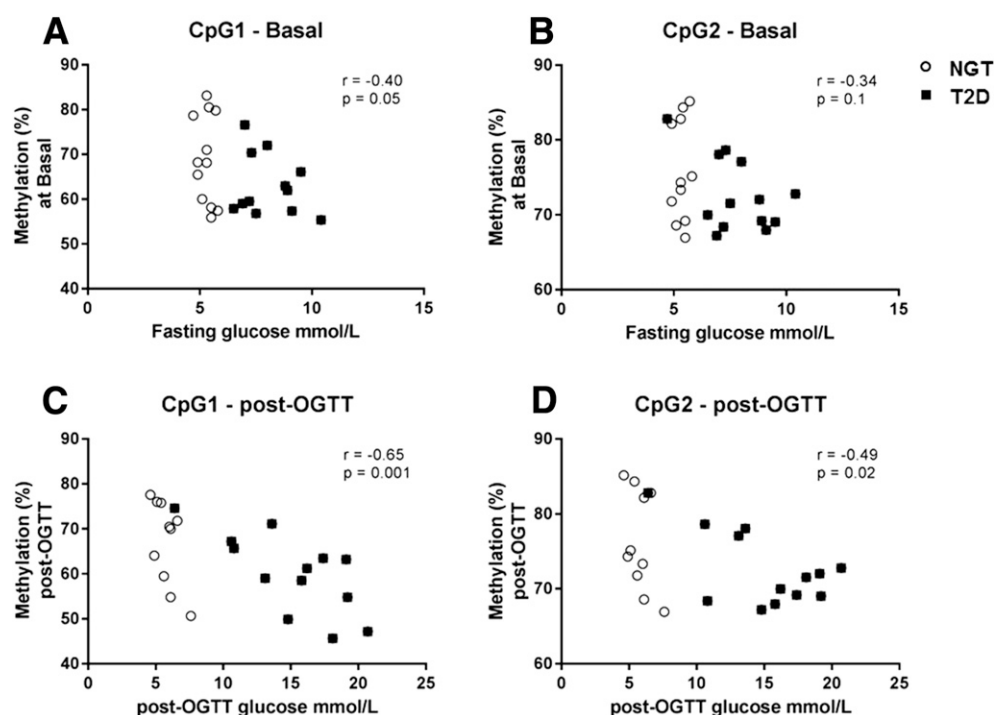


Figure 5—Spearman rank correlation coefficient between *DAPK3* DNA methylation and glucose concentration in people with normal glucose tolerance or type 2 diabetes. In men with normal glucose tolerance (NGT; open circles) and type 2 diabetes (T2D; filled squares), the relationship between fasting (Basal) glucose concentration and *DAPK3* CpG1 methylation ($P = 0.05$) (A) and *DAPK3* CpG2 methylation ($P = 0.1$) (B) did not reach statistical significance. *DAPK3* CpG1 methylation (C) and *DAPK3* CpG2 methylation (D) were inversely correlated with the 2-h glucose concentration measured during the oral glucose tolerance test (Post OGTT).

DNA methylation and gene expression profiles. Here we determined the direct effects of an acute insulin stimulation on genome-wide DNA methylation profiles in intact skeletal muscle from healthy donors incubated in vitro. We studied the epigenomic response to insulin in metabolically healthy people with normal glucose tolerance to gain insight into the development of insulin resistance. Our bioinformatic analysis of genome-wide DNA methylation revealed rapid and broad changes in CpG methylation patterns in skeletal muscle from healthy men in response to an acute insulin exposure. Furthermore, insulin-induced changes in CpG sites were identified in genes related to the insulin signaling pathway. Moreover, several pathways related to insulin action were enriched including those annotated as “metabolic pathways,” “inositol phosphate metabolism,” and “type 2 diabetes.” Thus, we reveal an insulin-induced epigenetic fingerprint in skeletal muscle on multiple pathways involved in glucose and lipid metabolism.

Our motivation for taking a genome-wide approach to assess DNA methylation was fueled in part by our desire to identify previously unrecognized insulin-responsive targets. Confirming the array results on a base-pair level, we found that insulin stimulation increased DNA methylation in the *DAPK3* gene. *DAPK3* (*ZIPK/DLK*) is a Ca^{2+} /calmodulin-regulated serine/threonine kinase that shares a high level of homology within its kinase domain with

the other two *DAPK* family members (*DAPK*), *DAPK1* and *DAPK2*. *DAPK3* plays a role in autophagy (34) and apoptosis (35). To further validate the effects of insulin on *DAPK3* methylation, we exposed primary human myotubes to insulin and assessed methylation at the identified CpG sites. We observed a trend for insulin-induced *DAPK3* methylation in primary human skeletal muscle that persisted over 4 h. Although not statistically significant, this effect mirrored the insulin-induced DNA methylation of *DAPK3* in incubated human muscle strips. Interestingly, *DAPK3* is a tumor suppressor that is frequently methylated or mutated in many cancer types (36) and is negatively regulated by Akt signaling (37). *DAPK3* is important for myosin light chain phosphorylation, leading to muscle contraction or cell mobility (34). While the role of *DAPK* family members in metabolic disease is unknown, acute exercise alters *DAPK2* phosphorylation in skeletal muscle from healthy untrained men (38).

One of the most dramatic shifts in fuel utilization throughout the day occurs during the transition between the use of lipids in the fasting state and the use of carbohydrates in the insulin-stimulated fed state. In this regard, skeletal muscle is a highly malleable tissue and can transition between these states with a high degree of metabolic flexibility (39). Moreover, activation of energy/nutrient-sensing pathways in the fasted versus fed state subsequently modifies gene expression (40). Thus, we determined whether an oral glucose challenge altered DNA

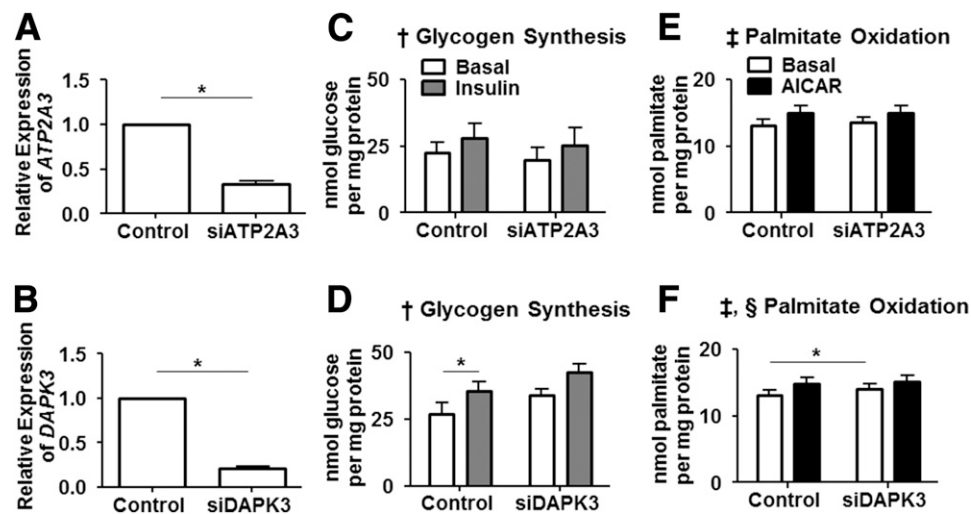


Figure 6—Effect of *ATP2A3* or *DAPK3* siRNA on glycogen synthesis and palmitate oxidation. Primary myotubes derived from people with normal glucose tolerance were transfected with siRNA against *ATP2A3* (A) or *DAPK3* (B), and mRNA expression of the respective gene was determined. Control experiments in which myotubes were transfected with a nontargeting negative control siRNA (Control) are shown for each gene as a reference. Untreated (Basal; open bars) and 120 nmol/L insulin-stimulated (Insulin; gray bars) glucose incorporation to glycogen was assessed in cultured myotubes transfected with siRNA against *ATP2A3* (C) or *DAPK3* (D). Untreated (Basal; open bars) or 2 mmol/L AICAR-stimulated (AICAR; filled bars) palmitate oxidation was assessed in cultured myotubes transfected with siRNA against *ATP2A3* (E) or *DAPK3* (F). *Indicates significant pairwise effect. †Indicates significant main effect of insulin. ‡Indicates significant main effect of AICAR. §Indicates significant main effect of silencing *DAPK3*. Results are mean \pm SEM and $n = 4$ –9 matched primary myotubes in all panels.

methylation of CpG sites in *DAPK3*. Contrasting the insulin-stimulated condition, we found that *DAPK3* DNA methylation was reduced in skeletal muscle from healthy men after the glucose tolerance test. The difference in *DAPK3* DNA methylation between the insulin- and glucose-stimulated conditions is difficult to reconcile, given that plasma insulin levels rise during a glucose challenge. However, metabolomic studies have revealed that thousands of metabolites from classes that include free fatty acids, acylcarnitines, bile acids, lysophosphatidylcholines, and amino acids are altered during an oral glucose tolerance test (41,42). Thus, the systemic milieu between the in vitro muscle incubation and the oral glucose tolerance test may account for the differences. During the glucose tolerance test, the rise in blood glucose, as well as any one of a number of metabolites, may act as a nutrient signal to suppress processes controlling *DAPK3* DNA methylation. However, we did not observe similar reductions in *DAPK3* DNA methylation in human muscle cells exposed to high glucose in vitro. Thus, other metabolites in addition to glucose may influence *DAPK3* DNA methylation. In mouse knockout models, *DAPK3* has an independent effect from ERK or Akt activation to negatively regulate mTOR-S6K-S6 signaling (43), implicating *DAPK3* in nutrient signaling pathways. We observed an inverse correlation between DNA methylation and the 2-h blood glucose concentration, suggesting dynamic regulation of the *DAPK3* gene with refeeding. Furthermore, methylation of the *DAPK3* gene at CpG1 but not CpG2 was lower in patients with type 2 diabetes. Even though the patients with type 2

diabetes are fasted, they are in a state of metabolic inflexibility, with peripheral tissues facing nutrient overload (39), which may account for the differences in DNA methylation.

We used siRNA against *DAPK3* or *ATP2A3* and assessed glucose or lipid metabolism. Silencing of either *DAPK3* or *ATP2A3* did not alter basal or insulin-stimulated glucose incorporation into glycogen in cultured myotubes. However, *DAPK3* silencing increased basal and AICAR-stimulated palmitate oxidation. Since carbohydrate ingestion acutely reduces lipid oxidation, our data suggests that glucose/carbohydrate feeding may lead to a long-lasting inhibition of lipid oxidation via reduced methylation of *DAPK3*, a gene which may normally modulate lipid oxidation, as evidenced by our siRNA experiments. Although we did not observe any changes in *DAPK3* mRNA at 2 h following the glucose challenge, changes in *DAPK3* expression may occur at a later time point. *DAPK3* has been implicated in the regulation of autophagy (34), and therefore we determined an abundance of the autophagy markers p62 and LC3. Silencing of *DAPK3* did not alter the abundance of these targets. We cannot exclude the possibility that *DAPK3* or *ATP2A3* may be involved in other aspects of metabolism and skeletal muscle physiology. For example, *ATP2A3* encodes a SERCA3 Ca^{2+} -ATPase, an intracellular pump located in the sarcoplasmic and endoplasmic reticulum that plays a role in excitation and contraction, and thus *ATP2A3* silencing may affect skeletal muscle contraction.

Pyrosequencing studies confirmed that insulin altered DNA methylation of *ATP2A3* in incubated human skeletal

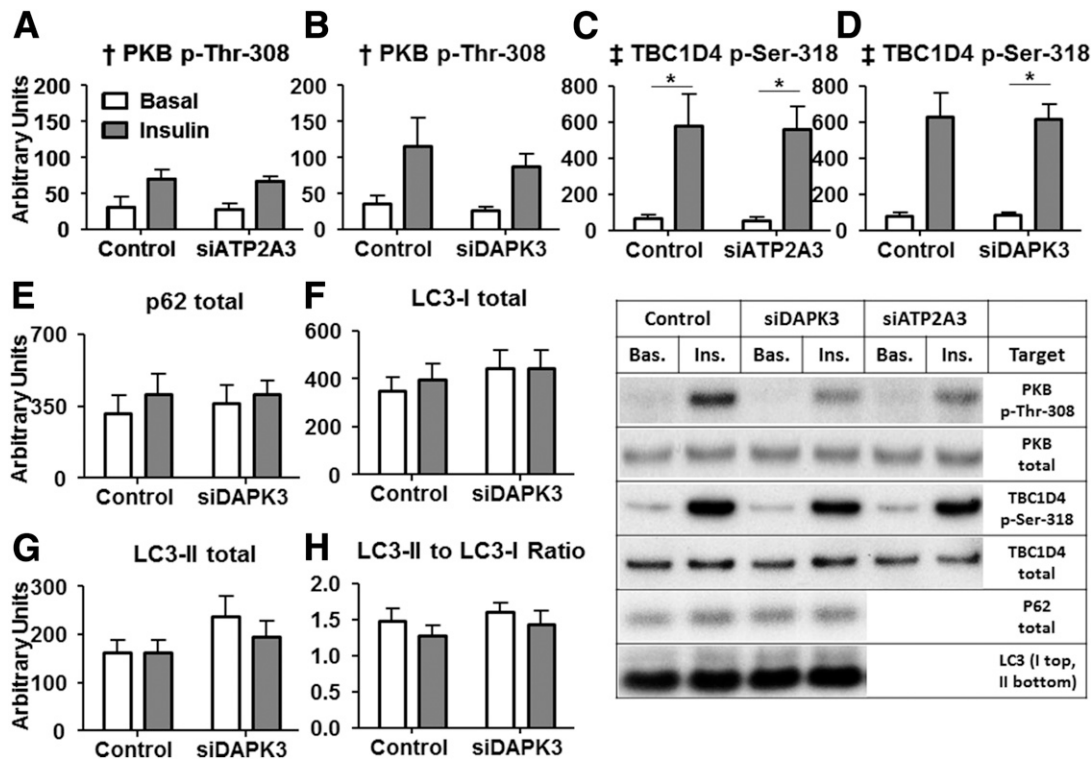


Figure 7—Effect of silencing *ATP2A3* or *DAPK3* on insulin signaling and abundance of autophagy markers. Primary myotubes derived from people with normal glucose tolerance were transfected with siRNA against *ATP2A3* or *DAPK3*. Myotubes were incubated in the absence (Basal [Bas.]; open bars) or presence of 120 nmol/L insulin (Insulin [Ins.]; gray bars) for 1-h control experiments in which myotubes were transfected with a nontargeting negative control siRNA (Control) are shown for each gene as a reference. PKB p-Thr-308 (A and B), TBC1D4 p-Ser-318 (C and D), P62 (E), LC3-I (F), LC3-II (G), and the ratio of LC3-I to LC3-II (H) were determined by Western blot analysis. *Indicates significant pairwise comparison. †Indicates significant main effect of insulin. ‡Indicates significant effect by Friedman test when assumptions of two-way repeated measures ANOVA were violated. Results are mean ± SEM, and *n* = 5–7 matched primary myotubes in all panels.

muscle from men with normal glucose tolerance. *ATP2A3* is implicated in glucose-activated β -cell regulation of calcium homeostasis in type 2 diabetes (44) and endoplasmic reticulum stress (45). Rare missense mutations in the *ATP2A3* gene are associated with type 2 diabetes (46). In cultured human muscle, we observed that insulin acutely decreased *ATP2A3* methylation, with levels normalized within 4 h after the stimulation, indicating that systemic factors play a role. Chronic hyperglycemia in rats subjected to partial pancreatectomy reduces *ATP2A3/SERCA3* mRNA, implicating that glucose may have a direct effect on gene expression (47). However, we found that *ATP2A3* DNA methylation was unaltered by a glucose challenge, and similar levels were noted between patients with type 2 diabetes and healthy subjects. Thus, insulin rather than glucose or type 2 diabetes appears to drive *ATP2A3* methylation changes.

DNA methylation can affect gene expression by altering histone interactions, influencing transcription factor binding, and/or influencing recruitment of methyl-binding proteins (48). Thus, a simple filter for analyzing genome-wide methylation and transcriptomic data is to search for inverse relationships between DNA methylation and mRNA

expression of the corresponding gene. In the current study, changes in DNA methylation did not correlate with changes in mRNA levels of *DAPK3* or *ATP2A3* in skeletal muscle at the time points investigated. However, DNA methylation may not always correlate with mRNA expression (17,18,49,50). Although epigenetic processes are one part of the complex system of regulation of gene transcription and a localized CpG methylation might be insufficient to control gene expression, changes in mRNA could occur at later time points than investigated in the current study.

In conclusion, insulin acutely alters the DNA methylation profile of human skeletal muscle. These findings advance the notion that DNA methylation is a rapidly adaptive epigenetic mark in somatic cells. Acute insulin exposure altered DNA methylation of *DAPK3* and *ATP2A3*, genes involved in apoptosis and endoplasmic reticulum stress, respectively. *ATP2A3* was unaffected by glucose challenge or type 2 diabetes, indicating that insulin has a direct role. In contrast, *DAPK3* DNA methylation was altered in type 2 diabetes and after an oral glucose challenge, with levels of DNA methylation inversely correlated with glucose levels. Moreover, insulin and glucose modulate skeletal muscle *DAPK3* DNA methylation in a

reciprocal manner, suggesting a feedback control on gene expression. Continued efforts to establish the epigenomic response to systemic factors associated with type 2 diabetes in metabolically healthy people with normal glucose tolerance will be useful to better predict genomic landscape involved in the development of skeletal muscle insulin resistance.

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