

# Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle

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## SUMMARY

DNA methylation is a covalent biochemical modification controlling chromatin structure and gene expression. Exercise elicits gene expression changes that trigger structural and metabolic adaptations in skeletal muscle. We determined whether DNA methylation plays a role in exercise-induced gene expression. Whole genome methylation was decreased in skeletal muscle biopsies obtained from healthy sedentary men and women after acute exercise. Exercise induced a dose-dependent expression of *PGC-1 $\alpha$* , *PDK4*, and *PPAR- $\delta$* , together with a marked hypomethylation on each respective promoter. Similarly, promoter methylation of *PGC-1 $\alpha$* , *PDK4*, and *PPAR- $\delta$*  was markedly decreased in mouse *soleus* muscles 45 min after *ex vivo* contraction. In L6 myotubes, caffeine exposure induced gene hypomethylation in parallel with an increase in the respective mRNA content. Collectively, our results provide evidence that acute gene activation is associated with a dynamic change in DNA methylation in skeletal muscle and suggest that DNA hypomethylation is an early event in contraction-induced gene activation.

## INTRODUCTION

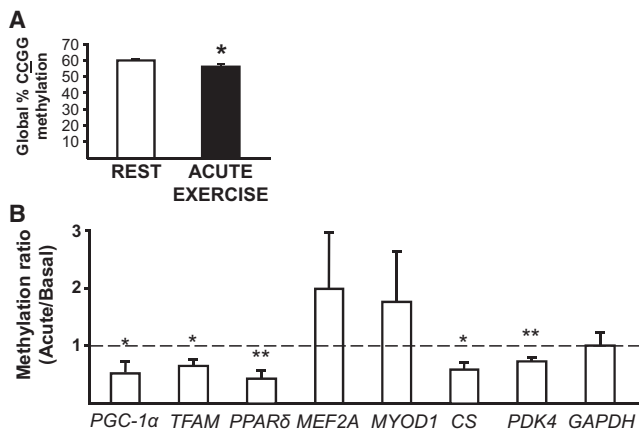
The regulation of gene expression is a fundamental process that establishes and impacts the phenotype of each tissue. Although the genetic code is identical in all cells of an organism, each cell type possesses its own gene expression pattern, driven by a specific epigenetic signature. DNA methylation is a major epigenetic modification that suppresses gene expression by modulating the access of the transcription machinery to the chromatin or by recruiting methyl binding proteins (Cedar and Bergman, 2009).

DNA methylation is generally thought to be mitotically stable. Consequently, environmental factors have been disregarded as driving substantial and sustained changes in DNA methylation

patterns in adult tissues. However, several studies support the notion that environmentally induced changes in DNA methylation patterns throughout life influence gene-expression signatures. For example, the naturally occurring short-chain fatty acid butyrate acutely alters histone deacetylase activity and DNA methylation status in normal (Parker et al., 1986) and cancer-cell lines (de Haan et al., 1986; Stoddart et al., 1989). Moreover, acute exposure of cultured human myotubes to either palmitate or oleate increases promoter methylation of the mitochondrial protein peroxisome proliferator-activated receptor gamma, coactivator 1  $\alpha$  (*PGC-1 $\alpha$* ) (Barrès et al., 2009). Evidence is emerging that epigenetic modifications through DNA methylation contribute to the increased risk and development of metabolic disease by modifying the expression of genes controlling whole body energy and glucose homeostasis (Barrès et al., 2009; Klose and Bird, 2006).

Skeletal muscle is distinguished by a high degree of plasticity in its adaptive response to environmental stressors that challenge the structural and metabolic demands of the tissue. Muscle contraction through physical exercise drives adaptive responses to improve metabolic efficiency, oxidative capacity, and contractile activity by altering gene expression profiles and protein levels (Coffey and Hawley, 2007). Exercise increases the messenger RNA (mRNA) expression and protein levels of a plethora of genes regulating mitochondrial function and fuel usage, including *PGC-1 $\alpha$* , transcription factor A, mitochondrial (*TFAM*); peroxisome proliferator-activated receptor  $\delta$  (*PPAR- $\delta$* ); and pyruvate dehydrogenase kinase, isoenzyme 4 (*PDK4*) (Egan et al., 2010; Fritz et al., 2006; Pilegaard et al., 2000; Pilegaard et al., 2003; Short et al., 2003). It remains unknown whether DNA methylation controls these genomic responses. Moreover, the unifying trigger that orchestrates the genomic response to exercise is incompletely defined. Working independently or synergistically, potential mechanisms include changes in calcium flux, the AMP:ATP ratio, or oxidative stress (Coffey and Hawley, 2007).

We tested the hypothesis that exercise, a physiological stressor that is known to alter whole body energy and glucose homeostasis, rapidly alters DNA methylation in skeletal muscle. The effects of a single, acute bout of exercise was studied using methylated DNA capture, followed by quantitative PCR (qPCR)



**Figure 1. Acute Exercise Remodels DNA Methylation**

(A) LUMA analysis of global DNA methylation. Global CpG methylation analysis of DNA extracted from muscle at baseline (REST) or 20 min after acute exercise (ACUTE EXERCISE). Results are mean  $\pm$  SE. \* $p < 0.05$  versus REST. (B) Promoter-specific analysis of methylation levels. Methylated DNA Immunoprecipitation followed by quantitative PCR analysis (MeDIP-qPCR) was performed. Ratio between methylated levels at rest and acute exercise is shown. Dashed line symbolically delimitate an equal quantity of methylation at rest and after acute exercise. Results are mean  $\pm$  SEM for  $n = 14$  subjects. \* $p < 0.05$ , \*\* $p < 0.01$ .

and bisulfite sequencing. We provide evidence that exercise-induced gene induction is associated with transient alterations in promoter methylation.

## RESULTS

### Acute Exercise Alters Global and Gene-Specific Promoter Methylation

Although acute exercise alters skeletal muscle mRNA and protein levels of genes involved in fuel utilization and mitochondrial function (Coffey and Hawley, 2007; Pilegaard et al., 2000), the mechanism remains unknown. To determine the effect of exercise on DNA methylation, we first analyzed global DNA methylation levels in biopsies of vastus lateralis skeletal muscle obtained from 14 healthy, young ( $25 \pm 1$  years), sedentary men and women before and after an acute bout of exercise. Clinical characteristics are presented in Table S1 (available online). The luminometric methylation assay (LUMA) interrogates methylation of the inner cytosine of all CCGG sites within the genome (Karimi et al., 2006). As assessed by LUMA, global methylation decreased after acute exercise (Figure 1A). Global DNA methylation changes did not correlate with hemoglobin mRNA content ( $R = 0.032$ ,  $p = 0.753$ ), excluding the possibility that blood contamination in the skeletal muscle biopsy contributed to the decreased global methylation observed after acute exercise.

To determine whether exercise-induced alterations in DNA methylation are truly global (i.e., all the genes are affected) or gene-specific, we studied a list of genes exerting different metabolic and structural functions in skeletal muscle. We evaluated the effect of acute exercise on DNA methylation levels of genes previously described to be differentially methylated in type 2 diabetes (Barrès et al., 2009) and whose transcript abundance is elevated after exercise (*PGC-1 $\alpha$* , *TFAM*, *PPAR- $\delta$* , *PDK4*, citrate

synthase [*CS*]), as well as factors involved in expression of muscle-specific genes (myocyte enhancer factor 2A [*MEF2A*], myogenic differentiation 1 [*MYOD1*] and one housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*]). Using methylated DNA Immunoprecipitation (MeDIP) followed by quantitative PCR (qPCR), we found that captured methylated promoters for metabolic genes were lower after acute exercise, whereas muscle-specific transcription factors, including *MYOD1* and *MEF2A*, as well *GAPDH* (Figure 1B), were unchanged. Collectively, our results provide evidence to suggest that acute exercise induces gene-specific DNA hypomethylation in human skeletal muscle.

### Exercise Intensity-Dependent Decrease in Promoter Methylation and Associated Gene Activation

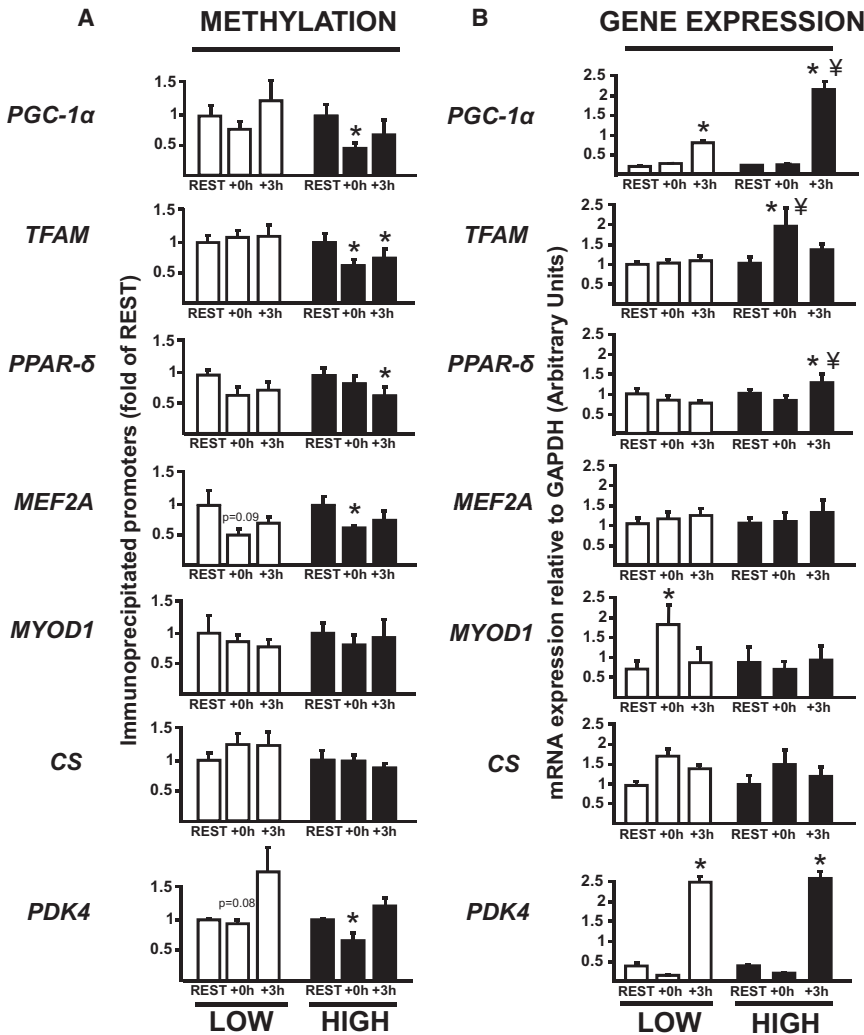
Aerobic exercise intensity drives gene transcription in a dose-dependent manner (Egan et al., 2010). Thus, we determined whether the exercise-induced decrease in promoter methylation was dependent on exercise intensity. A dose-response and time-course analysis of DNA methylation after an acute exercise was performed in biopsies of vastus lateralis skeletal muscle from a separate cohort of eight young healthy sedentary men, described earlier (Egan et al., 2010). Skeletal muscle biopsies were obtained before, immediately after, and 3 hr after an acute exercise bout at either 40% (low-intensity) or 80% (high-intensity) of maximal aerobic capacity, and DNA methylation levels were determined by MeDIP, followed by qPCR. High-intensity exercise markedly reduced promoter methylation of *PGC-1 $\alpha$* , *TFAM*, *MEF2A*, and *PDK4* immediately after exercise, whereas *PPAR- $\delta$*  methylation was decreased 3 hr after exercise (Figure 2A). Analysis of the corresponding mRNA expression revealed that decreases in DNA methylation were associated with elevations in relative mRNA levels either at the same or the next time point (Figures 2A and 2B). The decrease in DNA methylation, as assessed by MeDIP-PCR, was confirmed by bisulfite sequencing of the *PGC-1 $\alpha$*  promoter (Figure S1). Our results suggest that DNA methylation is a component of the exercise-induced effect on expression of these genes.

### Ex Vivo Muscle Contraction Induces Hypomethylation

Intracellular pathways associated with muscle contraction and exercise-induced gene expression can be activated by neurotransmitters and circulating factors. Thus, we studied isolated mouse *soleus* muscle to determine if extracellular factors are involved in exercise-induced hypomethylation. Gene expression of *Pgc-1 $\alpha$* , *Ppar- $\delta$* , and *Pdk4* increased 180 min after ex vivo contraction (Figure 3A). Promoter methylation of *Pgc-1 $\alpha$* , *Ppar- $\delta$* , *Pdk4*, *Myod1*, and *Mef2a* decreased 45 min after contraction (Figure 3B). Thus, contraction-induced gene expression is associated with promoter-methylation remodeling, independent of exercise-induced changes in neurotransmitters or circulating factors.

### Caffeine Induces Promoter Hypomethylation in Rat Myotubes

The amplitude of gene transcription in skeletal muscle in response to exercise is orchestrated by primary messengers such as changes in the AMP:ATP ratio, calcium release from the endoplasmic reticulum, or the intracellular redox state



**Figure 2. Exercise-Induced Promoter Hypomethylation Is Intensity-Dependent.**

(A and B) Promoter methylation (A) and respective mRNA level of genes (B) involved in fuel utilization and mitochondrial biogenesis as measured by MeDIP-qPCR at REST, 0 hr, or 3 hr after low- or high-intensity acute exercise (light or dark bars, respectively); n = 8 subjects. Results are mean ± SEM. \*p < 0.05 versus REST, †p < 0.05 versus low-intensity exercise. mRNA expression of *PGC-1α* in this cohort has been reported previously (Egan et al., 2010) but is shown here for comparative purposes against DNA methylation.

expression of *Pgc-1α*, *Tfam*, *Mef2a*, and *Cs*, but not *Pdk4* (Figure 4C). Promoter methylation was unaltered by ionomycin (Figure 4C). Activation of AMP kinase (using aminoimidazole-carboxamide-ribose nucleotide [AICAR]) did not affect promoter methylation. Conversely, reactive oxygen species (ROS) production (induced by H<sub>2</sub>O<sub>2</sub>) elicited hypermethylation (Figure S2). Thus, calcium release is necessary but not sufficient to induce DNA hypomethylation.

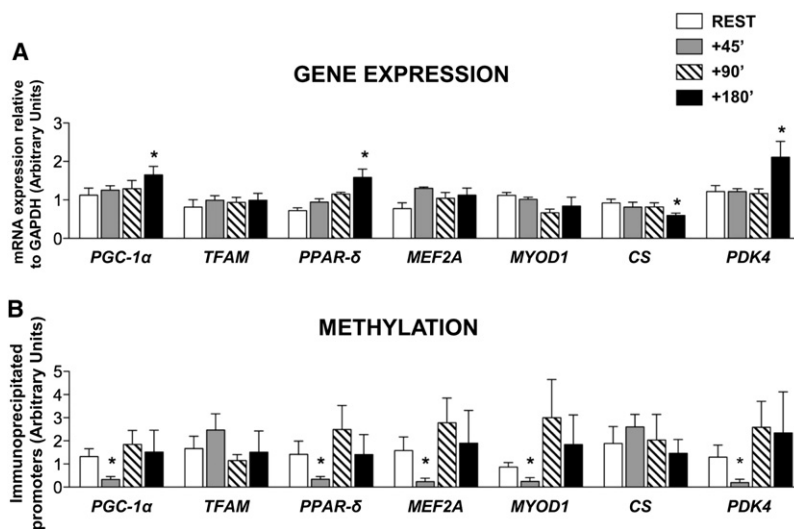
**DISCUSSION**

We determined the effect of exercise on DNA methylation in human skeletal muscle and provide evidence that acute exercise alters promoter methylation of exercise-responsive genes in a dose-dependent manner. Using isolated contracting muscle and cultured myotubes, we show DNA methylation remodeling

(Coffey and Hawley, 2007). By increasing the elevation of cytoplasmic Ca<sup>2+</sup> levels, caffeine mimics exercise-induced expression of genes related to mitochondrial function in L6 myotubes (Ojuka et al., 2003). We performed a time-course study in L6 myotubes to determine whether changes in gene expression are associated with a promoter demethylation. *Pgc-1α*, *Tfam*, *Mef2a*, *Cs*, and *Pdk4* mRNA expression was elevated upon exposure to caffeine (Figure 4A). Caffeine exposure decreased promoter methylation of *Pgc-1α*, *Tfam*, *Mef2a*, *Cs*, and *Pdk4*, as measured by methylcytosine capture followed by qPCR. Promoter methylation of *Pgc-1α*, *Tfam*, *Mef2a*, and *Cs*, but not *Pdk4*, decreased prior to gene expression changes (Figure 4A). Dantrolene blocks caffeine-induced gene expression by inhibiting Ca<sup>2+</sup> release from the sarcoplasmic reticulum (Ojuka et al., 2002; Van Winkle, 1976). Coincubation of L6 myotubes with caffeine and dantrolene markedly inhibited gene expression and suppressed promoter hypomethylation (Figure 4B), implicating the involvement of Ca<sup>2+</sup> release. L6 myotubes were also incubated in the presence of the Ca<sup>2+</sup> ionophore ionomycin (1 μM). Consistent with previous results (Kusuhara et al., 2007; Ojuka et al., 2002), ionomycin increased mRNA

parallels changes in mRNA expression. Acute gene activation is associated with a dynamic change in DNA methylation in skeletal muscle and suggests that DNA hypomethylation is an early event in contraction-induced gene expression.

DNA methylation is thought to be mitotically stable, and hence environmental factors were assumed to be unlikely candidates to induce substantial and sustained changes in DNA methylation in adult tissues (Reik et al., 2001). Nevertheless, indirect evidence for dynamic changes in DNA methylation has emerged from studies determining the effects of diets deficient in the methyl-donor folic acid (Van den Veyver, 2002). Rapid cycling in DNA methylation of the pS2/TFF1 gene promoter upon estrogen activation of breast cancer cells provides additional evidence that DNA methylation is dynamic (Kangaspeska et al., 2008; Métivier et al., 2008). DNA methyl transferases 3A and 3B (DNMT3A and DNMT3B, also called de novo methyl transferases) possess demethylase activity, but the biochemical sequence is unknown (Kangaspeska et al., 2008; Métivier et al., 2008). One proposed mechanism for exercise-induced demethylation is through hydroxylation of the methyl group on the 5-position of the cytosine (5-hydroxyl methyl), which is an



**Figure 3. Muscle Contraction Induces Promoter Hypomethylation**

(A and B) Ex vivo contracted or rested (REST) soleus muscle was incubated for 45 (+ 45'), 90 (+ 90') or 180 min (+ 180'). Gene expression (A) and promoter methylation (B) was analyzed. Results are mean ± SEM. \*p < 0.05 versus BASAL.

intermediate for demethylation. The CXXC domain-containing enzyme TET1 has been described to participate in DNA demethylation through modifying 5'-hydroxylase activity (Zhang et al., 2010). The bisulfite modification technique used in this study does not distinguish between methylated and hydroxyl methylated cytosines (Huang et al., 2010). Thus, the decrease in DNA methylation measured after acute exercise may occur from a loss of methyl groups rather than hydroxylation, suggesting that rapid demethylation has occurred. However, an increase in hydroxymethylcytosine content at promoter regions was not detected after caffeine exposure (data not shown), which may suggest that demethylation through hydroxylation is not involved in exercise-induced DNA demethylation. Nevertheless, the use of a distinct technical approach relying on methylated cytosine capture further supports our observation for acute exercise-induced DNA demethylation.

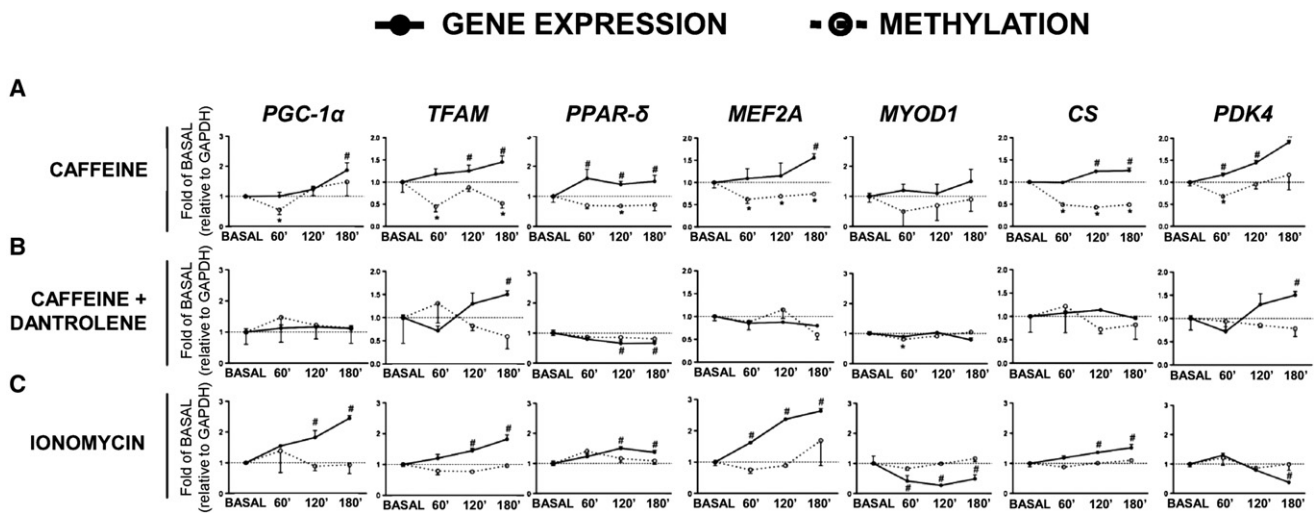
Bisulfite sequencing revealed that acute exercise mainly altered cytosine residues located within a CpA, CpT, or CpC context. Evidence has emerged to support a physiological role of non-CpG methylation (Lister et al., 2009; Ramsahoye et al., 2000; Woodcock et al., 1988), notably in stems cells, where drastic remodeling of the epigenome occurs (Bird, 2007). We have shown that systemic factors associated with insulin resistance, including elevated levels of free fatty acids or the cytokine TNF- $\alpha$ , acutely induced non-CpG methylation in human myocytes (Barrès et al., 2009). Non-CpG methylation may play a specialized role as compared to CpG methylation in mediating transient or rapid methylation.

We found that DNA methylation was unaltered 48 hr after a 3-week exercise training program, whereas RNA expression of *PGC-1 $\alpha$*  and *TFAM* promoters was elevated (data not shown), further suggesting that DNA hypomethylation is a transient mechanism involved in mRNA synthesis. Changes in DNA methylation after acute exercise are inversely associated with gene activation of some but not all genes studied here (Table S2). For instance, *PGC-1 $\alpha$*  mRNA expression was increased 3 hr after low-intensity exercise, whereas methylation was unchanged. Although we cannot exclude the possibility that changes in promoter methylation at this time point were below the limit

of sensitivity of the assay, exercise-induced DNA hypomethylation is an unlikely prerequisite for the activation of transcription. Our findings that ionomycin, AICAR, or ROS production increased mRNA expression without altering promoter methylation may support the notion that DNA methylation does not exclusively control exercise-induced gene expression. The exact role of exercise-induced DNA hypomethylation in vivo may be tested by selectively blocking the enzymatic machinery involved but the precise demethylation machinery is currently unknown, thus limiting further investigation into this potential mechanism. Our finding of gene-specificity in exercise-induced DNA hypomethylation suggests that methylation may serve as a selective mechanism to orchestrate the activation of a subset of genes but, clearly, other mechanisms, such as transcription factor activation and recruitment to the chromatin, are likely to be involved.

Regular physical activity reduces the risk for cardiovascular diseases, type 2 diabetes, multiple cancers, depression, obesity, and musculoskeletal diseases (Thune et al., 1997). Muscle contraction causes a plethora of intracellular perturbations that disturb metabolic homeostasis that, in turn, promote adaptive responses, including changes in mRNA and protein levels. The increase in cytosolic Ca<sup>2+</sup> following muscle contraction is an early intracellular signal that provokes changes in mRNA and the adaptive response to exercise training. We provide evidence that caffeine exposure decreased promoter methylation of *Pgc-1 $\alpha$* , *Tfam*, *Mef2a*, *Cs*, and *Pdk4* in cultured myotubes, and this effect was blocked by dantrolene, an inhibitor of Ca<sup>2+</sup> release. Ionomycin, a calcium release activator, induced gene expression without altering promoter hypomethylation, suggesting that Ca<sup>2+</sup> release is necessary but not sufficient to promote DNA hypomethylation. Consequently, caffeine may also induce Ca<sup>2+</sup>-independent mechanisms that participate in promoter hypomethylation. Notably, neither AICAR-induced AMPK activation nor ROS production were involved in promoter hypomethylation. Although mechanical stress, neural input, or circulating hormones alter skeletal muscle gene expression after exercise (Egan et al., 2010; Fritz et al., 2006; Pilegaard et al., 2000, 2003; Short et al., 2003), disturbances in intracellular homeostasis are sufficient to induce DNA methylation remodeling.

In conclusion, acute exercise leads to transient changes in DNA methylation in adult skeletal muscle. Our finding that the patterns of DNA methylation change in differentiated nondividing somatic cells provides further evidence that the epigenetic marks across the genome are subject to more dynamic variations than previously appreciated.



**Figure 4. Caffeine-Induced Gene Expression Is Associated with Promoter Hypomethylation in Differentiated L6 Myotubes**

(A–C) Promoter methylation (hatched line) and mRNA levels (solid line) were measured after exposure to 5 mM caffeine (A), 5 mM caffeine (B), and 10  $\mu$ M dantrolene or 1  $\mu$ M ionomycin (C). Results are mean  $\pm$  SE. #*P* < 0.05 versus BASAL for mRNA. \**P* < 0.05 versus BASAL for promoter methylation.

## EXPERIMENTAL PROCEDURES

### Study Participants

Experiments were performed with approval from the local ethical committee. All studies were performed according to the declaration of Helsinki. Informed written consent was obtained from all participants before testing was initiated. Two separate cohorts were used.

Clinical characteristics of the acute exercise cohort (*n* = 14 males and females) are presented in Table S1. Subjects were asked to complete a peak pulmonary oxygen uptake rate ( $\text{VO}_2$  peak) test under fasting conditions. This test consisted of incremental exercise to volitional fatigue on an electromagnetically braked cycle ergometer (SensorMedics). Muscle biopsies were collected 20 min after cessation of exercise. The exercise intensity cohort included eight sedentary males who were required to complete two isocaloric acute exercise trials at 40% (low-intensity) and 80% (high-intensity)  $\text{VO}_{2\text{peak}}$  on separate occasions and in random order separated by at least 1 week. Muscle biopsies from this cohort have been studied earlier for exercise intensity-dependent regulation of *PGC-1 $\alpha$*  mRNA abundance and activation of upstream signaling kinases (Egan et al., 2010). Subjects reported to the laboratory after an overnight fast and a resting muscle biopsy was taken (rest). Subjects then consumed a high carbohydrate breakfast and remained in the laboratory for 4 hr, at which point they started the exercise bout on a stationary ergometer (cadence at 70–75 rpm) and continued until 1,674 kJ were expended, as determined by indirect calorimetry monitored on a minute-by-minute basis. Muscle biopsies were taken immediately (+ 0 hr) and 3 hr after exercise (+ 3 hr).

### Skeletal Muscle Biopsy

Skeletal muscle biopsies (50–100 mg) were obtained under local anesthesia (lidocaine hydrochloride, 5 mg/mL) from the vastus lateralis portion of the quadriceps femoris using a Weils-Blakesley contochoime and were immediately frozen and stored in liquid nitrogen.

### Methylated DNA Immunoprecipitation and Methylcytosine Capture: qPCR

Purified genomic DNA prepared from cultured cells and tissue was digested overnight with AluI restriction enzyme (New England Biolabs Ipswich, MA) for MeDIP or sheared using a sonicator (Branson Ultrasonics Corporation, Danbury, CT) for methylcytosine capture (Methylminer, Invitrogen) to obtain fragments of 256 base-pairs on average. Digested DNA (4  $\mu$ g) was used for a standard MeDIP assay as described (Weber et al., 2005) and 1–2  $\mu$ g for methylcytosine capture. For MeDIP, DNA was immunoprecipitated using

10  $\mu$ g of monoclonal antibody against 5-methylcytidine (Eurogentec, Liège, Belgium) in 300  $\mu$ l IP buffer (10 mM sodium phosphate [pH 7.0], 140 mM NaCl, 0.05% Triton X-100) for 5 hr at 4°C and then washed 3 times with 800  $\mu$ l IP buffer. Immunoprecipitated DNA was recovered with proteinase K digestion followed by column based-purification (DNA wizard, Promega, Fitchburg, WI). Recovered DNA fractions were diluted 1/50 and measured using RT-PCR with an ABI PRISM 7000 sequence detector system and fluorescence-based SYBR-green technology (Applied Biosystems, Carlsbad, CA). The primers used in this study are described in Supplemental Information.

### Nucleic Acid Purification and RT-PCR

DNA from 10–20 mg of vastus lateralis muscle was extracted using DNeasy Blood & Tissue columns (QIAGEN, Hilden, Germany). The total amount of DNA recovered was determined by spectrophotometry. For RNA extraction, 10 mg of skeletal muscle tissue was homogenized in 1 ml of Trizol reagent (Sigma-Aldrich, Brønby, Denmark), and RNA was purified according to recommendations of the manufacturer. The RNAs from cultured cells were also purified using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was used as a template for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen) with random hexamers. cDNA quantity was measured using RT-PCR with the ABI PRISM 7000 sequence detector system and fluorescence-based SYBR-green technology (Applied Biosystems). PCR was performed in a final volume of 25  $\mu$ l, consisting of diluted cDNA sample, 1  $\times$  SYBR-green PCR Master Mix (Applied Biosystems), primers optimized for each target gene, and nuclease-free water. Samples were analyzed in duplicate. Primers were designed using Primer Express computer software (Applied Biosystems).

### Bisulfite Sequencing

Bisulfite treatment was performed as described (Olek et al., 1996), with the following adaptations: 1  $\mu$ g of genomic DNA was embedded in a 2% Low Melting Point Agarose solution and 10 beads were formed. A freshly prepared bisulfite solution (4 M sodium betabisulfite, Sigma; 250 mM hydroquinone, Sigma; pH 5.0) was added to each reaction tube containing one single bead. The reaction mixtures were incubated for 4 hr at 50°C under exclusion of light. Treatment was stopped by equilibrations against 1 ml of Tris-EDTA (TE) (4  $\times$  15 min) followed by desulphonation in 500  $\mu$ l of 0.2 M NaOH (2  $\times$  15 min). The reaction was neutralized and beads were washed with 1 ml TE (2 $\times$ 15 min). Prior to PCR, beads were equilibrated against 1 ml of ddH<sub>2</sub>O (2  $\times$  30 min). PCR fragments were purified from an agarose gel (MinElute Gel Extraction Kit; QIAGEN) and cloned into pDrive vector using PCR Cloning Kit (QIAGEN), according the manufacturer's protocol. Individual clones were

grown and plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN). For each subject, 10–50 clones were sequenced using T7 promoter primer on an ABI 3730xl DNA Analyzer platform at Cogenics (Hope End, UK).

### Luminometric Methylation Assay

For LUMA interrogation of CpG methylation within the CCGG sequence, experimental conditions are described (Karimi et al., 2006). Restriction enzymes (HpaII, MspI, and EcoRI) and Tango® buffer (33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.1 mg/ml BSA) was purchased from Fermentas (Fermentas Scandinavia, Stockholm). The PSQ 96 SNP reagents for pyrosequencing were from Biotage (Biotage AB, Uppsala, Sweden). Genomic DNA (500–1,000 ng) was submitted to a double digestion using either HpaII+EcoRI or MspI+EcoRI in Tango® buffer, 4 hr at 37°C. Pyrosequencing annealing buffer was then added to each reaction and samples were analyzed using a pyrosequencer machine with an assay sequence defined as AC/TCGA. The percentage of CCGG methylation level was calculated with the following equation:  $[1 - (HpaII/EcoRI)/(MspI/EcoRI)] \times 100$ .

### Ex Vivo Incubation of Isolated Soleus Muscle

Experiments were approved by the regional animal ethical committee. Soleus muscles were removed from anesthetized and fed female C57BL/6 mice and incubated at 30°C for 10 min as previously described (Treebak et al., 2007) in oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs-Henseleit bicarbonate buffer (containing 8 mM mannitol, 5 mM glucose, and 0.1% BSA) in a Myograph system (DMT A/S, Denmark). Muscles were incubated under control conditions or stimulated to contract as follows: 0.3 s trains (25 Hz, 0.1 ms impulse) repeated every 1 s for 5 min. This protocol was repeated every 10 min for a total stimulation time of 60 min. Thereafter, muscles recovered in new media for 0, 45, 90, or 180 min and frozen in liquid nitrogen.

### Statistics

Results are mean ± SEM. Data were tested for normal distribution using the Skewness and Kurtosis test in SPSS Statistics Software 17.0. Statistical differences were determined using a two-tailed paired Student's t-test.  $p < 0.05$  was considered significant.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at doi:10.1016/j.cmet.2012.01.001.

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