The microRNA Signature in Response to Insulin Reveals Its Implication in the Transcriptional Action of Insulin in Human Skeletal Muscle and the Role of a Sterol Regulatory Element-Binding Protein-1c/Myocyte Enhancer Factor 2C Pathway

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OBJECTIVE—Factors governing microRNA expressions in response to changes of cellular environment are still largely unknown. Our aim was to determine whether insulin, the major hormone controlling whole-body energy homeostasis, is involved in the regulation of microRNA expressions in human skeletal muscle.

RESEARCH DESIGN AND METHODS—We carried out comparative microRNA (miRNA) expression profiles in human skeletal muscle biopsies before and after a 3-h euglycemic-hyperinsulinemic clamp, with TaqMan low-density arrays. Then, using DNA microarrays, we determined the response to insulin of the miRNA putative target genes in order to determine their role in the transcriptional action of insulin. We further characterized the mechanism of action of insulin on two representative miRNAs, miR-1 and miR-133a, in human muscle cells.

RESULTS—Insulin downregulated the expressions of 39 distinct miRNAs in human skeletal muscle. Their potential target miRNAs coded for proteins that were mainly involved in insulin signaling and ubiquitination-mediated proteolysis. Bioinformatic analysis suggested that combinations of different downregulated miRNAs worked in concert to regulate gene expressions in response to insulin. We further demonstrated that sterol regulatory element–binding protein (SREBP)-1c and myocyte enhancer factor 2C were involved in the effect of insulin on miR-1 and miR-133a expression. Interestingly, we found an impaired regulation of miRNAs by insulin in the skeletal muscle of type 2 diabetic patients, likely as consequences of altered SREBP-1c activation.

CONCLUSIONS—This work demonstrates a new role of insulin in the regulation of miRNAs in human skeletal muscle and suggests a possible implication of these new modulators in insulin resistance. Diabetes 58:2555–2564, 2009

Insulin is one of the major hormones involved in the control of energy expenditure and carbohydrate, lipid, and protein metabolism. It also regulates a variety of biological processes such as protein turnover, cell growth and differentiation, and DNA synthesis. To perform these actions in a concerted manner, insulin coordinates a complex program of transcriptional changes in the human skeletal muscle.

Recently, our understanding of complex gene regulatory networks governing cell physiology has rapidly evolved with the discovery of microRNAs (miRNAs). They represent a large class of evolutionary conserved RNAs of 21–22 nt, which act as negative regulators of gene expression either by inhibiting mRNA translation or promoting mRNA degradation through base pairing to the 3′ untranslated region (UTR) of target miRNAs (3). These small noncoding RNAs are transcribed by RNA polymerase II–producing long primary transcripts (pri-miRNAs), which are then processed by Drosha and Pasha (also DGCR8), which result in a stem-loop precursor called premiRNA. The pre-miRNA is subsequently transported from the nucleus to the cytoplasm by exportin-5 (XPO5). Then, DICER1 (RNase III endonuclease) processes the stem-loop to produce a 21-bp RNA duplex. One strand of the duplex, the miRNA, enters the RNA-induced silencing complex (RISC) and directs the complex to target mRNA (4). In agreement with their important regulatory functions, global effects of miRNAs upon the transcriptional profile and tissue specificity of mRNA expression have been reported in response to experimental manipulations of miRNA levels (5–7), and the latest estimate indicates that they may regulate up to one-third of the mammalian genome (8).

Important roles for these miRNAs have emerged in the control of metabolic pathways, as suggested by studies implicating miRNAs in the regulation of fat metabolism, adipocyte differentiation, energy homeostasis, and glucose-stimulated insulin secretion (9).

Skeletal muscle is one of the largest tissues in the human body and is the major site for insulin-dependent glucose disposal. Changes in mRNA and protein abundance in this tissue are central to a large number of metabolic and other disorders, including insulin resistance. The discovery that some miRNAs are expressed specifically in skeletal muscle raises the question of their
potential involvement in the transcriptional action of insulin in this tissue (10). Here, we report that insulin regulates miRNA expressions in the human skeletal muscle in vivo, which in return participate in insulin transcriptional action in this tissue. We also demonstrated that miR-1 and miR-133a, specifically expressed in muscle tissues (11–13), are regulated by insulin via sterol regulatory element–binding protein (SREBP)-1c and myocyte enhancer factor 2C (MEF2C). Furthermore, we found that miR-1 and miR-133a have impaired insulin response in the skeletal muscle of type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Euglycemic-hyperinsulinemic clamps. Volunteers provided written consent, and protocols were approved by an ethics committee (Hospices Civils, Lyon, France). Fifteen subjects without history of diabetes were submitted to a 3-h euglycemic-hyperinsulinemic clamp to achieve supraphysiological plasma insulin concentrations (insulin infusion rate of 2 mU·min⁻¹·kg⁻¹). Five subjects among 15 were selected based on their ages (51 ± 2 years). Their response to insulin infusion was compared with the response of five insulin-resistant type 2 diabetic patients (aged 50 ± 3 years) who had interrupted their usual treatment with oral antidiabetes agents at least 5 days before investigations. Metabolic parameters are presented in the online appendix in Table S1 (available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0165/DC1). Percutaneous biopsies of the vastus lateralis muscle were performed under basal conditions and after the clamp study.

Hyperglycemic-euinsulinemic clamp. Seven healthy men were submitted to a 3-h hyperglycemic-euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release. Muscle biopsies of vastus lateralis muscles were performed under basal conditions and after the clamp study as described previously (14).

Streptozotocin-induced diabetic mice. Three groups of 10-week-old C57B6/6 mice were used (n = 6). The control group was given daily intraperitoneal injections of sodium citrate buffer for 3 days. The remaining two groups were made diabetic by daily intraperitoneal injection of streptozotocin (STZ) for 3 consecutive days. Glucose levels were monitored daily, and mice were studied when they achieved fed blood sugars >500 mg/dl for at least 3 days. One group was further treated with two injections of insulin (3 mU Insulatard; Novo Nordisk). Twenty-four hours after the first injection of insulin, all animals were killed and gastrocnemius muscles were removed.

miRNA expression profiles in human muscle cells or skeletal muscle biopsies. Differentiated myotubes were prepared from three different skeletal muscle biopsies from three healthy volunteers (15). We quantified the expression of 365 human miRNAs in myoblasts and differentiatated myotubes, or in skeletal muscle biopsies, by using the TaqMan low-density arrays with the Applied Biosystems 7900HT fast real-time PCR system.

Quantification of mature miRNAs. Mature human or mouse miRNA expression was quantified by using the TaqMan miRNA assays according to the manufacturer’s instructions. To account for possible differences in the amount of starting RNA, all samples were normalized to RNU48.

Quantitative real-time PCR. Primers were given in supplementary Table S2. Quantitative RT-PCR data were computed as fold using geometric means and normalized to the mean value of the control sample in each paradigm, defined as 1. All experiments were performed in triplicate, and comparisons were analyzed using Student’s t test. Data are expressed as means ± SE. Significance was defined as P < 0.05.

Microarray analysis. cDNA microarrays are from The Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/). The dataset is available from GEO database (GSE 11868). The 6 insulin-sensitive subjects used for microarray analysis were included in the group of 15 (supplementary Table S1). Signal intensities were log transformed, and normalization was performed by Lowess method. Only spots with recorded data on the six slides were selected for analysis. With these criteria, 26,108 spots were retrieved. Among them, 11,804 had a gene symbol and were considered in this study as the list of genes expressed in human muscle. Genes with fold changes ≥1.19 were considered as significantly regulated (i.e., corresponding to the 95th percentile of genes based on the magnitude of the fold changes), which represented 1,681 genes (844 upregulated and 737 downregulated). Correction for multiple testing was performed using the Benjamini and Hochberg procedure.

Modulation of SREBP-1c mRNA levels in primary cultures of human muscle cells. Differentiated myotubes were infected for 48 h with adenoviruses expressing either green fluorescent protein (control) or nuclear SREBP-1c, as described previously (15). To knockdown SREBP-1, differentiated human myotubes were transfected with siRNA against SREBP-1 (Qiagen) for 36 h. Then, they were treated with 100 nmol/l insulin (Sigma Aldrich) for 5 h. Chromatin immunoprecipitation. Differentiated C2C12 cell lines were infected for 48 h with recombinant adenoviruses. Protein-DNA complexes were fixed for 5 min with 1% formaldehyde and proceed according to Active Motif’s ChIP-IT Express Kit (Active Motif Europe) with MEF2C antibodies (E-17, sc-13266; Santa Cruz).

RESULTS

miRNAs are regulated by insulin in human skeletal muscle. To identify miRNAs regulated by insulin in human skeletal muscle, we carried out comparative miRNA expression profiling in biopsies from healthy subjects, before and after a 3-h euglycemic-hyperinsulinemic clamp, by using TaqMan low-density arrays combined with multiplex RT-PCR to quantify 365 mature human miRNAs from the miRBase database (16). A subset of 216 human miRNAs was found to be expressed in skeletal muscle biopsies (Fig. 1). Since miRNAs are expressed in a tissue-specific manner (10), we identified those specifically expressed in human muscle by using primary cultures of myoblasts and in vitro–differentiated myotubes prepared from human skeletal muscle biopsies. Among 216 miRNAs expressed in skeletal muscle, 192 were expressed in myoblasts and/or myotubes, confirming their presence in muscle cells (Fig. 1 and supplementary Table S3). Among them, 39 were found downregulated by insulin in skeletal muscle of healthy subjects (none were upregulated) (Fig. 1 and Table 1). They represent two different classes of miRNAs; 39% are synthesized from primary transcripts located in the noncoding regions (i.e., intergenic miRNAs) and 61% are expressed from introns of protein-coding transcripts (i.e., intronic miRNAs). Although they have two different modes of transcription (17,18), their coordinated downregulation during hyperinsulinemia indicated that they could share common features for regulation in response to insulin. Of note, seven other miRNAs were also regulated...
miRNAs, expressed in human muscle cells, regulated in vivo by insulin in human skeletal muscle, during a 3-h hyperinsulinemic-euglycemic clamp

| miRNA names (miRBase version 9) | new miRNA names* | Subject 1 | Subject 2 | Subject 3 | Means ± SE | Host genes | Genome context |
|-------------------------------|------------------|-----------|-----------|-----------|------------|------------|---------------|
| hsa-miR-95                   |                  | 0.75      | 0.81      | 0.6       | 0.72 ± 0.06 | ABLM2      | Intron Sense  |
| hsa-miR-324-3p               |                  | 0.54      | 0.61      | 0.91      | 0.69 ± 0.11 | ACADVL     | Intron Antisens|
| hsa-miR-324-5p               |                  | 0.19      | 0.44      | 0.41      | 0.35 ± 0.08 | ACADVL     | Intron Antisens|
| hsa-miR-24                   |                  | 0.64      | 0.91      | 0.68      | 0.74 ± 0.08 | C9orf3 (24-1) and C9orf155 (24-2) | Intron Sense |
| hsa-miR-27b                  |                  | 0.28      | 0.69      | 0.55      | 0.51 ± 0.12 | C9orf3     | Intron Sense  |
| hsa-miR-30a-3p hsa-miR-30a*  |                  | 0.83      | 0.84      | 0.64      | 0.77 ± 0.07 | C9orf155   | Intron Sense  |
| hsa-miR-30a-5p               |                  | 0.32      | 0.62      | 0.65      | 0.53 ± 0.11 | C9orf155   | Intron Sense  |
| hsa-miR-30c                  |                  | 0.6       | 0.84      | 0.66      | 0.7 ± 0.07  | NFYC (30c-1) and NFYC (30c-2) | Intron Sense |
| hsa-miR-423                  |                  | 0.62      | 0.84      | 0.78      | 0.75 ± 0.07 | CCDC55     | Intron Sense  |
| hsa-miR-532                  |                  | 0.66      | 0.91      | 0.8       | 0.79 ± 0.07 | CLCN5      | Intron Sense  |
| hsa-miR-660                  |                  | 0.5       | 0.49      | 0.83      | 0.61 ± 0.11 | CLCN5      | Intron Sense  |
| hsa-miR-152                  |                  | 0.5       | 0.68      | 0.7       | 0.63 ± 0.06 | COPZ2      | Intron Sense  |
| hsa-miR-26b                  |                  | 0.6       | 0.74      | 0.94      | 0.76 ± 0.1  | CTDSPL (26a-1) and CTDSPL (26a-2) | Intron Sense |
| hsa-miR-26a                  |                  | 0.52      | 0.78      | 0.89      | 0.73 ± 0.11 | CTDSPL (26a-1) and CTDSPL (26a-2) | Intron Sense |
| hsa-miR-616                  |                  | 0.34      | 0.8       | 0.73      | 0.62 ± 0.14 | DDFI3      | Intron Sense  |
| hsa-miR-126                  |                  | 0.56      | 0.93      | 0.86      | 0.78 ± 0.11 | EGFL7      | Intron Sense  |
| hsa-miR-330                  |                  | 0.92      | 0.51      | 0.82      | 0.75 ± 0.12 | EML2       | Intron Sense  |
| hsa-miR-181b                 |                  | 0.59      | 0.89      | 0.96      | 0.81 ± 0.11 | novel transcripts | Intron Sense |
| hsa-miR-615                  |                  | 0.18      | 0.77      | 0.82      | 0.59 ± 0.21 | HOXC4      | Intron Sense  |
| hsa-miR-491                  |                  | 0.37      | 0.85      | 0.96      | 0.73 ± 0.18 | KIAA1797   | Intron Sense  |
| hsa-miR-1                   |                  | 0.59      | 0.89      | 0.53      | 0.67 ± 0.11 | MIB1 (1-2) and C20orf166 (1-1) | Intron Sense or antisens |
| hsa-miR-133a                 |                  | 0.27      | 0.75      | 0.59      | 0.54 ± 0.14 | MIB1 (133a-1) and C20orf166 (133a-2) | Intron Sense or antisens |
| hsa-miR-30e-3p hsa-miR-30e*  |                  | 0.73      | 0.81      | 0.58      | 0.71 ± 0.07 | NFYC       | Intron Sense  |
| hsa-miR-107                  |                  | 0.49      | 0.7       | 0.77      | 0.65 ± 0.08 | PANK1      | Intron Sense  |
| hsa-miR-29c                  |                  | 0.3       | 0.64      | 0.44      | 0.46 ± 0.10 | Intergenic  | Intron Sense  |
| hsa-miR-29a                  |                  | 0.28      | 0.65      | 0.38      | 0.44 ± 0.11 | Intergenic  | Intron Sense  |
| hsa-miR-30b                  |                  | 0.62      | 0.7       | 0.74      | 0.69 ± 0.04 | Intergenic  | Intron Sense  |
| hsa-miR-30d                  |                  | 0.54      | 0.74      | 0.66      | 0.64 ± 0.06 | Intergenic  | Intron Sense  |
| hsa-miR-130a                 |                  | 0.35      | 0.88      | 0.84      | 0.69 ± 0.17 | Intergenic  | Intron Sense  |
| hsa-miR-210                  |                  | 0.76      | 0.57      | 0.31      | 0.54 ± 0.13 | Intergenic  | Intron Sense  |
| hsa-miR-376a                 |                  | 0.11      | 0.1       | 0.22      | 0.14 ± 0.04 | Intergenic  | Intron Sense  |
| hsa-miR-432                  |                  | 0.91      | 0.45      | 0.72      | 0.69 ± 0.13 | Intergenic  | Intron Sense  |
| hsa-miR-125a                 |                  | 0.64      | 0.89      | 0.92      | 0.82 ± 0.09 | Intergenic  | Intron Sense  |
| hsa-miR-181d                 |                  | 0.74      | 0.52      | 0.89      | 0.72 ± 0.11 | Intergenic  | Intron Sense  |
| hsa-miR-27a                  |                  | 0.28      | 0.4       | 0.75      | 0.48 ± 0.14 | Intergenic  | Intron Sense  |
| hsa-miR-296                  |                  | 0.44      | 0.9       | 0.4       | 0.58 ± 0.16 | Intergenic  | Intron Sense  |
| hsa-miR-206                  |                  | 0.8       | 0.63      | 0.34      | 0.59 ± 0.13 | Intergenic  | Intron Sense  |
| hsa-miR-125b                 |                  | 0.42      | 0.84      | 0.86      | 0.71 ± 0.14 | Intergenic  | Intron Sense  |
| hsa-miR-193a                 |                  | 0.68      | 0.74      | 0.78      | 0.73 ± 0.03 | Intergenic  | Intron Sense  |

*mRNA identifications are from http://microrna.sanger.ac.uk/.

by insulin in muscle samples (Table 2), but as they were not detected in muscle cells, we assumed that they arose from other cell types. The subset of miRNAs downregulated by insulin included muscle-specific miRNAs (i.e., miR-1, miR-133a, and miR-206) but also miRNAs broadly expressed in other tissues including insulin-sensitive tissues (i.e., adipose tissue and liver) (10).

To further characterize the mechanism involved in the downregulation of these miRNAs by insulin, we investigated the effects of insulin on the expression of key components of the miRNA synthesis machinery. We found a significant upregulation of the nuclear protein DROSHA mRNA ($P < 0.01$) and the cytoplasmic protein DICER1 ($P < 0.05$) (Fig. 2), whereas expressions of DGCR8 and XPO5 were unchanged. Regarding the expression of the Argonaute proteins, components of the RISC complex, mRNA levels of all except one (EIF2C1) were significantly increased by insulin. Together, these data indicated that insulin positively modulated a number of specific actors of the miRNA synthesis machinery.

**Insulin regulates miR-1 and miR-133a at the transcriptional level.** As we found that insulin induced a downregulation of 39 miRNAs, while at the same time expressions of genes involved in miRNA synthesis were upregulated, we postulated that insulin might directly affect miRNA levels either by regulating pri-miR transcrip-
INSULIN REGULATES microRNA IN SKELETAL MUSCLE

TABLE 2
Other miRNAs regulated in vivo by insulin in human skeletal muscle, during a 3-h hyperinsulinemic-euglycemic clamp not expressed in human muscle cells

| miRNA names (miRBase version 10)* | Subject 1 | Subject 2 | Subject 3 | Means ± SE | Host genes | Genome context |
|----------------------------------|-----------|-----------|-----------|------------|------------|---------------|
| hsa-miR-200c                     | 8.85      | 1.33      | 1.37      | 3.85 ± 2.50| Intergenic |               |
| hsa-miR-517c                     | 0.34      | 0.48      | 0.18      | 0.33 ± 0.09| Intergenic |               |
| hsa-miR-518b                     | 0.39      | 0.41      | 0.76      | 0.52 ± 0.12| Intergenic |               |
| hsa-miR-518c                     | 0.93      | 0.45      | 0.27      | 0.55 ± 0.20| Intergenic |               |
| hsa-miR-96                       | 15.01     | 15.47     | 25.83     | 18.77 ± 3.53| Intergenic |               |
| hsa-miR-575                      | 7.50      | 15.87     | 9.11      | 10.82 ± 2.56| SC5D       | Intergenic    |
| hsa-miR-646                      | 3.00      | 3.67      | 2.82      | 3.16 ± 0.26| Novel transcript | Intergenic    |

*miRNA identifications are from http://microrna.sanger.ac.uk/.

...tion or by inducing their degradation. To clarify these points, we decided to focus on two representative muscle-specific miRNAs, miR-1 and miR-133a (19). Their downregulation by insulin was confirmed by quantitative RT-PCR in muscle samples from a larger group of 15 healthy subjects (Fig. 3A). We also investigated miR-1 and miR-133a regulations in an insulin-deficient mice model obtained by streptozotocin treatment (20). Compared with control mice, STZ-induced diabetic mice showed increased miR-1 and miR-133a levels (Fig. 3B), whereas insulin treatment resulted in the decrease of both miRNAs (Fig. 3B). These data supported the downregulation of miR-1 and miR-133a expression by insulin, observed in human skeletal muscle. However, glucose uptake by skeletal muscle is robustly increased during the hyperinsulinemic clamp, and STZ-induced diabetic mice are also markedly hyperglycemic (20). To verify whether glucose, a strong transcriptional regulator (14), played a role in the observed effects of insulin on miRNA levels, we measured miR-1 and miR-133a expression in the skeletal muscle of seven healthy men during a 3-h hyperglycemic-euinsulinemic clamp, an experimental situation designed to distinguish between the transcriptional effects of glucose and those of insulin (14). Figure 3A showed that hyperglycemia alone, in absence of hyperinsulinemia, did not modify miR-1 or miR-133a levels in human muscle.

Since miR-1 and miR-133a are derived from introns of protein-coding transcripts (i.e., MIB1 and C20orf166), it could be conceivable that the observed downregulation of miR-1 and miR-133a by insulin might be a consequence of the downregulation of their host genes. Figure 4 indicated that two host genes were in fact upregulated during insulin infusion with a significant effect for MIB1 (P < 0.05) in muscle samples. Therefore, regulations of MIB1 and C20orf166 in response to insulin were unlikely to explain the decreased levels of miR-1 and miR-133a.

Another potent mechanism controlling miRNA expression is the editing of their precursors by adenosine deaminases acting on RNAs, which can inhibit their processing and thus can decrease mature miRNA levels (21). This mechanism has been well described for miR-376a (21) and included in the list of 39 downregulated miRNAs by insulin (Table 1). We therefore verified whether primary transcripts of miR-1 and miR-133a were edited in response to insulin by sequencing pri-miRNA-1-1, pri-miRNA-1-2, pri-miRNA-133a-1, and pri-miRNA-133a-2 cDNAs in muscle samples from three healthy subjects before and after hyperinsulinemia. Sequences were identical before and after insulin infusion (data not shown), indicating that the decreased levels of mature miR-1 and miR-133a were not related to RNA editing.

Decreased expressions of miR-1/miR-133a by insulin are correlated with MEF2C downregulation and require SREBP-1c. Previous studies have shown that the bicistronic transcript miR-1/miR-133a is regulated by MEF2C, myogenic differentiation 1 (MYOD1), and serum response factor (SRF), which regulate the activity of enhancers located either upstream of miR-1/133a locus or between the miR-1 and miR-133a coding regions (11,13,22). To determine whether these transcription factors could be involved in the action of insulin, we quantified their expression in human muscle in response to insulin. Results indicated that MEF2C mRNA level was significantly reduced after insulin infusion (P < 0.05, n = 15) (Fig. 5A), whereas expressions of MYOD1 and SRF were slightly increased or unchanged. Thus, one could postulate that reduction of MEF2C expression could contribute to the downregulation of miR-1 and miR-133a.

Recently, we have found that MEF2C was downregulated by SREBP-1c (15), a key mediator of insulin transcriptional action in human muscle (23,24). This observation suggested that insulin could downregulate MEF2C and subsequently miR-1 and miR-133a expressions through activation of SREBP-1c. Figure 5B demonstrated that overexpression of SREBP-1c in human myotubes robustly downregulates MEF2C mRNA levels. Furthermore, overexpression of SREBP-1c was also associated with a significant reduction of miR-1 and miR-133a expressions in myotubes (Fig. 5C). To determine whether this reduction was a consequence of the downregulation of MEF2C, we performed chromatin immunoprecipitation

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*Fig. 2. Transcript levels of components of the miRNA biogenesis pathway before and after insulin infusion in 15 healthy subjects, determined by qRT-PCR. *P < 0.05 and **P < 0.01 vs. control, respectively.*
assay by using specific antibodies against MEF2C combined with PCR amplification of the MEF2C-binding site in the enhancer region located between pri-miRNA-1-2 and pri-miRNA-133a-1 (22). Figure 5D shows that after 48 h of SREBP-1c overexpression in muscle cells, it was not possible to detect by PCR MEF2C binding on this enhancer. This result confirmed that SREBP-1c mediated miR-1 and miR-133a downregulation through the downregulation of MEF2C in muscle cells. Finally, to demonstrate that downregulations of miR-1 and miR-133a by insulin were mediated by SREBP-1c, we knocked down SREBP-1 expression in human myotubes before stimulation by insulin and quantified the expressions of miR-1 and miR-133a. Figure 5E showed that downregulations of miR-1 and miR-133a by insulin observed in muscle cells transfected with green fluorescent protein siRNA (control) was not detectable in cells transfected with SREBP-1 siRNA, confirming that insulin regulation of these two miRNAs was mediated by SREBP-1.

To determine whether the mechanism involving SREBP-1c/MEF2C in the regulation of miR-1/miR-133a expressions could be generalized to the downregulatory effect of insulin on the set of miRNAs observed in the muscle of healthy individuals, we quantified the expression of five of them in muscle cells overexpressing SREBP-1c. As shown in the supplementary Fig. S4, miR-107 and miR-95 displayed increased expressions, which is the opposite of the marked downregulation observed during the clamp. These results indicated that other cellular pathways than SREBP-1c/MEF2C are likely involved in the regulation of some of the 39 miRNAs in response to insulin.

Regulations of miR-1 and miR-133a are altered in skeletal muscle of type 2 diabetic patients. Because impaired insulin stimulation of SREBP-1c has been consistently observed in skeletal muscle of type 2 diabetic patients (25,26), we suspected that regulations of miR-1 and miR-133a by insulin could be altered in the muscle of these patients. Figure 6A showed that basal expressions of miR-133a and miR-1 were not significantly different in skeletal muscle from healthy individuals and insulin-resistant type 2 diabetic patients. However, the effect of insulin

![Figure 3](image-url3)

FIG. 3. A: Regulation of miR-1 and miR-133a during a hyperinsulinemic-euglycemic clamp (HIEG, ■) or during a hyperglycemic-euinsulinemic clamp (HGEI, □) in human skeletal muscle. *P < 0.05 vs. before clamp. B: miR-1 and miR-133a expressions in skeletal muscle of control, STZ-induced diabetic, and insulin-treated STZ mice. **P < 0.01 vs. control mice.

![Figure 4](image-url4)

FIG. 4. Quantification by qRT-PCR of miR-1 and miR-133a host genes, MIB1 and C20orf166, during insulin infusion in skeletal muscle (n = 15). *P < 0.05 vs. before clamp. HIEG, hyperinsulinemic-euglycemic clamp.
was altered in the skeletal muscle of diabetic patients. The response to insulin was completely abolished for miR-133a, and the effect on miR-1 expression was markedly reduced, showing only a modest decrease that did not reach significance ($P < 0.2$) (Fig. 6A). Concomitantly, the significant reduction in MEF2C expression observed during the clamp in insulin-sensitive individuals was not found in type 2 diabetic patients (Fig. 6B).

**Detection of miRNA signals within the set of genes regulated by insulin in human skeletal muscle.** Previous microarray analyses have shown that reduction of transcript levels can be observed following miRNA transfection (6,7,22,27). It has been demonstrated that insulin affects the expression level of ~1,000 genes in vivo in human skeletal muscle (1,2). It was therefore important to determine whether insulin-regulated miRNAs could contribute to the network of gene expression induced by insulin in skeletal muscle. To this aim, we first analyzed by cDNA microarrays the global changes in mRNA levels in the muscle of six healthy middle-aged subjects (including samples from the three subjects used for miRNA analysis) in response to insulin. Then we analyzed the 3' UTR region of the upregulated mRNAs, assuming that downregulated miRNAs by insulin would have their target genes upregulated during the clamp. Among 944 upregulated genes, 357 can be predicted as a target of at least one miRNA downregulated by insulin (predictions using TargetScan version 4.2) (28). Since we observed that several binding sites for distinct miRNAs were present on the same transcript, we analyzed more in detail the distribution of the number of miRNA-binding sites in this set of genes. Figure 7 showed that the proportions of genes upregulated by insulin that can be a target of at least one (or more) distinct miRNA downregulated by insulin were significantly greater than the proportions of upregulated genes that were not targets. Moreover, these proportions increased with the number of distinct miRNA-binding sites in the 3' UTR (Fig. 7). This observation suggested that single miRNA induces moderate changes in expression of their targets in response to insulin, while combinations of different miRNAs can work in concert to exert significant effects on gene expression. This result is in agreement with a previous study (29) showing that the number and arrangement of miRNA recognition sites can influence the degree and specificity of miRNA-mediated gene repression.

To further explore the role of miRNAs in the insulin transcriptional network, we analyzed the functions of the predicted target genes for 39 downregulated miRNAs by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (30). To take into account that miRNAs act as negative regulators of gene expression also by
inhibiting mRNA translation, we considered all potential targets including those that were not regulated at the mRNA level. Among 11,863 genes expressed in human skeletal muscle (see RESEARCH DESIGN AND METHODS), 4,481 were the target of at least one regulated miRNA. Among 4,481 potential target genes, 1,279 were recorded at least in one KEGG pathway. Table 3 shows KEGG pathways that are most significant in terms of containing more genes than expected (P < 0.05). This analysis revealed that several signaling pathways and ubiquitin-mediated proteolysis are the major pathways potentially affected by 39 insulin-regulated miRNAs.

**DISCUSSION**

miRNAs form a novel class of regulators that add a new level of regulation and fine-tuning in the control of gene expression. They are important for a wide range of cellular functions, and recent studies have provided evidence that miRNAs affect critical pathways of metabolic control, such as adipocyte and skeletal muscle differentiation, amino acid metabolism, lipid homeostasis, and insulin secretion from pancreatic β-cells (9). However, factors that govern miRNA synthesis and expression in response to changes in the cellular environment are still largely unknown. In this study, we demonstrate for the first time that insulin, the major hormone controlling whole-body energy homeostasis and metabolism, is involved in the regulation of miRNA expression in human skeletal muscle, providing evidence that regulation of miRNA expressions is a novel pathway of insulin action in vivo. The fact that this regulation is also observed in the murine model and that most of the regulated miRNAs are expressed in other insulin-sensitive tissues suggests that insulin action on miRNAs is not restricted to our experimental situation and occurs more generally in other tissues. Interestingly, 12 of 39 insulin downregulated miRNAs have been previously found dysregulated in primary muscular disorders (31) (i.e., miR-133a, miR-181d, miR-423, miR-30c, miR-95, miR-30b/d, miR-206, miR-30a-5p, miR-26a, and miR-29a/c), suggesting an important role of these miRNAs in specific physiological pathways in skeletal muscle.

Although hundreds of miRNAs have been cloned in various species, molecular actors involved in pri-miRNA transcription are largely unknown. Recently, it has been demonstrated that hepatocyte nuclear factor-1α induces miR-194 expression during intestinal epithelial cell differentiation (32), that p53 transactivates miR-34a in pancreatic cells (33), that high mobility group at-hook 1 (HMGA1) downregulates five different miRNAs in mouse embryonic fibroblasts (34), and that two different MEF2-dependent enhancers regulated miR-1 and miR-133a expressions during muscle differentiation (11,13,22). One important result of this study is the demonstration that SREBP-1c and MEF2C contribute to insulin action, at least on miR-1 and miR-133a, which were studied as representative miRNAs. The transcription factor SREBP-1 is mainly involved in cholesterol and fatty acid metabolism (35). Recently, we have published a microarray analysis of human muscle
cells overexpressing SREBP-1c and revealed several hundred potential new target genes of this transcription factor, including MEF2C. Here, we found that both insulin in vivo in skeletal muscle and SREBP-1c overexpression in muscle cells induced the coordinated downregulation of miR-1 and miR-133a expressions. Thus, these data led us to propose the following mechanism (Fig. 8): Insulin activates the translocation of the SREBP-1c active

**TABLE 3**
KEGG pathways potentially affected by the 39 insulin-regulated miRNAs during a 3-h hyperinsulinemic-euglycemic clamp in human skeletal muscle

| KEGG pathways                          | KEGG annotations | Number of genes annotated on microarray | Number of targeted gene (six or more miRNAs)* | \(P\) (Fisher test)† | Adjusted \(P\) (Benjamini) |
|----------------------------------------|------------------|----------------------------------------|-----------------------------------------------|-----------------------|----------------------------|
| MAPK signaling pathway                 | ko04010          | 352                                    | 42                                            | 0.000392              | 0.00503                    |
| VEGF signaling pathway                 | ko04370          | 93                                     | 16                                            | 0.000527              | 0.00503                    |
| Calcium signaling pathway              | ko04340          | 187                                    | 26                                            | 0.000613              | 0.00503                    |
| Ubiquitin-mediated proteolysis         | ko04120          | 206                                    | 27                                            | 0.001161              | 0.007934                   |
| mTOR signaling pathway                 | ko04150          | 90                                     | 13                                            | 0.009689              | 0.039725                   |
| Wnt signaling pathway                  | ko04310          | 255                                    | 28                                            | 0.01207               | 0.044987                   |
| Jak-STAT signaling pathway             | ko04630          | 179                                    | 21                                            | 0.015888              | 0.054284                   |

The 11,864 genes retrieved from microarray data analysis were classified into KEGG categories. We determined significant associations between the set of genes collectively targeted by the 39 insulin-downregulated miRNAs and specific KEGG pathways. *We considered genes targeted by at least six different miRNAs, which corresponded to percentile 95th of the distribution of predicted miRNA target sites in 3’ UTR, for the 11,864 expressed in human muscle, when using TargetScan 4.2 (conserved site across human, mouse, rat, and dog). †Enrichment was significant for \(P \leq 0.05\).

**FIG. 8.** Proposed mechanism to explain the downregulation of miR-1 and miR-133a by insulin in human skeletal muscle. Insulin activates the translocation of SREBP-1c (BHLH) active form from the endoplasmic reticulum (ER) to the nucleus and, concomittantly, induces SREBP-1c expression via the phosphatidylinositol (PI) 3-kinase (PI3K) signaling pathway (36). SREBP-1c mediates MEF2C downregulation (15) through a mechanism that remains to be determined. As a consequence of lower MEF2C binding on their enhancer region, the transcription of miR-1 and miR-133a is reduced, leading to decreased levels of their mature forms in muscle, after insulin treatment. Altered activation of PI 3-kinase and SREBP-1c may explain the defective regulation of miR-1 and miR-133a expression in response to insulin in muscle of type 2 diabetic patients (25).
form to the nucleus and also the induction of its own expression via the phosphoinositide 3-kinase signaling pathway (36). Then SREBP-1c induces MEF2C downregulation and reduction of its binding to the pri-miRNA-1-2 and pri-miRNA-133a-1 enhancer region. This leads to the reduction of miR-1 and miR-133a transcription and thus to the decreased levels of their mature forms. Testing this mechanism on five other miRNAs than miR-1 and miR-133a, we found that two of them (miR-107 and miR-95) were actually upregulated in muscle cells overexpressing SREBP-1c. This observation strongly suggests that other mechanisms than the pathway involving SREBP-1c/MEF2C are likely involved in the effects of insulin on miRNA levels. Particularly, we cannot rule out the fact that the decrease in miRNA concentrations may reflect possible regulations of the miRNA-processing machinery mediated by the miRNAs themselves. The insulin-induced decrease in miRNA levels should thus be followed by increased levels of target mRNAs, including those coding for the proteins involved in their processing. In addition, regulation of miRNA level during clamp could also be the results of changes in the protein level or activity of components of the miRNA processing machinery. Unfortunately, the small size of the muscle biopsies did not allow us to obtain enough material to verify these hypotheses in human samples. Further studies are now required to identify these alternative mechanisms.

One important result, which also supports the above-proposed mechanism of insulin action on miRNA expressions, is the observation that miR-1/miR-133a regulations are altered in the muscle of type 2 diabetic patients during the hyperinsulinemic clamp. Defective regulation of gene expressions in response to insulin in peripheral tissues of insulin-resistant type 2 diabetic patients has been reported, including a marked alteration in the induction of SREBP-1c expression (25,26). The present study demonstrates that one of the unexpected consequences of this defect is a marked alteration in the regulation of specific miRNAs. Therefore, our data could also support the implication of miRNAs in the pathophysiology of type 2 diabetes and insulin resistance in humans. Interestingly, in a murine model of diabetes, 15 miRNAs have been found differentially expressed in skeletal muscle when compared with control rats (37). Two of 15 are present in the list of insulin-regulated miRNAs (Table 1). Furthermore, overexpression of miR-29 has been shown to reduce insulin action on protein kinase B/AKT activation in adipocytes (37). Interestingly, miR-29a and miR-29c are among the most regulated miRNAs in response to insulin in our study. Taken together, these data strongly suggest that some miRNAs regulated by insulin might be implicated in insulin resistance.

Bioinformatic analysis showed that target genes of 39 miRNAs downregulated by insulin are mainly involved in signaling pathways and in ubiquitination-mediated proteolysis. Previous data (1) have suggested that the ubiquitin-proteasome system is an important component of insulin action. Regarding intracellular signaling pathways, our observation confirmed previous in silico studies indicating that miRNAs significantly target proteins involved in signal transduction (38,39). In agreement, a role for specific miRNAs has been described in the regulation of insulin receptor substrate-1 (40) and phosphatase and tensin homolog (41) proteins. Here, our data suggested that p85α phosphoinositide 3-kinase, a key mediator of insulin signaling is a potential target for six different miRNAs downregulated by insulin (i.e., miR-376a, miR-107, miR-30a-3p, miR-30e-3p, miR-29a, and miR-29c) (supplementary Table S5).

This study reveals the contribution of miRNAs in the transcriptional action of insulin in human skeletal muscle. Although further studies are now required to understand the biological impact of miRNAs in insulin action, the observed dysregulation in patients with type 2 diabetes may open potential perspectives for the understanding of insulin resistance and development of therapeutic strategies.

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