Sterol Regulatory Element Binding Protein-1c Expression and Action in Rat Muscles

Insulin-Like Effects on the Control of Glycolytic and Lipogenic Enzymes and UCP3 Gene Expression

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Sterol regulatory element binding protein-1c (SREBP-1c) is a transcription factor that mediates insulin effects on hepatic gene expression. It is itself transcriptionally stimulated by insulin in hepatocytes. Here we show that SREBP-1c mRNA is expressed in adult rat skeletal muscles and that this expression is decreased by diabetes. The regulation of SREBP-1c expression was then assessed in cultures of adult muscle satellite cells. These cells form spontaneously contracting multinucleated myotubes within 7 days of culture. SREBP-1c mRNA is expressed in contracting myotubes. A 4-h treatment with 100 nmol/l insulin increases SREBP-1c expression and nuclear abundance by two- to threefold in myotubes. In cultured myotubes, insulin increases the expression of glycolytic and lipogenic enzyme genes and inhibits the 9-cis retinoic acid–induced UCP3 expression. These effects of insulin are mimicked by adenovirus-mediated expression of a transcriptionally active form of SREBP-1c. We conclude that in skeletal muscles, SREBP-1c expression is sensitive to insulin and can transduce the positive and negative actions of the hormone on specific genes and thus has a pivotal role in long-term muscle insulin sensitivity. Diabetes 51:1722–1728, 2002

Insulin has a key role in metabolic adaptations linked to changes in the diet carbohydrate status. It not only rapidly modulates the activity of specific proteins but also, in the long term, changes their quantity by altering their transcription rate.

We proposed that in the liver, the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) mediates the transcriptional effects of insulin (1). SREBP-1c belongs to a family of transcription factors (together with SREBP-1a and SREBP-2) originally involved in the regulation of genes by the cellular availability in cholesterol (2,3). SREBPs are synthesized as a precursor protein bound to the endoplasmic reticulum and nuclear membranes, and their activation thus requires a proteolytic cleavage allowing the transcriptionally active part of SREBPs to be translocated into the nucleus.

In mouse liver, the expression of SREBP-1c and the presence of its mature form in nuclei are high in carbohydrate-refed compared with starved animals (4), and we have shown in primary cultured hepatocytes that insulin is able to activate SREBP-1c expression strongly (5). At the protein level, insulin induces an increase in the precursor form of SREBP-1c and a concomitant increase in the nuclear mature form, which is detectable 2–4 h after insulin addition (6). The effect of insulin on SREBP-1c has been corroborated by in vivo studies showing that SREBP-1c expression and nuclear abundance is low in the liver of diabetic rats and increased markedly after an insulin treatment (7). We have shown that SREBP-1c is involved in the stimulation by insulin of hepatic genes that are for their transcription either exclusively dependent on insulin, such as glucokinase, or dependent on insulin and glucose, such as fatty acid synthase (FAS), acetyl-CoA carboxylase, l-pyruvate kinase, or Spot14 (1,5). SREBP-1c can mimic the negative effect of insulin on phosphoenolpyruvate carboxykinase gene transcription (8). The similarity between insulin and SREBP-1c effects on hepatic gene expression is also true in vivo as shown recently in diabetic mice (9).

In adipose tissue, the expression of SREBP-1c and its nuclear abundance are also controlled positively by insulin (10). With the use of transfection studies of promoter/reporter constructs in cell lines, it has been reported that this factor mediates the effect of insulin on the FAS gene (10). This suggests that in this insulin-sensitive tissue, SREBP-1c has a role similar to that observed in the liver.

In muscles, the third major insulin-sensitive tissue, the presence of SREBP-1 has been detected in rodent and human species. On the basis of RNase protection assay experiments, the SREBP-1c isoform was found to be more abundant than the SREBP-1a isoform in the mouse and human species (11).

As a first clue to the understanding of the role of muscle SREBP-1c, we studied its expression in different muscle types and in primary cultures of muscle satellite cells.
under various experimental conditions. In addition, we analyzed the regulation of a number of genes in cultured muscle cells in which the dominant-positive or dominant-negative forms of SREBP-1c were overexpressed using adenoviral vectors.

**RESEARCH DESIGN AND METHODS**

**Animals.** Animal studies were conducted according to the French guidelines for the care and use of experimental animals. Rats were housed in plastic cages at a constant temperature (22°C) with light from 0700 to 1900 h. All studies were performed between 0900 and 1000 h on previously anesthetized rats. For diabetic rats and their controls, 2-month-old Wistar male rats (308 g)fed ad libitum with a standard laboratory diet were used. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg body wt in 0.1 mol/l sterile sodium citrate buffer [pH 4.5]; Sigma Chemical, St. Louis, MO). An injection of sodium citrate buffer was administered to control animals. Diabetes was diagnosed 48 h later and defined as a blood glucose level >14 mmol/l. Blood glucose was determined every other day on a blood drop obtained by tail vein puncture. Tissues were sampled after 6 days of overt diabetes.

**Tissue sampling.** In control and STZ-diabetic rats, liver, spleen, diaphragm, red sternomastoidus, soleus, gluteus, and extensor digitorum longus (EDL) were dissected on ice, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

**Preparation of recombinant adenovirus.** The adenovirus vector containing the transcriptionally active amino-terminal fragment (amino acids 1–403) of SREBP-1c was constructed according to He et al. (12) as described previously (1). The recombinant adenovirus containing the dominant-negative form of SREBP-1c was constructed as described previously (5). The adenovirus vector containing the major late promoter with no exogenous gene (ad null) was used as a control.

**Primary culture of muscle satellite cells and treatment with recombinant adenovirus.** Satellite cells from 8-week-old rat hindlimb muscle fibers were prepared and cultured as previously described (13). For the experiments involving adenovirus, contracting myotubes were incubated for 120 min with agitation at 37°C in 2 ml of Dulbecco’s modified Eagle’s medium containing adenovirus (50 pfu/nucleus). The medium was then completed to 5 ml with a fresh medium.

**Effect of 9-cis retinoic acid.** Contracting myotubes were treated for 16 h with 10 μmol/l 9-cis retinoic acid (Sigma). Thereafter, 100 μmol/l insulin or adenovirus was added, and cell cultures were studied after 8 h. Cells were harvested for total RNA extraction or mitochondria preparation.

**Isolation of total RNA and Northern blot hybridization.** Total RNA was extracted from frozen tissues according to Chomczynski and Sacchi (14), and specific expression was analyzed by Northern blot. Autoradiograms of Northern blots were scanned and quantified using an image processor program (NIH 1.62). Rat ATP-citrate lyase, UCP2, and UCP3 cDNA probes were synthesized by RT-PCR using, respectively, rat liver, white adipose tissue, and muscle total RNA as templates. The primers used were as follows: ATP-citrate lyase upper primer, 5’-CATGTGACGGAAGGCGATT-3’ (antisense); SREBP-1c, 5’-AGGAGGCTTCCAGAGGCGGAC-3’ (sense) and 5’-CAGGAGGCTTCCAGAGGCGGAC-3’ (antisense); SREBP-1c, 5’-AGGAGGCTTCCAGAGGCGGAC-3’ (antisense). A control CDNA was used as an interplate calibrator, and the variability in the initial quantities of CDNA was normalized by using ribosomal 18S RNA amplifications. Results were obtained from three to four independent RNA samples from individual experiments, each tested in triplicate. Results are expressed as arbitrary units indicating relative expressions normalized to 18S. This cutoff value allows a quantitative comparison of a similar mRNA under different conditions but also of different mRNAs.

**Preparation of membrane and nuclear extracts.** Membrane and nuclear protein extracts were prepared from cultured cells as described by Azzout-Marniche et al. (6).

**Isolation of mitochondria.** Mitochondria were isolated from contracting myotubes by homogenization in an ice-cold glass Dounce homogenizer in a sucrose buffer (300 mmol/l sucrose, 5 mmol/l Tris, 2 mmol/l EDTA [pH 7.4]) containing protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 5 μg/ml aprotonin). The homogenate was centrifuged at 1,600g for 10 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 9,200g for 10 min at 4°C to sediment the mitochondria. The supernatant (cytosolic fraction) was kept for additional studies. Mitochondria were rinsed twice in sucrose buffer and then further purified on self-forming Percoll gradients (33% [wt/vol] Percoll in sucrose buffer). Purified mitochondria were rinsed twice in sucrose buffer, and the protein concentration was determined by the Bradford’s method using BSA as a standard.

**Western blot analysis.** Thirty micrograms of membrane, nuclear, cytosolic, or mitochondrial proteins was separated on a 10% polyacrylamide gel and then electrotransferred onto a 0.2-μm nitrocellulose membrane. Membranes were blotted with the relevant antibody and then blots were revealed with the enhanced chemiluminescent system (Supersignal, Pierce).

**Antibodies.** The polyclonal anti-human 32A-UCP3 antibody was purchased from Alpha Diagnostic International. The polyclonal anti-human hexokinase II antibody raised against a peptide at the carboxy terminus (identical to the corresponding rat sequence) was purchased from Santa Cruz Biotechnology. FAS protein was probed with a previously characterized anti-rat FAS polyclonal antibody (16). SREBP-1 antibody was as in Azzout-Marniche et al. (6)

**Data analysis and statistics.** The data are presented as means ± SE. The data were analyzed using a one-way ANOVA, followed by the Newman-Keuls test for multiple comparisons. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Expression of SREBP-1 in adult rat tissues.** The expression of SREBP-1 mRNA (the 1a and 1c isoforms cannot be differentiated by the CDNA probe) was assessed by Northern blots in the liver and muscles of adult rats. SREBP-1 (Fig. 1A) was clearly expressed in the skeletal muscles tested, with a high expression in the gluteus muscle, a white muscle composed of fast-twitch glycolytic fibers, and a lower expression in the soleus and the red part of the sternomastoidus muscle, which is composed of slow-twitch oxidative fibers, and in the EDL, which is composed of intermediate fibers (fast-twitch oxidative glycolytic fibers). Interestingly, the expression of SREBP-1 in the gluteus muscle was comparable to that observed in the liver on the same blot. The respective contribution of SREBP-1a and SREBP-1c expression to total SREBP-1 mRNA was determined using real-time RT-PCR. As seen in Fig. 1B, we found that SREBP-1c is the predominant isoform in liver, whereas SREBP-1a is the predominant isoform in spleen as described previously (11). SREBP-1c mRNA was expressed in an 8- to 30-fold excess in different muscles when compared to SREBP-1a, demonstrating that Northern blots are representative of SREBP-1c expression.

**Effect of diabetes on muscle SREBP-1c expression.** SREBP-1c expression was then compared in tissues of control and STZ-diabetic rats. As shown in Fig. 1A, diabetes was concomitant in the liver with the predicted decrease of SREBP-1 expression (7). In all tested muscles, diabetes induced a clear-cut decrease in SREBP-1 expression. Diabetes was concomitant with upregulation of UCP3 as previously described (17,18), whereas UCP2 and PPAR-α expression were not affected (Fig. 1A). To be sure that the SREBP-1c isoform was indeed affected by the diabetic state, we analyzed changes in SREBP-1c expression by real-time RT-PCR (Fig. 1C). In all skeletal muscles studied, as in the liver, SREBP-1c expression was decreased by diabetes.
SREBP-1c expression in rat muscles

A

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<tr>
<th>Liver</th>
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Control of SREBP-1 expression in primary cultures of muscle cells. That SREBP-1c expression is decreased by diabetes is indicative but not direct evidence of a control of its gene by insulin. To document this aspect, we used primary cultures of muscle satellite cells (13). The relative abundance of the SREBP-1a and -1c isoforms was measured using real-time quantitative RT-PCR. SREBP-1a expression is fourfold lower than SREBP-1c expression (results not shown), and, thus, Northern blots reflect mainly SREBP-1c expression.

In cultured contracting myotubes, 100 nmol/l insulin does not modify α-actin expression but rapidly increases SREBP-1 mRNA content (Fig. 2A). The effect of insulin on the SREBP-1c isoform was verified by quantitative real-time RT-PCR (results not shown). The increased expression of SREBP-1c is concomitant with an increased amount of the SREBP-1 protein in both membranes and nuclear extracts (Fig. 2B).

Target genes of SREBP-1 in muscle cells. In the liver, the SREBP-1c isoform is involved in the stimulation by insulin of genes of the glycolytic/lipogenic pathway (5,19,20). We then sought to determine whether a similar function could be observed in cultured muscle cells. We first studied the action of insulin on the expression of two lipogenic-related enzymes, FAS and ATP-citrate lyase (ATP-CL). A clear-cut stimulation of the expression of FAS and ATP-CL genes after 4–6 h of culture in the presence of insulin was observed (Fig. 2A). We next infected contracting myotubes with an adenovirus containing a dominant-positive form of SREBP-1c. The mRNA content of the SREBP-1 transgene was increased after 4 h of infection, whereas endogenous SREBP-1 and α-actin expression were not affected (Fig. 2C). Forced expression of SREBP-1c induced the stimulation of FAS and ATP-CL gene expression (Fig. 2C).

Because the hexokinase II (HK II) gene has been described as a bona fide target of insulin in muscles (21–24), we studied the effect of insulin and SREBP-1c on the HK II protein concentration in the cytosol. Insulin markedly increases cytosolic HK II concentration, an effect that is counteracted when a dominant-negative form of SREBP-1c is overexpressed but mimicked by the expression of a dominant-positive form of SREBP-1c (Fig. 3). Similar results were obtained for the FAS protein (Fig. 3).

SREBP-1c mimics the effect of insulin on UCP3 gene expression. Insulin concentration varies in an opposite manner to UCP3 expression in diabetes (17,18) (Fig. 1A) and fasting (25,26). We then tested the hypothesis of a repressive effect of insulin on UCP3 expression in cultured contracting myotubes. Because in the basal state the expression of UCP3 is extremely low, it was first induced by 9-cis retinoic acid (Fig. 4A). Insulin clearly antagonizes the effect of 9-cis retinoic acid on UCP3 expression (Fig. 4A).

Infection of contracting myotubes with a dominant-positive form of SREBP-1c that increases FAS expression (Fig. 4A) antagonizes the effect of 9-cis retinoic acid on UCP3 expression. In contrast, a mutated form of SREBP-1c that is unable to bind to DNA response elements has no effect (Fig. 4A). Similar results are found at the protein level in mitochondria extracted from contracting myotubes, with insulin and the dominant-positive form of SREBP-1c inhibiting the retinoic acid–induced increase in UCP3 level (Fig. 4B).

Discussion

SREBP-1c expression and regulation in muscles. In muscles, SREBP-1c expression is clearly detectable at a level close to that observed in liver, with the highest expression found in white fast-twitch glycolytic fibers. The present in vivo experiments in diabetic rats suggest that as in the liver and adipose tissue, expression of SREBP-1c in skeletal muscles requires the presence of insulin. This is
corroborated by in vitro studies performed in contracting myotubes in which SREBP-1c expression and nuclear concentration are increased by insulin, although we cannot at this stage conclude whether insulin also has an effect on the cleavage of the precursor form of SREBP-1c. This finding is in accordance with studies in human skeletal muscles showing that SREBP-1c expression is increased threefold by an hyperinsulinemic-euglycemic clamp (27).

This set of experiments clearly shows that, as in the liver and adipose tissue, SREBP-1c expression in skeletal mus-
speci

Which genes are regulated by SREBP-1c in skeletal
tion of genes related to glucose/lipid metabolism second-
that SREBP-1c can be involved in muscles in the regula-
lices is linked to the nutritional environment. This suggests
that SREBP-1c can be involved in muscles in the regula-
tion of genes related to glucose/lipid metabolism second-
ary to changes in insulin/glucose concentration.

Which genes are regulated by SREBP-1c in skeletal
muscles? In muscles, studies on the effects of insulin on
specific muscle genes are limited. The best documented
target of insulin in muscles is the HK II gene. An effect of
insulin on muscle HK II gene has been reported both in
vivo in rodents and humans and in vitro in a muscle cell
line (21–24). Data concerning the effect of insulin on the
glucose transporter GLUT4 expression are less convincing
because no or only modest effects of insulin have been
described (28). Recently, it was also shown in human subjects
that a 3-h hyperinsulinemic-euglycemic clamp was concomitant with a significant increase of the p85α subunit of phosphatidylinositol 3-kinase and of the Rad
protein (27). In addition and as mentioned above, the
expression of SREBP-1c was clearly increased by insulin
in human muscle in vivo (27), whereas glycogen synthase
expression was not modified.

In cultured contracting myotubes, insulin has no effect
on GLUT4 and glycogen synthase expression (results not
shown). As shown previously (21–24), insulin is able to
increase the expression of HK II in muscle cells. Insulin is
also able to induce the expression of two key enzymes of
the lipogenic pathway, namely FAS and ATP-CL. Notably,
if endogenous SREBP-1c is ablated using a dominant-
negative form of the protein, then the effect of insulin on
HK II and FAS is abolished. In addition, the forced
expression of SREBP-1c reproduces insulin effects on HK
II and lipogenic enzyme expression. This set of experi-
ments strongly suggests that SREBP-1c is an intermediate
in muscle insulin action on gene expression. It could be
argued that the increased nuclear abundance of SREBP-1c
in the presence of insulin is modest in the face of the
strong induction of FAS. However, insulin, as suggested in
the liver, could also modify the transcriptional activity of
SREBP-1c at a posttranslational level by activating the
nuclear mature form through phosphorylation/dephos-
phorylation processes (29,30).

Activation of the lipogenic capacity by insulin is a
puzzling result because in vivo, lipogenic enzyme activities
are hardly detectable in muscles. It could, however, be
related to an enhanced lipogenic capacity of muscle
satellite cells, which are devoted to muscle repair and
growth. In this context, insulin-induced muscle lipogenesis
could be an important pathway in cases of muscle satellite
cell proliferation, for instance, after muscle injury or when
recovering from large muscle losses as seen after long-
term starvation.

In vivo, malonyl-CoA, an intermediate of the lipogenic
pathway, has been proposed as a regulator of fuel selection
because it can inhibit carnitine palmitoyl transferase
I, a key enzyme for fatty acid oxidation (31). One can then
wonder whether insulin, through SREBP-1c and lipogene-
sis modulation, could not be in the long term a regulator of
lipid utilization in muscles.

UCP3, a gene negatively regulated by insulin and
SREBP-1c in muscle cells. UCP3 expression in muscles is
increased under several conditions linked to the energy
status and often in conditions in which circulating free
fatty acids concentrations are elevated: fasting (25,26),
diabetes (17), provision of fatty acids or fatty acid deriv-
atives acid (32,33), thyroid hormones (34), exercise (35),
activation of AMP-dependent protein kinase (35), and also
by peroxisome proliferator ligands (36) and 9-cis retinoic
(37). These positive regulators do not rule out the possi-
bility of negative ones. In vivo studies are consistent with
the possibility of a negative effect of insulin on UCP3
expression. Indeed, fasting and diabetes strongly induce
the muscle expression of this protein. Refeeding carbohy-
drates after a starvation period downregulates the expres-
sion of UCP3, whereas refeeding a high-fat diet has no
effect (33). We clearly show here that insulin is able to
repress the expression of UCP3. A recent study suggested
a positive effect of insulin on UCP3 expression in in vitro
preparations of whole muscles (38). However, this is
clearly not in accordance with the previous in vivo studies
described above. As it is the case for the negative effect
of insulin on PEPCK expression in the liver (8), the negative
action of insulin on UCP3 expression is mimicked by the
overexpression of a dominant-positive form of SREBP-1c,
whereas a form of this transcription factor that cannot
bind to DNA has no effect, suggesting that this transcrip-
tion factor is also able to transduce negative effects of
insulin on muscle gene expression.

That UCP3 expression and protein content in mitochon-
dria are negatively regulated by insulin is consistent with
the idea that its role is linked to the switch from glucose to

FIG. 4. Effects of insulin or of dominant-positive or -negative forms
of SREBP-1c on UCP3 mRNA and protein expression in contracting
cultured myotubes. Contracting myotubes were treated or not for
16 h with 10 µmol/l of 9-cis-retinoic acid (RA). Thereafter, 100
nmol/l insulin (Ins) or a null adenovirus (Ad Null; 50 pfu/nucleus),
an adenovirus containing a dominant-positive form of SREBP-1c
(DP; 50 pfu/nucleus), and an adenovirus containing a dominant-
negative form of SREBP-1c (DN; 50 pfu/nucleus) were added and cell
cultures were harvested after 6 h. A: Total RNA was extracted and
analyzed for the expression of FAS, UCP3, and SREBP-1c genes and
18S rRNA. The Northern blot is representative of three independent
experiments. B: UCP3 protein abundance was quantified by Western
blot. The Western blot presented is representative of three independ-
ent experiments.
fat oxidation rather than to diet-modulated muscle thermogenesis. In a recent article (39), UCP3 was overexpressed in the mitochondria of cultured human muscle cells using an adenoviral vector. This overexpression led to an increase in the capacity of oleate oxidation and promoted oleate-induced inhibition of glucose utilization.

In skeletal muscles, SREBP-1c could, as in liver and adipose tissue, transduce the positive and negative actions promoted oleate-induced inhibition of glucose utilization.

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