Chronic Hyperinsulinemia Causes Selective Insulin Resistance and Down-regulates Uncoupling Protein 3 (UCP3) through the Activation of Sterol Regulatory Element-binding Protein (SREBP)-1 Transcription Factor in the Mouse Heart*

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The risk for heart failure and death after myocardial infarction is abnormally high in diabetic subjects. We and others have shown previously that mitochondrial uncoupling protein 3 (UCP3) improves functional recovery of the rodent heart during reperfusion. Here, we demonstrate that pharmacological induction of hyperinsulinemia in mice down-regulates myocardial UCP3. Decreased UCP3 expression was linked to the development of selective insulin resistance in the heart, characterized by decreased basal activity of Akt but preserved activity of the p44/42 mitogen-activated protein kinase, and overactivation of the sterol regulatory element-binding protein (SREBP)-1–mediated lipogenic program. In cultured myocytes, insulin treatment and SREBP-1 overexpression decreased, whereas SREBP-1 interference increased, peroxisome proliferator-activated receptor-stimulated expression of UCP3. Promoter deletion and site-directed mutagenesis identified three functional sterol regulatory elements in the vicinity of a known complex intronic enhancer. Increased binding of SREBP-1 to this DNA region was confirmed in the heart of hyperinsulinemic mice. In conclusion, we describe a hitherto unknown regulatory mechanism by which insulin inhibits cardiac UCP3 expression through activation of the lipogenic factor SREBP-1. Sustained down-regulation of cardiac UCP3 by hyperinsulinemia may partly explain the poor prognosis of type 2 diabetic patients after myocardial infarction.

Type 2 diabetes represents 95% of all diabetes cases, and its incidence is expected to soar in the next decades as a consequence of the obesity epidemic (1). The insulin resistance and hyperinsulinemia that characterize this category of patients are both strong predictors of ischemic heart disease (2, 3). In addition to increasing the incidence of myocardial infarction, diabetes is also associated with increased cardiac morbidity and mortality following revascularization interventions (4–7). Following pharmacological and surgical revascularization techniques, type 2 diabetic patients treated with insulin have more postoperative complications and have a mortality rate 2–3-fold higher than type 2 diabetic patients who are not on insulin (8, 9). This suggests that insulin treatment may unexpectedly worsen prognosis in type 2 diabetic patients following an ischemic insult, and the defect may reside in the myocardium.

Uncoupling protein 3 (UCP3)2 is an inner mitochondrial membrane protein predominantly expressed in brown adipocytes, skeletal muscle, and the heart (10, 11). Uncoupling proteins have a tripartite structure, which has been compared with that of the ADP/ATP carrier of the mitochondrial inner membrane (12). Despite its high sequence homology with the archetypal uncoupling protein 1 and the fact that it can mediate mitochondrial proton leak, the physiological function of UCP3 remains unknown. There is compelling evidence that UCP3 protects the heart from ischemia–reperfusion injury. For example, we and others have recently shown both ex vivo and in vivo that the decrease or the complete loss of UCP3 in the rodent heart results in poorer recovery of contractile function and increased tissue damage following ischemia (13–15). Moreover, UCP3 mRNA and protein levels increase more than 2-fold in the heart of Wistar rats after ischemia (16). There is currently little information about the regulation of Ucp3 expression. MyoD and the peroxisome proliferator-activated receptor (PPAR) transcription factors are responsible for the stimulation of its expression in myocytes and in response to fatty acids, respectively (17). The signaling and transcription factors involved in the repression of Ucp3 are less clear, although such signals probably have an important physiological function because the half-life of the protein has been estimated to be a few hours (18).

Because the expression of UCP3 is positively regulated by fatty acids (19) and because the uncoupling activity of the pro-

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2 The abbreviations used are: UCP3, uncoupling protein 3; CP, core promoter sequence; EP, extended promoter; FoxO, forkhead box O; GSK-3, glycogen synthase kinase 3; PPAR, peroxisome proliferator-activated receptor; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; VDAC, voltage-dependent anion channel.
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protein is stimulated by reactive oxygen species and by fatty acids, both of which are increased in cardiomyocytes with diabetes, it is generally believed that UCP3 levels increase in the heart of diabetic patients (20, 21). Although this has been confirmed in many occasions for rodent models of type 1 diabetes (22–26), evidence is lacking to infer the same with type 2 diabetes (20). Moreover, Schrauwen et al. (27) reported that UCP3 protein levels in vastus lateralis muscle of patients with type 2 diabetes were half the levels found in nondiabetic subjects. More studies are clearly needed to investigate the regulation of myocardial UCP3 expression in the dysregulated metabolic and hormonal states of type 2 diabetes.

The present study focused on the impact of normal and dysregulated insulin signaling on UCP3 expression in the murine heart. We hypothesized that the hyperinsulinemia and insulin resistance that characterize type 2 diabetes may contribute to a down-regulation of myocardial UCP3 levels. We are now able to demonstrate that the acute stimulation of insulin signaling pathways in the heart down-regulates Ucp3 in vivo. Moreover, we show that pharmacological hyperinsulinemia sustained for 15 days leads to a 40% decrease in UCP3 transcript and protein levels. Decreased UCP3 expression was linked to the development of selective insulin resistance and overactivation of the sterol regulatory element-binding protein (SREBP)-1-mediated lipogenic program in the heart. Reporter gene assays and DNA sequence analysis revealed that the inhibitory effect of SREBP-1 on UCP3 expression is mediated through sterol regulatory elements (SREs) located in the first intron of the Ucp3 locus. Taken together, the results presented below identify a novel regulatory mechanism governing UCP3 expression that becomes defective in the development of selective insulin resistance of the heart.

Experimental Procedures

Animal Experiments—The experiments were conducted on 8–12-week old C57BL/6) male mice. The animals were kept on a 12-h light/12-h dark cycle in the University of Texas-Houston Medical School Animal Care Center or in the Laboratory Animal Facilities of the University of Mississippi Medical Center and fed standard rodent laboratory chow (LabDiet® 5001 at the University of Texas-Houston; Teklad 22/5 Rodent Diet 8640 at the University of Mississippi). All procedures were reviewed and approved by the Animal Welfare Committees of the University of Texas Health Science Center and University of Mississippi Medical Center. In a first set of experiments, overnight-fasted mice received a single intraperitoneal injection of Humulin® U-100 (1 unit/kg of body weight; Lilly). Control groups included saline-injected mice and mice treated with the antilipolytic agent nicotinic acid (75 mg/kg body weight; Sigma-Aldrich). The effects of chronic hyperinsulinemia were investigated using subcutaneous injections of increasing doses of neutral protamine Hagedorn insulin (0.14–1.68 units/day; Novolin® N, Novo Nordisk) for 15 days (28, 29). Two-thirds of the dose was given at 2000 h and one-third at 0800 h. To prevent hypoglycemia, the rodent chow diet was supplemented with sugar cubes, and 5% (w/v) glucose was added to the drinking water (INS + CARB). A second group of mice was maintained on the same carbohydrate-supplemented diet/water and subjected to a similar schedule of saline injections (CARB). The control group consisted of noninjected animals maintained on chow diet and plain water (untreated). Plasma and tissue samples were recovered at 0800 h in the morning of the 16th day, 12 h after the last insulin injection. All animals were sacrificed by cervical dislocation and exsanguination prior to sample collection. Upon recovery, hearts and plasma were flash-frozen in liquid nitrogen and stored at −80 °C until further analyses were carried out.

Real-time PCR Measurements of Transcript Levels—Total RNA was extracted from tissue samples and cultured cells using TRIzol® (Life Technologies, Inc.), treated for potential DNA contamination with DNA-free™ (Life Technologies), and reverse transcribed into complementary DNA using RevertAid™ Reverse Transcriptase (Thermo Fisher Scientific). Relative quantification of target mRNA levels was performed with self-designed primers and TaqMan® probes (series available upon request).

Immunoblotting—Protein homogenates were prepared in the presence of protein phosphatase (Sigma-Aldrich) and protease (Roche Applied Science) inhibitors. Proteins were detected by immunoblotting using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Santa Cruz Biotechnology, Inc.). The phospho-Akt Thr-308 (catalog no. 4056), phospho-Akt Ser-473 (catalog no. 4058), phospho-GSK-3β Ser-9 (catalog no. 9323), GSK-3β (catalog no. 9315), phospho-p44/42 MAPK Thr-202/Tyr-204 (catalog no. 4377), p44/42 MAPK (catalog no. 4695), phosphotuberin/TSC2 Ser-939 (catalog no. 3615), tuberin/TSC2 (catalog no. 3990), and voltage-dependent anion channel (VDAC) (catalog no. 4661) antibodies were from Cell Signaling Technology. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 10R-G109a) antibody was from Fitzgerald Industries International. The UCP3 (ab3477) and PPARα (ab24509) antibodies were from Abcam. The synthetic UCP3 peptide (ab4994) used to produce the polyclonal antibody ab3477 was used in control experiments to confirm the specificity of the detected bands. The pan-Akt antibody (sc-8312) and the SREBP-1 antibodies (sc-8984 used for mouse heart tissue; sc-13551 used for L6 cells) were from Santa Cruz Biotechnology. The phospho-FoxO3a Thr-32 (07-695) and FoxO3a (06-951) antibodies were from Upstate (now part of EMD Millipore). Densitometry analyses were performed using ImageJ (version 1.49).

Plasma Parameters—Plasma insulin, glucose, and free fatty acid levels were measured by the Mouse Metabolism Core at the Diabetes Research Center, Baylor College of Medicine (Houston, TX).

Cell Culture and Treatments—In vitro experiments were carried out in L6 myocytes due to the ease of transfection of plasmid DNA in these cells, due to their sensitivity to insulin and to PPAR agonists, and because this is a widely accepted model to study the transcriptional regulation of UCP3 expression (30). L6 rat myogenic cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 584 mg/liter L-glutamine, 110 mg/liter sodium pyruvate, 4.5 g/liter D-glucose and supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. At confluence, the cells were switched to DMEM supplemented with 2% (v/v) horse
serum to induce differentiation. After 7 days of differentiation, the cells were treated for 24 h with 1 or 10 μM Wy-14,643 (PPAR-α agonist; Cayman Chemical), rosiglitazone (PPAR-γ agonist; Adipogen), or L-165,041 (PPAR/β/δ agonist; R&D Systems), alone or in the presence of 100 nM insulin (from bovine pancreas; Sigma-Aldrich). All PPAR agonists were initially dissolved in DMSO, and insulin was aliquoted in 0.01 M HCl. Therefore, control cells were incubated in culture medium containing 0.01% (v/v) DMSO and 0.1% (v/v) HCl 0.01 M to match the treatment conditions. Mitochondria were isolated by differential centrifugation as described previously (13, 31).

Vectors—Genomic DNA was purified from mouse heart using DNeasy® blood and tissue kit (Qiagen), and fragments of the Ucp3 genomic locus were PCR-amplified with Phusion® high fidelity DNA polymerase (New England Biolabs). Primers 5′-GGCACTCCGAGAATTGTTGCTCTATGTTGGCA-3′/5′-GGGCGAGATCTTCTTGGTGAGCTTTTGAG-CCATGTG-3′ were used to amplify the Ucp3 core promoter (hereinafter referred to as CP) spanning positions −511 to +90 relative to the beginning of the first exon. A second ampiclon spanning positions −590 to +6,452 and covering the core promoter, exon 1, intron 1, and a part of exon 2 was generated using primers 5′-GGCACTCCGAGAATTGTTGCTCTATGTTGGCA-3′/5′-GGGCGAGATCTTCTTGGTGAGCTTTTGAG-CCATGTG-3′ and is referred to hereafter as extended promoter 1 (EP1). Both ampiclons were double-digested with Xhol and EcoRV, purified and cloned into the respective restriction sites of pGL4.12[luc2CP] (Promega). The firefly luciferase reporter encoded by this vector contains a PEST protein degradation element. Both elements act in concert to accelerate the turnover of firefly luciferase to 0.6 h, thereby providing an ultrafast reporter whose levels change very quickly in response to changes in promoter activity. Vectors were fully sequenced to ensure that there were no PCR-introduced mutations.

Deletion constructs were generated by double digestion of the EP1 construct with EcoRV and a series of single cutter restriction enzymes. For each deletion construct, the restriction enzyme used and the position of the restriction site on the Ucp3 locus were as follows: EP2, PmlI at +3,526; EP3, AvrII at +3,941; EP4, SwaI at +2,581; EP5, EcoNI at +1,284; EP6, XcmI at +162. The digestion products were gel-purified and recircularized, and deletions were validated by restriction analysis and sequencing.

Chromatin Immunoprecipitation Assay—Chromatin was prepared from heart tissue samples using the ChIP-IT® High sensitivity kit (Active Motif). Due to the small amount of starting material, 3–4 mouse hearts had to be pooled to perform a single ChIP reaction, thereby allowing the preparation of 3 independent ChIP experiments/treatment condition. The chromatin was fragmented with a Covaris M220 ultrasonicator, and 10-μg chromatin aliquots were incubated overnight at 4 °C with 6 μg of SREBP-1 antibody (Santa Cruz Biotechnology, sc-13551). Specific primer pairs were designed for each of the putative SREs to be tested. The negative control primers span a DNA region in the first intron of the Ucp3 locus located 3.4 kb downstream of the analyzed SREs. A primer pair spanning two known SREs in the proximal promoter of mouse SCD1 was used as a positive control (33). Primer sequences and their position in DNA sequences are provided in Table 1. Immunoprecipitated DNA was analyzed by real-time PCR on a Viia™7 instrument using iTaq™ Universal SYBR® Green Supermix (Bio-Rad), and enrichment was represented as a percentage of input DNA.

Results

Acute and Chronic Insulin Stimulation Down-regulate UCP3 at the Transcriptional Level in Mouse Heart—We first sought to determine the time course effect of acute insulin stimulation on cardiac UCP3 expression in vivo. Overnight-fasted mice received a single intraperitoneal injection of insulin (1 unit/kg of body weight). Increased phosphorylation of Akt and of its downstream target glycogen synthase kinase 3β (GSK-3β) revealed that activation of the PI3K/Akt pathway in

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Insulin Resistant in the Heart—respectively (Fig. 2).

Insulin treatment also induced a linear decrease of myocardiuc UCP3 transcript levels over time (Fig. 1C). Insulin blocked UCP3 expression for at least 4 h (Fig. 1D) and led to a 20% decrease in UCP3 protein levels after this treatment duration (Fig. 1E). The fact that the antilipolytic agent nicotinic acid failed to decrease myocardial UCP3 transcript levels suggests that insulin inhibits UCP3 expression through a direct action on the heart rather than through a transient decrease in plasma free fatty acid levels (Fig. 1F). The plasma free fatty acid rebound observed with nicotinic acid is typical of this compound (Fig. 1G) (34).

The effect of chronic hyperinsulinemia on UCP3 expression in the heart was then investigated by injecting mice with a stepwise increase in the concentration of intermediate-acting insulin over 15 days (Fig. 2A). During the treatment, mice had free access to sugar cubes and 5% glucose in the drinking water to prevent hypoglycemia. As reported previously for mice under this protocol (28), carbohydrate supplementation (CARB) led to a significant reduction in chow consumption and to increased water consumption that was independent of the insulin treatment (Table 2). At the time of sacrifice, mean plasma insulin levels were significantly increased by 135% in the INS group when compared with the untreated animals (Table 3). Carbohydrate supplementation was sufficient to prevent hypoglycemia and led to a 20% decrease in UCP3 protein levels after this treatment duration (Fig. 1E). This was accompanied in both cases by a decrease of myocardial UCP3 transcript levels over time (Fig. 3B). Consistent with the fact that chronic Akt activation is implicated in early alterations of myocardial metabolism in the prediabetic, insulin-resistant stage (40). Consistent with the fact that chronic Akt activation down-regulates PPARα in the heart (41), we observed an increase in PPARα levels for treated mice (Fig. 4A). However, the transcript levels of the PPARα target enzymes medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and pyruvate dehydrogenase kinase 4 (PDK4) were unchanged or even slightly reduced in the case of carnitine palmitoyltransferase 1B (CPT1B) for the INS + CARB group (Fig. 4B). The results indicate that a change in PPARα activity is unlikely to account for the dramatic decrease in UCP3 expression.

Increased activity of the forkhead box O (FoxO) family of transcription factors has also been proposed to mediate lipotoxicity and contractile dysfunction in the diabetic heart (42).

### Table 1

Primers used in the chromatin immunoprecipitation assay

| Target name | Ensembl gene sequence | Primer sequences | Location |
|-------------|-----------------------|------------------|----------|
| *Scal* positive control | ENSMUSG0000000037071 | 5'-TTGCAACAAATCTCCTACTCTGGAAT-3' (forward) 5'-CCCTGCTCCCTCTTCTTTCCAT-3' (reverse) | 5'-UTR |
| *Scal* negative control | ENSMUSG0000000033942 | 5'-ATCATGGCTCTGGASAGAAGAAAGG-3' (forward) 5'-GCGAGCCTGAGCAAATG-3' (reverse) | Intron 5 |
| Ucp3 SRE1 | ENSMUSG0000000032942 | 5'-GGCCGTCTTCTCTTCTGCTGAGG-3' (forward) 5'-GCGCTGCTCTTCTCTGCTGAGG-3' (reverse) | Intron 1 |
| Ucp3 SRE2 | ENSMUSG0000000032942 | 5'-GGCCGTCTTCTCTTCTGCTGAGG-3' (forward) 5'-GCGCTGCTCTTCTCTGCTGAGG-3' (reverse) | Intron 1 |
| Ucp3 SRE3 | ENSMUSG0000000032942 | 5'-GGCCGTCTTCTCTTCTGCTGAGG-3' (forward) 5'-GCGCTGCTCTTCTCTGCTGAGG-3' (reverse) | Intron 1 |

Chronic Hyperinsulinemia Selectively Activates the Lipogenic Program in the Heart—Because hepatic glucose and lipid metabolism are altered by selective insulin resistance (35) and because UCP3 regulates glucose and fatty acid metabolism in myocytes (38, 39), we investigated whether the down-regulation of UCP3 in the heart could be linked to a metabolic remodeling induced by the dysregulation of one or several insulin-regulated transcription factors. Increased activity of the transcription factor PPARα is implicated in early alterations of myocardial metabolism in the prediabetic, insulin-resistant stage (40). Consistent with the fact that chronic Akt activation down-regulates PPARα in the heart (41), we observed an increase in PPARα levels for treated mice (Fig. 4A). However, the transcript levels of the PPARα target enzymes medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and pyruvate dehydrogenase kinase 4 (PDK4) were unchanged or even slightly reduced in the case of carnitine palmitoyltransferase 1B (CPT1B) for the INS + CARB group (Fig. 4B). The results indicate that a change in PPARα activity is unlikely to account for the dramatic decrease in UCP3 expression.
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A. Time course analysis of insulin signaling activation in the heart of overnight fasted mice (n = 37). Representative immunoblots for the phosphorylation of Akt at Thr-308 and Ser-473, the phosphorylation of the direct Akt target GSK-3β at Ser-9, and the phosphorylation of the p44/42 MAPK at both Thr-202 and Tyr-204 are shown. β-Tubulin levels are shown as a loading control. *, p < 0.05; **, p < 0.01; †, p < 0.05; ††, p < 0.01 versus 0 min. †, p < 0.05; ††, p < 0.01; †††, p < 0.001 versus 5–15 min (phospho-Akt Thr-308 and phospho-Akt Ser-473), 5–45 min (phospho-GSK-3β), or 15 min (phospho-MAPK). Blood glucose levels (B) and cardiac UCP3 transcript levels (C) were also measured in the same animals. Data are means ± S.E. (error bars) of 3–5 animals/time point. *, p < 0.05; **, p < 0.01 versus time 0.

D. Acute insulin treatment was repeated in another set of mice, and cardiac UCP3 transcript levels were quantified and compared with those of saline-treated mice 1, 2, and 4 h after injection (n = 4–6). E, cardiac UCP3 protein levels were determined by immunoblotting 4 h after insulin treatment (n = 4), *, p < 0.05. Cardiac UCP3 mRNA levels (F) and plasma free fatty acids (G) were quantified 4 h after intraperitoneal injection with saline, insulin, nicotinic acid, or a combination of insulin and nicotinic acid. Data are means ± S.E. of 7 animals/group. *, p < 0.05; **, p < 0.01 versus saline. †, p < 0.05; ††, p < 0.01 versus nicotinic acid. A.U., arbitrary units.

FIGURE 1. Acute stimulation of insulin signaling in mouse heart inhibits UCP3 expression. A, time course analysis of insulin signaling activation in the heart of overnight fasted mice (n = 37). Representative immunoblots for the phosphorylation of Akt at Thr-308 and Ser-473, the phosphorylation of the direct Akt target GSK-3β at Ser-9, and the phosphorylation of the p44/42 MAPK at both Thr-202 and Tyr-204 are shown. β-Tubulin levels are shown as a loading control. *, p < 0.05; **, p < 0.01; †, p < 0.05; ††, p < 0.01 versus 0 min. †, p < 0.05; ††, p < 0.01 versus 5–15 min (phospho-Akt Thr-308 and phospho-Akt Ser-473), 5–45 min (phospho-GSK-3β), or 15 min (phospho-MAPK). Blood glucose levels (B) and cardiac UCP3 transcript levels (C) were also measured in the same animals. Data are means ± S.E. (error bars) of 3–5 animals/time point. *, p < 0.05; **, p < 0.01 versus time 0. D, acute insulin treatment was repeated in another set of mice, and cardiac UCP3 transcript levels were quantified and compared with those of saline-treated mice 1, 2, and 4 h after injection (n = 4–6). E, cardiac UCP3 protein levels were determined by immunoblotting 4 h after insulin treatment (n = 4), *, p < 0.05. Cardiac UCP3 mRNA levels (F) and plasma free fatty acids (G) were quantified 4 h after intraperitoneal injection with saline, insulin, nicotinic acid, or a combination of insulin and nicotinic acid. Data are means ± S.E. of 7 animals/group. *, p < 0.05; **, p < 0.01 versus saline. †, p < 0.05; ††, p < 0.01 versus nicotinic acid. A.U., arbitrary units.
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Chronic hyperinsulinemia decreases UCP3 mRNA and protein levels in the heart. A, mice were rendered hyperinsulinemic with injections of intermediate-acting insulin twice daily and fed a carbohydrate-supplemented diet and water to prevent hypoglycemia (INS + CARB). Other treatment groups included saline-treated mice fed with the same carbohydrate-supplemented diet and water (CARB) and uninjected mice maintained on normal rodent diet and plain water (untreated). B, cardiac UCP3 transcript levels were quantified by real-time PCR. Data are shown as means ± S.E. (error bars) of 8–11 animals/group. *, p < 0.05 versus untreated. C, cardiac UCP3 protein levels were quantified by immunoblotting and normalized against the mitochondrial protein VDAC. Data are means ± S.E. of 10–11 animals/group. **, p < 0.01 versus untreated. Representative immunoblots of 3–4 animals/group are shown. D, the three bands detected between 25 and 37 kDa correspond to UCP3 because binding of the antibody (1 µg/ml) was inhibited when preincubated with the immunizing peptide (2 µg/ml). Nonspecific binding of the antibody is unaffected by preincubation with the immunizing peptide (arrowheads). A.U., arbitrary units.

### TABLE 2

Daily consumption of rodent chow, sugar cubes, and drinking water per mouse during the chronic hyperinsulinemia treatment

| Parameter               | Untreated (n = 11) | CARB (n = 11) | INS + CARB (n = 10) |
|-------------------------|--------------------|---------------|---------------------|
| Rodent chow (g)         | 5.4 ± 0.2          | 2.4 ± 0.2*    | 2.5 ± 0.1*          |
| Sugar cubes (g)         | 1.5 ± 0.0          | 1.3 ± 0.1     |                     |
| Drinking water (ml)     | 5.5 ± 0.1          | 8.8 ± 0.3**   | 7.0 ± 0.2**         |

* p < 0.001 versus untreated. ** p < 0.05 versus INS + CARB.

### TABLE 3

Plasma and body parameters of mice at the end of the chronic hyperinsulinemia treatment

| Parameter               | Untreated (n = 11) | CARB (n = 11) | INS + CARB (n = 10) |
|-------------------------|--------------------|---------------|---------------------|
| Plasma insulin (ng/ml)  | 0.88 ± 0.16        | 1.58 ± 0.45   | 2.07 ± 0.48*        |
| Plasma glucose (mg/dl)  | 180 ± 11           | 205 ± 17      | 200 ± 14            |
| Plasma free fatty acids (milliequivalents/liter) | 0.99 ± 0.05        | 1.22 ± 0.13   | 1.18 ± 0.12         |
| Body weight (g)         | 22.9 ± 0.7         | 23.8 ± 0.9    | 24.8 ± 0.9          |
| Tibial length (mm)      | 16.6 ± 0.2         | 16.9 ± 0.3    | 17.2 ± 0.2          |
| Heart weight/body weight (mg/g) | 4.60 ± 0.10         | 4.51 ± 0.11   | 4.58 ± 0.12         |
| Heart weight/tibial length (g/mm) | 6.34 ± 0.20         | 6.34 ± 0.12   | 6.37 ± 0.19         |

* p < 0.05 versus untreated.
when used at 100 nM (Fig. 5A). Similar to what was observed in the mouse heart, the insulin-mediated down-regulation of UCP3 was accompanied by an increased expression of lipogenic enzymes. Next, we used various PPAR agonists to stimulate UCP3 expression in L6 myocytes. Wy-14,643 (a PPARα agonist) and rosiglitazone (a PPARγ agonist) induced a 1.6- and 1.9-fold increase, respectively, in UCP3 transcript levels when used at 10 µM (Fig. 5B). The PPARβ/δ-selective agonist L-165,041 increased UCP3 mRNA levels 17- and 25-fold when used at 1 and 10 µM, respectively (Fig. 5B). These results are consistent with previous findings reporting that PPARβ/δ is the most abundant PPAR isoform expressed in L6 cells (43). The addition of insulin (100 nM) to the culture medium strongly inhibited the induction of UCP3 expression by all three PPAR agonists (Fig. 5B). Insulin treatment also resulted in a 26 and 14% decrease in baseline and L-165,041-induced UCP3 protein levels, respectively (Fig. 5C). Taken together, the results demonstrate that rat L6 myocytes constitute a valid model to study the molecular basis of UCP3 down-regulation by insulin.

### The First Intron of Ucp3 Contains the Cis-acting Element(s) Responsible for Ucp3 Repression by SREBP-1

The core promoter sequence (CP) of the mouse Ucp3 gene was PCR-amplified from cardiac DNA and cloned upstream of a firefly luciferase reporter gene. Because it has recently been shown that the first intron of the mouse Ucp3 gene contains several enhancer elements necessary for the stimulation of UCP3 expression in brown adipose tissue (44), we generated an extended version of the promoter (EP1) covering the core promoter, exon 1, intron 1, and a part of exon 2 from the Ucp3 locus (Fig. 6A). Both vectors led to a significant increase in basal luciferase activity when transfected into L6 myocytes (Fig. 6B). The cells were subsequently treated for 24 h with insulin (100 nM), L-165,041 (10 µM), or a combination of both drugs (Fig. 6C). Similar to what was observed with the promoterless vector (basic), reporter gene expression did not vary with the different treatment conditions when under the control of CP. However, L-165,041 successfully stimulated firefly luciferase expression from EP1. These observations are in line with previous findings showing that a UCP3 enhancer contained in the first intron regulates the activity of the core promoter (44). The positive effect of the PPARβ/δ agonist was significantly attenuated when co-incubated with insulin (Fig. 6C).

To confirm the involvement of SREBP-1 in the inhibition of UCP3 expression, the EP1 vector was cotransfected with one of three siRNAs targeting distinct regions of the SREBP-1 transcript. All three siRNAs led to a significant decrease in both the precursor and cleaved forms of SREBP-1 (Fig. 6D). At the mRNA level, the knockdown of SREBP-1 was accompanied by decreased expression of its known target genes and by an increase in endogenous UCP3 expression (Fig. 6E). Compared with stearoyl-CoA desaturase 1, the decrease in acetyl-CoA carboxylase 1 and fatty acid synthase expression may have been compensated for by SREBP-2 (45), whose expression in L6 myocytes was unaffected by the siRNAs (data not shown). The knockdown of SREBP-1 also led to enhanced EP1 promoter activity under L-165,041 stimulation (Fig. 6F). Next, we investigated the impact of SREBP-1c overexpression on firefly luciferase activity for both CP and EP1 vectors. As expected, SREBP-1c overexpression strongly inhibited EP1 promoter activity, independent of the pharmacological treatment used (Fig. 6G). Conversely, SREBP-1c overexpression potentiated CP activity under all treatment conditions (Fig. 6G). Thus, the data indicate that one or several SREs are located in the first intron of the Ucp3 locus and contribute to SREBP-1-mediated inhibition of UCP3 expression. Furthermore, binding of overexpressed SREBP-1 to the core promoter sequence may occur in our in vitro assay and drive enhanced firefly luciferase expression in the absence of the functional inhibitory element(s). Indeed, bioinformatics analysis also revealed the presence of a putative SRE in the core promoter (Table 4). We then decided to perform a serial deletion analysis to identify more precisely the location of the inhibitory SRE(s). Portions of the first intron were gradually deleted, starting from the 3’-end of the EP1 construct, and the effects on firefly luciferase activity were analyzed in the presence of SREBP-1c overexpression. Luciferase activity was lower or similar to that observed with the promoterless vector for all constructs containing the first
2,479 base pairs of the first intron (constructs EP1–EP4). However, luciferase activity was increased for the constructs containing only the first 1,184 bp of the first intron and below (EP5 and EP6; Fig. 7A). This indicates that one or several binding sites for SREBP-1 are located between nucleotides 1,289 and 2,584 relative to the beginning of the first exon. Three potential SREs were identified in this part of the intronic sequence by bioinformatics analysis (Table 4). Interestingly, these putative binding sites are located in the vicinity of the elements that form the UCP3 complex enhancer (Fig. 7B). The functionality of these binding elements was investigated by site-directed mutagenesis. The loss of a single element or two elements had no effect on the ability of insulin to inhibit PPAR-mediated expression of the reporter gene. However, simultaneous loss of
all three elements significantly reduced the effect of insulin (Fig. 7C). Last, we investigated whether hyperinsulinemia is associated with increased binding of SREBP-1 to this intronic DNA region in the heart \textit{in vivo}. Chromatin immunoprecipitation demonstrated increased recruitment of SREBP-1 to known SREs located in the proximal promoter of stearoyl-CoA desaturase 1 for the heart of INS/H11001 CARB-treated mice (Fig. 7D). Whereas no significant change could be detected for the CARB group, we also confirmed increased binding of SREBP-1 at the SREs contained in the first intron of the \textit{Ucp3} locus for the INS/H11001 CARB group.

\textbf{Discussion}

We have shown that the acute stimulation of insulin signaling pathways in the heart of male mice leads to the rapid down-regulation of \textit{UCP3}. In the same animal model, experimental hyperinsulinemia led to the development of a pathway-selective insulin resistance characterized by decreased Akt signaling but preserved p44/p42 MAPK activity. This selective insulin resistance was associated with a 40% decrease in \textit{UCP3} transcript and protein levels in the heart. The decrease in \textit{UCP3} was mirrored by increased expression and maturation of SREBP-1 and increased transcript levels of the transcription factor’s target genes. Reporter gene assays performed in cultured myocytes confirmed the inhibition of PPAR-mediated \textit{UCP3} expression by insulin and SREBP-1. Last, we found that the sterol regulatory elements responsible for this inhibition are located in the first intron of the \textit{Ucp3} locus, in a complex regulatory region known as the \textit{UCP3} enhancer.

Although \textit{UCP3} was discovered more than 15 years ago, much remains to be learned about its function and the extent of its regulation in physiological and pathological conditions. \textit{UCP3} is known for being up-regulated by fatty acids through activation of the PPAR transcription factors. Indeed, rodent studies have shown that cardiac \textit{UCP3} expression increases in conditions where the supply of free fatty acids to the heart is increased, such as heart failure (46), starvation (47), and high fat feeding (48). In adult rat cardiomyocytes, long-chain fatty acids...
**FIGURE 6.** The first intron of mouse Ucp3 contains the cis-acting element(s) responsible for UCP3 down-regulation by insulin and SREBP-1. A, the core promoter of mouse Ucp3 and an extended version of the promoter including the core promoter, exon 1, intron 1, and a part of exon 2 (EP1) were cloned upstream of the firefly luciferase (FLuc) reporter gene as described under “Experimental Procedures.” B, relative luciferase activity measured from the constructs transfected in L6 myocytes is shown. Data are means ± S.E. of seven experiments. ***, p < 0.001 versus basic. ††, p < 0.01 versus CP. C, transfected cells were treated with insulin (100 nM), L-165,041 (10 μM), or both drugs together. Data are means ± S.E. of six experiments. **, p < 0.01; ***, p < 0.001 versus basic. E, the mRNA expression levels of SREBP-1, SREBP-1 known target genes, and UCP3 were quantified by real-time PCR. Data are means ± S.E. of five experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control. F, cells were transfected with the EP1 vector and a non-targeting siRNA sequence (control) or one of three distinct siRNAs against SREBP-1 (s134943, s134944, or s134945). D, protein levels for both the precursor and the cleaved form of SREBP-1 were determined by immunoblotting. A representative immunoblot of two independent experiments is shown. Data are means ± S.E. of five experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control. A.U., arbitrary units.

**TABLE 4**
Prediction of SREBP-1 binding sites in the core promoter and the first intron of Ucp3
The binding sites were predicted with the JASPAR CORE database of experimentally defined transcription factor binding sites for eukaryotes (version 5.0, ALPHAB).

| Model ID    | Model name (SRE 1) | Score | Relative score | Start | End | Strand | Site sequence |
|-------------|--------------------|-------|----------------|-------|-----|--------|--------------|
| MA0595.1    | SREBF1             | 7.908 | 0.84252        | −87   | −78 | −1     | ATCAGTTGAC   |
| MA0595.1    | SREBF1 (SRE 1)     | 7.611 | 0.83600        | +1829 | +1838| −1     | CTCTCTCAT    |
| MA0595.1    | SREBF1 (SRE 2)     | 6.363 | 0.80860        | +1994 | +2003| 1      | CTCAGCCAAG   |
| MA0595.1    | SREBF1 (SRE 3)     | 10.488| 0.89918        | +2251 | +2260| 1      | ATCACCACAC   |

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in general and oleate in particular strongly activate UCP3 expression (49). Furthermore, work performed in rat hearts has demonstrated a diurnal oscillation of UCP3 transcript levels that is coordinated with the expression of fatty acid oxidation genes and with the up-regulation of transcriptional activators of the PPARα system (50). Therefore, it seemed at first more than likely that in our animal models, the down-regulation of UCP3 by insulin was indirectly caused by the hormone’s antilipolytic action. However, this does not seem to be the case for the following reasons. First, the decrease in UCP3 mRNA levels induced by acute insulin treatment could not be reproduced with the antilipolytic agent nicotinic acid. Second, free fatty acids levels were not decreased following the acute insulin treatment; nor were they decreased with chronic hyperinsulinemia. Third, chronic hyperinsulinemia induced a 40% decrease in cardiac UCP3, whereas PPARα levels concomitantly increased, and the expression of other PPARα-activated genes was mostly unaltered. All of these observations suggest a different mode of action for insulin signaling in the transcriptional regulation of UCP3 in the heart.

The expression of the lipogenic factor SREBP-1 is stimulated by insulin and further enhanced by hyperinsulinemia in the liver, white adipose tissue, and skeletal muscle (35, 51, 52). Furthermore, SREBP-1c has been proposed to antagonize the stimulation of UCP3 expression by 9-cis-retinoic acid in skeletal myocytes (53). Accordingly, we observed a decrease in UCP3 protein levels and a concomitant increase in SREBP-1 precursor and cleaved forms in the soleus muscle of our experimental model of chronic hyperinsulinemia (data not shown). It has also been shown that the activation of the p44/42 MAPK pathway is responsible for insulin-mediated up-regulation of total and nuclear SREBP-1 in the heart.

FIGURE 7. Stepwise deletions and site-directed mutagenesis identify the binding sites for SREBP-1. A, 3′ portions of the EP1 vector were excised to gradually delete the totality of the first intron. The constructs were transfected in the absence (baseline) or in the presence of pSV Sport SREBP-1c. Basic, promoterless vector. Red boxes, first exon and part of the second exon of Ucp3. Broken lines, deleted intronic sequences. Vertical dotted lines, cutting sites in the sequence, numbered relative to the beginning of the first exon. Data are means ± S.E. of three experiments performed in duplicates. ***, p < 0.001 versus basic, EP1, EP2, EP3, and EP4. B, mapping of the three putative binding sites for SREBP-1 (SRE 1, SRE 2, and SRE 3; highlighted in blue) comprised in the intronic sequence between nucleotides +1,289 and +2,584. Previously identified binding sites for the transcription factors SP1/SP3, PPARγ/RXRα, and MyoG/MyoD (44) are highlighted in yellow. Changes in the DNA sequence induced by site-directed mutagenesis are indicated in red. C, EP1 vectors with mutation of one, two, or all three putative SREs were transfected into rat L6 myocytes, and the cells were treated with L-165,041 (10 μM) in the absence or in the presence of insulin (100 nM). Data are presented as a percentage of the PPAR-induced firefly luciferase activity inhibited by insulin. Crossed circles represent mutation of the respective elements as depicted in B. Data are means ± S.E. (error bars) of five experiments. *, p < 0.05 versus EP1. D, ChIP analysis of pooled heart tissue samples. Immunoprecipitated DNA was analyzed by real-time PCR, and enrichment is represented as percentage of input DNA. Pos and Neg, positive and negative control sequences, respectively. Data are means ± S.E. of two real-time PCR assays performed on 3 independent ChIP experiments/treatment group.
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Investigations in perfused rodent hearts have revealed both protective and detrimental effects for UCP3 on cardiac function, effects that seem to be linked to the equivocal uncoupling activity of the protein. On the one hand, UCP3 protects from ischemia-reperfusion injury by preserving mitochondrial function through a decrease in reactive oxygen species generation and inhibition of the mitochondrial permeability transition pore opening (14, 15, 60). Genetic deletion of UCP3 also favors a glycolytic shift after myocardial infarction, an observation that further underlines the permissive role that the mitochondrial protein has in fatty acid oxidation in the stressed heart (61). On the other hand, increased UCP3 expression in response to high fat feeding is associated with decreased cardiac efficiency, and UCP3 deletion results in an increase in both cardiac efficiency and cardiac power (48, 62). However, the role of the oxygen-wasting effect of UCP3 in contractile dysfunction of the failing heart remains to be clearly established. Interestingly, loss of UCP3 increases sudden cardiac death in high fat-fed mice treated with etomoxir, suggesting another cardioprotective role for UCP3 in lipid-challenged conditions (63).

An accumulation of triglycerides in the heart is a common feature of patients and animal models with obesity and type 2 diabetes (64–66). Intracardiac lipid accumulation is thought to result mainly from a mismatch between an increased supply of circulating lipids and a decreased oxidative capacity of the heart amplified by mitochondrial dysfunction (67). However, increased SREBP-1c expression in cardiac myocytes from patients with metabolic syndrome and aortic stenosis has also previously been reported to correlate with increased lipid accumulation in these cells (68). Based on this report and on our present findings, the activation of lipogenesis in the heart in response to hyperinsulinemia may also play a significant role in intracardiac lipid accumulation. Because UCP3 promotes fatty acid oxidation in muscle cells (13, 39), its down-regulation by insulin may be part of the adaptive mechanism by which the heart switches from predominant fat utilization to increased carbohydrate consumption between the fasted and fed states (69). Besides the regulation of UCP3 and lipogenic enzymes, the global role played by SREBPs in cardiac physiology remains to be investigated. Recent microarray studies performed in skeletal myocytes suggest that the functions of SREBPs expand beyond metabolic regulation and involve the activation of stress pathways and muscle growth (70–72).

Last, our findings suggest an additional explanation for the poor prognosis of type 2 diabetic patients after myocardial infarction. It is already known that impaired activity of the Akt kinase worsens tissue damage following ischemia-reperfusion through a plethora of factors, including reduced NO bioavailability, the loss of blockade of pro-apoptotic factors, and increased opening of the mitochondrial permeability transition pore (73–75). We and others have shown recently that UCP3 is cardioprotective in the setting of ischemia-reperfusion (13–15). Therefore, we propose that the down-regulation of UCP3 through increased p44/42 MAPK activity may be a further mechanism by which hyperinsulinemia and the ensuing selective insulin resistance sensitize the heart to ischemia-reperfusion injury and impair the recovery of myocardial energetics and contractile function.
We acknowledge that the present study is not devoid of certain limitations. First, in order to optimize transfection efficiency, our promoter analyses were performed in an immortalized skeletal myoblast cell line, a model that does not fully reproduce the physiology of cardiac myocytes. Moreover, although still representing the accepted standard in gene promoter analysis, studies investigating the cis-acting motifs contained in the UCP3 promoter have demonstrated that in vitro systems may not fully reproduce regulatory events occurring in vivo (30). Last, it is possible that decreased UCP3 expression is also linked to increased mRNA or protein turnover, and the involvement of such mechanisms in our model will require further investigation.

In conclusion, our work has exposed insulin as a major inhibitor of UCP3 expression in the rodent heart. We have also demonstrated that experimental hyperinsulinemia induces a pattern of pathway-selective insulin resistance in the heart associated with the activation of the lipogenic program and a sustained decrease in UCP3 levels. In physiological conditions, the SREBP-1-mediated down-regulation of UCP3 may be part of the mechanism by which myocardial metabolism adapts to daily fluctuations in substrate supply. However, we propose that the sustained repression of UCP3 induced by chronic hyperinsulinemia contributes to myocardial metabolic inflexibility in obesity, the metabolic syndrome, and type 2 diabetes. More investigations are needed to determine whether the impairment of cardiac UCP3 expression contributes to the poor prognosis of type 2 diabetic subjects after myocardial infarction.

Author Contributions—R. H. and H. T. designed the study and wrote the paper. D. L. H. performed and analyzed the experiments shown in Figs. 2 and 3. K. A. W. performed and analyzed the experiments shown in Fig. 5. R. H. designed and constructed the plasmids and performed and analyzed the experiments shown in Figs. 1, 4, 6, and 7. All authors reviewed the results and approved the final version of the manuscript.

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