SIRT1 mRNA Expression May Be Associated With Energy Expenditure and Insulin Sensitivity

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OBJECTIVE—Sirtuin 1 (SIRT1) is implicated in the regulation of mitochondrial function, energy metabolism, and insulin sensitivity in rodents. No studies are available in humans to demonstrate that SIRT1 expression in insulin-sensitive tissues is associated with energy expenditure and insulin sensitivity.

RESEARCH DESIGN AND METHODS—Energy expenditure (EE), insulin sensitivity, and SIRT1 mRNA adipose tissue expression (n = 81) were measured by indirect calorimetry, hyperinsulinemic-euglycemic clamp, and quantitative RT-PCR in 247 non-diabetic offspring of type 2 diabetic patients.

RESULTS—High EE during the clamp (r = 0.375, P = 2.8 × 10−3) and high ΔEE (EE during the clamp – EE in the fasting state) (r = 0.602, P = 2.5 × 10−24) were associated with high insulin sensitivity. Adipose tissue SIRT1 mRNA expression was significantly associated with EE (r = 0.289, P = 0.010) and with insulin sensitivity (r = 0.334, P = 0.002) during hyperinsulinemic-euglycemic clamp. Furthermore, SIRT1 mRNA expression correlated significantly with the expression of several genes regulating mitochondrial function and energy metabolism (e.g., peroxisome proliferator–activated receptor γ coactivator-1α, estrogen-related receptor α, nuclear respiratory factor-1, and mitochondrial transcription factor A), and with several genes of the respiratory chain (e.g., including NADH dehydrogenase [ubiquinone] 1α subcomplex 2, cytochrome c, cytochrome c oxidase subunit IV, and ATP synthase).

CONCLUSIONS—Impaired stimulation of EE by insulin and low SIRT1 expression in insulin-sensitive tissues is likely to reflect impaired regulation of mitochondrial function associated with insulin resistance in humans. Diabetes 59:829–835, 2010

SIRT1 mRNA Expression May Be Associated With Energy Expenditure and Insulin Sensitivity

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Compromised mitochondrial function in skeletal muscle predisposes to insulin resistance and type 2 diabetes (1,2). In contrast, physical activity and weight loss in obese and sedentary subjects stimulate mitochondrial biogenesis and improve insulin sensitivity (3). Animal and human studies have shown that mitochondrial function is associated with insulin sensitivity, but the mechanisms explaining this association are largely unknown (4).

The mammalian sirtuins (SIRT1–SIRT7) are implicated in gene silencing, mitochondrial function, energy homeostasis, insulin sensitivity, and longevity (5). We previously demonstrated that treatment with SIRT1 activator, resveratrol, enhanced mitochondrial activity and protected mice from diet-induced obesity and insulin resistance (4). The effects of resveratrol were seen in both muscle and adipose tissue and resulted in an increase in mitochondrial function, which translated into an increase in energy expenditure (EE) and insulin sensitivity. Small molecule activators of SIRT1, that are structurally unrelated to resveratrol, have also been shown to improve insulin sensitivity, lower plasma glucose, and increase mitochondrial capacity (6). In many rodent models (4,7), the upregulation of the oxidative phosphorylation (OXPHOS) pathway is coordinated by peroxisome proliferator–activated receptor γ coactivator (PGC) 1α, which is a target of SIRT1 (8). Similarly, insulin resistance in human skeletal muscle has been associated with decreased mitochondrial oxidative capacity and ATP synthesis and decreased expression of genes that control mitochondrial activity, including PGC-1α (9–11).

The offspring of type 2 diabetic subjects are known to be insulin resistant, and they have defects in mitochondrial OXPHOS associated with increased intramyocellular lipid content (9). The association of EE and insulin sensitivity with SIRT1 and PGC-1α mRNA expression has not been previously investigated. Therefore, we studied here the association of EE, insulin sensitivity, and adipose tissue SIRT1 and PGC-1α mRNA expression in 247 non-diabetic offspring of subjects with type 2 diabetes.

RESEARCH DESIGN AND METHODS

The subjects were selected from an ongoing study and included healthy nondiabetic offspring of patients with type 2 diabetes, as previously described (12). The diabetic patients (proband) were randomly selected among type 2 diabetic subjects living in the region of the Kuopio University Hospital. Spouses of the probands had to have a normal glucose tolerance in an oral glucose tolerance test (OGTT). The study protocol was approved by the ethics committee of the University of Kuopio. All study subjects gave an informed consent. A total of 247 offspring (one to three from each family) were studied. Their mean ± SD age was 35.1 ± 6.3 years and BMI 26.3 ± 4.7 kg/m².
Clinical and laboratory methods. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. BMI was calculated as weight (in kilograms) divided by square of height (in meters). On the first day, an OGTT was performed with 75 g of glucose. Subjects with normal glucose tolerance (n = 210), isolated impaired fasting glucose (n = 5), or impaired glucose tolerance (n = 5) were recruited on the basis of the World Health Organization criteria (13), were included in further studies. An intravenous glucose tolerance test (IVGTT) was performed to determine the first-phase insulin release after an overnight fast. After baseline blood collection, a bolus of glucose (300 mg/kg in a 50% solution) was given within 30 s into the antecubital vein. Samples for the measurement of blood glucose and plasma insulin (all blood) were drawn at baseline and 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 min. After an IVGTT, the degree of insulin sensitivity was evaluated with the hyperinsulinemic-euglycemic clamp technique (insulin infusion rate of 40 mU · min⁻¹ · m⁻² body surface area) as previously described (14). Blood glucose was clamped at 5.0 mmol/l for the next 120 min by infusion of 20% glucose at various rates according to blood glucose measurements performed at 5-min intervals. The mean amount of glucose infused during the last 20 min of the clamp was used to calculate the rates of whole-body glucose uptake (WBGU) and divided with lean body mass (LBM) for statistical analyses. Nonoxidative glucose metabolism (per LBM) was calculated as the difference between the rates of WBGU/LBM and glucose oxidation (per LBM). Indirect calorimetry was performed with a computerized flow-through canopy gas analyzer system (Deltatrac; Dateix) as previously described (14). The mean value of the data during the last 20 min of the clamp was used. Basal and glucose-stimulated [3H]-GTP uptake into total protein and glucose oxidation were calculated according to the method reported by Ferrannini (15). A computed tomography scan (Siemens Volume Zoom) at the level of the fourth lumbar vertebra was performed to evaluate the amount of intra-abdominal and subcutaneous fat as previously described (14). Blood and plasma glucose were measured by the glucose oxidase method (Glucose and Lactate Analyzer 2300 Stat Plus; Yellow Springs Instruments), and plasma insulin was determined by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics; 125I RIA Kit, Incstar, respectively). Serum free fatty acids (FFAs) were determined by an enzymatic method from Wako Chemicals. Nonprotein urinary nitrogen was measured by the automated Kjeldahl method as previously described (14). Plasma concentrations of tumor necrosis factor-α and cytokines (interleukin [IL]-1 receptor antagonist [IL-1RA], IL-6, IL-8, IL-10, IL-18, and interferon γ and serum levels of soluble adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, e-selectin, and p-selectin) were measured with high-sensitivity assay kits from R&D Systems. IL-8 was measured with a kit from Biosource International. High-sensitivity C-reactive protein (hs-CRP) was measured with an Immulite analyzer and a DPC hs-CRP assay.

Gene expression studies in offspring of type 2 diabetic parents. Subcutaneous fat samples were taken after an overnight fast in a supine position at the level of the umbilicus during local anesthesia. Total RNA from adipose tissue (n = 81) was isolated with an RNasey Lysis Tissue Mini kit (Qiagen) and treated with DNase (DNA-free; Ambion). Total RNA from skeletal muscle biopsies (n = 11) was isolated with QIAzol (Qiagen) and cleaned with an RNasey Plus Micro kit (Qiagen). RNA was transcribed to cDNA using a TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR experiments were performed in a seven 500 Real-Time PCR System (Applied Biosystems) using 6 ng (RNA equivalents) of cDNA as template, gene-specific primers, and probes (Applied Biosystems) (information on primers and probes available upon request). Expression levels of different genes were normalized to large ribosomal protein P0 (adipose tissue: Hs99999902_ml; Applied Biosystems) or to β-actin (skeletal muscle: Hs00909000_m1; Applied Biosystems) using the standard curve method.

Sirt1 mRNA expression studies in mice. Total RNA was isolated from adipose tissue samples of 5-month-old female transgenic (DBA/2 × BALB/c) mice using TRI reagent (Ambion), according to the manufacturer’s guidelines. The resulting total RNA was subjected to DNase treatment using RNase-free DNase (Ambion). The purity of isolated RNA was measured by a NanoDrop spectrophotometer. A set concentration of RNA was reverse transcribed into cDNA, and quantitative PCR was performed on ABI Prism 7500 Sequence Detection System (Applied Biosystems). Inventoried Taqman primers for Sirt1 (Mm01186821_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm00465120_m1) were purchased from Applied Biosystems. Sirt1 expression was normalized to the copy number of GAPDH.

Sirt1 protein expression studies in mice. Adipose tissue samples were homogenized and proteins extracted using Q-PER (no. 78510; Pierce) along with protease inhibitors and phosphatase inhibitors (Roche) from the same mice as in mRNA expression studies. After homogenization, tissue lysates were centrifuged for 30 min at high speed, and supernatant was collected and stored at −70°C until further analysis. Protein concentrations were measured with bichromatic acid protein assay kit (no. 23225; Pierce, Rockford, IL). A total of 20 μg/lane of protein samples containing NuPAGE LDS sample buffer (Invitrogen) and reducing agent were loaded into 4–12% NuPAGE Bis-Tris gels (Invitrogen) and were subjected to gel electrophoresis and transferred to Trans-Blot paper (Bio-Rad). After drying, bands were visualized using chemiluminescence (ECL plus; GE Health Care), and images were finally washed with TBS-0.1% Tween-20 for 3 min before incubating them with secondary anti-rabbit horseradish peroxidase–conjugated immunoglobulin (no. NA0047; GE Health Care, Amersham, U.K.) (1:40,000) for 2 h at room temperature. The membranes were finally washed with TBS-0.05% Tween-20 for 3 × 5 min. For GAPDH, the membranes were blocked in TBS with 5% milk and 0.1% Tween-20 for 2 h at room temperature, washed with TBS-0.1% Tween-20 for 3 × 5 min, and incubated with GAPDH (no. ab8245; Abcam) primary antibodies (1:5,000) overnight at +4°C. The membranes were washed with TBS-0.1% Tween-20 for 3 × 5 min before incubating them with secondary anti-mouse horseradish peroxidase–conjugated immunoglobulin (no. NA031V; GE Health Care) (1:10,000) in TBS-0.1% Tween-20 for 1 h at room temperature. The membranes were finally washed with TBS-0.1% Tween-20 for 3 × 5 min. The bands were visualized using chemiluminescence (ECL plus; GE Health Care), and images were captured in a Image Quant RT-ECL machine (version 1.0.1; GE Health Care). Quantification of the bands was done by applying Quantity One software (Bio-Rad). Sirt1 protein expression was normalized to GAPDH protein levels. The experiments were repeated four times.

Statistical analysis. Data analyses were carried out with SPSS 14.0 for Windows. The results for continuous variables are given as means ± SD. Variables with skewed distribution (glucose, insulin, FFAs, and subcutaneous and intra-abdominal fat) were logarithmically transformed for statistical analyses. Linear regression was used to calculate the correlations. Uni-and multivariate regression models were applied to assess the determinants of the association of EE and insulin sensitivity, we analyzed the rates of WBGU among the tertiles of fasting EE, glucose oxidation, or nonoxidative glucose disposal (data not shown). In contrast, subjects in the lowest tertile of EE during the hyperinsulinemic clamp had highest EE/LBM during the hyperinsulinemic clamp according to the tertiles of EE (Fig. 1). We did not find differences in WBGU among the tertiles of fasting EE, glucose oxidation, or nonoxidative glucose disposal (data not shown). In contrast, subjects in the highest tertile of EE/LBM during the hyperinsulinemic clamp had highest WBGU/LBM (49.85 ± 15.43 vs. 55.02 ± 15.46 vs. 63.44 ± 18.76 μmol/kg LBM/min, P = 2.2 × 10⁻⁶), which was attributable to both high-glucose oxidative (19.54 ± 5.42 vs. 20.96 ± 5.62 vs. 22.75 ± 6.11 μmol/kg LBM/min, P = 0.007) and nonoxidative (30.31 ± 12.81 vs. 34.06 ± 13.24 vs. 40.68 ± 16.52 μmol/kg LBM/min, P = 1.2 × 10⁻⁵) glucose disposal. These differences were even more pronounced across the tertiles of ΔEE/LBM, where subjects in the highest tertile had highest WBGU/LBM (43.82 ± 13.25 vs. 55.75 ± 13.64 vs. 67.96 ± 16.31 μmol/kg LBM/min, P = 2.5 × 10⁻⁶), attributable to both high-glucose oxidative (17.51 ± 4.34 vs. 20.81 ± 5.45 vs. 24.58 ± 5.31 μmol/kg LBM/min, P = 5.9 × 10⁻⁵) and nonoxidative (20.31 ± 12.08 vs. 34.94 ± 12.24 vs. 43.38 ± 15.16 μmol/kg LBM/min, P = 3.6 × 10⁻⁵) glucose disposal.

Subjects in the highest ΔEE tertile used more glucose for energy production than did subjects in the lower ΔEE tertile...
tertiles, as indicated by their higher respiratory quotient in the fasting state \( (P = 0.010) \) and during the hyperinsulinemic clamp \( (P = 1.2 \times 10^{-12}) \) (Fig. 2). Subjects with the highest \( \Delta EE \) had the lowest lipid oxidation in the fasting state \( (P = 9.2 \times 10^{-12}) \). In the fasting state, FFA levels were not different among the tertiles \( (P = 0.417) \), whereas during the hyperinsulinemic clamp, subjects with the highest \( \Delta EE \) had the lowest levels of FFAs \( (0.05 \pm 0.03 \text{ vs. } 0.04 \pm 0.02 \text{ vs. } 0.03 \pm 0.03 \text{ mmol/l}, P = 8.7 \times 10^{-7}) \).

To evaluate variables associated with the rates of WBGU/LBM during the hyperinsulinemic clamp, we per-
formed univariate linear regression analysis (Table 1). High ΔEE exhibited the strongest association with high WBGU/LBM, followed by low levels of low intra-abdominal adipose tissue mass and low total triglycerides. Low lipid oxidation during the hyperinsulinemic clamp and low subcutaneous adipose tissue mass were also associated with insulin sensitivity. In multivariate regression analyses, a model including ΔEE and intra-abdominal adipose tissue as independent variables explained a higher proportion of the variance of WBGU/LBM (R^2 = 0.431, P = 1.7 × 10^{-24}) than did ΔEE alone (R^2 = 0.362), but adding age and sex into this model did not improve substantially the R^2 value (R^2 = 0.436, P = 5.4 × 10^{-23}). A model that included ΔEE and subcutaneous adipose tissue mass as independent variables was not more strongly associated with WBGU/LBM (R^2 = 0.379, P = 8.0 × 10^{-21}) than was ΔEE alone.

**SIRT1 mRNA expression correlation with EE, insulin sensitivity, and SIRT1 target genes.** To explore the determinants of insulin-stimulated EE and WBGU/LBM, we measured adipose tissue mRNA expression of SIRT1 and PGC-1α. SIRT1 mRNA expression correlated significantly with EE (r = 0.289, P = 0.010) and with WBGU/LBM (r = 0.334, P = 0.002) during the euglycemic clamp (Fig. 3). No statistically significant correlation was found between SIRT1 expression and EE in the fasting state (r = 0.142). The correlation between SIRT1 expression and PGC-1α expression was 0.448 (P = 3.5 × 10^{-5}). PGC-1α expression correlated significantly only with WBGU/LBM (r = 0.387, P = 3.9 × 10^{-4}) but not with EE during the clamp (r = 0.167).

We also measured adipose tissue mRNA levels of several target genes of SIRT1 and PGC-1α (Table 2). SIRT1 mRNA expression correlated significantly with PGC-1β expression, estrogen-related receptor (ERR) α, nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (TFAM), catalase (CAT), and with several genes of the respiratory chain, including NADH dehydrogenase (ubiquinone) 1a subcomplex 2, cytochrome c, cytochrome c oxidase subunit IV, and ATP synthase. SIRT1 mRNA expression also correlated with the expression of soluble superoxide dismutase 1. The correlations of mRNA expression of these genes with PGC-1α expression were quite similar but somewhat weaker. Neither SIRT1 mRNA expression nor PGC-1α mRNA expression correlated with superoxide dismutase 2.

**SIRT1 mRNA expression correlation in adipose tissue and skeletal muscle.** In 11 subjects who had both adipose tissue and skeletal muscle biopsy, the correlation of SIRT1 mRNA expression in these tissues was 0.655 (P = 0.029). Mitochondrial DNA in skeletal muscle also correlated positively with SIRT1 mRNA expression in adipose tissue (r = 0.519) and skeletal muscle (r = 0.533), although the correlations were not statistically significant due to a small sample size (supplemental Table 2 in the online appendix [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1191/DC1]).

**SIRT1 mRNA expression correlation with cytokines and adhesion molecules.** SIRT1 mRNA expression negatively correlated with hs-CRP (r = −0.241, P = 0.039), but...
Sirt1 mRNA and protein correlation. Subcutaneous adipose tissue from 5-month-old female mice was obtained and used for quantitative RT-PCR and Western blot analyses. We observed a strong correlation between Sirt1 mRNA and Sirt1 protein expression levels \((r = 0.882, P < 0.001)\) (Fig. 4).

**TABLE 2**

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<th>SIRT1</th>
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<td>PGC-1β</td>
<td>(r = 0.358)</td>
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<tr>
<td>NRF1</td>
<td>(r = 0.286)</td>
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<tr>
<td>ERRα</td>
<td>(r = 0.339)</td>
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<td>TFAM</td>
<td>(r = 0.379)</td>
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| SIRT1 mRNA expression correlated significantly with EE and insulin sensitivity in offspring of type 2 diabetic patients. Additionally, high EE during hyperinsulinemia, and particularly high ΔEE, were strongly associated with insulin-stimulated increase in EE was strongly associated with insulin sensitivity in offspring of patients with type 2 diabetes. Furthermore, we showed for the first time that adipose tissue SIRT1 mRNA expression correlated with EE and insulin sensitivity during hyperinsulinemia. Moreover, SIRT1 expression correlated with the expression of several genes regulating the mitochondrial function. In our study, high EE during hyperinsulinemia, and particularly high ΔEE, were strongly associated with insulin-stimulated increase in EE during the hyperinsulinemic clamp. Furthermore, we showed for the first time that adipose tissue SIRT1 mRNA expression correlated with EE and insulin sensitivity during hyperinsulinemia. Moreover, SIRT1 expression correlated with the expression of several genes regulating the mitochondrial function.

**DISCUSSION**

Our study demonstrated that insulin-stimulated increase in EE was strongly associated with insulin sensitivity in offspring of type 2 diabetes. Furthermore, we showed for the first time that adipose tissue SIRT1 mRNA expression correlated with EE and insulin sensitivity during hyperinsulinemia. Moreover, SIRT1 expression correlated with the expression of several genes regulating the mitochondrial function. In our study, high EE during hyperinsulinemia, and particularly high ΔEE, were strongly associated with insulin-stimulated increase in EE during the hyperinsulinemic clamp. Furthermore, we showed for the first time that adipose tissue SIRT1 mRNA expression correlated with EE and insulin sensitivity during hyperinsulinemia. Moreover, SIRT1 expression correlated with the expression of several genes regulating the mitochondrial function.
PGC-1β expression, which has several overlapping functions with PGC-1α in inducing genes related to OXPHOS (24). Significant correlations of SIRT1 mRNA expression were also observed with ERRα, which mediates many of the downstream effects of activated PGC-1α on mitochondrial function (25), with NRF-1, which is an ERRα/PGC-1α target, and with TFAM, which is a target of NRF-1 (26). Furthermore, SIRT1 mRNA levels correlated with expression of several genes regulating the respiratory chain. SIRT1 mRNA expression level also correlated with the expression of soluble superoxide dismutase 1, which protects the cell from superoxide toxicity, and with catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen (27). Because the accumulation of reactive oxygen species can induce insulin resistance (28), the role of SIRT1 in governing superoxide dismutase 1 and catalase expression could cooperate with the effects of SIRT1 on the control of OXPHOS to improve insulin sensitivity. Our findings are also in agreement with recent studies in mice (4, 29). Transgenic mice overexpressing SIRT1 (30) or mice having SIRT1 activity enhanced by the administration of the SIRT1 agonist, resveratrol (4, 29), or a small molecule activator of SIRT1 (6), were both leaner, more hypermetabolic, and showed favorable effects on glucose and lipid metabolism. Mice treated with SIRT120, a potent synthetic activator of SIRT1, enhanced insulin sensitivity (31). Furthermore, in human studies a SIRT1 activator (SIRT501) has been shown to improve glucose control (32). Adipose tissue SIRT1 mRNA expression was negatively associated with hs-CRP level, which is in agreement with the anti-inflammatory effect of SIRT1 (33). The correlation of adipose tissue SIRT1 mRNA with other inflammatory markers was modest. This may reflect a poorer correlation of SIRT1 mRNA with plasma levels of cytokines compared with cytokine expression in adipose tissue.

SIRT1 mRNA expression in adipose tissue had a high correlation with skeletal muscle SIRT1 mRNA ($r = 0.655$). Therefore, we believe that our results obtained in adipose tissue reflect metabolic changes in skeletal muscle, which is the main tissue for EE and insulin sensitivity during insulin stimulation. Skeletal muscle mitochondrial DNA correlated closely with SIRT1 mRNA expression in skeletal muscle and adipose tissue, giving evidence that upregulation of the genes regulating mitochondrial biogenesis in adipose tissue and likely to reflect corresponding changes in skeletal muscle (Table 2). Finally, we demonstrated in mice that SIRT mRNA expression and SIRT1 protein levels were highly correlated (Fig. 4), demonstrating that our results are likely to be valid also at protein level. However, the limitation of our study is that we could not determine SIRT1 protein level from adipose tissue biopsies due to a small amount of tissue that we can obtain using needle biopsy techniques.

In summary, we demonstrated that insulin-stimulated EE is strongly associated with insulin-stimulated glucose uptake in offspring of subjects with type 2 diabetes. Impaired stimulation of EE by insulin is likely to reflect impaired regulation of mitochondrial function in insulin-resistant states. This could be at least partially explained by low expression of SIRT1 and PGC-1α, two important master regulators of mitochondrial activity. Even though it is not possible to determine the primary defect from our cross-sectional data, disturbance in mitochondrial function and low EE were strongly associated with impaired insulin-stimulated glucose uptake. Our results give evidence that activating SIRT1 could be one of the potential mechanisms to treat insulin resistance and patients with type 2 diabetes.

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D.A.S. is a consultant to Sirtris Pharmaceuticals (a GlaxoSmithKline company developing sirtuin-based drugs) and an inventor on Harvard patents licensed to GlaxoSmithKline. P.E. was and C.W. is employed by Sirtris Pharmaceuticals. No other potential conflicts of interest relevant to this article were reported.

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