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

Jarno Rutanen,1 Nagendra Yaluri,1 Shalem Modi,1 Jussi Pihlajamäki,1 Markku Vänttinen,1 Paula Itkonen,1 Sakari Kainulainen,2 Hiroyasu Yamamoto,3,4 Marie Lagouge,3 David A. Sinclair,5 Peter Elliott,6 Christoph Westphal,6 Johan Auwerx,3,4 and Markku Laakso1

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 nondiabetic 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 = 0.062, 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, 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

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From the 1Department of Medicine, University of Kuopio and Kuopio University Hospital, Kuopio, Finland; the 2Department of Clinical Radiology, University of Kuopio, Kuopio, Finland; the 3Institut de Génétique et de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale/Université Louis Pasteur, Illkirch, France; the 4Laboratory of Integrative and Systems Physiology, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; the 5Department of Pathology and Glenn Labs for Aging Research, Harvard Medical School, Boston, Massachusetts; and the 6Sirtris Pharmaceuticals, Cambridge, Massachusetts.

Corresponding author: Markku Laakso, markku.laakso@kuh.fi.

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 = 0) were based on the criteria 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 (insulin (PK) blood) were drawn at 0, 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; Datex) as previously described (14). The mean value of the data during the last 20 min of the clamp was used. Fatty acids and lipolysis rates were determined by using equilibrium and liquid chromatography. Proteins were extracted using T-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 bichromonic 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 nitrocellulose. Membranes were blocked in Tris-buffered saline (TBS) with 3% milk and 0.05% Tween-20 1 h at room temperature, washed with TBS-0.05% Tween-20 for 3 × 5 min, and incubated overnight at +4°C with SIRT1 (no. 07-131; Millipore) primary antibodies (1:1,000). The membranes were washed with TBS-0.05% Tween-20 for 3 × 5 min before incubating them with secondary anti-rabbit horseradish peroxidase–conjugated immunoglobulin (no. NA9004; 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-0.01% 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-0.01% Tween-20 for 3 × 5 min. The membranes were incubated with secondary anti-mouse horseradish peroxidase–conjugated immunoglobulin (no. NA0011; 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 an 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. For mixed-model analysis, we included the pedigree (coded as a family number) as a random factor, the tertiles as fixed factors, and age as a covariate.

RESULTS

Energy expenditure and insulin sensitivity. EE during the clamp positively correlated with insulin sensitivity (r = 0.375, P = 2.8 × 10⁻⁷) (Fig. 1). Even stronger correlation was found between insulin-stimulated energy expenditure (EE) (defined as EE during the clamp − EE in the fasting state) and insulin sensitivity (r = 0.602, P = 2.5 × 10⁻²⁴). In contrast, fasting EE was not correlated with insulin sensitivity (r = −0.004). To further investigate the association of EE and insulin sensitivity, we analyzed the rates of WBGU/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 EE/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 × 10⁻⁹), attributable to both high-glucose oxidative (17.51 ± 5.42 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 tertiles...
tertiles, as indicated by their higher respiratory quotient in the fasting state ($P < 0.010$) and during the hyperinsulinemic clamp ($P < 1.2 	imes 10^{-12}$) (Fig. 2). Subjects with the highest ΔEE had the lowest lipid oxidation in the fasting state ($P < 1.6 	imes 10^{-4}$) and during the hyperinsulinemic clamp ($P = 9.2 	imes 10^{-8}$). In the fasting state, FFA levels were not different among the tertiles ($P = 0.417$), whereas during the hyperinsulinemic clamp, subjects with the highest ΔEE had the lowest levels of FFAs (0.05 ± 0.03 vs. 0.04 ± 0.02 vs. 0.03 ± 0.03 mmol/l, $P = 8.7 	imes 10^{-7}$).

To evaluate variables associated with the rates of WBGU/LBM during the hyperinsulinemic clamp, we per-

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**FIG. 1.** A: Correlation between the rates of WBGU and fasting EE (univariate linear regression). Correlation between the rates of WBGU and EE during the hyperinsulinemic clamp (B) and correlation between the rates of WBGU and ΔEE (defined as EE during the clamp – EE in the fasting state) (C). Rates of WBGU in the lowest (■), middle (□), and highest (▲) EE tertiles according to fasting EE (D), EE during the hyperinsulinemic clamp (E), and the ΔEE (F). Data are means ± SD (D–F).
formed univariate linear regression analysis (Table 1). High \( \Delta EE \) 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 \( \Delta EE \) and intra-abdominal adipose tissue as independent variables explained a higher proportion of the variance of WBGU/LBM (\( R^2 = 0.431, P = 1.7 \times 10^{-24} \)) than did \( \Delta EE \) alone (\( R^2 = 0.362 \), but adding age and sex into this model did not improve substantially the \( R^2 \) value (\( R^2 = 0.436, P = 5.4 \times 10^{-23} \)). A model that included \( \Delta EE \) and subcutaneous adipose tissue mass as independent variables was not more strongly associated with WBGU/LBM (\( R^2 = 0.379, P = 8.0 \times 10^{-21} \)) than was \( \Delta EE \) 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 (\( P = 3.5 \times 10^{-5} \)). PGC-1α expression correlated significantly only with WBGU/LBM (\( r = 0.387, P = 3.9 \times 10^{-4} \)) 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) \( \alpha \), 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 SIRT 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.** SIRT mRNA expression negatively correlated with hs-CRP (\( r = -0.241, P = 0.039 \), but

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**FIG. 2. A**: Respiratory quotient in the fasting state and during the hyperinsulinemic-euglycemic clamp in the tertiles of \( \Delta EE \). Data are means ± SD in the lowest (■), middle (□), and highest (●) tertile. Lipid oxidation in the fasting state and during the hyperinsulinemic-euglycemic clamp in the tertiles of \( \Delta EE \) (B) and FFAs in the fasting state and during the hyperinsulinemic-euglycemic clamp in the tertiles of \( \Delta EE \) (C). Data are means ± SD.
analyses. We observed a strong correlation between 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).

**DISCUSSION**

Our study demonstrated that 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 WBGU/LBM. These results agree with previous studies (16,17) including relatively small samples of lean and obese subjects. Hyperinsulinemic clamp simulates the postprandial state with high insulin levels that promote the glucose flux from circulating blood into insulin sensitive tissues. An 8-h insulin infusion in humans has been shown to increase mitochondrial mRNA transcript levels, mitochondrial protein synthesis, and ATP production (18). This response was, however, blunted in type 2 diabetic patients. In another study (19), diabetic patients exhibited lower ATP production rate in response to high-dose insulin infusion compared with that in non-diabetic individuals. Thus, impaired mitochondrial fitness could be a consequence of impaired insulin action as supported by a study in mice fed a high-fat, high-sucrose diet showing that mitochondrial alterations do not precede the onset of insulin resistance (20). In agreement with this notion, a recent study (21) in mice demonstrated a direct effect of SIRT1 on insulin sensitivity by repressing PTP1B. Whether this mechanism is working also in humans needs to be shown.

Alternatively, primary mitochondrial dysfunction could lead to insulin resistance. An attractive possibility to explain a causal link between impaired mitochondrial function and insulin resistance is the hypothesis that impaired OXPHOS capacity leads to intramyocellular lipid accumulation (9) and thus impaired insulin signaling and insulin resistance (22). High lipid levels in the circulating blood impair insulin-stimulated ATP production in humans (23). We observed that subjects with low insulin-stimulated EE also had higher levels of FFAs, higher lipid oxidation, and lower respiratory quotient during the hyperinsulinemic clamp, reflecting changes in fuel selection in these subjects, which often lead to insulin resistance.

Further evidence supporting the hypothesis that mitochondrial activity stimulated by SIRT1 might be important for energy metabolism and insulin action are high correlations of adipose tissue SIRT1 mRNA expression with expression of genes regulating mitochondrial function. SIRT1 mRNA expression correlated significantly with otherwise the correlations with cytokines and adhesion molecules were almost entirely nonsignificant (supplementary Table 1).

**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>Gene</th>
<th>SIRT1</th>
<th>PGC-1α</th>
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<tr>
<td>PGC-1β</td>
<td>$r = 0.358$</td>
<td>$r = 0.152$</td>
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<tr>
<td>NRF1</td>
<td>$r = 0.286$</td>
<td>$r = 0.235$</td>
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<tr>
<td>ERRα</td>
<td>$r = 0.339$</td>
<td>$r = 0.260$</td>
</tr>
<tr>
<td>TFAM</td>
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<td>NDUFA2</td>
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<td>COXIV1</td>
<td>$r = 0.332$</td>
<td>$r = 0.262$</td>
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<tr>
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<td>$r = 0.248$</td>
<td>$r = 0.196$</td>
</tr>
<tr>
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<td>$r = -0.009$</td>
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<tr>
<td>CAT</td>
<td>$r = 0.350$</td>
<td>$r = 0.422$</td>
</tr>
</tbody>
</table>

Expression of all genes was normalized to RPL0 expression. ATP5G1, ATP synthase; H + transporting, mitochondrial F0 complex, subunit C1; CAT, catalase; COXIV1, cytochrome c oxidase subunit IV isoform 1; CYCS, cytochrome c, somatic; NDUFA2, NADH dehydrogenase (ubiquinone) 1 α subcomplex 2; SOD1, superoxide dismutase 1, soluble; SOD2, superoxide dismutase 2, mitochondrial.

**FIG. 3.** A: Correlation of adipose tissue SIRT1 mRNA expression level with EE during the hyperinsulinemic clamp. B: Correlation of adipose tissue SIRT1 mRNA expression level with the rates of whole-body glucose uptake in offspring of type 2 diabetic patients.
poorer correlation of SIRT1 mRNA with plasma levels of inflammatory markers was modest. This may reflect a correlation of adipose tissue SIRT1 mRNA with other negatively associated with hs-CRP level, which is in agreement (32). Adipose tissue SIRT1 mRNA expression was activated (SRT501) has been shown to improve glucose and lipid metabolism. Mice treated with SRT1720, a small molecule activator of SIRT1 (6), were both leaner, more hypermetabolic, and showed favorable effects on glucose and lipid metabolism. Mice treated with SIRT1720, a potent synthetic activator of SIRT1, enhanced insulin sensitivity (31). Furthermore, in human studies a SIRT1 activator (SRT501) 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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