Down-Regulation of Insulin Receptor Substrates (IRS)-1 and IRS-2 and Src Homologous and Collagen-Like Protein Shc Gene Expression by Insulin in Skeletal Muscle Is Not Associated with Insulin Resistance or Type 2 Diabetes

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To examine whether altered gene expression of insulin receptor substrates (IRS)-1 and IRS-2 and Src homologous and collagen-like protein Shc is an inherited trait or contributes to muscle insulin resistance in type 2 diabetes, we measured mRNA levels of these genes by a relative quantitative RT-PCR method in muscle biopsies taken before and after an insulin clamp from 12 monozygotic twin pairs discordant for type 2 diabetes and 12 control subjects. Insulin-stimulated glucose uptake was decreased both in the diabetic and nondiabetic twin, compared with healthy control subjects (5.2 ± 0.7 and 8.5 ± 0.8 mmol/kg/min; P < 0.01 and P < 0.02, respectively). Basal mRNA levels of IRS-1, IRS-2, and Shc were similar in the diabetic and nondiabetic twins as well as in the control subjects. Insulin decreased mRNA expression of IRS-1 by 72% (from 0.75 ± 0.06 to 0.21 ± 0.04 relative units; P < 0.01) and IRS-2 by 71% (from 0.55 ± 0.10 to 0.16 ± 0.08 relative units; P < 0.03), and Shc by 25% (from 0.95 ± 0.04 to 0.71 ± 0.04 relative units; P < 0.01) vs. baseline as demonstrated in the control subjects. The postclamp Shc mRNA level was slightly higher in the diabetic twins (P = 0.05) but similar in the nondiabetic twins, as compared with the control subjects, whereas postclamp IRS-1 and IRS-2 mRNA levels were similar between the study groups. There was an inverse correlation between postclamp Shc mRNA concentration and glucose uptake (r = −0.53, P = 0.01; n = 22) in the controls and nondiabetic twins. However, the decrease in Shc gene expression by insulin was not significantly different between the study groups. In conclusion, because insulin down-regulates IRS-1, IRS-2, and Shc gene expression in skeletal muscle in diabetic and nondiabetic monozygotic twins and control subjects to the same extent, it is unlikely that expression of these genes is an inherited trait or contributes to skeletal muscle insulin resistance. (J Clin Endocrinol Metab 87: 255–259, 2002)

Insulin binds to and activates the insulin receptor kinase, which, in turn, phosphorylates insulin receptor substrates (IRSs). The major substrates of the insulin receptor are IRS-1, IRS-2, and Src homologous and collagen-like protein Shc (1–3), which are expressed in various tissues, including skeletal muscle. Upon phosphorylation, these substrates act as docking proteins for downstream complexes, leading to downstream mitogenic and metabolic effects, including insulin-mediated glucose uptake. Both IRS-1 and IRS-2 are necessary for insulin-stimulated Glut4 translocation via PI 3-kinase pathway in adipocytes and in skeletal muscle (1, 4, 5). Also, IRS-1 and Shc can interact with Son-of-sevenless/Grb-2 complex and thus activate the Ras/MAPK pathway. The Ras/MAPK pathway has also been shown to enhance membrane Glut4 intrinsic activity in adipose cells and L6 myotubes (6). To date, no consistent mutations have been found in IRS-2 and Shc genes responsible for muscle insulin resistance in type 2 diabetes, apart from a polymorphism in the IRS-1 gene implicated in β-cell dysfunction (7–9). Insulin has been ascribed a regulatory effect on the expression of some genes involved in insulin action and glucose metabolism in human skeletal muscle, including up-regulation of PI 3-kinase, Glut4, hexokinase II, Rad, and glycogen synthase (10–14), and down-regulation of lipoprotein lipase (10). Altered gene expression of hexokinase II, PI 3-kinase, and glycogen synthase has been reported in muscle of type 2 diabetic patients (12–15). Little is known about whether altered IRS-1, IRS-2, and Shc gene expression is an inherited trait or associated with muscle insulin resistance or type 2 diabetes. To address this question, we analyzed IRS-1, IRS-2, and Shc gene expression in muscle biopsies taken before and after an insulin clamp from 12 control subjects and from 12 monozygotic twins pairs discordant for type 2 diabetes (16, 17). The study of monozygotic twins discordant for diabetes allowed us to study whether the potential defect of gene expression is under genetic control.

Subjects and Methods

Subjects

Twelve Caucasian monozygotic twin pairs discordant for type 2 diabetes and 12 healthy subjects without family history of diabetes participated in the study. Type 2 diabetes had been diagnosed after the age of 40 yr, based on a standardized 75-g oral glucose tolerance test (18). The control subjects were matched to the nondiabetic twins for age, sex, and body mass index (Table 1). Monozygosity of the twins was confirmed by genetic markers (16). Insulin sensitivity was measured by a 3-h euglycemic hyperinsulinemic clamp with prior infusion of insulin (16, 17). Muscle biopsies were obtained in the basal state (0 min) and at...
 TABLE 1. Clinical characteristics of the diabetic and nondiabetic monozygotic twins and the control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic twins</th>
<th>Nondiabetic co-twins</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (female/male)</td>
<td>12 (5/7)</td>
<td>12 (5/7)</td>
<td>12 (5/7)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>64 ± 3</td>
<td>64 ± 3</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.1 ± 1.3</td>
<td>27.5 ± 1.3</td>
<td>26.0 ± 1.0</td>
</tr>
<tr>
<td>Fasting plasma insulin (pm)</td>
<td>86.8 ± 108a</td>
<td>52.4 ± 4.3</td>
<td>49.5 ± 5.7</td>
</tr>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>11.4 ± 1.1b</td>
<td>6.0 ± 0.2b</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose uptake (mg/kg FFMin)</td>
<td>5.2 ± 0.7b</td>
<td>8.5 ± 0.8b</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>Glucose storage (mg/kg FFMin)</td>
<td>2.8 ± 0.6b</td>
<td>4.8 ± 0.6b</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>Glucose oxidation (mg/kg FFMin)</td>
<td>2.4 ± 0.2b</td>
<td>3.7 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Insulin (pm), baseline</td>
<td>69.6 ± 16.5</td>
<td>50.9 ± 6.4</td>
<td>45.9 ± 5.0</td>
</tr>
<tr>
<td>Insulin (pm), clamp</td>
<td>528.1 ± 51.6</td>
<td>497.9 ± 40.9</td>
<td>559.6 ± 32.3</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.  

a P < 0.01, b P < 0.05, c P < 0.02, vs. controls.

end the of the clamp (+180 min), frozen immediately in liquid nitrogen, and stored at −80°C until analyzed. Informed consent was obtained from all subjects. The protocol was approved by the regional ethics committee, and the study was conducted according to the principles of the Declaration of Helsinki.

Quantitation of IRS-1, IRS-2, and Shc gene expression

RNA expression was examined using a modified primer-dropping RT-PCR method (19). Total RNA was isolated from the muscle biopsies by the acid guanidinium thiocyanate method (20) and was subjected to DNase I (Promega Corp., Madison, WI) treatment, according to the manufacturer’s instruction, to avoid genomic DNA contamination. The treated total RNA (400 ng) was then reverse-transcribed in a 40-μl reaction with a 5-μM oligo(dT)₁₈ primer in the presence of 200 U SUPERSCRIPT II reverse transcriptase (RT) (Life Technologies, Inc., Glasgow, Scotland) and 25 μM deoxynucleotide triphosphate for 60 min at 37°C. After heat inactivation of the RT at 95°C for 5 min, total cDNA was subjected to PCR coamplification of IRS-1, IRS-2, Shc genes, together with cyclophilin as a reference gene. Cyclophilin was used as a reference gene because it is unaffected by insulin and the diabetic state (21). Validation of this method has been described in a previous study (11). The primer pairs for IRS-1 were (from 5’ to 3’): GTCTTCTGACGCTCCTGCTCCTCC and CTCTGACGACTACCGAG and CTGCTTTTCCTGAGAGAGAC; and for Shc, GGGCACTTTTGACATGAAG and TAGGGAATAGGGTGGAAAGG. The Shc primers define a region that is included in all the 3 Shc isoforms p66, p52, and p46. The primer pairs for cyclophilin (cycloF-cycloR or cycloF-cycloR2) were: GCTCTCTTTGGCCTTGCTGC (cycloF), TGGCCTTCCAATATTCATGC (cycloR), CTGGGACCATTTGGCTGGTG (cycloR2). To study the effect of insulin on gene expression, we measured IRS-1, IRS-2, and Shc mRNA levels in skeletal muscle biopsies from 12 control subjects, before and after a euglycemic clamp, using the following conditions: For IRS-1, the PCR reaction (20 μl) contains 2 μl RT reaction, 1X PCR buffer, 200 μM deoxynucleotide triphosphate, 5% dimethylsulfoxide, 5% formamide, 0.5 U Taq polymerase, 0.25 μM IRS-1 primers, and 0.6 μM cyclophilin primers (cycloF-cycloR2). The PCR was run for 36 cycles (94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec) and followed by a final extension at 72°C for 10 min. For IRS-2 and Shc, the PCR conditions were similar to that of IRS-1 except for cycle numbers and primer concentrations (44 for 25 μM IRS-2 primers, 40 for 2 μM cycloF-cycloR2; 34 for 0.2 μM Shc primers, 34 for 0.2 μM cycloF-cycloR). The PCR conditions were optimized according to the primer-dropping method (19) for each individual gene. PCR products were separated on a 2% agarose gel containing ethidium bromide, photographed with an UPP-110HA printing paper (Sony, Tokyo, Japan), and quantitated using a Personal Densitometer SI scanner together with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). The mRNA signals were expressed relative to that of cyclophilin. To examine whether altered gene expression of IRS-1, IRS-2, and Shc is associated with type 2 diabetes, we measured basal and postclamp mRNA concentrations of these genes in all three groups (Table 1). We used the same conditions as described above for measuring basal IRS-1, IRS-2, and Shc mRNA concentrations. Postclamp Shc mRNA concentrations were also measured under the same conditions as above, but postclamp IRS-1 and IRS-2 mRNA concentrations were measured using different conditions. This was because the IRS-1 and IRS-2 mRNA concentrations in some postclamp samples were too low to measure using the conditions described above. To measure such low mRNA concentrations and to allow a direct comparison of them among the groups, we had to change the conditions for postclamp IRS-1 and IRS-2 concentration measurements.

Analytical measurements

Plasma glucose was determined with a glucose oxidase method (Glucose Analyzer 2; Beckman Coulter, Inc. Instruments, Fullerton, CA). Plasma insulin was measured using a double-antibody RIA (Pharmacia Diagnostics, Uppsala, Sweden) (16, 17).

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using an NCSS 6.0.21 statistical package (NCSS Statistical Software, Kaysville, UT). The significance of differences within or between groups was tested by Wilcoxon or Mann-Whitney rank tests. The relationship between various variables was analyzed by Spearman correlations.

Results

Clinical characteristics of the subjects

Fasting plasma glucose levels were higher in the diabetic and the nondiabetic twins, whereas fasting plasma insulin levels were only higher in diabetic twins, compared with those of the control subjects (Table 1). Plasma insulin concentrations in the basal state and during the clamp were similar in the diabetic (69.6 ± 16.5 and 528.1 ± 51.6 pm) and the nondiabetic twins (50.9 ± 6.4 and 497.9 ± 40.9 pm) twins as well as in the control subjects (45.9 ± 5.0 and 559.6 ± 32.3 pm). The diabetic twins had 55% lower rates of insulin-stimulated glucose uptake (P < 0.01), 63% lower rates of glucose storage (P < 0.01), and 37% lower rates of glucose oxidation (P < 0.01), compared with the control subjects (Table 1). The nondiabetic monozygotic co-twins also had a 25% lower rate of insulin-stimulated glucose uptake (8.5 ± 0.8 vs. 11.4 ± 0.9 mg/kg fat free mass (FFM) per min; P < 0.03), which was particularly caused by a 37% decrease in the rate of glucose storage (4.8 ± 0.6 vs. 7.6 ± 0.9 mg/kg FFM per min; P < 0.02), compared with the control subjects (Table 1).

IRS-1, IRS-2, and Shc gene expression

Basal IRS-1, IRS-2, and Shc mRNA levels were similar in the diabetic and nondiabetic twins as well as in the control subjects (Table 2). Insulin infusion decreased IRS-1 mRNA

levels 3.6-fold (from 0.75 ± 0.06 to 0.21 ± 0.04 relative units; 
P < 0.001); IRS-2, 3.4-fold (from 0.55 ± 0.10 to 0.16 ± 0.08 
relative units; P < 0.03); and Shc, 1.3-fold (from 0.95 ± 0.04 
to 0.71 ± 0.04 relative units; P < 0.01) vs. baseline as dem-
onstrated in the control subjects (Fig. 1). The postclamp Shc 
mRNA level was slightly higher in the diabetic twins (P = 0.05) 
but similar in the nondiabetic twins, as compared with the 
control subjects (Table 2), whereas postclamp IRS-1, IRS-2 
mRNA levels were similar in all three groups (Table 2). There 
was an inverse correlation between the postclamp Shc 
mRNA concentration and the insulin-mediated glucose up-
take (M-value) in all subjects (r = −0.52, P < 0.01). This 
correlation was also observed in the combined group of the 
control subjects and the nondiabetic twins (r = −0.53, P = 0.01), 
whereas no correlation was observed between the basal 
Shc mRNA concentration and the M-value. However, the 
change in Shc gene expression between basal and postclamp 
states was not significantly different among the three groups.

TABLE 2. IRS-1, IRS-2, and Shc mRNA expression in the 
diabetic and nondiabetic monozygotic twins and the control 
subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic twins</th>
<th>Nondiabetic co-twins</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>IRS-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.15 ± 0.03</td>
<td>1.16 ± 0.08</td>
<td>1.03 ± 0.08</td>
</tr>
<tr>
<td>Clamp</td>
<td>1.11 ± 0.30</td>
<td>0.89 ± 0.11</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>IRS-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.12 ± 0.16</td>
<td>0.94 ± 0.17</td>
<td>0.68 ± 0.19</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.84 ± 0.34</td>
<td>0.70 ± 0.39</td>
<td>0.93 ± 0.28</td>
</tr>
<tr>
<td>Shc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.97 ± 0.04</td>
<td>0.99 ± 0.05</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.82 ± 0.03a</td>
<td>0.75 ± 0.05</td>
<td>0.71 ± 0.04</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. The mRNA level is expressed relative to that of cyclophilin in relative units. Postclamp mRNA levels of IRS-1 and 
IRS-2 were measured under different conditions from those in Fig. 1, 
and are not directly comparable with the data shown in Fig. 1. 

Neither basal nor postclamp IRS-1 and IRS-2 mRNA con-
centrations showed any significant correlation with the M-
values, either in all subjects or in the subgroups (data not 
shown).

Discussion

In this study, we examined the gene expression of three 
substrates of the insulin receptor kinase, i.e. IRS-1, IRS-2, and 
Shc, in skeletal muscle from monozygotic twins discordant 
for diabetes and healthy control subjects. Whereas insulin 
clearly decreased expression of these substrates, no differ-
cence was seen between diabetic and control subjects, nor 
between diabetic and nondiabetic twins. It is well established 
that insulin stimulates phosphorylation of IRS-1, IRS-2, and 
Shc through binding to and activation of the insulin receptor 
kinase, thereby activating downstream effectors. Phosphor-
ylation occurs rapidly, within a few minutes after insulin 
stimulation. However, after prolonged insulin treatment, de-
sensitization to insulin action occurs at multiple steps, in-
cluding both protein and mRNA changes. Insulin-induced 
receptor down-regulation, as reflected by a decrease in in-
sulin binding, has been reported in murine NIH 3T3 HIR3.5 
hepatocytes (22). In this cell model, the decrease in insulin 
binding is associated with decreased cellular insulin receptor 
content (22). In rat AR42J pancreatic acinar cells (23) and in 
human HepG2 hepatocytes (24), the decrease in the insulin 
receptor protein concentration could be, at least in part, at-
tributed to decreased insulin receptor mRNA concentration. 
However, in rat hepatoma cells, insulin down-regulation of 
the receptor may not be sufficient to completely account for 
insulin desensitization (25). Therefore, a down-regulation of 
postreceptor substrates has been proposed (25) and demon-
strated in rat hepatoma cells (26). Song et al. (27) showed that 
IRS-1 and IRS-2 phosphorylation was initially increased and 
subsequently decreased, in a time-dependent manner, in 
three types of rat skeletal muscles incubated with insulin for
40 min. In keeping with these results, we observed a decrease in mRNA concentrations of IRS-1, IRS-2, and Shc in human skeletal muscle after a 3-h insulin clamp, achieving plasma insulin concentrations of about 500 pm. Similar results have been reported by Andreelli et al. (15), who observed a 2-fold reduction in IRS-1 mRNA expression in human skeletal muscle after a 3-h clamp, with somewhat higher insulin concentrations (approximately 900 pm). More recently, Ducluzeau et al. (14) reported down-regulation of IRS-1 mRNA in skeletal muscle after a 3-h euglycemic insulin clamp, without differences between control subjects and patients with type 2 diabetes. Also compatible with these results, decreased mRNA and protein levels of IRS-1 and IRS-2 have been reported in skeletal muscle of the ob/ob mice (28), Zucker fatty rats (29), and Sprague Dawley rats fed with high-fat diet (30). In addition, a recent study by Shimomura et al. (31) reported that chronic hyperinsulinemia also reduces the amount of IRS-2 mRNA and protein in mouse liver, both in vivo and in vitro. No data are, to our knowledge, available on the effect of insulin on Shc gene expression in human skeletal muscle. Together, the data clearly show that insulin down regulates gene expression of IRSs in skeletal muscle. If this down-regulation is associated with a similar reduction in protein levels, this could, of course, present a mechanism by which insulin signaling is down-regulated, providing that phosphorylation of these substrates also is impaired by insulin. Given the recent identification of calpain 10 as a putative candidate gene for type 2 diabetes (32), it is interesting to note that calpains have been ascribed a role in insulin-mediated breakdown of the IRS protein (33). The mechanism by which insulin decreases IRS mRNA concentrations is unknown. Recently, it has been demonstrated that the PKC isoform, PKC-δ, positively regulates IRS-1 gene transcription in human breast cancer cells (34). It has also been shown that insulin causes translocation of PKC-δ from the cytosol to the cell membrane in both rat and human skeletal muscle (35,36). Whether insulin-mediated PKC-δ translocation could influence transcription of IRSs in human skeletal muscle is currently unknown.

It is also clear, from our results, that the capacity of insulin to down-regulate expression of the IRS-1, IRS-2, and Shc genes is not resistant to the effect of insulin. We did not find any correlation between the degree of IRS-1 and IRS-2 gene down-regulation and the M-value, either in the diabetic or in the control subjects. In contrast, the postclamp Shc gene expression was inversely correlated with the M-value, particularly in the nondiabetic subjects. It is unclear whether the slight increase in Shc mRNA concentrations seen in the diabetic twins really has a physiological meaning. Similar results were reported by Andreelli et al. (15) and Ducluzeau et al. (14), who observed that insulin down-regulation of the IRS-1 gene was not impaired in muscle from obese and type 2 diabetic patients. Although fasting hyperinsulinemia and insulin resistance are hallmarks of IGT and mild diabetes, we did not see any impairment in the down-regulatory effect of insulin on expression of IRS-1 and IRS-2 in these subjects. One could hypothesize that chronic hyperinsulinemia could cause a reduction in mRNA expression similar to that of the insulin infusion. On the other hand, we do not know the dose-response characteristics of the effect of insulin on expression of these genes, i.e. whether fasting hyperinsulinemia in the range of 50–80 pm is sufficient to do the same as 500 pm insulin during the clamp. Although protein levels were not examined in this study, some other studies have shown decreased levels of IRS-1 and IRS-2 proteins in muscle from obese humans (37) and insulin-resistant animals (28, 29, 38). Moreover, chronic insulin treatment of adipocytes increased degradation of IRS-1 protein but did not change mRNA expression of IRS-1 (33, 39–41). In conclusion, because insulin down-regulates IRS-1, IRS-2, and Shc gene expression in skeletal muscle in the diabetic and nondiabetic monzygotic twins and the control subjects to the same extent, it is unlikely that expression of these genes is a heritable trait or contributes to skeletal muscle insulin resistance.

Acknowledgments

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