Increased collagen content in insulin-resistant skeletal muscle

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Am J Physiol Endocrinol Metab 290: E560–E565, 2006. First published October 25, 2005; doi:10.1152/ajpendo.00202.2005.—Oversupply of lipids has long been known to produce experimental insulin resistance (3, 16, 31). Recent evidence (24) suggests that an experimental increase in FFA can decrease the expression of nuclear-encoded mitochondrial genes, as well as peroxisome proliferator activated receptor-γ coactivator (PGC)-1, the transcriptional coactivator responsible for much of mitochondrial biogenesis. In addition, lipid oversupply can result in a dramatic increase in the expression of extracellular matrix genes in skeletal muscle from healthy subjects (24). These genes included several collagen genes, fibronectin, proteoglycans, and connective tissue growth factor (24). These changes were accompanied by alterations in the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases. This pattern of changes is typical of an inflammatory response and is characteristic of nonalcoholic steatohepatitis (18, 20), diabetic kidney disease (25), atherosclerotic plaque formation (27), and pancreatitis (7). In addition, an intriguing observation made using a collagen VI knockout mouse model of Bethlem muscular dystrophy suggests that mitochondrial abnormalities can be induced by the interaction of skeletal muscle cells with an abnormal extracellular matrix (12). This raises the possibility that an inflammatory response that changes the composition of the extracellular matrix could result in mitochondrial dysfunction. This mitochondrial abnormality could, in turn, alter fat utilization, leading to accumulation of fatty acyl-CoAs and ceramides, and thus produce insulin-signaling abnormalities and insulin resistance. The present study was undertaken to determine whether alterations in the extracellular matrix are present in insulin-resistant skeletal muscle. Because high glucose itself is widely known to produce matrix abnormalities, both nondiabetic and type 2 diabetic insulin-resistant patients were examined.
METHODS

Subjects. A total of 30 volunteers (10 lean control subjects, 10 obese nondiabetics, and 10 patients with well-controlled type 2 diabetes) participated in the research study. Their clinical characteristics are shown in Table 1. All subjects received a 75-g oral glucose tolerance test using American Diabetes Association criteria. The diabetic patients were newly diagnosed (n = 5) or had been treated with sulfonylureas (n = 5), which were discontinued 72 h before the study. Other than diabetes, none of the subjects had any significant medical problems, and none were taking any medications that are known to affect glucose metabolism. Subjects were instructed to maintain their usual diet for ≥3 days and not to engage in vigorous exercise for ≥2 days before the study. The purpose, nature, and potential risks of the study were explained to all subjects, and written consent was obtained before their participation. The protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Study design. All studies were conducted in the General Clinical Research Center of the University of Texas Health Science Center at San Antonio and began at 0700 after a 10-h overnight fast. Subjects underwent a euglycemic hyperinsulinemic clamp (6) using an insulin infusion of 80 mU·min⁻¹·m⁻². To perform the clamp, an antecubital vein was cannulated for infusion of insulin and glucose, and a hand vein was cannulated retrogradely and placed in a heated box (60°C) for sampling of arterialized blood. A primed (25 μCi) continuous infusion of [³H]glucose (0.25 μCi/min) was started, and 2 h (3 h for diabetics) were allowed for isotopic equilibration. The priming dose of tritiated glucose was increased in the diabetics in proportion to the increase in their fasting plasma glucose concentration. Sixty minutes before the beginning of the insulin infusion, a percutaneous biopsy of the vastus lateralis muscle was obtained. Muscle biopsy specimens (75–200 mg) were immediately blotted free of blood, frozen, and stored in liquid nitrogen until use. After 60 min, a primed continuous infusion of insulin was started at a rate of 80 mU·min⁻¹·m⁻², and plasma glucose was measured with a glucose analyzer (Beckman Instruments, Fullerton, CA) at 5-min intervals throughout the euglycemic clamp. A variable infusion of 20% glucose was used to maintain euglycemia. Thirty minutes after the start of the insulin infusion, a second muscle biopsy was obtained from a site 4 cm distal to the first. The insulin infusion was continued for a total of 120 min to obtain an estimate of the rate of glucose disposal (90- to 120-min period). Glucose specific activity was determined on barium hydroxide-zinc sulfate extracts of plasma. Plasma insulin concentration was determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA).

Muscle fractionation. Skeletal muscle biopsies were homogenized while still frozen using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY) in a buffer (hydroxethyl starch) consisting of 20 mM HEPES, 1 mM EDTA, 250 mM sucrose, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM ammonium molybdate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 250 μM PMSF. The resulting homogenate was centrifuged at 4°C in a Beckman J21 centrifuge at 1,500 g for 10 min. The supernatant was saved and the pellet rehomogenized in the same buffer used for initial homogenization. This second homogenate was centrifuged again at 1,500 g for 10 min at 4°C, and the supernatant from this centrifugation was combined with that from the first. The resulting pellet, containing primarily nuclei, mitochondria, and cell debris, was discarded. The combined supernatants were centrifuged in a Beckman L8 ultracentrifuge at 4°C at 200,000 g for 60 min. The supernatant was saved for analysis.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as described previously (5). IRS-1 was immunoprecipitated, and proteins were resolved on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. The extent of tyrosine phosphorylation of IRS-1 was assessed using an anti-phosphotyrosine antibody. Membranes were stripped and re-probed with antibodies against IRS-1 and the p85 regulatory subunit of PI 3-kinase to measure content of those proteins in the fractions. Detection was accomplished using enhanced chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL), and bands were quantified by digital scanning and image analysis.

Hydroxyproline content. Hydroxyproline content of skeletal muscle was assayed colorimetrically on acid hydrolysates of 20–30 mg of muscle (wt/wt) by previously published methods (30, 35).

Reagents. Sepharose beads cross-linked to protein A or protein G were obtained from Sigma Chemical (St. Louis, MO). All reagents for electrophoresis were obtained from Bio-Rad Laboratories, (Richmond, CA). All other chemicals were obtained from Sigma.

Antibodies. Antibodies against IRS-1, insulin receptor, and p85 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine (PY99) and horseradish peroxidase-linked goat anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology.

Calculations and statistics. Basal rates of glucose disposal were calculated using the isotopic dilution technique and steady-state equations. Rates of glucose disposal during insulin infusion were calculated using the non-steady-state equations of Steele et al. (29). Comparisons of basal and insulin-stimulated values of protein tyrosine phosphorylation and expression were made using repeated-measures analysis of variance (StatView, SAS Institute, Cary, NC).

RESULTS

Subjects and insulin action in vivo and in vitro. The subject characteristics are given in Table 1. The groups were matched for ethnicity and sex, although the patients with type 2 diabetes mellitus were slightly older than the lean or obese nondiabetic subjects. Both the diabetic and diabetic subjects had a significantly greater body mass index and lower

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Ethnicity</th>
<th>Sex (F/M)</th>
<th>BMI, kg/m²</th>
<th>Lean Body Mass, %</th>
<th>FPG, mg/dl</th>
<th>Hb A₁c, %</th>
<th>Insulin-Stimulated Glucose Disposal (mg/kg FFM · min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean control</td>
<td>35±4</td>
<td>5 MA/4 C/1 AA</td>
<td>4/6</td>
<td>24.9±0.8</td>
<td>78±2</td>
<td>98±2</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>Obese nondiabetic</td>
<td>41±3</td>
<td>5 MA/4 C/1 AA</td>
<td>6/4</td>
<td>30.6±0.8*</td>
<td>67±3*</td>
<td>92±2</td>
<td>5.0±0.2</td>
</tr>
<tr>
<td>Type 2 diabetic</td>
<td>45±3</td>
<td>6 MA/3 C/1 AA</td>
<td>6/4</td>
<td>33.0±1.0*</td>
<td>64±3</td>
<td>142±14*</td>
<td>6.3±0.6*</td>
</tr>
</tbody>
</table>

Data are given as means ± SE, MA, Mexican American; C, Caucasian; AA, African American; BMI, body mass index; FPG, fasting plasma glucose; FFM, fat-free mass. *P < 0.05 vs. lean control; †P < 0.05 vs. obese nondiabetic.
percentage of lean body mass than the lean control subjects ($P < 0.05$). As expected, fasting plasma glucose and Hb A1c were greater in patients with type 2 diabetes than in either lean or obese nondiabetics. To assess the level of insulin resistance of the subjects who took part in this study, euglycemic clamp experiments with muscle biopsies were performed. The rate of insulin-stimulated glucose disposal, as expected, was highest in the lean control subjects, intermediate in the obese nondiabetic, and lowest in patients with type 2 diabetes mellitus (Table 1). On the other hand, insulin stimulation of IRS-1 tyrosine phosphorylation, as well as of the association of the p85 regulatory subunit of PI 3-kinase with IRS-1, was decreased similarly (compared with lean controls) in the nondiabetic obese subjects and the diabetic patients (Fig. 1, A and B). These data confirm the severe systemic and cellular insulin resistance present in the obese and diabetic subjects.

**Muscle collagen abundance.** Having established the level of insulin resistance in these patients, the overall level of collagen protein in muscle biopsies was estimated using the content of hydroxyproline as a marker for collagen. Muscle biopsy specimens were hydrolyzed using 6 N HCl, and the resulting hydrolysates were assayed colorimetrically. Hydroxyproline content was significantly increased in skeletal muscle from patients with type 2 diabetes and in muscle from obese nondiabetic subjects compared with lean controls ($P < 0.01$; Fig. 2). These data were corrected by analysis of covariance for age and fasting plasma glucose concentration.

In a previous study (24), expression of collagens I and III were increased during experimental insulin resistance produced by a lipid infusion. Expression of these two proteins in skeletal muscle biopsies was examined by immunofluorescence staining of thin sections of muscle biopsies. There was greater immunoreactive collagen III and I protein in the insulin-resistant subjects, which was revealed by immunostaining (Fig. 3).

**DISCUSSION**

Years of investigation have led to an increased understanding of the molecular mechanisms of insulin action. A number of hypotheses have been put forth to explain the nature of defects in these mechanisms that are involved in insulin resistance. Currently, abundant evidence points to an accumulation of fatty acids or fatty acid metabolites in skeletal muscle, and perhaps other tissues, as a proximal defect that can adversely affect insulin signaling and produce insulin resistance (1, 9, 28, 37). Although an accumulation of fatty acids in muscle could result simply from an oversupply due to increased plasma FFA and triglyceride levels, it would appear that defects also exist in the utilization of fatty acids (28). At least two factors, and probably more, could contribute to a decreased ability of muscle to utilize, that is, oxidize, fatty acids. First, insulin resistance is associated with an increase in the resting respiratory exchange ratio of skeletal muscle, indicating a preference for utilization of carbohydrate rather than fat (15). Such a change could be brought about by a decrease in adiponectin signaling, resulting in decreased activation of AMP-dependent protein kinase, thereby lessening the phosphorylation state and increasing the activity of acetyl-CoA carboxylase, leading to an increase in fatty acid synthesis and the subsequent inhibition of carnitine-acyltransferase (26). A number of laboratories have shown that there are decreases in both plasma adiponectin concentrations (2, 11, 34) and expression of the adiponectin receptors (4) in insulin resistance. There also appear to be defects in the expression of nuclear-encoded mitochondrial genes, perhaps due to decreased expression of PGC-1. It has recently been shown (24) that oversupply of lipids per se can decrease expression of PGC-1. Moreover, there are anatomic (14) and functional (22, 23) data that provide evidence of mitochondrial abnormalities in insulin-resistant skeletal muscle. It is possible that all of these factors combine to produce a decrease in fat.
utilization and accumulation of so-called ectopic fat in insulin-resistant skeletal muscle.

What has been lacking is an explanation for the underlying basis of mitochondrial abnormalities in insulin-resistant muscle. We (24) recently showed that a lipid infusion that produces insulin resistance in healthy subjects can decrease the expression of PGC-1 and nuclear-encoded mitochondrial genes. These changes, in and of themselves, could lead to mitochondrial abnormalities. However, in that study (24), the most profound changes in gene expression produced by a lipid infusion were marked increases in mRNA and protein expression of a number of extracellular matrix genes, such as those for several collagen chains, fibronectin, and a proteoglycan, and these changes were accompanied by increased expression of mRNAs for matrix metalloproteinases and tissue inhibitors of metalloproteinases. These changes suggested that there had been a response to an inflammatory stimulus and were reminiscent of inflammatory responses in other tissues related to the insulin resistance syndrome, such as fibrosis in atherosclerotic plaques (27) or nonalcoholic steatohepatitis (20), as well as diabetic complications such as glomerular expansion and sclerosis (25). Moreover, insulin-resistant adipose tissue is characterized by inflammatory changes (33, 36).

Therefore, because chronic inflammation is now recognized to be present in insulin resistance and type 2 diabetes, in the present study we asked whether collagen expression was increased in naturally occurring insulin resistance, as well as in lipid-induced experimental insulin resistance. The obese non-diabetic and type 2 diabetic subjects in the present study were characterized by insulin resistance at both the systemic and biochemical levels. Compared with lean healthy controls, the obese nondiabetic and diabetic subjects had decreased the insulin-stimulated glucose disposal and the decreased insulin stimulation of IRS-1 tyrosine phosphorylation and association of PI 3-kinase with IRS-1 that are characteristic of insulin-resistant patients (5, 17). To obtain a reflection of total collagen abundance in muscle biopsies, we assayed hydroxyproline content after acid hydrolysis of total muscle protein. This analysis showed that hydroxyproline content was increased two- to threefold in muscle not only from patients with type 2 diabetes, but also from obese nondiabetic subjects. This indicates that the increase in hydroxyproline, a surrogate measurement for collagen, was not due to hyperglycemia alone, as the obese nondiabetic subjects had normal glucose tolerance, normal fasting glucose, and normal Hb A1c. Therefore, such a change should not be viewed as merely a manifestation of a diabetic complication in a tissue that had not previously been recognized to be prone to complications. Rather, this increase in collagen abundance may be viewed as being associated with insulin resistance. Moreover, immunofluorescence staining and immunoblot analysis showed an increase in the expression of types I and III collagen protein. These data suggest that, just like in lipid-induced experimental insulin resistance, naturally occurring insulin resistance is associated with increased collagen expression in skeletal muscle and an altered extracellular matrix.

The results of a recent study (12) using a collagen VI knockout mouse model for inherited muscle disorders caused by mutations in the Col6a1 gene revealed an underappreciated connection between alterations in the extracellular matrix and
mitochondrial abnormalities. In the case of the Col6a/−/− mouse, the muscle dysfunction is caused by apoptosis induced by the release of mitochondrial proteins (12). In culture, myoblasts derived from the Col6a/−/− mouse cultured on collagen VI behaved normally, and inhibition of the mitochondrial permeability transition pore with cyclosporin reversed the phenotype of muscular dystrophy (12). These investigators hypothesized an integrin- and Rac-mediated connection between the extracellular matrix and mitochondrial function. Whether or not such an abnormality on a milder, more chronic scale exists in insulin resistance is unknown. In addition, the mouse model has a complete lack of one matrix component, rather than a less severe change in the proportions of matrix components characterized by an increase in some. Nevertheless, the experiments with the Col6a/−/− mouse provide proof of the principle that a change in the composition of the extracellular matrix in skeletal muscle can lead directly to mitochondrial abnormalities. In the case of the Col6a/−/− mouse cultured on collagen VI, dysregulated gene expression and insulin sensitivity, decreased plasma adiponectin concentrations are closely related to hepatic fat content and insulin resistance. Dysfunctional adiponectin receptors gene expression and insulin sensitivity are coordinately downregulated in human diabetes. Adiponectin plays a role in free fatty acid metabolism in human skeletal muscle in type 2 diabetes. Diabetes 51: 2944–2950, 2002.


