Expression of FOXC2 in adipose and muscle and its association with whole body insulin sensitivity

Gina B. Di Gregorio, Rickard Westergren, Sven Enerback, Tong Lu, and Philip A. Kern

1The Central Arkansas Veterans Healthcare System; Department of Medicine, Division of Endocrinology; 2University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; and 3Medical Genetics, Department of Medical Biochemistry, Goteborg University, SE 405 30 Goteborg, Sweden

Submitted 2 April 2004; accepted in final form 9 June 2004

Numerous studies have demonstrated a relationship between obesity and insulin resistance, and recent epidemiologic studies have linked the increasing prevalence of diabetes to the obesity epidemic (9). Insulin resistance occurs with obesity, and is a prominent early feature of subjects who later develop type 2 diabetes. The link between obesity and insulin resistance, however, is not clear, because peripheral insulin resistance is a process that predominantly involves glucose transport into skeletal muscle (6). Much research has demonstrated the expression by adipose tissue of proteins that are associated with peripheral insulin sensitivity, including TNF-α, IL-6, resistin, and adiponectin (10–12), however, the mechanisms underlying these associations are not known.

Data from recent studies have suggested a possible role of the forkhead/winged helix transcription factors in obesity and insulin sensitivity (4). This family of proteins has been shown to play a role in embryonic pattern formation, regulation of tissue-specific gene expression, and tumorigenesis (14). One member of this protein family, FOXC2, was recently overexpressed in the adipose tissue of mice (4). FOXC2 overexpression altered the subunit composition of PKA, resulting in a greater sensitivity of the PKA-signaling pathway. As a result of these changes in gene expression, FOXC2 decreased total body lipid content and levels of nonesterified fatty acids (NEFA), along with increased insulin sensitivity and increased thermogenesis in white adipose tissue, resulting in a mouse that was protected from diet-induced obesity and insulin resistance.

To determine whether FOXC2 expression was regulated in humans, previous studies measured FOXC2 mRNA in human adipose tissue (16, 20). In a study of obese subjects, fasting insulin levels were inversely correlated with visceral adipose tissue FOXC2 mRNA expression (16), suggesting that insulin-sensitive subjects have high levels of FOXC2. Because of the FOXC2 overexpression studies in mice (4), these studies suggested that FOXC2 may be protective against insulin resistance. However, in another study, involving nonobese subjects, there was no correlation between FOXC2 and insulin resistance (20).

In the present study, we examined the relationship between FOXC2 gene expression in subcutaneous adipose tissue and muscle from nondiabetic lean and obese subjects with varying degrees of insulin resistance, measured using the frequently sampled intravenous glucose tolerance test (FSIVGTT). Although muscle FOXC2 expression was not related to obesity or insulin resistance, adipose tissue FOXC2 mRNA levels were significantly inversely correlated with insulin sensitivity, such that the most insulin-resistant subjects had the highest FOXC2 levels.

METHODS

Subjects. This study involved 35 weight-stable subjects aged 26–58 yr. All subjects gave informed consent, and the research was approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. Subjects initially underwent a 75-g oral glucose tolerance test, and subjects with diabetes (fasting glucose ≥126 mg/dl, 2-h glucose >200 mg/dl) were excluded. Of the 35 subjects, 16 had impaired glucose tolerance, based on a 2-h glucose value of 140–199 mg/dl. Subjects then underwent an FSIVGTT and an adipose and muscle tissue biopsy, which were performed on separate days. In some cases, FOXC2 expression data were not available for both adipose and muscle tissue in the same subjects.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
FOX2 EXPRESSION IN ADIPOSE AND MUSCLE

Table 1. Baseline characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>Women</th>
<th>Men</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>39±1.6</td>
<td>38±3.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>129±16.3</td>
<td>138±30.4</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>123±6.6</td>
<td>142±14.6</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>51±2.1</td>
<td>45±0.5</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>5.1±0.12</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>35±1.8</td>
<td>27±2.8</td>
</tr>
<tr>
<td>Fat, %</td>
<td>44±1.3</td>
<td>20±3.9</td>
</tr>
<tr>
<td>S₁</td>
<td>3±0.5</td>
<td>3±0.7</td>
</tr>
<tr>
<td>NEFA, mEq/l</td>
<td>0.58±0.25</td>
<td>0.32±0.16</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. LDL and HDL, low- and high-density lipoprotein, respectively; BMI, body mass index; S₁, insulin sensitivity index; NEFA, nonesterified fatty acids.

Characteristics of the study subjects are shown in Table 1. Blood lipids, glucose, and Hb A₁c were measured using standard clinical assays. Of the 35 subjects studied, 29 were women and 6 were men, and the subjects ranged from lean to very obese (body mass index [BMI] range 22–61 kg/m²). Some subjects demonstrated mild dyslipidemia, but no subject demonstrated fasting triglycerides of >400 mg/dl. Body composition was determined using bioelectric impedance, which correlates well with other measures of body fat (17).

Adipose tissue and muscle biopsies. Approximately 5 g of abdominal subcutaneous adipose tissue were removed from each patient by incision under local anesthesia. Immediately after the fat biopsy, a muscle biopsy was performed by needle biopsy from the vastus lateralis muscle (18). Some of the tissue was snap-frozen in liquid N₂ and the subjects ranged from lean to very obese [body mass index (BMI) range 22–61 kg/m²]. Some subjects demonstrated mild dyslipidemia, but no subject demonstrated fasting triglycerides of >400 mg/dl. Body composition was determined using bioelectric impedance, which correlates well with other measures of body fat (17).

Insulin sensitivity measurements. The measurement of in vivo insulin sensitivity was performed in the fasting state, using the tolbutamide-modified minimal-model analysis of the FSIVGTT (1, 2), which has been validated against the euglycemic clamp (3, 19). Four basal blood samples were obtained at time 0. Patients were then given an intravenous glucose bolus (11.4 g/m²) and, 20 min later, an injection of tolbutamide (125 mg/m²). Frequent blood sampling was then performed according to the standard protocol. Glucose was measured using the glucose oxidase method in a glucose analyzer, and insulin was measured using a chemiluminescent enzyme-linked immunosorbent assay (MLT Insulin Assay; MLT Research, Cardiff, Wales, UK). The insulin sensitivity index (S₁) was calculated using the MINMOD program, along with the acute insulin response to glucose (AIRGlu) (1). To assess β-cell compensation to insulin resistance, the disposition index (D₁) was calculated as the product of S₁ times AIRGlu, as described previously (7).

Real-time quantitative RT-PCR. RNA was extracted from both muscle and adipose tissue as described previously (5) and were made DNA free by treatment with DNase I before the reverse transcription reaction. Reverse transcription was carried out using 2 μg of total RNA, and 0.5 μl (7.8 ng of cDNA) was used in each 25-μl PCR reaction (Tagman Universal PCR Master Mix, Applied Biosystems) to determine FOX2 expression levels and 18S ribosomal RNA levels. Real-time PCR was performed using the ABI Prism 7900HT sequence detection system. All samples were analyzed twice with and without reverse transcriptase, and no amplification was seen in the samples in the absence of reverse transcriptase. The change in (Δ) cycle threshold (ΔCₜ) method was used to calculate FOX2 mRNA levels, and the Cₜ values were generally between 20 and 30. The primer sequences used to detect FOX2 were forward 5’-GCCAGCAGAACAATTTCC-3’ and reverse 5’-CGGGAGTCTGGTCTCACA-3’; for 18S ribosome the forward primer sequences was 5’-ATGCCGTGCTGCTTGATCAACA-3’, and the reverse primer sequence was 5’-GATCCGGACCCCTACTAAC-3’. The probe sequences for FOX2 and 18S ribosome were 5’-6FAM-TGTCGAACCTCCACGGCTG-GGC-TAMRA-3’ and 5’-6FAM-CGCCCTGCTACTAGGAT-TTG-TAMRA-3’, respectively.

Measurement of cytokines and adipokines. All cytokines and adipokines were measured as previously described (12). Adipose-secreted and serum IL-6 and TNF-α were measured using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN). Leptin and adiponectin were measured by RIA (Linco Research, St. Charles, MO).

Statistics. All data were expressed as means ± SE. FOX2 mRNA levels were expressed in relation to 18S RNA. Because the FOX2 data were not normally distributed, the data were transformed by first adding 1 to all the values. Then the log transformation of this value resulted in a positive integer, which was multiplied by 10⁶ to facilitate plotting the data. Analysis of trends was performed using linear regression. Student’s t-test was used to compare mean FOX2 expression levels between obese and lean subjects or between insulin-sensitive and insulin-resistant subjects. One-way analysis of variance was used to compare the means of three or more subject groups. When appropriate, Tukey’s test was used to determine significant differences among subject groups.

RESULTS

FOX2 mRNA expression and obesity. Fat and muscle biopsies were obtained from the subjects described in Table 1, and FOX2 mRNA levels were measured. Figure 1 shows the relationship between FOX2 and obesity with BMI as an indicator of obesity. There was no significant relationship between BMI and FOX2 in either adipose tissue (Fig. 1A) or muscle (Fig. 1B) (r = 0.18, P = 0.5; r = −0.10, P > 0.5; adipose and muscle, respectively), and this lack of correlation between FOX2 and BMI was not gender dependent. When obesity was expressed as percent body fat or as total fat mass, there was again no significant relationship with either adipose tissue or muscle FOX2. As noted in Fig. 1, the level of FOX2 mRNA, expressed in relation to 18S RNA, was ~20-fold higher in adipose tissue than in muscle.

FOX2 expression and its relation to whole body insulin sensitivity. The relationship between insulin sensitivity, using the insulin sensitivity index (S₁) and adipose tissue and muscle FOX2 mRNA was examined. There was a strong inverse relation between FOX2 mRNA and S₁ in adipose tissue (r = −0.78, P < 0.001, n = 19; Fig. 2A), and this association did not change when men were eliminated from the analysis. In contrast to the significant inverse relationship in adipose tissue, there was no relationship between muscle FOX2 and S₁ (r = −0.29, P < 0.20, n = 26; Fig. 2B). Moreover, adipose, but not muscle, FOX2 expression was positively correlated with fasting serum insulin (r = 0.49, P < 0.05). From the FSIVGTT, the AIRGlu was obtained, along with the D₁, which is an indicator of β-cell compensation to insulin resistance. Neither adipose nor muscle FOX2 mRNA expression was correlated with either AIRGlu or D₁ (data not shown).
FOXC2 mRNA is related to S_I independently of BMI. As described above, adipose tissue FOXC2 expression was associated with insulin resistance and with some measures of obesity. As expected, there was a significant relationship between obesity and insulin sensitivity in our subjects (BMI vs. S_I, r = 0.48, P < 0.005). To demonstrate the relationship between adipose FOXC2 mRNA expression and insulin sensitivity independent of obesity, we examined FOXC2 expression in subjects who had similar degrees of adiposity but were discordant for S_I. By use of the median S_I values of our population, subjects were divided into those with an S_I greater than or less than 2.0. From these groups, we matched subjects according to BMI (4 kg/m²). As shown in Fig. 3, by design there was no significant difference in BMI, but there was a significant difference in S_I. Plasma NEFA levels were similar in insulin-sensitive and insulin-resistant subjects (0.47 ± 0.13 and 0.53 ± 0.11 meq/l, respectively). In addition, this matching paradigm resulted in no differences in age, percent body fat, or serum leptin but significant differences in serum adiponectin. As shown in Fig. 3, the insulin-resistant subjects with the low S_I demonstrated significantly higher levels of adipose tissue FOXC2, but no significant differences in muscle FOXC2. Thus these data demonstrate that adipose, but not muscle, FOXC2 is related to insulin sensitivity independently of obesity.

FOXC2 mRNA and cytokines. In other studies, TNF-α induced the expression of FOXC2 in adipocytes (4, 8). Both serum- and adipose tissue-secreted measures of TNF-α, IL-6, and adiponectin were available on these subjects, and cytokine expression was analyzed in relation to FOXC2. We found no association of either adipose or muscle FOXC2 mRNA with either circulating or adipose-secreted TNF-α, IL-6, leptin, or adiponectin.

FOXC2 and plasma NEFA. In previous studies, FOXC2 was associated with PKA sensitivity. Because increased PKA activity in adipose tissue would be expected to result in increased lipolysis, we examined the relationship between adipose FOXC2 and plasma NEFA. We found no significant association between adipose FOXC2 mRNA and plasma NEFA when all subjects were analyzed together (r = −0.19, P < 0.20).
DISCUSSION

The potential role of FOXC2 in obesity and insulin resistance was first highlighted in studies in transgenic mice over-expressing FOXC2 in adipose tissue (4). These mice were leaner and more insulin sensitive and did not become obese with high-fat feeding, suggesting that FOXC2 was a defense against diet-induced obesity. In addition, FOXC2 overexpression led to a pleiotropic effect on gene expression. The subunit composition of PKA was altered, leading to a regulatory subunit that would confer increased PKA sensitivity to cAMP. Such an effect would presumably lead to increased basal lipolysis and, hence, leanness. A subsequent study in humans examined FOXC2 mRNA expression in visceral fat and muscle of obese subjects (16). The most insulin-resistant subjects in that study (16) demonstrated lower levels of FOXC2 expression in both fat and muscle. Those data tended to confirm the mouse studies and suggested that increased FOXC2 levels may protect against insulin resistance in humans, as it did in the transgenic mice (4). However, another human study involving only nonobese subjects observed no correlation between FOXC2 and insulin resistance, wherein insulin resistance was measured by the euglycemic clamp (20). Although this latter study observed no correlation between FOXC2 and glucose disposal rate, insulin-resistant subjects demonstrated lower FOXC2 expression than insulin-sensitive subjects.

Our study was intended to examine FOXC2 levels in humans in relation to obesity and insulin resistance. In contrast to the previous human study (16), this study involved both obese and lean subjects, and the adipose tissue was derived entirely from the subcutaneous depot by biopsy. In addition, the measurement of insulin sensitivity was performed using the FSIVGTT, which is a more robust measurement of insulin resistance than measurements based on a fasting insulin measurement such as the homeostasis model of assessment of insulin resistance (HOMA-IR). In contrast to the previous study, we found that insulin resistance was associated with higher levels of adipose tissue FOXC2 expression. In addition, we found no significant relationship between muscle FOXC2 and any clinical or metabolic parameters. The inverse association between adipose FOXC2 and S1 was particularly strong, and when we separated insulin-sensitive and insulin-resistant subjects and matched them for obesity, we found that adipose tissue FOXC2 continued to be threefold higher in the most insulin-resistant subjects. To determine whether the association between FOXC2 and insulin resistance could be explained by other factors known to be associated with insulin resistance, we measured the expression or plasma levels of TNF-α, IL-6, adiponectin, and NEFA. However, we found no significant correlations with adipose FOXC2 expression.

Skeletal muscle is a key player in the development of insulin resistance, and the previous study of FOXC2 in humans (16) showed that skeletal muscle, as with adipose tissue FOXC2 mRNA, was also higher in leaner, more insulin-sensitive subjects. In the present study, we detected FOXC2 mRNA in human skeletal muscle, but the levels of expression were 10-fold lower than in adipose tissue. However, we found no association between FOXC2 mRNA and S1 in muscle. Moreover, there was no association with muscle FOXC2 mRNA levels and obesity or other parameters of insulin sensitivity such as fasting serum insulin or HOMA-IR.

There are a number of possible reasons why these studies differ from those of others (16, 20). In addition to the differences in the population groups, Ridderstrale et al. (16) studied only obese subjects (mean BMI 42), and Yang et al. (20) studied only nonobese subjects, whereas this study involved subjects covering a wide range of BMI, from lean to obese. It is possible that a high BMI in some individuals will evoke an increase in FOXC2 mRNA steady-state levels to counteract the obesity, whereas a lower BMI would not, hence displaying a different correlation pattern. In addition, the fat depot was different between this and previous studies. Ridderstrale et al. used only visceral fat obtained during surgery, and subcutaneous fat was biopsied under local anesthesia in the present study. It is certainly possible that visceral adipose tissue demonstrates properties different from subcutaneous adipose tissue, although it is surprising that the regulation with regard to insulin resistance is opposite. The technique for measurement of insulin resistance was different between these studies, but the data from our study were qualitatively the same regardless of whether insulin resistance was expressed as S1 or fasting insulin.

The physiological role of FOXC2 in human glucose metabolism and energy balance is not known. On the basis of the pleiotropic effects on gene expression, there is a wide spectrum of possible effects, which may vary between mice and humans and which may differ among different subjects and adipose...
FOXC2 EXPRESSION IN ADIPOSE AND MUSCLE

E803
depots. If the predominant result of increased FOXC2 expression is an increased expression of a more sensitive PKA regulatory subunit, then one would expect to see increased adipocyte lipolysis as a result of increased FOXC2 expression. Obese subjects generally demonstrate increased lipolysis, and this study observed a tendency for higher FOXC2 expression in obese subjects, although we did not observe a correlation between adipose FOXC2 expression and plasma NEFA in this study. In addition, a previous study demonstrated increased FOXC2 mRNA levels in adipocytes that were treated in vitro with insulin (16), and mice overexpressing FOXC2 expressed higher levels of hormone-sensitive lipase (4). Insulin-resistant subjects demonstrate higher plasma insulin levels but increased lipolysis. Hence, it is possible that insulin induces FOXC2 expression in obese subjects, resulting in an increased sensitivity of the adipocyte to basal catecholamine levels, increased hormone-sensitive lipase activity, and ultimately yielding increased lipolysis. On the other hand, it is possible that elevated FOXC2 in adipose tissue is but one more manifestation of the insulin resistance syndrome, perhaps due to adipocyte insulin resistance. On the basis of the lean, insulin-sensitive phenotype of FOXC2 overexpressing mice, increased lipolysis from elevated FOXC2 could represent a protective effect, attempting to reduce adipocyte volume through increased lipolysis in the face of insulin resistance.

ACKNOWLEDGMENTS

We thank Sarah Dunn for administrative assistance.

GRANTS

This study was supported by a Merit Review Grant from the Veterans Administration, Grant no. M01-RR-14288, of the General Clinical Research Center, and DK-39176 from the National Institute of Diabetes and Digestive and Kidney Diseases. In addition, this work was made possible with support from the Swedish Research Council (K2002-99B1-14383-01A, K2002-31X-12186-06A), EU Grants QLK3-CT-2002-02149 and LSII-CT-2003-503041, Swedish Cancer Society Grant 254270013, the Arne and IngaBritt Lundberg Foundation, the Wallenberg Foundation, the Swedish Foundation for Strategic Research (Nucleic Acid Program) and the Juvenile Diabetes Research Foundation.

REFERENCES