Numerous studies have demonstrated a relationship between obesity and insulin resistance. One family of proteins has been shown to play a role in embryonic pattern formation, regulation of tissue-specific gene expression, and tumorigenesis. 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.

The present study examined the relationship between FOXC2 expression and adipose tissue from obese patients 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. These subjects were matched for BMI but who were discordant for insulin sensitivity.
Characteristics of the study subjects are shown in Table 1. Blood lipids, glucose, and Hb Alc 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 &gt;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 for extraction of RNA afterward, and the remaining tissue was placed in Dulbecco’s modified Eagle’s medium (DMEM) for other assays. To measure the secretion of cytokines and adiponectin, 500 mg of adipose tissue were minced and placed into serum-free DMEM (pH 7.4, 10 nmol/l HEPES) at 37°C for varying times, as described previously (12, 13). To compare cytokine secretion among different subjects, we measured cytokine levels in the medium after 2 h at 37°C. All data were normalized to DNA content to control for differences in fat cell size. DNA content was measured as described previously (15).

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 insulin bolus (0.05 U/kg). Basal blood samples were obtained at time 0, 30, 60, 120, and 180 min. The relationship between insulin sensitivity, using SI as an indicator of obesity, and obesity with BMI as an indicator of obesity. There was no significant relationship between BMI and FOXC2 in either adipose tissue (Fig. 1A) or muscle (Fig. 1B). Moreover, adipose, but not muscle, FOXC2 expression was positively correlated with fasting serum insulin. In contrast to the significant inverse relationship in adipose tissue, there was no relationship between muscle FOXC2 and SI (r = −0.29, P < 0.20; n = 26; Fig. 2B). Moreover, adipose, but not muscle, FOXC2 expression was positively correlated with fasting serum insulin. Neither adipose nor muscle FOXC2 mRNA expression was correlated with either AIRGlc or D₄ (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/m^2). 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-\( \alpha \) induced the expression of FOXC2 in adipocytes (4, 8). Both serum- and adipose tissue-secreted measures of TNF-\( \alpha \), 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-\( \alpha \), 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 mRNA expression and insulin resistance, it is possible that visceral adipose tissue demonstrates properties different from subcutaneous adipose tissue, although it is surprising that the regulation with regard to insulin sensitivity was 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 SI or fasting serum insulin.

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 SI 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 SI 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 SI 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
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 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.

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