Overexpression of Foxf2 in adipose tissue is associated with lower levels of IRS1 and decreased glucose uptake in vivo

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The pathogenesis of type 2 diabetes involves insulin resistance in peripheral tissue such as skeletal muscle and adipose tissue. The insulin resistance syndrome, or as it also is called, the metabolic syndrome, refers to several findings including glucose intolerance, obesity, dyslipidemia, and hypertension. The importance of insulin receptor signaling pathway in development of insulin resistance has been shown by studies of genetic mouse models deficient in insulin receptor and its downstream signaling components, such as insulin receptor substrate 1 (IRS1) (3, 20). Although mice lacking IRS1 do not develop overt type 2 diabetes, they have impaired glucose tolerance, hyperlipidemia, and hypertension, a phenotype reminiscent of the prediabetic insulin-resistant stage of type 2 diabetes (1, 18). In humans, some studies have shown that Irs1 mutations are linked to impaired insulin-stimulated signaling (2, 17), whereas others have failed to show this (7, 8). In obese subjects, an Irs1 G972R allele has been associated with a 50% reduction in insulin sensitivity (6), and in Pima Indians of Arizona, which have very high incidence of type 2 diabetes, an association exists between variants in Irs1 and type 2 diabetes (14). Furthermore, in human adipocytes, low expression of IRS1 mRNA and protein has been shown to predict insulin resistance and type 2 diabetes (4).

It had previously been shown that several members of the forkhead gene family of transcription factors were involved in metabolism (5, 16, 22). Since Foxf2 has been reported to be expressed in adipocytes (9), we decided to investigate its putative role as a regulator of adipocyte metabolism. In this report, we find that mice with transgenic overexpression of the forkhead transcription factor Foxf2 in adipose tissue have altered levels of mRNA encoding proteins involved in metabolism. In particular, mRNA as well as protein levels of IRS1 are significantly downregulated. Such mice also secrete significantly more insulin in response to an intravenous glucose load, implicating Foxf2 as a negative upstream regulator of IRS1. Thus negative Foxf2-mediated regulation of IRS1 mRNA and/or IRS1 protein levels in adipocytes may contribute to development of glucose intolerance and support the view of low IRS1 protein levels as a risk factor for development of insulin resistance syndrome.

MATERIALS AND METHODS

All of the experiments in the present studies were performed in accordance with the permission issued by the ethics committee of southern and western Sweden.

Cloning and DNA construct. An incomplete human Foxf2 cDNA (Foxf2-pEVRF0) missing a part of the 5′ end was used (11). To complete the 5′ end, we introduced a 978-bp PCR product, using a BamHI-introducing forward primer (5′-ATAGGATCCCATGAGC-ACCGAGGCG-3′) and reverse primer (5′-GGCGACTCATAC-CTGCCTGCT-3′) on PAC clone RP4–66824, into the BamHI-KpnI sites of Foxf2-pEVRF0. Full-length Foxf2 cDNA was cloned into the BamHI-HindIII sites of pBluescript and thereafter moved with HindIII-XbaI into pCB6+ vector. A 5.4-kb NotI-SmaI fragment containing the ap2 promoter/enhancer from mouse was cloned upstream of Foxf2 in the Foxf2-pCB6+ plasmid using NotI-EcoRV sites. After these procedures, the resulting 8.3-kb fragment, harboring the ap2-Foxf2 construct with polyadenylation signal, was flanked by the unique sites NotI and AgeI. The plasmid was sequenced over ligation sites. Construct DNA, purified using a Qiagen kit according to the manufacturer’s instructions, was injected into the male pronucleus of C57BL/6J x CBA F1 zygotes, cultured overnight, and transferred to pseudopregnant females. Transgenic founder lines were backcrossed to C57BL/6J for four generations.

Cell culture. 3T3-L1 (American Type Culture Collection, Middlesex, UK) and mouse embryonic fibroblast (MEFs) were cultured in...
Dulbecco’s modified Eagle’s medium (DMEM), 4.5 g/l glucose, 10% heat-inactivated calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA). For differentiation into adipocytes, the MEFs were grown until confluence (day 2) and then stimulated (day 0) with a differentiation medium [DMEM, 10% FBS, antibiotics, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 10 µg/ml insulin, 20 ng/ml FGF (all from Sigma Aldrich, Stockholm, Sweden), and 1 µM rosiglitazone (Alexis Biochemical, Lausen, Switzerland)]. New differentiation medium was added at day 3. At day 5, the differentiation medium was removed and the cells were cultured in a medium containing DMEM, 10% FBS, antibiotics, 10 µg/ml insulin, 1 µM rosiglitazone, and 20 ng/ml FGF. This medium was changed every second day. From day 10, the cells were kept in DMEM supplemented with 10% FBS and antibiotics for 24 h as a washout period before the experiments were initiated.

Intravenous glucose tolerance test. The mice were anesthetized with an intraperitoneal injection of midazolam (0.4 mg/mouse) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse) (Janssen Pharmaceutical, Beerse, Belgium). Thereafter, a blood sample was taken from the retrobulbar capillary plexus in heparinized tubes, whereafter α-glucose (1 g/kg; British Drug Houses, Poole, UK) was injected rapidly intravenously. New blood samples were taken after 1, 5, 20, 50, and 75 min. After immediate centrifugation at 4°C, plasma was separated and stored at −20°C or until analysis.

Insulin tolerance test. Blood was withdrawn from the tail before a load of human insulin was administered (0.5 U/kg ip; Sigma-Aldrich). Further samples were collected 15, 30, 60, and 90 min after the insulin challenge. Blood glucose levels were determined using an AccuCheck Compact Plus glucometer (Roche).

Isolation of stromal-vascular fraction progenitor cells and adipocytes. Six-month-old female C57BL/6 mice were used to obtain adipocytes and stromal-vascular fraction (SVF) progenitor cells essentially as previously described (10). Briefly, the epidydimal fat pads were removed, washed from the blood, minced, and digested using collagenase A (1 mg/g tissue) at 37°C for 1 h in DMEM containing 4% bovine serum albumin (BSA). Samples were filtered through a 250-µm nylon mesh, and adipose cells and SVF were separated by centrifugation at 700 g for 7 min at room temperature. Both cell fractions were washed three times with 40 ml of DMEM containing 4% BSA and used directly for RNA isolation. Total RNA was prepared using TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions and treated with the Turbo-DNA kit (Ambion, Austin, TX).

Reverse transcription and quantitative real-time PCR. Tissues were dissected, and total RNA was isolated using the Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer’s instructions and treated with Turbo-DNA. Similar isolation was also used for RNA preparation of MEF and 3T3-L1 cells. Reverse transcription of 1 µg of total RNA was carried out using a First-Strand cDNA synthesis kit for quantitative real-time PCR (qRT-PCR) (Roche). For specificity, selected samples were analyzed twice with and without reverse transcriptase, and no amplification was seen in samples in the absence of reverse transcriptase. To scan genes of interest in white adipose tissue (WAT) from Foxf2 transgenic mice and wild-type littermates, we used the RT² Profiler PCR array (SuperArray Bioscience, Frederick, MD) according to the manufacturer’s instructions. The following arrays were used: mouse insulin signaling pathway (PAMM-030) and mouse diabetes (PAMM-023). Based on array data, specific genes were chosen and further analyzed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). All expression was normalized to the expression of 36B4. For primer sequences, see supplemental information. (Supplemental data for this article is available online at the American Journal of Physiology-Endocrinology and Metabolism website.)

Immunoprecipitation and Western blotting. WAT were homogenized, and MEFs differentiated to adipocytes were scraped in lysis buffer [50 mM Tris · HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1× Complete protease inhibitor (Roche), and 1× PhosStop (Roche)]. The samples were then centrifuged at 12,000 g for 10 min, and the supernatant was collected. The supernatant was repeatedly centrifuged until totally clear, and protein concentrations were measured using the BSA protein assay kit (Pierce). The proteins were separated by SDS-PAGE (Invitrogen NUPAGE 4–12% bis gel) and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ), and the specific proteins were detected by immunoblotsing with the indicated antibody using the ECL Plus Western blotting detection system (GE Healthcare, Waukesha, WI).

To detect phosphorylation of the IRS1 protein, we performed immunoprecipitation followed by Western blotting on isolated adipocytes from WAT from Foxf2 transgenic mice and wild-type littermates in lysis buffer [25 mM Tris · HCl, pH 7.4, 0.5 mM EDTA, 25 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM orthovanadate, 0.01 mg/ml leupeptin, 1 mM benzamidine, and 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (Peninsula), and equal amounts (according to BCA protein measurement) of total lysate were precleared three times with protein A-Sepharose (Amersham Biosciences) and lysis buffer. Immunoprecipitation using anti-IRS1 (Upstate, Millipore) at 4°C overnight followed. The beads were washed three times in lysis buffer and boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris · HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 0.05% bromphenol blue). The solubilized proteins were separated by SDS-PAGE (Invitrogen NUPAGE 4–12% bis gel), transferred to PVDF membrane (Amersham Biosciences), and detected by immunoblotting with the indicated antibody using the ECL PLUS Western blotting detection system (GE Healthcare). Antibodies used for detection were anti-IRS1, anti-p307 IRS1 (pSer307), anti-p612 IRS1 (pSer612), and antiphosphotyrosine (pYtr), all from Millipore, and β-actin, from Abcam. Some membranes were subsequently incubated at 55°C for 30 min in stripping buffer (100 mM mercaptoethanol, 2% SDS, and 62.5 mM Tris · HCl, pH 6.7) to prepare them for a second round of immunoblotting. The signals were quantified using LAS-1000 Plus or LAS-4000 (Fuji Photo Film, Tokyo, Japan).

2-Deoxyglucose uptake. [3H]2-Deoxyglucose (Perkin Elmer) uptake in differentiated MEFs was measured; MEF cells were grown and differentiated in 12-well plates. The cells were serum-starved for 3 h in Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, pH 7.4, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO4, 1 mM CaCl2, 0.4 mM NaH2PO4, and 0.6 mM Na2HPO4) supplemented with 0.2% BSA and then stimulated with 50, 100, or 200 nM insulin for 15 min. Glucose uptake was determined in triplicate after addition of [3H]2-deoxyglucose (0.1 µCi/well) for 5 min. Nonspecific glucose uptake was examined with addition of 25 µM cytochalasin B and subtracted from values for glucose uptake. Washing cells three times with ice-cold PBS terminated uptake. Cells were harvested using 1% Triton-X-100 in PBS for 15 min at room temperature. 1H was measured in a liquid scintillator (Beckman LS 6000TA; Coulter, Fullerton, CA).

Foxf2 expression in diet-induced obesity and insulin resistance. C57BL/6J mice were divided into two groups (n = 6), one given standard chow (4.8% fat, D12310; Research Diets, New Brunswick, NJ) and the other a high-fat diet (35.9% fat, D12309; Research Diets) for 8 wk. WAT was dissected for further analysis.

Statistics. All values are means ± SEM. Student’s t-test was used for statistical analysis. A P value of <0.05 was considered to be significant.

RESULTS

Foxf2 is expressed in adipose tissue. In a screen for adipocyte-expressed forkhead genes, we used the highly conserved DNA-binding domain of this gene family as a probe to screen adipocyte cDNA libraries under nonstringent hybridization conditions. This led to identification of Foxf2 as an adipose
tissue-expressed forkhead gene. To confirm this, we used qRT-PCR, enabling identification of significant Foxf2 mRNA levels in abdominal, inguinal, and perirenal adipose depots (Fig. 1A). From this experiment it also is clear that only very low or background levels of Foxf2 mRNA are present in pancreas, skeletal muscle, and liver. During differentiation of 3T3-L1 cells, there is a gradual induction of Foxf2 mRNA levels during the first phase of adipogenesis. At approximately day 10, after confluence, there is a reduction compared with day 4. Prolonged culturing of 3T3-L1 adipocytes demonstrates a second phase of induction around day 18 that appears to be constant at least to day 27. Thus Foxf2 mRNA is significantly expressed in 3T3-L1 cells and displays a biphasic profile during adipogenesis (Fig. 1B). Furthermore, Foxf2 mRNA is significantly more abundant in bona fide adipocytes compared with adipose tissue stromal-vascular cells (Fig. 1C).

Systemic glucose tolerance. To study the role of Foxf2 in adipocytes, we generated transgenic mice with enhanced expression of Foxf2 in adipose tissue (see MATERIALS AND METHODS). As can be deduced from Fig. 2A, transgenic mice have approximately fourfold higher Foxf2 mRNA levels than wild-type mice. Mice overexpressing Foxf2 in adipose tissue display significantly elevated levels of insulin in response to an intravenous glucose load. A: real-time analysis showing mRNA levels for Foxf2, Foxa2, Foxc2, and Foxo1 in abdominal white tissue from Foxf2 transgenic (tg) animals and wild-type (wt) littermates. B: wt and Foxf2 tg mice were fed a standard diet for 14 wk. After intravenous injection of glucose (1 g/kg), blood samples were drawn immediately before and at 1, 5, 20, 50, and 75 min for analysis of glucose and insulin. Plasma glucose (B) and plasma insulin levels (C) from wt (n = 5) and tg mice (n = 8) were measured and plotted. For further details, see MATERIALS AND METHODS. D: blood glucose levels at time 0, before a load of human insulin was administered (0.5 U/kg ip). Further samples were collected 15, 30, 60, and 90 min after the insulin challenge (n = 4). Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001.
littermates. We found no significant difference in steady-state mRNA levels for three unrelated adipocyte-expressed forkhead genes, *Foxa2*, *Foxc2*, and *Foxo1*, indicating that increased *Foxf2* expression did not affect expression of these genes (Fig. 2A). *Foxf2* transgenic mice developed normally; there was no significant difference in food consumption, degree of adiposity, total body weight, adipocyte size, or circulating adiponectin levels compared with wild-type littermates (not shown). However, although there were only very limited differences in how an intravenously given glucose load was handled, the level of insulin secretion evoked in response to this was significantly different (Fig. 2, B and C). Mice overexpressing *Foxf2* in adipose tissue displayed severalfold higher levels of systemic insulin levels, most pronounced in the early initial phase of insulin secretion (Fig. 2C). There was an approximately fourfold increase in insulin secretion during the first 20 min after an intravenous glucose load. To further investigate systemic insulin and glucose turnover, we performed an insulin tolerance test demonstrating a significantly more rapid elimination of circulating glucose in wild-type compared with transgenic mice (Fig. 2D). Thus it appears that transgenic mice require more insulin to eliminate a given load of glucose (Fig. 2C) and that they also display a less pronounced sensitivity to insulin in terms of its glucose-lowering capacity (Fig. 2D).

*Foxf2*-regulated genes. We used qrtPCR to quantify steady-state levels of mRNA of genes known to regulate adipocyte metabolism according to commercially available primer sets (see MATERIALS AND METHODS). Compared with *Foxc2*, another adipocyte expressed forkhead gene, which in most instances acts as a positive regulator of downstream target genes (5), we found the opposite to be true for *Foxf2*. As shown in Fig. 3, there were significantly lower levels of *Irs1*, *Perilipin*, *Acrp30* (adiponectin), and *Cpt2* (carnitoyl palmitoyltransferase 2), compared with wild-type littermates, indicating that increased *Foxf2* expression did not affect expression of these genes (Fig. 2A). Mice overexpressing *Foxf2* in adipose tissue displayed severalfold higher levels of systemic insulin levels, most pronounced in the early initial phase of insulin secretion (Fig. 2C). There was an approximately fourfold increase in insulin secretion during the first 20 min after an intravenous glucose load. To further investigate systemic insulin and glucose turnover, we performed an insulin tolerance test demonstrating a significantly more rapid elimination of circulating glucose in wild-type compared with transgenic mice (Fig. 2D). Thus it appears that transgenic mice require more insulin to eliminate a given load of glucose (Fig. 2C) and that they also display a less pronounced sensitivity to insulin in terms of its glucose-lowering capacity (Fig. 2D).

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whereas *Srebp1* (sterol regulatory element binding protein 1) was induced in response to enhanced expression of *Foxf2* in adipose tissue. Several of these genes are known to be involved in pathogenesis of insulin resistance. The rather isolated effect on the early phase of insulin secretion together with previous studies showing that low *Irs1* mRNA levels in adipocytes are associated with increased susceptibility for type 2 diabetes and type 2 diabetes-related phenotypes (4) made us particularly interested in *Irs1*. To study this, we used WAT from wild-type and transgenic mice overexpressing *Foxf2* in adipocytes. We also analyzed MEFs that were differentiated into adipocytes in vitro and made comparisons using wild-type and *Foxf2* transgenic MEF adipocytes cultured under identical conditions (see MATERIALS AND METHODS). *Irs1* protein level, as judged by Western blots, was significantly downregulated in response to enhanced levels of FOXF2, in both WAT (Fig. 4A) and MEF adipocytes (Fig. 4B).

To gain insight into the phosphorylation pattern of IRS1, we used the phospho-specific antibodies pSer\(^{307}\) (Fig. 5B), pSer\(^{612}\) (Fig. 5C), and pTyr (Fig. 5D). IRS1 phosphorylation detected by these antibodies was found to be reduced in proportion to the lowered level of IRS1 (Fig. 5, A–D). This is compatible with a reduction in *Irs1* transcription with reduced *Irs1* steady-state mRNA levels, followed by similarly lowered IRS1 protein levels and an unaffected phosphorylation pattern. Using Western blotting, we analyzed levels of GLUT1 and GLUT4 in both MEF adipocytes and WAT without detecting any significant difference in expression levels of these proteins (not shown).

**Reduced glucose uptake in Foxf2 MEF adipocytes compared with wild type.** To measure directly in adipocytes the role of enhanced *Foxf2* expression in terms of insulin-mediated glucose uptake, we set up a 2-deoxyglucose assay using MEFs that had been maintained in vitro and differentiated to adipocytes (see MATERIALS AND METHODS). In a range of physiological insulin values, a significant reduction in glucose uptake was registered for adipocytes with increased FOXF2 levels (Fig. 6). This experiment supports the view that, in vivo, the most likely cell type to confer the *Foxf2*-induced reduction in glucose tolerance is the adipocyte (Fig. 2, B and C).

To investigate the possibility that *Foxf2*, with its negative effect on insulin-mediated glucose uptake in adipocytes, could play a role in obesity-induced insulin resistance, we compared wild-type C57BL6/J mice that had received either standard chow or a high-fat diet (see MATERIALS AND METHODS) for 8 wk. We previously showed that this regime renders the high fat-fed mice obese and insulin resistant (5, 13). Using qRT-PCR, we were able to demonstrate a fourfold induction of *Foxf2* steady-state mRNA levels in adipose tissue from high fat-fed mice (Fig. 7). This is compatible with the view that induction of *Foxf2* could play a role in mediating diet-induced insulin resistance.

**DISCUSSION**

We have demonstrated that *Foxf2* is expressed in adipose tissue and 3T3-L1 adipocytes (Fig. 1). Interestingly, other tissues important for peripheral metabolism, such as skeletal...
The fact that mice lacking Tanne and type 2 diabetes (4). This notion also is supported by human adipocytes have been proposed to predict insulin resistance in phosphorylation on Ser307 and Ser612 (Fig. 5, A). MEF adipocytes generated from these mice showed Foxf2 protein levels as judged by Western blot analysis in WAT from insulin (18). Further analysis revealed significantly lower protein levels (Fig. 5, B and C). Transgenic mice were also significantly less sensitive to insulin in terms of its glucose-lowering capacity (Fig. 2D). We became particularly interested in the downregulation of Irs1 mRNA (Fig. 3A), since low Irs1 mRNA and IRS1 protein levels in human adipocytes have been predicted to predict insulin resistance and type 2 diabetes (4). This notion also is supported by the fact that mice lacking Irs1 have higher circulating insulin levels and display resistance to the glucose-lowering effects of insulin (18). Further analysis revealed significantly lower protein levels as judged by Western blot analysis in WAT from Foxf2 transgenic mice compared with wild-type littermates (Fig. 4A). MEF adipocytes generated from these mice showed a similar pattern of IRS1 protein expression (Fig. 4B). Immuno-precipitated IRS1 displayed a degree of significant reduction in phosphorylation on Ser607 and Ser612 (Fig. 5, B and C) and total tyrosine residues (Fig. 5D), as one would expect due to lower total protein levels (Fig. 5A). This is most likely an effect of adipose tissue origin, since the promoter used drives expression in adipose tissue. This view is also strengthened by the fact that IRS1 is downregulated in adipose tissue, whereas mice lacking Irs1 display an ~50% reduction at the same insulin concentration (12). Thus data presented in the present study support the notion that a direct effect on insulin-mediated glucose uptake is to be expected as a consequence of reduced IRS1 levels. It is interesting to note that high fat-fed mice induced Foxf2 steady-state mRNA levels approximately fourfold (P < 0.001, Fig. 7). This indicates that diet-induced alterations in FOXF2 levels could contribute to development of adipose tissue, and possibly also systemic, insulin resistance.

Although human genetic studies in some instances have shown association between polymorphisms in the Irs1 locus and type 2 diabetes or related phenotypes, others have failed to do so. On the other hand, it appears that low levels of Irs1 mRNA and/or IRS1 protein in both humans and animal models are associated with defects in insulin-mediated glucose uptake, which leads to enhanced susceptibility to develop type 2 diabetes. Although genetic studies using IRS1-related markers analyze the degree of involvement derived from this particular locus, such approaches fall short of identifying unrelated regulators of Irs1 expression levels. Epistatic interactions contributing to reduced levels of IRS1 through altered activation of genes regulating Irs1 expression could very well be linked to a particular phenotype such as type 2 diabetes, even though the Irs1 locus as such is not. This gains support from a previous study in which human adipocytes from individuals with increased risk of developing type 2 diabetes were analyzed for Irs1 mRNA and IRS1 protein levels. Significantly lower levels of Irs1 mRNA and IRS1 protein were found in those individuals with manifest insulin resistance. The authors demonstrated that lower Irs1 mRNA and IRS1 protein levels in adipocytes predict insulin resistance and type 2 diabetes (4). Interestingly, no association with the Irs1 G972R polymorphism was found in this study, emphasizing the possibility that genes regulating IRS1, like Foxf2, could play a role as regulators of glucose and insulin turnover.

In this study we present data that support a role for adipocyte-expressed Foxf2 as a functionally important regulator of Irs1 expression and insulin-mediated glucose uptake in adipose tissue and that indicate this is of importance for systemic glucose tolerance. Foxf2 regulation and its downstream target genes may play a previously unrecognized role in systemic insulin-mediated glucose uptake and possibly also in the pathogenesis of the insulin resistance syndrome.
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DISCLOSURES

No conflicts of interest are declared by the author(s).

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