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

Rickard Westergren,1 Daniel Nilsson,1 Mikael Heglind,1 Zahra Arani,1 Mats Grände,1 Anna Cederberg,1 Bo Ahren,2 and Sven Enerbäck1

1Department of Medical and Clinical Genetics, Institute of Biomedicine, The Sahlgrenska Academy, University of Göteborg, Göteborg; and 2Department of Clinical Sciences, Division of Medicine, Lund University, Lund, Sweden

Submitted 22 June 2009; accepted in final form 10 December 2009


Many members of the forkhead genes family of transcription factors have been implicated as important regulators of metabolism, in particular, glucose homeostasis, e.g., Foxo1, Foxa3, and Foxo2. The purpose of this study was to exploit the possibility that yet unknown members of this gene family play a role in regulating glucose tolerance in adipocytes. We identified Foxf2 in a screen for adipose-expressed forkhead genes. In vivo overexpression of Foxf2 in an adipose tissue-restricted fashion demonstrated that such mice display a significantly induced insulin secretion in response to an intravenous glucose load compared with wild-type littermates. In response to increased Foxf2 expression, insulin receptor substrate 1 (IRS1) mRNA and protein levels are significantly downregulated in adipocytes; however, the ratio of serine vs. tyrosine phosphorylation of IRS1 seems to remain unaffected. Furthermore, adipocytes overexpressing Foxf2 have a significantly lower insulin-mediated glucose uptake compared with wild-type adipocytes. These findings argue that Foxf2 is a previously unrecognized regulator of cellular and systemic whole body glucose tolerance, at least in part, due to lower levels of IRS1. Foxf2 and its downstream target genes can provide new insights with regard to identification of novel therapeutic targets.

forkhead genes; transcriptional regulation; insulin signaling; insulin resistance; type 2 diabetes; glucose uptake; adipocyte metabolism

In the world of today, ~170 million people suffer from type 2 diabetes, and predictions postulate that by 2030, 366 million will be affected (21). 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 show 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′-ATAGGATCCCATGATGCACCCAGGCGC-3′) and reverse primer (5′-GGACAGGTTACCAGTCTGTACGGGCTG-3′) on PAC clone RP4–66824, into the BamHI-KpnI sites of Foxf2-pEVRF0. Full-length Foxf2 cDNA was cloned in the BamHI-XbaI 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/Jd × CBA F1 zygotes, cultured overnight, and transferred to pseudopregnant females. Transgenic founder lines were backcrossed to C57BL/Jd 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 0) 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 supplied 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, Beere, 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 Accu-Check 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 epididymal 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 animals and wild-type littermates, we used the RT2 Profiler PCR array (SuperArray Bioscience, Frederick, MD) according to the manufacturer’s instructions. The following array sets 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·HCI, 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 immunoblotting with the indicated antibody using the ECL Plus Western blotting detection system (GE Healthcare, Waukesha, WI).

To detect phosphorylation of the IR51 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·HCI, 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 (SAB)]. The phosphorylated protein (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·HCI, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 0.005% 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 anti-phosphotyrosine (pTyr), 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·HCI, 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. 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 Na2HPO4, and 0.6 mM NaH2PO4) 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 2H2O (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. 3H was measured in a liquid scintillator (Beckman LS 6000TA; Coulter, Fullerton, CA).

Foxf2 expression in diet-induced obesity and insulin resistance. C57BL6/J 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 ± SE. 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
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 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 differentiating 3T3-L1 cells. C: Foxf2 expression was 7-fold higher in the adipocyte (Ad) than stromal-vascular (SV) fraction in Awat. Values are means \pm SE. **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 qRT-PCR 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),

![Fig. 3. Expression of genes in white adipose tissue (WAT) of Foxf2 tg mice. Gene expression analysis of WAT from Foxf2 tg mice compared with wt littermates showed a significant change in gene expression for several important WAT-expressed genes involved in adipocyte metabolism, differentiation, and insulin signaling (n = 3). Lowered gene expression of Irs1 (A), Perilipin (B), Acrp30 (adiponectin; C), and Cpt2 (carnitoyl palmitoyltransferase 2; D) was detected, whereas increased expression was observed for Srebp1 (sterol regulatory element binding protein 1; E). *P < 0.05.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00905.2009)
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 pSer307 (Fig. 5B), pSer612 (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.

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).

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 qrtPCR, 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
Human adipocytes have been proposed to predict insulin resistance and type 2 diabetes (4). Interestingly, 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 it 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.

**Fig. 6.** Impaired insulin-stimulated glucose uptake observed in MEFs differentiated to adipocytes derived from Foxf2 tg mice. Differentiated MEFs were incubated with different concentrations of insulin and [3H]deoxyglucose, according to MATERIALS AND METHODS. MEF cells overexpressing Foxf2 show decreased insulin-stimulated glucose uptake compared with the wt cells. All values are represented as fold induction from non-insulin-treated cells. **P < 0.01.

**Fig. 7.** Foxf2 expression in response to high fat feeding. Foxf2 expression is shown in WAT from mice fed normal chow (4.8% fat; LF) and high-fat chow (35.9% fat; HF) for 8 wk. ***P < 0.001.
ACKNOWLEDGMENTS

We thank Dr. Peter Carlsson for valuable discussions.

GRANTS

This work was supported by Swedish Research Council Grant K2005-32BI-15324-01A, European Union Grants QLK3-CT-2002-02149 and LSHM-CT-2003-503041, the Arne and Inga Britt Foundation, the Söderberg Foundation, and the Swedish Foundation for Strategic Research through the Center for Cardiovascular and Metabolic Research.

DISCLOSURES

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

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