Degradation of IRS1 leads to impaired glucose uptake in adipose tissue of the type 2 diabetes mouse model TALLYHO/Jng

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Abstract

The TALLYHO/Jng (TH) mouse strain is a polygenic model for type 2 diabetes (T2D) characterized by moderate obesity, impaired glucose tolerance and uptake, insulin resistance, and hyperinsulinemia. The goal of this study was to elucidate the molecular mechanisms responsible for the reduced glucose uptake and insulin resistance in the adipose tissue of this model. The translocation and localization of glucose transporter 4 (GLUT4) to the adipocyte plasma membrane were impaired in TH mice compared to control C57BL/6J (B6) mice. These defects were associated with decreased GLUT4 protein, reduced phosphatidylinositol 3-kinase activity, and alterations in the phosphorylation status of insulin receptor substrate 1 (IRS1). Activation of c-Jun N-terminal kinase 1/2, which can phosphorylate IRS1 on Ser307, was significantly higher in TH mice compared with B6 controls. IRS1 protein but not mRNA levels was found to be lower in TH mice than controls. Immunoprecipitation with anti-ubiquitin and western blot analysis of IRS1 protein revealed increased total IRS1 ubiquitination in adipose tissue of TH mice. Suppressor of cytokine signaling 1, known to promote IRS1 ubiquitination and subsequent degradation, was found at significantly higher levels in TH mice compared with B6. Immunohistochemistry showed that IRS1 colocalized with the 20S proteasome in proteasomal structures in TH adipocytes, supporting the notion that IRS1 is actively degraded. Our findings suggest that increased IRS1 degradation and subsequent impaired GLUT4 mobilization play a role in the reduced glucose uptake in insulin resistant TH mice. Since low-IRS1 levels are often observed in human T2D, the TH mouse is an attractive model to investigate mechanisms of insulin resistance and explore new treatments.

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Introduction

Understanding the pathogenesis of type 2 diabetes (T2D) is necessary to identify therapeutic targets as well as to generate prognostic information, which ultimately should lead to improved outcomes in affected individuals (O'Rahilly et al. 2005). The etiology of T2D likely involves genetic predisposition and nongenetic risk factors such as high calorie diets and reduced physical activity (Leahy 2005, O'Rahilly et al. 2005). It is commonly held that most forms of T2D in humans follow polygenic inheritances; i.e. predisposing alleles from multiple genes contribute to the development of the disease. Furthermore, T2D is genetically heterogeneous and various pathologic pathways underlie the disease in different affected individuals (Leahy 2005, O'Rahilly et al. 2005). Possibly owing to the etiologic complexity, only recently common molecular factors have been identified that affect pathogenesis of human T2D (McCarthy & Zeggini 2007).

A hallmark of T2D is insulin resistance. During normal glucose homeostasis, insulin suppresses hepatic glucose production and increases glucose uptake in muscle and adipose tissues. The latter results from insulin-induced translocation to the cell surface of glucose transporter 4 (GLUT4), the major insulin-responsive GLUT. Insulin binding to its receptor initiates a cascade of events resulting in translocation of GLUT4 from an intracellular compartment and insertion into the plasma membrane (PM). The insulin resistance of T2D mellitus includes a blunting of the insulin-stimulated increase in glucose uptake into adipocytes and muscle. Decreased GLUT4 expression in adipocytes can affect glucose homeostasis (Minokoshi et al. 2003), and adipose-selective targeting of the Glut4 gene leads to impaired insulin action in muscle and liver (Abel et al. 2001).

Low-insulin receptor substrate 1 (IRS1) expression and protein levels have been linked to the development of insulin resistance and T2D in humans (Carvalho et al. 1999) and mice heterozygous for IR and IRS1 null alleles (Bruning et al. 1997). Hirosumi et al. (2002) proposed that activation of c-Jun N-terminal kinase (JNK) leads to a reduction in IRS1 levels and thus to insulin resistance in the Lep(ob) mouse and a diet induced obesity model. What factors activate this pathway and whether it is a general principle in T2D has not yet been fully established.

Genetic animal models have been valuable resources for T2D research, and several polygenic rodent models have been developed (Rees & Alcolado 2005). The TALLYHO/Jng model...
(TH) mouse strain is a newly established polygenic model for T2D characterized by moderate obesity, impaired glucose tolerance and uptake, insulin resistance, hyperinsulinemia, and male limited hyperglycemia. The TH strain originated from phenodervant mice with polyuria discovered in a colony of outbred Thelier original mice (Kim et al. 2001). Several phenodervants were imported into The Jackson Laboratory and underwent inbreeding by an intercross/backcross scheme with selection for hyperglycemia in male mice. Although hyperglycemia initially segregated as a single recessive trait, subsequent mapping studies in backcrosses with B6 and CAST/Ei mice found several genetic loci contributing to hyperglycemia in the crosses with a major locus mapping to mouse chromosome 19 (Kim et al. 2001). To better validate the TH mouse as a model for human T2D, we examined GLUT4 protein levels, translocation and localization in adipose tissue, as well as components of the insulin signaling pathway. We show not only dysregulated GLUT4 translocation as in other T2D models (Farese et al. 2007), but also a defect in phosphatidylinositol 3-kinase (PI3K) activation, and low-IRS1 levels. We found that IRS1 localizes aberrantly to proteasomal structures in TH adipocytes. Our study identifies IRS1 degradation as a contributor to insulin resistance in TH mice.

Materials and Methods

Materials

Anti-IRS1 and anti-phospho-S307 IRS1 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRS1 ELISA kits, the antibodies for phospho-tyrosine (anti-PY), and PI3K (p85) were purchased from Upstate (Lake Placid, NY, USA). Anti-GLUT4 and anti-suppressor of cytokine signaling 1 (SOCS1) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-ubiquitin antibodies were purchased from Sigma. Anti-20S proteasome α/β antibodies were obtained from Novus (Littleton, CO, USA). Catch and Release kit for PI3 kinase assays were obtained from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ubiquitin antibodies were obtained from Sigma. Anti-20S proteasome α/β antibodies were obtained from Novus (Littleton, CO, USA). Capture and Release kit for PI3 kinase assays were obtained from Upstate. Porcine insulin was purchased from Eli Lilly (Indianapolis, IN, USA).

Animals

The TH inbred mouse strain has been described in previous studies (Kim et al. 2001, 2006). Ten- to 12-week-old male TH mice were used in this study. Mice were bred and maintained in the Research Animal Facility at The Jackson Laboratory with free access to food (NIH31 diet with 6% fat) and water on a 12 h light:12 h darkness cycle. All animal studies were performed with the approval of The Jackson Laboratory Animal Care and Use Committee. Male C57BL6/J (B6) mice (10–12 weeks old) were used as normoglycemic controls as described (Kim et al. 2001, 2006, Sung et al. 2005).

Real-time quantitative PCR

Real-time PCR assays were performed as previously described (Wang et al. 2006) on an ABI PRISM 7500 SDS instrument. Samples were analyzed in triplicate in three independent runs. To quantify the gene expression profiles, we used the comparative threshold cycle method. PCR primers that amplified 150–180 bp fragments of mouse Irs1 cDNA (NM010570) were used (F: CGCTACATCCCCAGTGC-TAAC, R: GCCGAAGTGAAGTCTCTTTCGA).

Immunohistochemistry and confocal microscopy

Immunohistochemical analyses were performed as described previously with modifications (Liu et al. 1993). Briefly, mice were either fasted overnight and left untreated or allowed to eat ad libitum and then given an i.p. injection of glucose (1 g/kg) and insulin (8 U/kg). Thirty minutes after the injections, the mice were anesthetized with tribromoethanol and perfused with PBS followed by 4% paraformaldehyde in PBS as fixative. Abdominal adipose tissue was removed and postfixed in the same fixative overnight at room temperature. A microwave procedure was used for antigen retrieval in paraffin–embedded tissue sections (8 min in citrate buffer, pH 6–6.5). Slides were then incubated in 0-3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. After incubation with blocking solution (3% normal goat serum), slides were incubated overnight with rabbit polyclonal antisem against GLUT4 protein, mouse monoclonal anti-IRS1 antibodies, and rabbit polyclonal anti-proteasome 20S α+β antibodies. Fluorescence microscopy (Leica CTR6000, Bannockburn, IL, USA) was used to detect GLUT4 distribution, and fluorescence microscopy (Leica DMLB) was employed for IRS1 and 20S proteasome protein localization. For single or double immunofluorescence staining, FITC- and rhodamine-conjugated secondary antibodies were employed. Analysis and photo-documentation were described in our previous studies (He et al. 2000, Wang et al. 2006). The specificity of staining was confirmed using negative controls in which the primary antibody was omitted.

Immunoprecipitations and western blot analysis

Immunoprecipitations and western blot analyses of insulin signaling proteins were performed on abdominal adipose tissue homogenates as previously described (Araki et al. 1994, Hu et al. 2004). Briefly, mice were fasted overnight and intraperitoneally injected with insulin (0-5 U/kg body weight) or saline. Thirty minutes after injection, epididymal fat pads were dissected and saved for immunoprecipitation and immunoblot (IB) analysis of insulin signaling molecules. Adipose tissue samples were homogenized in buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0-5% NP40, 0-1 mM Na3VO4, and protease inhibitors) and particulates removed by centrifugation. The supernatants were incubated overnight at 4 °C with antibodies against...
IRS1, p85, or ubiquitin, followed by the addition of protein A- or G-sepharose and antibodies against IRS1 and phosphotyrosine. Immunoprecipitated proteins were resolved by PAGE and detected by chemiluminescence after western blotting. Quantitative densitometric analyses of the chemiluminescence photographs were carried out on a LAS-1000 plus densitometer and Image Gauge V3.45 software (Fuji Photo Film Co., Stamford, CT, USA). Results were expressed as percentage of signal intensity seen in samples from insulin-stimulated B6 animals.

**Phosphatidylinositol kinase activity assay**

Mice were fasted overnight (18 h) and then given insulin (0.5 U/kg) or saline intraperitoneally. Thirty minutes after injection, adipose tissue was homogenized on ice with a 5 mm Omni ultrasound probe. PI3K assays were performed as previously described (Krook et al. 1997, Storgaard et al. 2001, Farese et al. 2007). Briefly, to 500 μl aliquots of diluted cell lysate (300 μg) in fresh tubes were added 4 μg PY20 antibody and 10 μl (1 μg) antibody capture affinity ligand (Fisher Scientific, Pittsburgh, PA, USA). The samples were briefly mixed and incubated for 5–15 min at room temperature. Proteins were captured in an Eppendorf spin column at 1500 g for 2 min. The spin columns were washed twice with 500 μl 1X Catch and Release lysis/wash buffer and centrifuged for 3 min at 2000 g. The bound protein was eluted with 500 μl of assay dilution buffer followed by 30–60 μl water to obtain the final combined eluates. Phosphatidylinositol (PI), 1 mg/ml, was dispersed by sonication for 15 min in 5 mM HEPES. PI was preincubated with the immunoprecipitants at 4 °C for 20 min at a final concentration of 0.2 mg/ml. The phosphorylation reaction was started by adding 20 μCi (γ-32P)ATP and MgCl2 to final concentration of 50 μM ATP (cold) and 5 mM MgCl2 in a volume of 50 μl. After incubation for 20 min at 37 °C, the reaction was terminated by adding 100 μl 1 M HCl. Phospholipids were extracted immediately with 200 μl CHCl3/MeOH (1:1). The organic phase was washed with 80 μl MeOH/HCl (1:1) and 15 μl of the organic phase were spotted onto a silica gel thin layer chromatography (TLC) plate. Phosphorylated products were separated by TLC in a CHCl3/MeOH/4 M NH4OH (9:7:2) developing solvent for 1 h and visualized by autoradiography.

**Measurement of JNK activation**

The phosphorylation status at threonine 183 and tyrosine 185 of JNK1/2 was assayed using a commercial antibody bead kit for phosho-JNK1/2 (pTpY 183/185) (Cat. LHO0081, Biosource, Camarillo, CA, USA).

**Adipocyte isolation and glucose uptake**

Adipocytes were isolated from overnight fasted mice by a protocol modified from Rodbell (1964). The epididymal fat pads were removed and minced into rice-sized pieces and then placed in pregressed (95% O2, 5% CO2) Krebs–Ringer buffer with HEPES (KRBH, 117 mM NaCl, 4.72 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 10 mM NaHCO3, 20 mM HEPES, 200 mM adenosine, filtered and adjusted to pH 7.4) with 3% BSA and 2.5 mg/ml collagenase. The cells were incubated in a 37 °C water bath for 1 h with shaking at 100 r.p.m. After digestion, the cell suspension was then dispersed equally by volume into 5 ml cell culture tubes. Cells were treated with saline (basal) or 70 nM insulin for 30 min before the addition of 0.2 μCi of 2-[^3]H]-deoxyglucose. After 10 min, glucose uptake was stopped by centrifugation of the cells over dinonyl phthalate oil at 350 g for 2 min. Cells were lysed with a 3% SDS (v/v) buffer and the lysates were solubilized in Ultima-Gold scintillation cocktail. Cytochalasin B was used as a control for non-insulin dependant glucose uptake. An aliquot of each cell lysate was taken prior to the addition of scintillation fluid and all counts were normalized for protein concentration. Final glucose uptake values are presented as pmol/mg per min (Zisman et al. 2000).

**Subcellular fractionation of isolated adipocytes**

Mice were fasted for 4 h after the darkness period and then given insulin (0.5 U/kg) or saline by i.p. injection. Thirty minutes later, the epididymal adipose tissue was collected. Subcellular fractions were prepared as previously described (Tsuji et al. 2001), with some modifications. In brief, the isolated adipocytes were suspended in 1 ml homogenization buffer A (20 mM Tris–HCl, 1 mM EDTA, pH 7.4) with protease inhibitors. They were homogenized on ice and were centrifuged at 4000 g for 5 min at 4 °C to remove fat droplets. The homogenates were centrifuged for 20 min at 19 000 g. The initial supernatant contained the microsomal membrane fraction. The PM-rich fractions were prepared as follows: the initial pellet was resuspended in 3 ml buffer B (20 mM Tris–HCl, 1 mM EDTA, pH 7.4 with protease inhibitors), then layered onto a 6 ml sucrose cushion (38% sucrose in buffer B) and centrifuged at 100 000 g for 70 min. The PMs collected at the interface on top of the sucrose cushion were resuspended in buffer B and centrifuged at 40 000 g for 20 min and yielded a pellet of the PM fraction. The 100 000 g pellet was resuspended by homogenization in buffer A, yielding the mitochondrial and nuclear fractions. The 19 000 g initial supernatant was centrifuged for 20 min at 41 000 g, yielding a pellet of the high-density microsome (HDM) fraction. The 41 000 g supernatants were centrifuged at 180 000 g for 70 min, yielding a pellet of the low-density microsome (LDM) fractions. For western blotting, 10 μg of each membrane fraction was solubilized in sample buffer (2·3 M urea, 1·5% (w/v) SDS, 15 mM Tris/HCl and 100 mM dithiothreitol, pH 6·8) at room temperature for 30 min, subjected to SDS/PAGE, and transferred to.

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nitrocellulose membranes. The nitrocellulose membranes were immunoblotted with anti-GLUT4 antibody, and detected with enhanced chemiluminescence as described previously (Wang et al. 2006).

Ex vivo insulin signaling studies in isolated adipocytes

Isolated adipocytes were incubated for 30 min with 0 (basal), 10, 50, and 100 nM insulin respectively in Krebs–Ringer buffer containing 3% BSA. Adipocytes were then harvested for immunoprecipitation and IB analysis of insulin signaling proteins. Because of the low abundance of IRS1 protein in mouse adipose tissue, ELISA assays were performed to compare total IRS1 protein levels in TH and B6 mice.

Statistical analysis

All data are presented as means ± S.E.M. The statistical analyses were conducted in StatView (v.4.5; Abacus Concepts, Berkeley, CA, USA). The differences between two groups was assessed by Student’s t-test, P<0.05 was considered statistically significant.

Results

Reduced glucose transport in TH mice

In comparison with B6 mice, adipocytes from TH mice displayed comparable basal but significantly reduced insulin-stimulated 2-deoxyglucose uptake (Fig. 1, 12% reduction under basal conditions and 60% reduction after insulin stimulation). The decrease in glucose uptake by adipocytes from TH versus B6 correlated with a significant 20% reduction in both basal and insulin-stimulated cellular GLUT4 content (Fig. 2A) as determined by western blot analysis of whole adipocyte lysates, and a diminished localization of the GLUT4 protein at the adipocyte cell surface as assessed by immunofluorescence in adipose tissue (Fig. 2C).

Figure 1 Reduced glucose uptake in TH mice. Epididymal adipocytes were isolated from 10-week-old TH mice (n=8–10 mice per group) as well as B6 controls (n=8 mice per group), and incubated in the absence or presence of insulin (70 nM) for 30 min. Glucose uptake was assessed by adding trace amounts of 2-[3H]-deoxyglucose.

Figure 2 Reduced GLUT4 translocation in TH mice. (A) Western blot analysis of GLUT4 protein from adipose tissue in B6 and TH mice. (B) Subcellular distribution of GLUT4 protein from adipocytes of B6 and TH mice. Plasma membrane (PM), low-density microsome (LDM), and high-density microsome (HDM) fractions were prepared from adipocytes treated with insulin or saline, and GLUT4 was detected by western blot as described in Materials and Methods (n=6 per group). Data from three experiments were quantitated by densitometry, and graphed relative to the value for insulin-stimulated LDM B6 control, which was assigned a value of 100%. (C) Conofocal fluorescent localization of adipocyte GLUT4 protein in B6 control mice (left panel) and TH mice (right panel). Mice were either fasted overnight and left untreated, Ins (−), or allowed to feed ad libitum and given an i.p. injection of insulin and glucose, Ins (+). Adipose tissue was cryopreserved. Arrows mark several locations of punctate cytoplasmic staining in adipocytes. Arrows mark locations of extensive membrane-associated GLUT4 in adipocytes of insulin-stimulated B6 mice. Images are representative of three independent experiments (n=3 per group). *P<0.05 in fractions of PM and LDM different from basal versus respective insulin-stimulated states.

To further explore the mechanism of impaired glucose uptake in TH mice, we carried out a fractionation of the cellular membranes of adipocytes from B6 and TH mice.
In unstimulated adipose tissue of control B6 mice, 10% of GLUT4 was located at the cell surface in the PM and > 90% in intracellular compartments, LDM and HDM fractions. As expected, insulin treatment (0.5 U/kg) of B6 adipocytes resulted in 2.5-fold increase in GLUT4 protein in the PM fraction along with a 30% decrease in GLUT4 protein in the LDM fraction, reflecting the translocation from the intracellular pools of GLUT4 transporter protein to the PM. In TH mice, in concordance with the reduction of total GLUT4 protein content, the combined membrane fraction (PM, LDM and HDM) GLUT4 showed approximately a 20% decrease compared with B6 controls (quantitation not shown). GLUT4 levels in the PM fraction slightly increased from basal levels after insulin stimulation. GLUT4 content in the LDM fraction from TH mice after insulin stimulation remained unchanged (Fig. 2B), reflecting an impairment of GLUT4 transport in the adipose tissue of TH mice.

Histologically, adipose tissue from fasted B6 mice exhibited a punctate, nonlinear cytoplasmic GLUT4 immunofluorescence as well as a weak immunofluorescence associated with the PM in the basal state (Fig. 2C top left panel). GLUT4 immunofluorescence was redistributed from the condensed cytoplasmic staining to the PM after treatment with insulin and glucose in B6 mice (Fig. 2C bottom left panel). GLUT4 staining in TH mice showed a similar distribution as B6 in the basal state but with less staining of the PM (Fig. 2C top right panel). However, in TH mice treated with insulin and glucose the localization of GLUT4 remained unchanged, with the immunofluorescence signal in punctate staining in the cytoplasm with little signal present on the cell membrane (Fig. 2C bottom right panel).

Impaired insulin signaling in TH mice

Binding of insulin to its receptor results in autophosphorylation of the intracellular domain of the receptor. The phosphorylated receptor associates with IRS1, phosphorylated IRS1 binds and activates the lipid kinase PI3K and triggers GLUT4 translocation and insulin–stimulated glucose uptake (Buret et al. 2002). We found that basal PI3K activity in adipose tissue of TH was lower than in B6, although not significantly so. Stimulation of PI3K activity by insulin was significantly reduced in TH mice (Fig. 3A). The association of the PI3K regulatory subunit p85 with IRS1 was markedly less in the adipose tissue of TH mice. IRS1 binding to PI3K in TH was reduced both before (B6: 50.0 ± 0.3% versus TH: 10.0 ± 5.2%, P < 0.01) and after insulin stimulation (B6: 100.0 ± 8.6% versus TH: 8 ± 3.6%, P < 0.01; Fig. 3B).

Decreased levels of IRS protein have been reported in insulin-resistant subjects as well as diabetic patients (Carvalho et al. 1999). We measured the protein and phosphorylation levels of IRS1 in TH mice by western blotting. IRS1 protein levels in adipose tissue of TH mice were significantly reduced compared with B6 in both basal and insulin-stimulated states (Fig. 4A). The reduction of IRS1 protein was paralleled by a similar reduction in IRS1 tyrosine phosphorylation (Fig. 4B).

Since Irs1 mRNA levels were not different between B6 and TH adipose tissue as measured by real–time PCR (data not shown), we considered protein degradation as a mechanism for the reduced IRS1 levels in TH mice. IRS1 degradation is mediated by the proteasome degradative pathway and is primed by phosphorylation of serine residues on IRS1, particularly Ser307 (Gual et al. 2005). In total protein lysates, the level of IRS1 phosphorylated at Ser307 was significantly lower in TH mice in the basal state and significantly higher after insulin stimulation compared with B6 (Fig. 4C left panel). However, importantly, the fraction of total IRS1 protein that was phosphorylated at Ser307 was higher in TH mice compared with B6 both in the basal state (1.5-fold) as well as after insulin stimulation (17-fold, Fig. 4C right panel).

To determine whether the defects of IRS1 in adipocytes of TH mice are intrinsic to adipose tissue or caused by the systemic metabolic milieu, we examined IRS1 protein levels.
and tyrosine phosphorylation in primary-culture adipocytes derived from TH and B6 mice. Similar results compared to the in vivo data were obtained. Insulin increased total IRS1 protein in both B6 and TH mice in a concentration-dependent manner, however, to a lesser degree in TH (25% reduction in basal level and 40% reduction after 10 nM insulin stimulation, Supplementary Fig. 1A, ELISA and

Figure 4 Reduced levels of IRS1 protein and aberrant phosphorylation of IRS1 in TH mice. (A) Western blot analysis of IRS1 protein from adipose tissue in B6 and TH mice. Animals (n=12 per group) were fasted overnight and then given insulin (0.5 U/kg) or saline. Lysates (20 μg/well) were resolved on a 3–8% polyacrylamide gradient Tris–Acetate gel, transferred to nitrocellulose and probed with anti-IRS1 antibody. Figures are representative of three independent experiments. (B) Mice were fasted overnight and given insulin (0.5 U/kg) or saline and adipocyte lysates were prepared for immunoprecipitation. Immunoprecipitates of IRS1 were probed for phosphotyrosine (P–Y) by immunoblotting (IB). Membranes were stripped and reprobed with anti-IRS1 to confirm IRS1 levels. (C) Western blot analysis of IRS1 S307 from adipose tissue lysates. IRS1 pSer307 was measured as fraction of total protein lysates (left panel) and total IRS1 contents (right panel) respectively. Data from three experiments were quantified by densitometry, and graphed relative to the value for insulin-stimulated B6 control. *P<0.05, **P<0.01 for TH samples versus the B6 samples in basal or insulin stimulation state respectively.

Figure 5 Degradation of IRS1 protein from adipose tissue of TH mice. (A) Activation of JNK1/2 (pTyr183/185) in adipose tissue of B6 and TH mice at 10 weeks of age (n=6–8). (B) Immunoprecipitation with antibodies against ubiquitin and subsequent western blot analysis of the precipitate with anti-IRS1 antibodies showed increased amounts of polyubiquinated IRS1 in TH adipose tissue. (C) Western blot analysis of SOCS1 from adipose tissue lysates. Data from three experiments were quantified by densitometry, and graphed relative to the value for insulin-stimulated B6 control, which was assigned a value of 100%.*P<0.05; **P<0.01 for TH samples versus the B6 samples in basal or insulin stimulation state respectively.

Fig. 1B, western blot available in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/cgi/content/full/JOE-09-0026/DC1). High-insulin concentrations (100 nM) markedly suppressed IRS1
protein in both TH mice and B6 controls (Supplementary Fig. 1A). Similar results were observed in T2D patients (Rondinone et al. 1997). Although tyrosine phosphorylation of IRS1 was similar in TH and B6 without insulin, in contrast to B6 derived adipocytes, insulin at 10 and 50 nM failed to stimulate IRS1 tyrosine phosphorylation in TH adipocytes (Supplementary Fig. 1C).

Activation of JNK has been implicated in phosphorylating the IRS1 Ser307 residue in insulin resistant mice (Ozcan et al. 2006). JKN1/2 activation (pTpY183/185) was measured and shown to be 1.5-fold higher in the basal state, and 2-fold higher after insulin stimulation in adipose tissue from TH mice compared with B6 controls (Fig. 5A).

**Colocalization of IRS1 and the 20S proteasome subunit, and degradation of IRS1**

Targeting of many proteins for proteasomal degradation is mediated by their modification with polyubiquitin. To assess whether the reduction of IRS1 was associated with enhanced degradation, immunoprecipitation with antibodies against ubiquitin and subsequent western blot analysis of the precipitate with anti-IRS1 antibodies was performed. The decrease in IRS1 content due to degradation is associated with retarded migration of the protein band during SDS/PAGE (Potashnik et al. 2003). In agreement with this, a higher molecular weight IRS1-immunoreactive band was observed in adipose tissue from TH mice but not in B6 (Fig. 5B).

SOCS1 has previously been shown to bind IRS1 protein and promote its ubiquitination and subsequent degradation (Rui et al. 2002), and expression of SOCS proteins has been reported to be elevated in rodent genetic models of obesity and diabetes. SOCS1 protein levels were significantly higher in TH mice compared with B6 (~1.7-fold, Fig. 5C). SOCS1 localizes to the complex-associated 20S proteasome (Vuong et al. 2004). To obtain cellular evidence for proteasomal targeting of IRS1, we stained adipose tissue samples from B6 and TH mice for the 20S core α and β subunits of the proteasome. The 20S α and β subunits of the proteasomes were localized throughout the cytoplasm of B6 adipocytes as shown in Fig. 6A. In TH adipose tissue, considerably more punctate staining for proteasomal structures was observed (Fig. 6A). In contrast to B6 where IRS1 staining was observed in the nucleus and cytoplasm (Fig. 6B top panel), in TH mice IRS1 staining colocalized with staining for the 20S subunit in cytoplasmic proteasomal structures (Fig. 6B bottom panel).

**Discussion**

Previously we have described a new mouse model for T2D, the inbred TH strain. TH mice are moderately obese, hyperinsulinemic, glucose intolerant, and insulin resistant (Kim et al. 2001, 2006). Although occasionally diabetic females are observed in our colony, hyperglycemia is largely male limited as is typical for mouse models of T2D (Leiter et al. 1991).
The insulin resistance of T2D mellitus leads to a blunting of the insulin-stimulated increase in glucose uptake into fat and muscle. In muscle, this is due to defective stimulation of the translocation of the insulin sensitive GLUT4 from intracellular storage pools to the cell surface (Shepherd & Kahn 1999, Watson et al. 2004). In adipocytes, the decreased insulin sensitivity is due to both this defect in insulin-stimulated translocation, as well as to decreased expression of GLUT4 (Shepherd & Kahn 1999, Minokoshi et al. 2003, Watson et al. 2004). Reduced adipocyte GLUT4 protein levels have also been found in human obesity and T2D, as well as in numerous rodent models of insulin resistance (Shepherd & Kahn 1999). We previously had shown that there was a slight, but not significant reduction of GLUT4 content in soleus muscle from TH male mice (Kim et al. 2006). In the current studies, we found that there was 20% reduction in the total GLUT4 content in adipose tissue from TH compared with B6 mice. Importantly, GLUT4 content in the PM fraction and the LDM fraction in TH mice after insulin stimulation remained unchanged (Fig. 2B), reflecting an impairment of GLUT4 transport in the adipose tissue of TH mice.

IRS1 is required for normal induction of the signaling pathway that is key to many of the metabolic actions of insulin. Induction of IRS1 (3-fold) in rodents occurs within 20–40 min following feeding or insulin injection (Bruning et al. 1997, Ruiz-Alcaraz et al. 2005). The induction is not due to changes in posttranslational phosphorylation, but related to increased protein synthesis (Ruiz-Alcaraz et al. 2005). This short term insulin stimulation of IRS1 is also observed in human muscle (Ruiz-Alcaraz et al. 2005). After prolonged insulin exposure (4–48 h) or in insulin resistant states, a reduction in IRS1 levels is observed (Renstrom et al. 2005). In the present study, IRS1 content in TH adipose tissue is significantly lower than in B6 controls (P<0.001, Fig. 4A). In both strains of mice insulin leads to a significant induction of IRS1 protein, however to a lesser degree in TH compared with B6 (~ 2 vs 4-fold respectively).

The GLUT4 translocation defect in adipose tissue could be ascribed to reduced levels of IRS1 in adipocytes, which consequently cause alterations in the phosphorylation status, and the activity of intermediates in the insulin signaling pathway through PI3 kinase in TH mice. The lower levels of IRS1 and reduced insulin signaling explain at least in part the insulin resistance observed in the TH model. It makes TH an attractive model for diabetes research since similar defects have been observed in human patients with T2D (Rondonone et al. 1997).

Our data showed an increase in serine phosphorylation and a decrease in tyrosine phosphorylation of IRS1 in the insulin resistant TH mice compared with the more insulin sensitive B6 controls. While the phosphorylation of IRS1 on tyrosine residues is required for insulin-stimulated responses, the role of serine phosphorylation of IRS1 is primarily to terminate the insulin effects (Kim et al. 1999). Increased serine phosphorylation (Ser307) of IRS1 was also reported in other models of insulin resistance in mice (Hirosumi et al. 2002, Rui et al. 2002, Ueki et al. 2004). Gual et al. (2003) and others have shown that stimulation with insulin increases the phosphorylation of Ser307 (mouse) in IRS1. Furthermore, Morino et al. (2005) found that in insulin resistant offspring of T2D parents reduced muscle glucose uptake was associated with increased IRS1 Ser312 (human) phosphorylation. Bikman et al. (2008) found that in morbidly obese gastric bypass surgery patients increased IRS1 Ser312 phosphorylation was associated with insulin resistance, which improved after surgery accompanied by a lowering of IRS1 Ser312 phosphorylation. The argument has been raised that phosphorylation of IRS1 at Ser312 (human)/Ser307 (mouse) following insulin stimulation may be a physiologically normal response, possibly implicated in the determination of the correct amplitude and length of insulin action (Frojdo et al. 2009).

One key signaling mechanism through which IRS1 can become phosphorylated at Ser307(mouse)/Ser312(human) is the stress and inflammatory response pathway mediated by JNK1/2. As observed in dietary obesity and the Lep^m model (Hirosumi et al. 2002), we find significant activation of JNK1/2 in the TH model compared with B6 controls. Since JNK can be activated by free fatty acids (FFA; Nguyen et al. 2005), the elevated FFA levels in TH mice may play a role (Kim et al. 2001, 2006).

Hyperphosphorylation of IRS1 on serine/threonine residues after chronic stimulation with insulin results in its targeted degradation by the proteasome (Rice et al. 1993, Sun et al. 1999). Important mediators in this process are the SOCS proteins (Howard & Flier 2006), which target IRS1 and IRS2 for proteasomal degradation via an interaction with their SOCS box (Emanuelli et al. 2001, Rui et al. 2002, Ueki et al. 2004). Higher levels of SOCS1 in TH mice suggest that the low IRS1 levels and concomitant insulin resistance in TH mice are, at least in part, due to SOCS mediated degradation of IRS1.

Degradation of IRS1 as a cause for insulin resistance in TH mice is also supported by our finding of IRS1 colocalization with proteasomal structures in TH mice, but not in B6. It had been described that polyubiquitin chains are essential for proteolytic targeting, whereas mono-ubiquitination might only affect subcellular localization (Beal et al. 1996). Polyubiquitination, as found for IRS1 in TH mice (Fig. 5B), cellular localization (Fig. 6B), and reduced protein levels of IRS1 (Fig. 4A) are, therefore, consistent with IRS1 being targeted for proteasomal degradation, leading to the insulin resistance observed in TH mice.

The impaired glucose tolerance and reduced glucose uptake in muscle and fat of TH mice are a plausible consequence of lower insulin signaling due to low-IRS1 levels. This correlates well with findings in humans that low-IRS1 expression and protein levels are predictive of insulin resistance and T2D (Carvalho et al. 1999). However, the cause for the IRS1 abnormalities including the potentially initiating event of JNK1/2 activation in TH mice as well as other models and humans remain to be determined. Nevertheless, the similarity...
of events leading to impaired glucose uptake and insulin resistance in the polygenic TH mouse with mechanisms proposed to mediate human T2D makes TH an excellent model for investigating T2D in general.

Declaration of interest

The authors have no conflicting interests to disclose.

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