Attenuation of Insulin-stimulated Insulin Receptor Substrate-1 Serine 307 Phosphorylation in Insulin Resistance of Type 2 Diabetes*

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Insulin resistance is a primary characteristic of type 2 diabetes and likely causally related to the pathogenesis of the disease. It is a result of defects in signal transduction from the cell surface receptor of insulin to target effects. We found that insulin-stimulated phosphorylation of serine 307 (corresponding to serine 302 in the murine sequence) in the immediate downstream mediator protein of the insulin receptor, insulin receptor substrate-1 (IRS1), is required for efficient insulin signaling and that this phosphorylation is attenuated in adipocytes from patients with type 2 diabetes. Inhibition of serine 307 phosphorylation by rapamycin mimicked type 2 diabetes and reduced the sensitivity of IRS1 tyrosine phosphorylation in response to insulin, while stimulation of the phosphorylation by okadaic acid, in cells from patients with type 2 diabetes, rescued cells from insulin resistance. EC50 for insulin-stimulated phosphorylation of serine 307 was about 0.2 nM with a t1/2 of about 2 min. The amount of IRS1 was similar in cells from non-diabetic and diabetic subjects. These findings identify a molecular mechanism for insulin resistance in non-selected patients with type 2 diabetes.

The incidence of type 2 diabetes is rapidly increasing in all parts of the world that allows a sedentary and affluent lifestyle, as the disease is closely associated with obesity. It has been estimated that over 200 million people will be afflicted with the disease by the end of this decade. The pathogenesis of diabetes is not understood in any great detail, but it is generally believed that insulin resistance in skeletal muscle and especially in adipose tissue is an early, if not primary, event. The peripheral insulin resistance is compensated for by increased concentrations of circulating insulin, masking development to diabetes. Eventually the pancreatic β-cells fail to compensate for the insulin resistance, and type 2 diabetes can be diagnosed. The insulin resistance of muscle and fat tissues is the result of impaired signal transduction from the cell surface receptor of insulin to metabolic effects, such as to increase glucose transport and inhibit lipolysis in adipocytes (1, 2). Epidemiological studies have failed to provide a strong link between the disease in general and genetic polymorphisms in the genes coding for the signaling proteins. The occupied insulin receptor auto-phosphorylates and then phosphorylates the insulin receptor substrate-1 (IRS1)1 protein on tyrosine residues to provide docking sites for downstream activation of signal-transducing proteins (1).

Recently, the tyrosine phosphorylation of IRS1 was shown to be the first signaling step to exhibit reduced sensitivity to insulin in adipocytes from patients with type 2 diabetes (3). The finding depended on the realization that adipocytes obtained from human beings become insulin-resistant from the surgical cell isolation procedures and that the resistance is reversed by overnight incubation of the cells (3). This insulin resistance is manifest downstream of IRS1, and if cells from diabetic patients are not allowed to recover overnight the mix of two different types of insulin resistance will be examined. IRS1 has also been identified as a key step in insulin resistance of human skeletal muscle (4–6). Naturally occurring mutations in the IRS1 protein are present in some subjects with type 2 diabetes (7–11), and obese individuals and relatives of diabetic patients have been reported to present with lower adipocyte expression of IRS1 (12).

Along a different line of research, in cell-free systems and cell cultures, phosphorylation of IRS1 on serine residues has been found to affect, usually inhibit, insulin receptor-catalyzed phosphorylation/activation of IRS1 on tyrosine residues (13–22). Specifically phosphorylation of murine serine 307 (corresponding to serine 312 in human IRS1) has been reported to block IRS1 interaction with the insulin receptor (18) or to enhance its proteolytic degradation in the cell (23). This site has also been reported to be phosphorylated in response to tumor necrosis factor-α (24) and in animal models of type 2 diabetes (19, 25). Recently, phosphorylation of IRS1 on serine 302 in murine 32D cells (corresponding to serine 307 in human IRS1) was reported to be stimulated by insulin and abolishing of this phosphorylation by introduction of an alanine residue in place of serine 302 impaired insulin-stimulated tyrosine-specific phosphorylation of IRS1 (26). However, negative effects by serine 302 phosphorylation on insulin stimulation of IRS1 tyrosine phosphorylation (27) and disruption of insulin receptor-IRS1 interaction using a yeast two-hybrid method (19) have also been reported.

Herein we have examined the effect of IRS1 serine 307 phosphorylation on IRS1 tyrosine phosphorylation in response to insulin and the ability of insulin to induce phosphorylation of serine 307 in primary adipocytes from patients with type 2 diabetes.

EXPERIMENTAL PROCEDURES

Subjects—Subject characteristics are described in the respective figure legends. Informed consent from patients and approval by the Ethics Committee were obtained.

Materials—Mouse anti-phosphotyrosine (PY20) monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Rabbit anti-IRS1 and goat anti-actin-(1–19) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-serine 307-IRS1 antibodies were from Cell Signaling Technology (Beverly, MA). Insulin, rapamycin, okadaic acid, and other chemicals were from Sigma as indicated in the text.

Isolation and Incubation of Adipocytes—Subcutaneous adipose tissue was obtained as described in the figure legends to Figs. 1 and 4. Adipocytes were isolated by collagenase (type I, Worthington) digestion as described (28). Tissue pieces from biopsies performed with local anesthesia using xylocaine were extensively washed in large volumes of 0.15 M NaCl before digestion with collagenase. At a final concentration of 100 μl packed cell volume per ml, cells were incubated in Krebs-Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 20 mM Heps, pH 7.40, 1% (w/v) fatty acid-free bovine serum albumin, 0.5 mM phenylisopropyladenosine, 0.5 μM 1-adenosine deaminase with 2 mM glucose, at 37 °C on a shaking water bath. For analysis after 20–24-h incubation cells were incubated at 37 °C; 10% CO2 in the same solution mixed with an equal volume of Dulbecco’s modified Eagle’s medium containing 7% (w/v) albumin, 20 mM phenylisopropyladenosine, 20 mM Hepes, 50 U/ml penicillin, 50 μg/ml streptomycin, pH 7.40 (3). Before analysis cells were washed and transferred to the Krebs-Ringer solution.

SDS-PAGE and Immunoblotting—Cell incubations were terminated by separating cells from medium by centrifugation through dinonylphthalate. To minimize postincubation signaling modifications in the cells and protein modifications during immunoprecipitation, the cells were immediately dissolved in SDS and β-mercaptoethanol with protease and protein phosphatase inhibitors, frozen within 10 s, and thawed in boiling water (28). Equal amounts of cells as determined by lipid content, which is total cell volume, was subjected to SDS-PAGE and immunoblotting. After SDS-PAGE and electrotransfer membranes were incubated with the indicated antibodies that were detected using ECL+ (Amersham Biosciences, Amersham, UK) with horseradish peroxidase-conjugated anti-IgG as secondary antibody and evaluated by chemiluminescence imaging (Las 1000, Image-Gauge, Fuji, Tokyo, Japan).

By two-dimensional electrophoresis, pH 3–10, SDS-PAGE analysis, and immunoblotting against phosphotyrosine and IRS1, >95% of the tyrosine-phosphorylated 180-kDa band was determined to represent IRS1 (3).

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2 The abbreviations used are: IRS1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; BMI, body mass index.

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RESULTS

Insulin-stimulated Phosphorylation of IRS1 Serine 307—In primary subcutaneous adipocytes from a group of non-diabetic subjects insulin stimulated the phosphorylation of IRS1 on serine 307 (Fig. 1A), thus establishing the physiological relevance of this phosphorylation event in primary human cells. However, in adipocytes from an unselected group of obese female (Fig. 1B) or male (Fig. 1D) patients with type 2 diabetes this insulin-induced serine 307 phosphorylation of IRS1 was curtailed, suggesting its involvement in the disease. In these same diabetic patients the insulin-stimulated steady-state phosphorylation of IRS1 on tyrosine was inhibited at submaximal insulin concentrations (cf. Ref. 3; Fig. 2).

Amount of IRS1 in Cells—It has been suggested that the amount of IRS1 protein is reduced in adipocytes from patients with diabetes (12). We found the amount of IRS1 protein to be identical in the adipocytes from the group of non-diabetic and diabetic subjects. The amount of IRS1 protein was determined by immunoblotting with site-specific antibodies and chemiluminescence imaging and normalized for the amount of IRS1 protein in each sample (3).

Comparison of the level of insulin-induced phosphorylation of serine 307 in cells from the female non-diabetic and diabetic subjects. A, effect of insulin on cells from female patients with type 2 diabetes. B, comparison of the level of insulin-induced phosphorylation of serine 307 in cells from non-diabetic control subjects. C, comparison of the level of insulin-induced phosphorylation of serine 307 in cells from female non-diabetic and diabetic subjects. D, effect of insulin on cells from male patients with type 2 diabetes. E, comparison of the amount of IRS1 protein in cells from the female non-diabetic and diabetic subjects. The amount of IRS1 protein was determined by immunoblotting and normalized for the amount of actin. In the comparisons all samples were analyzed from the female non-diabetic and diabetic subjects. All samples were analyzed from the female non-diabetic and diabetic subjects.

Phosphorylation of Serine 307 in Cells from Diabetics Makes Cells Insulin-sensitive—The phosphoserine/threonine protein phosphatase inhibitor okadaic acid completely inhibits protein phosphatase-2A at 1 nM and protein phosphatase-1 within 1 min (Fig. 3C). This is considerably more insulin-sensitive than the insulin receptor autophosphorylation (EC50 = 1–2 nM) (3) indicating a downstream effect involving enzymatic signal amplification by e.g. additional protein kinases; see Ref. 3. Time course studies in cells from non-diabetic control subjects demonstrated a half-maximal phosphorylation of serine 307 after about 2 min (Fig. 3B), which was slower than the insulin-induced tyrosine phosphorylation of IRS1, exhibiting half-maximal effect already within 1 min (Fig. 3C).

Phosphorylation of Serine 307 in Cells from Diabetics Makes Cells Insulin-sensitive—The phosphoserine/threonine protein phosphatase inhibitor okadaic acid completely inhibits protein phosphatase-2A at 1 nM and protein phosphatase-1 at 1 μM concentrations in intact cells, including adipocytes (29). Okadaic acid enhanced the phosphorylation of serine 307 in adipocytes from control subjects and also caused a general increase in the extent of serine/threonine phosphorylation of IRS1 as indicated by a shift in its gel mobility (data not shown). In adipocytes from patients with type 2 diabetes okadaic acid enhanced the phosphorylation of serine 307 on average 1.7 ± 0.4-fold (mean ± S.E., n = 4) and at the same time enhanced the sensitivity to insulin for tyrosine-specific phosphorylation of IRS1 (Fig. 4). It is interesting that okadaic acid has been reported to enhance

FIGURE 1. Insulin-induced phosphorylation of IRS1 serine 307 in primary adipocytes from non-diabetic subjects and from patients with type 2 diabetes. Abdominal subcutaneous adipose tissue was obtained during elective surgery in seven consecutively recruited non-diabetic Caucasian female control subjects (62 ± 3 years; BMI: 35 ± 3 kg/m², HbA1c: 6.2 ± 0.6%) or during local anesthesia with xylocain and needle biopsy through the skin from five Caucasian males with type 2 diabetes (56 ± 5 years; BMI: 36 ± 2.5 kg/m², HbA1c: 6.2 ± 0.6%). Isolated adipocytes were incubated with or without 100 nM insulin for 10 min, then cells were separated from medium, lysed in SDS and protein kinase, protein phosphatase, and proteinase inhibitors, and frozen within 10 s (3). IRS1 serine 307 phosphorylation was determined by immunoblotting with site-specific antibodies and chemiluminescence imaging and normalized for the amount of IRS1 protein in each sample (3). A, effect of insulin on cells from female patients with type 2 diabetes. B, comparison of the level of insulin-induced phosphorylation of serine 307 in cells from female non-diabetic and diabetic subjects. C, comparison of the level of insulin-induced phosphorylation of serine 307 in cells from male patients with type 2 diabetes. D, effect of insulin on cells from male patients with type 2 diabetes. E, comparison of the amount of IRS1 protein in cells from the female non-diabetic and diabetic subjects. The amount of IRS1 protein was determined by immunoblotting and normalized for the amount of actin.

In the comparisons all samples were analyzed from the female non-diabetic and diabetic subjects.
insulin-induced glucose uptake by human adipocytes from patients with type 2 diabetes (30, 31).

Inhibition of Serine 307 Phosphorylation in Cells from Control Subjects Makes Cells Insulin-resistant—Rapamycin has been reported to inhibit insulin-induced phosphorylation of the corresponding residue (serine 302) in mouse 32D myeloid cells and mouse embryo fibroblasts (26, 27). In the human adipocytes from non-diabetic subjects in which insulin increased the phosphorylation of serine 307, this increase was inhibited by rapamycin (Fig. 5A). Importantly, rapamycin inhibition of phosphorylation at serine 307 impeded insulin-induced phosphorylation of IRS1 on tyrosine residues, resulting in decreased insulin sensitivity (Fig. 5B) without affecting the maximal response (data not shown). Thus rapamycin inhibited serine 307 phosphorylation of IRS1 and concomitantly transformed the cells into a diabetes-like phenotype (3).

Rapamycin has been found to enhance insulin stimulation of glucose uptake in adipocytes (32, 33), demonstrating other effects of rapamycin downstream of IRS1 (34), which makes it impossible to extend the analysis of rapamycin effects on serine 307 phosphorylation in human adipocytes to effects on glucose transport (Fig. 5C).
tyrosine phosphorylation in response to insulin (26). This mechanism is clearly different from the acute insulin-induced phosphorylation of serine 307 in human adipocytes, which sensitizes IRS1 to activation by the insulin receptor. It should be stressed that earlier experiments described for phosphorylation of the cluster of serines 265, 302, 325, and 358 (murine sequence) in IRS1 of CHO-T cells (36). This is a first demonstration of a critical function of serine phosphorylation controlled by insulin and the nature of the interference in type 2 diabetes. The inability of insulin to enhance the phosphorylation of serine 307 may be a distinguishing feature of insulin resistance and predict type 2 diabetes. The protein kinase(s) and the corresponding protein phosphatase(s) involved in controlling serine 307 phosphorylation are potential targets for novel pharmaceutical intervention.

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