Enhanced insulin signaling in human skeletal muscle and adipose tissue following gastric bypass surgery

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Albers PH, Bojesen-Møller KN, Dirksen C, Serup AK, Kristensen DE, Frystyk J, Clausen TR, Kiens B, Richter EA, Madsbad S, Wojtaszewski JF. Enhanced insulin signaling in human skeletal muscle and adipose tissue following gastric bypass surgery. Am J Physiol Regul Integr Comp Physiol 309: R510–R524, 2015. First published June 10, 2015; doi:10.1152/ajpregu.00228.2014.—Roux-en-Y gastric bypass (RYGB) leads to increased peripheral insulin sensitivity. The aim of this study was to investigate the effect of RYGB on expression and regulation of proteins involved in regulation of peripheral glucose metabolism. Skeletal muscle and adipose tissue biopsies from glucose-tolerant and type 2 diabetic subjects at fasting and during a hyperinsulinemic-euglycemic clamp before as well as 1 wk and 3 and 12 mo after RYGB were analyzed for relevant insulin effector proteins/signaling components. Improvement in peripheral insulin sensitivity mainly occurred at 12 mo postsurgery when major weight loss was evident and occurred concomitantly with alterations in plasma adiponectin and in protein expression/signaling in peripheral tissues. In skeletal muscle, protein expression of GLUT4, phosphorylated levels of TBC1D4, as well as insulin-induced changes in phosphorylation of Akt and glycogen synthase activity were enhanced 12 mo postsurgery. In adipose tissue, protein expression of GLUT4, Akt2, TBC1D4, and acetyl-CoA carboxylase (ACC), phosphorylated levels of AMP-activated protein kinase and ACC, as well as insulin-induced changes in phosphorylation of Akt and glycogen synthase activity were enhanced 12 mo postsurgery. Adipose tissue from glucose-tolerant subjects was the most responsive to RYGB compared with type 2 diabetic patients, whereas changes in skeletal muscle were largely similar in these two groups. In conclusion, an improved molecular insulin-sensitive phenotype of skeletal muscle and adipose tissue appears to contribute to the improved whole body insulin action following a substantial weight loss after RYGB.

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ROUX-EN-Y GASTRIC BYPASS (RYGB) surgery induces weight loss and a high clinical remission rate of type 2 diabetes (8). In fact, RYGB is reported to be superior compared with conventional treatments of type 2 diabetes (16). Altered gut hormone secretion resulting in improved β-cell function in combination with enhanced hepatic insulin sensitivity contributes to an improved glycemic control already within days after surgery (15). In contrast, peripheral insulin sensitivity is unchanged (5, 10, 11, 17, 29, 38) or slightly increased (46) at 1–4 wk after RYGB, while marked improvements are seen after 3–12 mo (5, 7, 10, 11, 20). This suggests that improved peripheral insulin sensitivity is important for sustained remission of type 2 diabetes, but likely less important for the immediate improvement in glucose tolerance observed already days after RYGB.

Decreased peripheral insulin sensitivity is a common phenotypic characteristic of both obesity and type 2 diabetes (14) and has been attributed to impairment in insulin-stimulated glucose uptake/storage primarily in skeletal muscle (61). The adipokine, adiponectin, has been linked to insulin sensitivity in various populations (24, 50, 59), and weight loss-induced improvement in insulin sensitivity is associated to increased plasma levels of adiponectin (9, 28, 51). Furthermore, alterations in adipose tissue glucose handling can affect whole body insulin sensitivity in mice (1), and it is plausible that adipose tissue in morbidly obese subjects [body mass index (BMI) > 40 kg/m2] contributes significantly to the overall peripheral insulin-stimulated glucose clearance (40, 44).

Diet-induced weight loss of 7–20% of initial body weight over a period of 11–17 wk has been shown to increase peripheral insulin sensitivity in most (22, 30, 33, 48, 56), but not all (42) studies. In mice, AMP-activated protein kinase (AMPK) has been implicated in the improved insulin-stimulated glucose uptake in skeletal muscle following caloric restriction (58). Further, enhanced AMPK signaling has been reported in human skeletal muscle 6 mo after RYGB (26), implying a role of AMPK in postsurgery changes in peripheral insulin sensitivity. In line with this observation, Gauthier et al. (21) found improved AMPK signaling in adipose tissue of morbidly obese insulin-sensitive subjects compared with weight-matched, insulin-resistant subjects. Whether enhanced peripheral insulin sensitivity following RYGB is associated with altered AMPK signaling in adipose tissue is unknown.

Improved peripheral insulin sensitivity after RYGB could be mediated by enhanced insulin signaling in target tissue. In rats, RYGB has been shown to increase levels of the insulin receptor substrate 1 (IRS1) and membrane-bound GLUT4 in adipose tissue and skeletal muscle (6, 37). This suggests an improved insulin signaling and hence glucose handling in these tissues. In humans, an increased protein level of the insulin receptor (41) but not the membrane-bound fraction of GLUT4 (18) was detected in skeletal muscle 1 yr after RYGB. In a cross-sectional study, elevated ex vivo skeletal muscle glucose transport was associated with lesser inhibitory serine phosphorylation of IRS1, 1 yr after RYGB, compared with a BMI-matched group (4). Also, following caloric restriction for 11–17 wk...
resulting in body mass decreases of 7–20%, an elevated insulin signaling through Akt and the Akt-substrate TBC1D4 has been reported in human skeletal muscle (30, 33). The ability of insulin to engage these insulin-signaling elements is decreased in skeletal muscle from type 2 diabetic patients (31, 53) as is the activity of the major regulator of glycogen synthesis, glycogen synthase (GS) (13, 25, 54).

We hypothesized that the improved whole body insulin sensitivity seen after RYGB surgery is associated with improved molecular insulin sensitivity to regulate cellular metabolism in primary insulin-targeted tissues—skeletal muscle and adipose tissue. Thus whole body and molecular insulin sensitivity was examined prior to RYGB surgery, and after surgery during conditions where peripheral insulin sensitivity was unchanged (1 wk), partly enhanced (3 mo), and significantly improved (12 mo) in obese glucose-tolerant and type 2 diabetic subjects (5). To illuminate potential differences in the response to RYGB surgery this study was performed in a group of obese glucose-tolerant as well as obese type 2 diabetic subjects.

MATERIALS AND METHODS

Study Design

Ten obese normal glucose-tolerant subjects (NGT group, 7 women/3 men, age 40.1 ± 2.8 yr) and ten obese type 2 diabetic patients (T2D group, 6 women/4 men, age 43.6 ± 3.4 yr, diabetes duration 3.3 ± 1.0 yr) scheduled for laparoscopic RYGB at Hvidovre Hospital (Denmark) were examined after a mandatory diet-induced weight loss of minimum 8% (weight loss was 9.2 ± 1.2% presurgery) and 1 wk, 3 mo, and 12 mo postsurgery. The majority of this preoperative weight loss was achieved >3 mo prior to surgery. Diabetes was controlled with diet alone (n = 2), metformin alone (n = 4) or combined with liraglutide (n = 2) or NPH insulin (n = 2). The preoperative diet did not influence the treatment modality substantially, although insulin dose was reduced. Detailed description of the study design and subject characteristics has been reported elsewhere (5). In short, all studies were performed after an overnight fast and after ≥3 days discontinuation of antidiabetic medication. During a 2-h resting period and again immediately after a 4-h hyperinsulinemie-euglycemic clamp (40 mU·m⁻²·min⁻¹), Insulin, Actrapid, Novo Nordisk, Denmark) was injected. Blood samples were drawn in EDTA and plasma was collected. Direct calorimetry was performed using an open-circuit indirect calorimeter (Deltatrac II Metabolic Monitor, Datex-Ohmeda). Plasma adiponectin was determined by a time-resolved immunofluorometric assay (TRIFMA) based on commercial reagents from R&D Systems (Abingdon, UK) as previously described (19). The assay has been thoroughly validated, and it recognizes all three major forms of plasma adiponectin, i.e., low-, middle-, and high-molecular weight adiponectin (2). All samples were analyzed in duplicate, with a detection limit of 1.5 μg/l and intra-assay and interassay coefficients of variation averaging less than 5% and 7%, respectively.

Clinical experiments were approved by the Ethics Committee of Copenhagen and complied with the ethical guidelines of the Declaration of Helsinki II 2000. Written informed consent was obtained from all participants prior to entering the study. The clinical study is registered at www.ClinicalTrials.gov (NCT01202526).

Muscle and Fat Tissue Preparation

Before homogenization, muscles were freeze-dried and dissected free of visible blood, connective tissue, and fat. Homogenization of skeletal muscle and adipose tissue biopsies was carried out as described previously (34, 52). Lysates were prepared from homogenates by centrifuging 20 min at 16,000 g at 4°C. Total homogenate (muscle only) and lysate protein content were determined in triplicates by the bicinchoninic acid method using BSA as a standard.

Muscle Glycogen and Glycogen Synthase Activity

Muscle glycogen content and glycogen synthase (GS) activity were measured in muscle homogenates as previously described (53). GS activity was measured in the presence of 0.02, 0.17, and 8 mM glucose-6-phosphate (G6P; allosteric activator of glycogen synthase). GS activities are reported as total activity (8 mM G6P), % independent activity (%I-Form; 0.02/8 mM G6P × 100) and % fractional velocity (%FV; 0.17/8 mM G6P × 100). For details, see Ref. 53.

Immunoblotting

For determination of total and phosphorylated protein levels equal amounts of tissue lysate protein solubilized in Laemmli sample buffer were separated by SDS-PAGE with suitable (5–8%) self-cast Tris-HCl gels and transferred (semidy) to PVDF-membranes after which membranes were blocked in TBS with 0.05% Tween 20 and 2% skimmed milk for 45 min at room temperature (RT). Membranes were incubated with primary antibody overnight at 4°C followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibody (1 h, RT) (Jackson ImmunoResearch). Primary antibodies used were from Cell Signaling Technology: Integrinase II (no. 2867), TBC1D4Ser318 (no. 8619), TBC1D4Ser588 (no. 8730), AktThr308 (no. 9275), AktSer473 (no. 9271), Akt2 (no. 3063), AMPKThr172 (no. 2531), AMPKSer485 (no. 2537), and ACCSer221 (no. 3661); Thermo Scientific (Pierce, IL): GLUT4 (no. PAI-1065); Millipore: TBC1D4 (no. 07–741); Symansis (New Zealand): TBC1D4Thr642 (no. 3028-P1); Santa Cruz Biotechnology (Santa Cruz, CA): insulin receptor β (no. SC-711); Molecular Probes, Invitrogen: complex II (no. A11142); DAKO: acetyl-CoA carboxylase (ACC) (streptavidin, no. P0397); kindly donated by L. J. Goodyear (Joslin Diabetes Center and Harvard Medical School, Boston, MA): TBC1D4Ser704; and kindly donated by G. Hardie (Univ. of Dundee, Dundee, UK).

Table 1. Number of tissue biopsies at various time points

<table>
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<tr>
<th></th>
<th>NGT</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>1 wk</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Muscle biopsies (basal/insulin)</td>
<td>9/8</td>
<td>7/6</td>
</tr>
<tr>
<td>Adipose tissue biopsies (basal/insulin)</td>
<td>10/10</td>
<td>8/8</td>
</tr>
</tbody>
</table>

Values are number of subjects having muscle and adipose tissue biopsies taken during basal or insulin (clamp) conditions throughout the different time points in the two groups. Few subjects had postoperative complications (n = 4). A presurgery basal fat biopsy from one patient was lost, but a surgical biopsy from the same abdominal area was taken a few days later during the surgical Roux-en-Y gastric bypass (RYGB) procedure. NGT, group of normal glucose-tolerant subjects; T2D, group of patients with type 2 diabetes.
AMPKα1 and -α2. Bands were visualized using a ChemiDoc MP Imaging System (Bio-Rad) and enhanced chemiluminescence. Band densitometry was performed using Image Lab (version 4.0). On each gel an internal control was loaded to minimize assay variation and on each membrane a standard curve loading different amount of protein was performed to ensure that the quantification was within the linear range for each particular protein probed for. Membranes were reprobed with an alternate antibody after incubating the membrane for 1 h at 58°C in stripping buffer [63 mM Tris·HCl (pH 6.7), 2% SDS, 0.8% β-mercaptoethanol].

Calculations and Statistical Analyses

Rate of glucose disappearance (Rd) and net glucose oxidation rates were calculated from the last 30 min of the basal and clamp period using Steele’s equation (49) and standard in-direct nonprotein calori-metric equations (47), respectively. Nonoxidative glucose metabolism (NOGM) was calculated as the difference between Rd and net glucose oxidation rates.

The statistical model was a two-way ANOVA in a linear mixed model (LMM) always applying individual subjects as random effect but using different factors of interest as fixed effects. This model is an appropriate statistical model when dealing with missing data points (refer to Table 1 for variation in the number of tissue biopsies throughout the study) (35). In this model, the fixed effects correspond to the factors of interest.

For whole body measures and total protein expression (in which basal and insulin-stimulated values were collapsed into one averaged value after applying the linear mixed model to test for differences in each group [fixed effects were clamp and time from surgery; results are reported in the figure legends]); postoperative changes were evaluated using group and time from surgery as fixed effects (LMM1).

For protein phosphorylation and GS activity measures, differences in the response to insulin (basal vs. clamp) and between groups were evaluated by four two-way ANOVAs in linear mixed models with fixed effects for clamp and group (only presurgery values, LMM2), and in both groups at 12 mo (both groups, P < 0.001) posturgery compared with presurgery (Table 3). Trends were seen for adiponectin levels being lower in the T2D compared with the NGT group both at basal (P = 0.07) and during clamp (P = 0.06) (Table 3). Basal rate of glucose disappearance (Rd) was lowered 1 wk after surgery, but leveled back to presurgery values at 3 and 12 mo posturgery (Table 3, P < 0.05). During the clamp, Rd increased at 3 mo in the T2D group (P < 0.01) and in both groups at 12 mo (P < 0.01) posturgery (Table 3). Net glucose oxidation rates during clamp declined 1 wk after surgery (P < 0.05), but leveled back to presurgery values at 3 mo (Table 3, P > 0.05). At 12 mo both insulin-stimulated glucose oxidation and NOGM were increased above presurgery levels (Table 3, P < 0.05).

Effect of RYGB on Protein Expression and Insulin Signaling in Skeletal Muscle

Significant changes in protein expression and signaling in skeletal muscle and adipose tissue are summarized in Table 4. No statistically significant group differences before surgery were evident for any of the protein expression, protein phosphorylation, or GS activity measurements (P > 0.05).

### RESULTS

#### Subject and Metabolic Characteristics

Body composition and whole body metabolic characteristics following RYGB are presented in Tables 2 and 3. Total body weight, fat-free mass, fat mass, and fasting plasma glucose decreased posturgery, most markedly after 3 and 12 mo (Tables 2 and 3, P < 0.05). Serum insulin at fasting and during the clamp was lowered at all time points posturgery (Table 3, P < 0.05). Plasma adiponectin increased at 3 (only NGT group, P < 0.05) and 12 mo (both groups, P < 0.001) posturgery compared with presurgery (Table 3). Trends were seen for adiponectin levels being lower in the T2D compared with the NGT group both at basal (P = 0.07) and during clamp (P = 0.06) (Table 3). Basal rate of glucose disappearance (Rd) was lowered 1 wk after surgery, but leveled back to presurgery values at 3 and 12 mo posturgery (Table 3, P < 0.05). During the clamp, Rd increased at 3 mo in the T2D group (P < 0.01) and in both groups at 12 mo (P < 0.01) posturgery (Table 3). Net glucose oxidation rates during clamp declined 1 wk after surgery (P < 0.05), but leveled back to presurgery values at 3 mo (Table 3, P > 0.05). At 12 mo both insulin-stimulated glucose oxidation and NOGM were increased above presurgery levels (Table 3, P < 0.05).

### Table 2. Subject characteristics before and after RYGB-induced weight loss

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>NGT</th>
<th>T2D</th>
<th>Mixed Effect Model ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>1 wk</td>
<td>3 mo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MF)*</td>
<td>10 (3/7)</td>
<td>8 (3/5)</td>
<td>10 (3/7)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>116.9 ± 4.9</td>
<td>112.3 ± 6.2</td>
<td>96.6 ± 4.8**</td>
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<tr>
<td>Fat-free mass, kg</td>
<td>64.4 ± 4.1</td>
<td>58.2 ± 3.7**</td>
<td>56.3 ± 3.5**</td>
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<tr>
<td>Fat mass, kg</td>
<td>52.5 ± 2.3</td>
<td>38.4 ± 2.6</td>
<td>26.3 ± 3.2</td>
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<td>Hba1c, %a</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.3 ± 0.1</td>
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<tr>
<td>Hba1c, mmol/mol</td>
<td>3.6 ± 1.1</td>
<td>3.4 ± 1.1</td>
<td>3.4 ± 1.1</td>
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</table>

Values are means ± SE. *Rows in which data have been published previously (5). For clarity, symbols and statistics were performed as described (5). †P < 0.05, ††P < 0.01 for the change from preoperative level within the group (post hoc estimates from mixed-effect model). ‡P < 0.05, ‡‡P < 0.01 for differences between the groups at a given study session (post hoc unpaired t-test). †Rows in which data were analyzed as described in Calculations and Statistical Analyses. Significant findings are reported as *P < 0.05 overall main effect of time from preoperative level.
Expression of insulin receptor, GLUT4, hexokinase II, and complex II as well as glycogen synthase activity. In muscle, a minor increase in protein expression of the insulin receptor was seen at all time points after surgery in both groups (Figs. 1A and 6, P < 0.01). GLUT4 (both groups) and total GS activity (T2D group) were upregulated 12 mo after surgery (Figs. 1B and 6, P < 0.001, and 2A, P < 0.001), whereas hexokinase II expression was unchanged (Figs. 1C and 6, P > 0.05). Also, at 12 mo the ability of insulin to activate glycogen synthase was improved compared with presurgery (Fig. 2C, P < 0.05), concomitantly with enhanced insulin-induced NOG (Table 3, P < 0.01). While net glucose oxidation rate during the clamp was increased 12 mo postsurgery (Table 3, P < 0.05), the mitochondrial oxidative capacity, as measured by the complex II expression, was unchanged following RYG (Figs. 1D and 6, P > 0.05).

Glycogen levels. In skeletal muscle, glycogen levels were unchanged by surgery and unaffected by group (Fig. 2D, P > 0.05). Thus in this study the postoperative changes in muscle protein signaling seem unrelated to glycogen levels.

Akt and TBC1D4 signaling. In skeletal muscle a slight increase was found in protein expression of Akt2 at 3 mo (T2D group, Figs. 3A and 6, P < 0.01) and of TBC1D4 at 1 wk (both groups, Figs. 4A and 6, P < 0.05) compared with presurgery. Regulation of Akt was evaluated by phosphorylation on sites Thr308 and Ser473, both sites being insulin-responsive (Figs. 3, B and C, and 6, P < 0.001). RYG induced no improvement in the response to insulin on p-AktThr308 in the NGT group (P > 0.05). In contrast, a gradual higher response to insulin was evident in the T2D group, which was significantly higher at 12 mo postsurgery compared with presurgery values (P < 0.05). The phosphorylation of Akt site Thr308 was unaffected by surgery (P > 0.05). The phosphorylation of Akt site Ser473 was evaluated in the T2D group (P > 0.05). The increase in p-AktSer473 in the NGT group was evident in the T2D group, which was significantly higher at 12 mo postsurgery compared with presurgery values (P < 0.05). The regulation of p-AktSer473 in the NGT group was unaffected by surgery (P > 0.05).

AMPKα2 signaling. The phosphorylation level of AMPKα2 on site Thr172 (Figs. 5C and 6) and ACC on
Overview of significant RYGB-induced changes in protein measures compared with presurgery levels

Table 4.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Skeletal Muscle</th>
<th>Adipose Tissue</th>
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<tr>
<td></td>
<td>LMM</td>
<td>T2D</td>
</tr>
<tr>
<td></td>
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<td>3 mo</td>
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<td>GLUT4</td>
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<tr>
<td>Insulin-Rβ</td>
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<tr>
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<tr>
<td>Complex II</td>
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</tr>
<tr>
<td>GS activity (%I-Form)</td>
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</tr>
<tr>
<td>GS activity (%FV)</td>
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<td>+</td>
</tr>
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<tr>
<td>Δp-ACCSer221</td>
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</table>

Post surgery changes in protein measures in skeletal muscle (left) and adipose tissue (right) from normal glucose-tolerant subjects (NGT group) and patients with type 2 diabetes (T2D group). The LMM column specifies the statistical model used for particular analysis (for statistical details see Calculations and Statistical Analyses). The overall (NGT+T2D) column indicates when a significant main effect of time but no group differences was observed compared with preoperative levels. Total protein expression is an average of values from basal and insulin-stimulated tissue biopsies. Delta symbols represent the response to insulin (insulin minus basal values). Horizontal arrows indicate no difference at that specific time point compared with presurgery values. Arrows pointing upward/downward indicate an increase/decrease at that specific time point compared with presurgery values. Two arrows indicate a larger change in this group compared with the other group. ND, not determined.

Effect of RYGB on Protein Expression and Insulin Signaling in Adipose Tissue

Expression of insulin receptor, GLUT4, hexokinase II, and complex II. Insulin receptor protein expression increased 12 mo after RYGB in the NGT group only (Figs. 1E and 6, P < 0.001). RYGB increased adipose tissue GLUT4 expression at 12 mo in both groups (Figs. 1F and 6, P < 0.001). At this time point, GLUT4 levels were higher in the NGT group compared with the T2D group (P < 0.05). Hexokinase II protein expression was upregulated at 12 mo in the NGT group only (Figs. 1G and 6, P < 0.05). The mitochondrial oxidative capacity, as measured by the complex II expression, was unaffected by RYGB (Figs. 1H and 6, P > 0.05).

Akt and TBC1D4 signaling. In adipose tissue, Akt2 (Figs. 3D and 6) and TBC1D4 (Figs. 4F and 6) protein expressions were increased 12 mo postsurgery (P < 0.01). Furthermore, insulin-stimulated phosphorylation of Akt on Thr308 and Ser473 (Figs. 3, E and F, and 6) was increased 12 mo postsurgery in the NGT group only (P < 0.001). Consequently, at this time point the insulin-stimulated Akt response was higher in the NGT than the T2D group (P < 0.05). These changes were accompanied by an increased insulin-induced phosphorylation of TBC1D4 on sites Ser318 and Ser388 and increased Thr442 levels 12 mo postsurgery in the NGT group (Figs. 3, G–I, and 6, P < 0.001). The response to insulin on p-TBC1D4Ser588 was also increased after 12 mo in the T2D group (P < 0.001), but the change was less pronounced (P < 0.01) compared with the NGT group.

AMP-activated protein kinase and acetyl-CoA carboxylase signaling. Adipose tissue level of AMPKα1 was unchanged following RYGB (Figs. 5E and 6, P > 0.05), whereas expression of ACC increased in the NGT group 12 mo postsurgery (Fig. 5F, P < 0.001). At 12 mo postsurgery compared with presurgery, increased phosphorylation of AMPK on site Thr722 (NGT group; P < 0.001, Figs. 5G and 6) and Ser385 (NGT group; P < 0.001, Figs. 5I and 6) as well as of ACC on site Ser221 (both groups; P < 0.01, Figs. 5H and 6) was observed. Furthermore, irrespective of time from surgery, insulin decreased the phosphorylation of AMPKThr722 (P < 0.01), ACCSer221 (P < 0.001), and increased phosphorylation of AMPKSer485 (P < 0.05, NGT group).

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Fig. 1. Skeletal muscle and adipose tissue protein expression of GLUT4, insulin receptor, hexokinase II, and complex II following RYGB. Protein levels of insulin receptor (A and E), GLUT4 (B and F), hexokinase II (C and G), and complex II (D and H) in skeletal muscle (A–D) and adipose tissue (E–H) following Roux-en-Y gastric bypass (RYGB) in normal glucose-tolerant (NGT) subjects and type 2 diabetic (T2D) patients. Quantified values are in arbitrary units (AU) with a reference value of 100 in the NGT group preoperatively. Gray bars represent an average of the basal and insulin-stimulated values. No significant main effect of insulin in either group was evident (P > 0.05). Horizontal lines above bars indicate that the main effect of time was significantly different from preoperative levels at this time point. A Tukey post hoc test was used to evaluate significant main effects of time and significant interactions. Data are means ± SE. (†)P = 0.06, ††P < 0.05, †††P < 0.01, ††††P < 0.001 vs. presurgery; §P < 0.05 vs. NGT group.
Correlations Comparing Whole Body and Protein Signaling Measurements

Based on the criteria for applying correlation analyses (described in Calculations and Statistical Analyses), the following associations were significant when correlating changes from presurgery to 12 mo postsurgery: in the NGT/H11001 T2D group, Rd during insulin-stimulated conditions vs. GLUT4 expression in adipose tissue ($r = 0.51, P < 0.05$; Fig. 7A); in the NGT group, basal plasma adiponectin levels vs. Rd during insulin-stimulated conditions ($r = 0.83, P < 0.01$; Fig. 7B) and vs. NOGM ($r = 0.79, P < 0.05$; Fig. 7C) [these two associations were not significant within the T2D group alone or when the two groups were analyzed together ($P > 0.05$)]; in the NGT + T2D group, basal plasma adiponectin levels vs. basal p-AMPKThr172 ($r = 0.56, P < 0.05$; Fig. 7D) and vs. basal p-ACCSer221 ($r = 0.77, P < 0.001$; Fig. 7E) levels in adipose tissue.

Discussion

Insulin resistance is a phenotype of obesity and type 2 diabetes. One possible mediator of this improvement in insulin sensitivity is adiponectin. Adiponectin has been associated with insulin sensitivity in various populations (24, 50, 59), and our observation of increased plasma adiponectin levels after RYG is in line with previous findings (9, 51). Interestingly, we observed an association between increased (presurgery to 12 mo) basal adiponectin levels and Rd clamp, as well as NOGM in the NGT group only. These observations support that changes in adiponectin following RYG independently contribute to increased insulin sensitivity as observed by Swarbrick et al. (51). Our data further suggest normal glucose-tolerant subjects to be more sensitive (Rd and NOGM) to changes in adiponectin compared with type 2 diabetic patients.

On a cellular level, we showed that RYG leads to an increased protein expression of the key glucose transporter, GLUT4. Furthermore, expression of insulin signaling molecules such as the insulin receptor Akt2 and TBC1D4 was increased following surgery in both skeletal muscle and adipose tissue. Also, we demonstrate this to be accompanied by improved insulin-regulated Akt, TBC1D4, and GS signaling in skeletal muscle and Akt and TBC1D4 signaling in adipose tissue. These novel findings were most evident after a significant improvement in insulin sensitivity and a substantial weight loss 12 mo postsurgery. Thus improvements in periph-

Fig. 2. Skeletal muscle glycogen synthase activity and glycogen content following RYG. Activity of glycogen synthase (GS) was measured in skeletal muscle homogenate and expressed as total activity (8 mmol/l glucose-6-phosphate; G6P) (A), percent independent form (%I-Form) (B), percent fractional velocity (%FV) (C). Glycogen content (D) measured in skeletal muscle homogenate was expressed as nmol/mg protein. All measurements were performed in skeletal muscle from NGT subjects and T2D patients undergoing RYG. White and black bars represent basal and insulin-stimulated values, respectively. Horizontal lines above bars indicate that the main effect of time was significantly different from preoperative levels at this time point. Delta symbols represent the difference between insulin-stimulated and basal conditions. A Tukey post hoc test was used to evaluate significant main effects of time. Data are means ± SE. †$P < 0.05$, ††$P < 0.001$ vs. presurgery.
eral glucose disposal rates and peripheral protein expression/insulin-induced signaling are likely most important for sustained regulation of glucose metabolism, but likely less important for the immediate improvement in glucose tolerance observed already days following RYGB (5).

In adipose tissue, GLUT4 protein expression is lowered with obesity and type 2 diabetes (27, 32) and has been found to increase with exercise training (27). Furthermore, adipose tissue-specific knock-out of GLUT4 in mice not only affected glucose metabolism in adipose tissue, but also in skeletal muscle and the
liver (1). Thus our findings of increased GLUT4 content in adipose tissue 12 mo postsurgery and the significant association with $R_d$ could at least in part explain the improved whole body glucose disposal. Furthermore, regulation of Akt and TBC1D4 by insulin was improved in adipose tissue at this time point. Studies investigating the importance of TBC1D4 in adipose tissue found that in primary adipocytes from whole body TBC1D4 knock-out mice the insulin-stimulated glucose uptake was partly blunted (36, 37).

Fig. 4. Effects of RYGB on TBC1D4 protein signaling and expression in skeletal muscle and adipose tissue. Protein expression of TBC1D4 (A and F) and phosphorylation of TBC1D4 on sites Ser$^{318}$ (B and G), Ser$^{588}$ (C and H), Thr$^{642}$ (D and I), and Ser$^{704}$ (E) in skeletal muscle (A–E) and adipose tissue (F–I) following RYGB in NGT subjects and T2D patients. Quantified values are in arbitrary units (AU) with a reference value of 100 in the NGT group preoperatively. White, black, and gray bars represent basal, insulin-stimulated, and the averaged basal + insulin-stimulated values, respectively. For TBC1D4 protein expression (skeletal muscle and adipose tissue), no significant main effect of insulin in either group was evident ($P > 0.05$). Horizontal lines above bars indicate that the main effect of time was significantly different from preoperative levels at this time point. Delta symbols represent the difference between insulin-stimulated and basal conditions. A Tukey post hoc test was used to evaluate significant main effects of time and significant interactions. Data are means $\pm$ SE. *$P < 0.05$, **$P < 0.001$ vs. basal conditions; $†P < 0.05$, $‖P < 0.01$, $‖‖P < 0.001$ vs. presurgery; §§$P < 0.01$ vs. NGT group.
Thus improved signaling through Akt and TBC1D4 in adipose tissue 12 mo postsurgery potentially contributes to the enhanced glucose disposal rate at this time point. The importance of each individual phosphorylation site on TBC1D4 to regulate its own activity is not well described. Our observation of an unchanged p-TBC1D4Thr642 signaling following RYGB supports the notion that this site is of minor importance in regulating adipose tissue glucose uptake (12).

Fig. 5. Effects of RYGB on AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) protein signaling and expression in skeletal muscle and adipose tissue. Protein expression of AMPKα2 (A), AMPKα1 (E), and ACC (B and F) as well as phosphorylation of AMPKThr172 (C and G), ACCSer221 (D and H), and AMPKSer485 (I) in skeletal muscle (A–D) and adipose tissue (E–I) following RYGB of NGT subjects and T2D patients. Quantified values are in arbitrary units (AU) with a reference value of 100 in the NGT group preoperatively. White, black, and gray bars represent basal, insulin-stimulated, and the averaged basal + insulin-stimulated values, respectively. For AMPK and ACC protein expression (skeletal muscle and adipose tissue), no significant main effect of insulin in either group was evident (P > 0.05). Horizontal lines above bars indicate that the main effect of time was significantly different from preoperative levels at this time point. Delta symbols represent the difference between insulin-stimulated and basal conditions. A Tukey post hoc test was used to evaluate significant main effects of time and significant interactions. Data are means ± SE.

†P < 0.05, ††P < 0.01, †††P < 0.001 vs. presurgery.
Interestingly, at 12 mo postsurgery the effect of insulin on regulation of Akt and TBC1D4 as well as GLUT4 expression in adipose tissue was higher in the NGT compared with the T2D group concomitantly with a higher insulin-stimulated glucose disposal rate. Thus, at this time point, these molecules might contribute to the difference observed in whole body glucose uptake during insulin-stimulated conditions between the NGT and T2D group.

In skeletal muscle GLUT4 protein levels were enhanced together with improved regulation by insulin of Akt and TBC1D4 and increased phosphorylated levels of TBC1D4 at 12 mo postsurgery. We speculate that this may lead to a higher plasma membrane-bound fraction of GLUT4, which in turn would contribute to the observed improvement in the glucose disposal rate by insulin. Apparently, these changes may not increase basal glucose uptake, since basal membrane-bound
fraction of GLUT4 was reported to be unaltered following surgery-induced weight loss in human skeletal muscle (18). In line with this observation, we found that basal rate of glucose disposal was unchanged 12 mo after surgery.

Insulin-induced regulation of Akt, TBC1D4, and GS has been shown to be impaired in skeletal muscles from patients with type 2 diabetes (25, 31, 53) and partly restored by exercise training (54) or diet-induced weight loss (30, 33). In agreement, we show improved regulation of these proteins by insulin in skeletal muscle, but seemingly not until a considerable weight loss at 12 mo postsurgery. At this time point, insulin-stimulated phosphorylation of Akt was higher in skeletal muscle (T2D group) consistent with previous findings of Kirk et al. (33) in which insulin-stimulated p-AktSer473 was increased in skeletal muscle following diet-induced weight loss of 11% in obese subjects. Furthermore, it has been shown in mice that improvements in insulin-stimulated glucose uptake following caloric restriction are Akt2 dependent (39). These findings support a role of Akt regulation by insulin in enhancing insulin-induced glucose disposal rate following RYGB.

Improved TBC1D4 regulation by insulin in skeletal muscle could be a mediator of the enhanced peripheral insulin sensitivity following RYGB. Supporting this contention, a similar adaptation of TBC1D4 regulation and insulin sensitivity by exercise training has been shown previously (55). We also found a general increase in skeletal muscle levels of phosphorylated TBC1D4, which could be interpreted as a constitutive lowering of TBC1D4 activity. Our findings are in agreement
with Jazet et al. (30), who found that a 20% diet-induced weight loss was succeeded by higher p-TBC1D4Thr642 levels at basal and insulin-stimulated conditions in skeletal muscle from severely obese subjects.

The activity of GS has been shown to correlate with NOGM during insulin-stimulated conditions (43). In support, we found an improved ability of insulin to stimulate GS activity, concomitantly with an increased NOGM during insulin-stimulated conditions 12 mo postsurgery. Since NOGM primarily reflects glycogen synthesis (47), it is likely that enhanced GS activation contributes to the increase in insulin-stimulated NOGM following RYGB.

AMPK is considered an important energy sensor and reduced activity has been linked to insulin resistance in adipose tissue, whereas its role in skeletal muscle insulin resistance is less clear (45). Holmes et al. (26) reported that RYGB led to a marked increase in AMPK phosphorylation/protein-ratio in skeletal muscle from obese nondiabetic subjects. In contrast, we found increased AMPK signaling in adipose tissue but not in skeletal muscle following RYGB. It could be speculated whether results by Holmes et al. (26) were influenced by fat contamination of their muscle preparation, since no dissection of excess fat from the muscles biopsies was reported. Interestingly, we found a positive association between the increase (presurgery to 12 mo) in plasma adiponectin and basal phosphorylation of AMPK and ACC in adipose tissue, supporting the idea of AMPK signaling being regulated by adiponectin (24, 60). Although speculative, enhanced levels of AMPK signaling in adipose tissue postsurgery could lead to a decreased turnover of acetyl-CoA to malonyl-CoA, thereby decreasing lipogenesis.

Studies of primary rat adipocytes have previously suggested that the lipogenic effect of insulin in part may be dependent on AMPK inhibition and thus activation of the lipogenic enzyme ACC by insulin (3). In the present study, in particular in the NGT group, several aspects of Akt and TBC1D4 signaling suggest that the molecular insulin sensitivity is enhanced in the adipocytes. The changes in insulin signaling occur concurrently with tendencies to enhanced AMPK phosphorylation by insulin at Ser485 ($P = 0.1$). This site is inhibitory for phosphorylation at the activating Thr172 on AMPK (23) and might be involved in the improved ($P = 0.07$) ability for insulin to dephosphorylate AMPK at Thr172. Thus our data provide indications to suggest a scenario in which the increased insulin sensitivity in adipocytes after RYGB leads to a greater AMPK deactivation and ACC activation by insulin, which may improve the lipogenic effect of insulin in human adipose tissue.

Study Limitations

Since most postsurgery improvements were evident only after 12 mo, we suggest that weight loss determines changes in peripheral insulin signaling following RYGB. However, from the present study it is not possible to discriminate between weight loss-induced changes or whether the RYGB procedure per se has specific effects on peripheral glucose metabolism. Although not statistically tested, postsurgery changes in protein expression/regulation by insulin seem most evident in adipose tissue compared with skeletal muscle. We hypothesize that this could be linked to a remarkable decrease in body fat 12 mo postsurgery (means ± SE: NGT −25 ± 3 kg; T2D −19 ± 3 kg), whereas the reduction in fat-free mass (including skeletal muscle) (NGT −8 ± 2 kg; T2D −8 ± 1 kg) was considerably smaller ($P < 0.01$ (by paired t-tests) for both groups).

It is important to note that the majority of the preoperative weight loss (in total 9.2 ± 1.2% of body weight) was achieved >3 mo prior to surgery (5). In fact, in the T2D group mean BMI at 2 mo before surgery and at the time of the preoperative clamp was 38.7 and 38.9, respectively. In the NGT group mean BMI at 2 mo before surgery and at the time of the preoperative clamp was 41.4 and 40.2, respectively. Yet, we cannot exclude that the results presented are potentially influenced by a preoperative weight loss as discussed previously (5). However, the influence on signaling of weight loss (15–17%) at 3 mo postsurgery is minor and limited to a few of the factors measured. Thus the lower presurgery weight loss (∼9%) may be expected to induce even smaller changes in the two cohorts. Moreover, the insulin concentration obtained during the clamp was 15–30% lower at all three time points following RYGB compared with preoperative concentrations, likely as a consequence of increased hepatic insulin clearance (5). The reduced insulin concentration postoperatively possibly lowers the insulin signaling response in peripheral tissues. All together, these limitations likely made it more difficult to detect relevant differences in the present study.

Improved insulin sensitivity is a common feature of exercise training and results could be influenced by a higher rate of leisure time physical activity postsurgery. We did not measure training status before and after surgery, but protein expression of exercise-responsive proteins such as hexokinase II and complex II in skeletal muscle was unaltered following RYGB. This suggests that altered physical activity is likely not a major contributing factor to RYGB-induced changes in the current study.

In conclusion, an improved molecular insulin-sensitive phenotype of skeletal muscle and adipose tissue appears to contribute to the improved whole body insulin action following RYGB. These adaptations are seen in both obese NGT and T2D subjects after a significant weight loss only. Of notice, changes in the adipose tissue, rather than skeletal muscle, are associated with the changes in insulin sensitivity.

Perspectives and Significance

Our data suggest that molecular insulin sensitivity of adipose tissue plays a significant and perhaps a larger role than hitherto thought in regulating whole body insulin sensitivity to stimulate glucose disposal in humans. Although adiponectin is a possible player, the mechanism for an interaction between adipose tissue and other tissues awaits further studies. Our study suggests novel adaptations in the interplay between insulin and AMPK signaling with RYGB, which may improve the lipogenic effect of insulin and contribute to increased whole body insulin action.

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DISCLOSURES
P. H. Albers is an employee at Novo Nordisk A/S and owns stocks in Novo Nordisk A/S. T. R. Clausen is an employee at Novo Nordisk A/S and owns stocks in Novo Nordisk A/S and Zealand Pharma A/S.

AUTHOR CONTRIBUTIONS

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