Upregulation of IRS-1 Expression in Goto-Kakizaki Rats Following Roux-en-Y Gastric Bypass Surgery: Resolution of Type 2 Diabetes?

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Type 2 diabetes mellitus (T2DM) is an endocrine disorder that is rapidly growing in prevalence within China and throughout the world. Roux-en-Y gastric bypass (RYGB) surgery, widely used in the treatment of obesity, has been recognized as an effective and long-term treatment for T2DM in recent years. However, the underlying mechanisms responsible for glycemic control remain unclear. This study was designed to investigate the roles of insulin receptor substrates (IRSs) in glucose tolerance and insulin resistance following RYGB surgery. Goto-Kakizaki (GK) rats, a model of T2DM, were randomly allocated into three groups: RYGB surgery, sham surgery, and control (10 animals/group). Wistar rats were also used as non-diabetic control. Daily food intake, body weight, glucose and insulin were measured pre- and post-operatively. Insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) content, the main subtypes of IRSs, were measured in skeletal muscle, adipose tissue and liver using western immunoblot analyses on postoperative day 28. Following surgery, RYGB-treated rats showed markedly improved oral glucose tolerance, as judged by lower peak and area-under-the-curve glucose values (p < 0.01 vs. GK or GK sham). Improved insulin resistance was also observed in RYGB-treated rats. Western immunoblot analyses showed that IRS-1 and its phosphorylation levels were significantly increased in skeletal muscle and adipose tissues in RYGB group (p < 0.01 vs. GK or GK sham), whereas IRS-2 levels were downregulated in liver. These findings suggest that improvements in glucose tolerance and insulin resistance following RYGB surgery are associated with upregulation of IRS-1.

Keywords: Goto-Kakizaki; insulin receptor substrate 1; insulin receptor substrate 2; Roux-en-Y gastric bypass; type 2 diabetes mellitus


Type 2 diabetes mellitus (T2DM) is the most common endocrine disorder in China, affecting more than 92.4 million adults (Yang et al. 2010). Current management of T2DM is focused on the prevention of disease progression through lifestyle modifications and medical management. Although there have been tremendous advances in the pharmacological treatment of this disease, it remains a poorly controlled endocrine disorder (Turner et al. 1999; Liebl 2002).

Today, Roux-en-Y gastric bypass (RYGB) surgery is recognized as an effective and long-term treatment for T2DM (Liebl 2002; Buchwald et al. 2004; Meneghini 2007). This phenomenon was first discovered in 1980 when Water, Pories and colleagues observed improved glycemic control prior to the start of any significant weight loss following the RYGB procedure (Couzin 2008). They later reported an 83% remission rate in 146 RYGB patients with T2DM over 14 year time period (Pories et al. 1995). These findings have been subsequently confirmed by others with more than an 80% remission rate (Schauer et al. 2003; Buchwald et al. 2004, 2009). Although two hypotheses have been proposed to explain the positive effects of RYGB on T2DM: “hindgut hypothesis” (Pories and Albrecht 2001; Rubino and Maescaux 2004) and “foregut hypothesis” (Cummings et al. 2004; Patriti et al. 2004), the specific mechanisms remain unclear. We therefore attempted to determine potential mechanisms by investigating the changes in key proteins of the insulin signaling pathway following RYGB surgery, including insulin receptor substrates (IRSs).

IRSs are key mediators in insulin signaling and play a central role in maintaining glucose metabolism (Sesti et al. 2001). Insulin initiates metabolic effects by binding to the insulin receptor and activating its intrinsic tyrosine kinase.
This event leads to phosphorylation cascades of IRSs. Activation of these IRSs initiate signaling cascades, leading to the activation of multiple downstream effectors that ultimately transmit the insulin signal to a branching series of intracellular pathways that regulate glucose metabolism (Saltiel and Kahn 2001). To date, four members of this family have been identified, including IRS-1, IRS-2, IRS-3, and IRS-4. Current data indicate that IRS-1 functions primarily in skeletal muscle and adipose tissue, while IRS-2 has been shown to regulate hepatic insulin action as well as pancreatic β cell development and survival (Sun et al. 1991; Schuppin et al. 1998; Brady 2004; Takamoto et al. 2008). By contrast, IRS-3 and IRS-4 appear to play redundant roles in the insulin signaling pathway (Liu et al. 1999; Fantin et al. 2000).

We performed this study to determine expression levels of IRS-1 and IRS-2 in insulin target tissues (liver, skeletal muscle, adipose tissue) in order to better understand the role of IRSs activation in the regulation of glucose metabolism following RYGB surgery. These findings will provide a new theoretical basis for the treatment of T2DM.

Materials and Methods

Animals

Experiments were performed using aged-matched (10 week old) male Goto-Kakizaki (GK) and Wistar rats (SLAC, Ltd., Shanghai, China). All procedures were approved by the Committee for the Humane Use of Animals at China Medical University. Prior to surgery, all animals were individually housed in a climate controlled room with a 12-hour light/dark cycle and fed a standard rodent chow diet and allowed water ad libitum. Thirty GK rats, an inbred, lean model of T2DM derived from the Wistar rat (Goto et al. 1976), were randomly allocated into three groups: RYGB surgery, sham surgery and control (10 animals/group). Wistar rats were also used as non-diabetic control.

Surgical procedures

In GK RYGB group, rats were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). The junction of the esophagus and stomach was transected and the stump of the stomach was sewn closed using 6-0 sutures. The jejunum was divided 10 cm below the ligament of Treitz, an esophageojunostomy was created, and a side-to-side jejunocutaneousstomy was performed 10 cm below the esophageojejunostomy. GK sham operations consisted of a gastrointestinal incision at the same site where gastrostomy and enterotomy were performed in the RYGB surgery. Liquid diet (10% glucose) was provided for the first three days following all surgical procedures, then the rats were returned to regular chow. Additionally, GK and Wistar rats (10 animals each group) were used as the diabetic and non-diabetic control groups (respectively) without any surgical intervention.

Food intake and Body weight

Food intake was determined according to body weight, calculated using the following equation: food intake rate = daily food consumption (g)/rat body weight (kg). Postoperatively, GK, GK sham and Wistar rats were fed with controlled chow according to the individual weight and the average food intake of RYGB rats in order to eliminate the impact of decreased caloric intake. Body weight and food intake were measured daily.

Oral Glucose Tolerance Test (OGTT)

OGTT was performed 1 week before and 2 and 4 weeks after surgery. After 12 to 14 hours of fasting, blood samples were collected from the tail vein at 0, 30, 60, and 120 min following administration of 2 g/kg oral glucose gavage. Glucose levels were assessed using a handheld glucometer (Bayer, Germany). Additional blood samples were spun at 4,000 g for 10 min at 4°C, plasma was removed and stored at −20°C until assaying for insulin concentration using ELISA kit (GBD Ltd., San Diego, CA, USA).

Insulin resistance (IR) analysis

Insulin resistance was quantified using the homeostatic model assessment of insulin resistance (HOMA-IR) using the following formula: HOMA-IR (Wallace et al. 2004) = (G0 × I0) / 22.5, where G0 is the fasting blood glucose level (mmol/l) and I0 is the fast plasma insulin level (mU/l).

Tissue Collection

All rats were sacrificed on postoperative day (POD) 28. The rats were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). Soleus skeletal muscle (right and left), liver and adipose tissue were collected, weighed, and stored at −80°C until further analysis.

Western blot analysis

Whole cell protein was extracted based on the instructions of the lysis buffer (Keygen total protein extraction kit, Keygen Biotech., Nanjing, Jiangsu, China), and assayed quantitatively using the BCA method. Protein extracts derived from each rat were separated using 7.5% SDS-PAGE, transferred to PVDF membranes, and blocked with 10% skim milk in Tris-buffered saline containing Tween 20 (TBS-T). The membranes were incubated at 4°C overnight with rabbit anti-phosphorylated-IRS-1 (Tyr 632) (Santa Cruz, USA; 1:400 dilution), anti-IRS-1 (Santa Cruz, USA; 1:400 dilution) and anti-IRS-2 (Santa Cruz, USA; 1:400 dilution) antibodies. Secondary horseradish peroxidase-conjugated anti-rabbit IgG was used at a dilution of 1:2,000 for 1 hour at room temperature. Protein bands were revealed using the Immun-Star Chemiluminescent Kit according to the manufacturer’s protocol. Films were digitized and bands were quantified using Image J software (National Institute of Mental Health, USA). Detection of phosphorylated-IRS-1 (p-IRS-1) was performed first. After the targeted bands of p-IRS-1 were exposed, PVDF membranes were stripped with eluent (Beyotime Biotech., Haimen, Jiangsu, China). The second hybridism was carried out to obtain IRS-1 or IRS-2 data, respectively. The band intensity ratio (IRS-1, p-IRS-1) was calculated using the trapezoidal rule. All data were expressed as means ± s.d. and analyzed using SPSS 11.5 statistical software (SPSS, Inc., Chicago, IL, USA).Statistically significant differences were determined using one-way analysis of variance. P < 0.05 was recognized as statistically significant.
Results

Body Weight

Following surgery, only one rat from the RYGB group died due to anastomotic leakage. The remaining rats completed the 28-day experiment. There were no significant differences among groups of GK rats in body weight prior to the start of the experiment, and all GK rats weighed less than the same age-matched Wistar rats (Fig. 1). Two weeks following surgery, body weight in the RYGB group decreased significantly compared to the other GK groups (Fig. 1) \((p < 0.01)\).

Glucose Tolerance

To evaluate the influence of RYGB on glucose tolerance, OGTT was performed. Compared to that of the GK groups, preoperative glucose tolerance was higher in the Wistar group following OGTT \((p < 0.01)\) (Fig. 2A, D). Blood glucose in the postoperative RYGB group showed a significant decrease \((p < 0.01\text{ vs. GK or GK sham})\), and had no apparent difference compared with the Wistar group on POD 28 (Fig. 2B, C, D). These results suggest that RYGB efficiently improved glucose tolerance in GK rats.

Insulin

As circulating insulin levels \emph{in vivo} are a reflection of \(\beta\)-cell survival and function, insulin levels were also examined. There were no significant differences in fasting plasma insulin levels among all groups pre- and postoperatively. Interestingly, the postgavage insulin levels in GK groups were significantly lower than those in the Wistar group preoperatively \((p < 0.01)\) (Fig. 3A, D). Although postgavage insulin levels in the RYGB group showed no significant alterations on POD 14, an upward trend was noted (Fig. 3B). Over time, postgavage insulin secretion increased. On POD 28, the postgavage insulin levels in RYGB group were significantly higher than those of the GK or GK-sham group \((p < 0.05)\) (Fig. 3C, D). The above results suggest that the glucose-stimulated insulin response is markedly increased in GK rats following RYGB surgery.

HOMA-IR

To confirm the hypothesis that RYGB improves insulin resistance in GK rats, HOMA-IR values were examined. As shown in Table 1, HOMA-IR values in GK groups were significantly higher than those in the Wistar group preoperatively \((p < 0.05)\). As expected, HOMA-IR values in postoperative RYGB group were significantly lower than those in the preoperative and other GK groups \((p < 0.05)\), and there were no significant differences between the RYGB group and Wistar group on POD 28. These results suggest that RYGB could improve insulin resistance in GK rats.

Expression of IRS-1 and IRS-2

To determine the effects of RYGB on IRSs, we investigated the expression of IRS-1 and p-IRS-1 in insulin target tissues (skeletal muscle, liver and adipose tissue) by western immunoblot analyses. Interestingly, expression of IRS-1/p-IRS-1 was significantly increased in skeletal muscle and adipose tissue following RYGB surgery \((p < 0.01\text{ vs.}\)
Fig. 2. Glucose tolerance.
Blood glucose was measured at 0, 30, 60, and 120 min following administration of 2 g/kg oral glucose gavage in Wistar (n = 10), GK (n = 10), GK-sham (n = 10) and GK-RYGB (n = 9) groups. RYGB-treated group showed markedly improved glucose tolerance post-operatively. \( * p < 0.01 \) compared to GK and GK-sham groups. Data are presented as means ± s.d.
(A) - (C) Blood glucose was respectively measured at 1 week before, 2 weeks and 4 weeks post-surgery.
(D) The area under the curve (AUC) of glucose.

Fig. 3. Insulin levels.
Plasma insulin levels were measured at 0, 30, 60, and 120 min following administration of 2 g/kg oral glucose gavage in Wistar (n = 10), GK (n = 10), GK-sham (n = 10) and GK-RYGB (n = 9) groups. \( * p < 0.01 \) compared to all GK groups; \( ** p < 0.05 \) compared to GK and GK-sham groups. \( \# p < 0.05 \) compared to GK-RYGB group. Data are presented as means ± s.d.
(A) - (C) Insulin levels were measured at 1 week before, 2 weeks and 4 weeks post-surgery.
(D) The area under the curve (AUC) of insulin.
vs. GK or GK sham) (Fig. 4A, B), although there was no difference in liver among any of the groups (Fig. 4C). We also investigated IRS-2 expression in the above tissues. IRS-2 expression in liver was lower in the RYGB group ($p < 0.01$ vs. GK or GK sham), but remained higher than that in the Wistar group ($p < 0.01$) (Fig. 5). However, IRS-2 expression in skeletal muscle and adipose tissue was not different among all the groups.

**Discussion**

The pathogenesis of T2DM is characterized by a combination of peripheral insulin resistance and impaired insulin secretory capacity of pancreatic $\beta$ cells. Initially, in order to compensate for insulin resistance, the pancreas produces more insulin. However, when this hyperinsulinemic response is unable to sufficiently compensate, overt diabetes ensues.

The results of our study confirmed that RYGB is an effective treatment for T2DM (Liebl 2002; Buchwald et al. 2004; Meneghini 2007). Following RYGB, GK rats had an improvement in insulin sensitivity and glucose tolerance; however, GK control and GK-sham rats did not experience similar changes. Since all groups have the same food intake rate, the resolution of T2DM was independent of effects from caloric intake. Our data also demonstrated postagavage insulin levels in the RYGB group increased gradually compared to that in other GK groups. As pancreatic $\beta$ cells are the sole source of insulin production place, we believe that the increased insulin levels in the RYGB group are suggestive of the promotion of $\beta$-cell survival and function.

Since the RYGB surgical procedure has proven effective in improving glucose tolerance and insulin resistance, we aimed to understand potential mechanisms associated with these effects and detect key proteins involved in the regulation of glucose metabolism, such as IRSs. Currently, four members of the IRSs family of proteins have been identified, including IRS-1, IRS-2, IRS-3, and IRS-4. Current data indicate that IRS-1 plays a major role in regulating insulin sensitivity and glucose metabolism in skeletal muscle and adipose tissue. Primary adipocytes from knockout mice lacking IRS-1 (IRS-1$^{−/−}$) show a decrease in glucose transport and glucose transporter type 4 (GLUT4) translocation to the plasma membrane in response to insulin (Tamemoto et al. 1994). IRS-1$^{−/−}$ mice have increased resistance to the glucose-lowering effects of insulin and a marked defect in insulin-stimulated glucose transport in skeletal muscle. The actions of IRS-1 are further clarified by work from Hribal et al. (2000). They demonstrated overexpression of recombinant human IRS-1 in L6 rat myocytes could stimulate glucose transport, glucose transporter translocation, and glycogen synthesis by affecting the activities of phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (PKB) and glycogen synthase kinase-3 (GSK-3) (Hribal et al. 2000).

Following RYGB surgery, our data showed IRS-1 and its phosphorylation levels in skeletal muscle and adipose tissues were significantly increased in GK rats. These changes correlated with a decrease in glucose levels and improvement in insulin resistance (not significantly different from Wistar rats). The fact that IRS-1 is the main docking protein for the binding and activation of PI3-kinase in response to insulin (Rondinone et al. 1997), we suspect the expression changes of IRS-1 may enhance the PI 3-kinase/PKB/GSK-3 signaling pathway, which contributes to the treatment of T2DM.

Furthermore, these findings verify that low expression of IRS-1 in insulin sensitive target tissues (e.g., skeletal muscle and adipose tissue) could be considered a molecular marker of insulin-resistant states such as type 2 diabetes (Goodyear et al. 1995; Yamauchi et al. 1996; Previs et al. 2000). Targeting the upregulation of IRS-1 could increase insulin sensitivity and glucose tolerance, resulting in a treatment instead of management of the disease (Hribal et al. 2000).

We also investigated IRS-2 in insulin target tissues (skeletal muscle, liver and adipose tissue). Our data suggest that the expression levels of IRS-2 in liver were obviously lower in RYGB group than that of the GK and GK-sham groups ($p < 0.01$), yet still higher than the Wistar group ($p < 0.01$). As IRS-2 plays a key role in hepatic insulin signal transduction pathways that mediate the metabolic effects of insulin (Previs et al. 2000; White 2002; Brady 2004; Takamoto et al. 2008). Therefore, we believe the overexpression of hepatic IRS-2 in GK rats may act as a compensatory mechanism for insulin resistance. As insulin resistance improved in the RYGB group, the expression levels of hepatic IRS-2 decreased gradually.

Table 1. Changes in pre- and postoperative HOMA-IR.

<table>
<thead>
<tr>
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<th>Preoperation</th>
<th>14-day postoperation</th>
<th>28-day postoperation</th>
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</thead>
<tbody>
<tr>
<td>Wistar ($n = 10$)</td>
<td>1.97 ± 0.21*</td>
<td>1.68 ± 0.23**</td>
<td>1.91 ± 0.29***</td>
</tr>
<tr>
<td>GK ($n = 10$)</td>
<td>3.55 ± 0.26</td>
<td>3.19 ± 0.41</td>
<td>3.6 ± 0.52</td>
</tr>
<tr>
<td>GK-sham ($n = 10$)</td>
<td>3.58 ± 0.31</td>
<td>3.03 ± 0.32</td>
<td>3.45 ± 0.48</td>
</tr>
<tr>
<td>GK-RYGB ($n = 9$)</td>
<td>3.59 ± 0.29</td>
<td>2.25 ± 0.24**</td>
<td>2.05 ± 0.18***</td>
</tr>
</tbody>
</table>

HOMA-IR, homeostatic model assessment of insulin resistance.

*p < 0.05 compared to pre-operation GK groups; **, ***p < 0.05 compared to GK and GK-sham groups; $p < 0.05$ compared to preoperative GK-RYGB group. Data are expressed as means ± s.d.
Fig. 4. Expression levels of IRS-1/p-IRS-1 in skeletal muscle, adipose tissue and liver. Shown is a representative immunoblot for IRS-1 and p-IRS-1. Quantitative densitometric analyses of the amount of IRS-1 and p-IRS-1 normalized to β-actin (IRS-1/β-actin; p-IRS-1/β-actin). Data are presented as means ± s.d.

(A) Expression levels of IRS-1/p-IRS-1 in skeletal muscle. *p < 0.01 compared to GK and GK-sham groups. #p < 0.05 compared to the Wistar group.

(B) Expression levels of IRS-1/p-IRS-1 in adipose tissue. *p < 0.01 compared to GK and GK-sham groups.

(C) Expression levels of IRS-1/p-IRS-1 in liver. No differences were found in IRS-1 and p-IRS-1 expression in liver among any of the groups.
In conclusion, our results suggest that RYGB could downregulate plasma glucose levels, improve insulin resistance, and increase postgavage insulin levels in T2DM. Upregulation of IRS-1 in skeletal muscle and adipose tissue is a potential mechanism. Further studies are required to detect other key proteins in insulin signaling pathway, such as PI 3-kinase and GLUT-4. Additional studies at the molecular level should be performed to explore whether expression of IRS-1 or IRS-2 is connected with the changes of gastrointestinal hormones, including glucagon-like peptide-1, peptide YY, or ghrelin.

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Conflict of Interest

All authors declare no conflict of interest.

References


