Improved insulin sensitivity 3 months after RYGB surgery is associated with increased subcutaneous adipose tissue AMPK activity and decreased oxidative stress

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Abstract

Morbidly obese individuals are predisposed to a wide range of disorders including type 2 diabetes, atherosclerotic cardiovascular disease (ASCVD), fatty liver disease, and certain cancers. Remarkably, all of these disorders can be improved or prevented by \textit{Roux-en-Y} gastric bypass (RYGB) surgery. We have reported that decreased AMP-activated protein kinase (AMPK) activity, together with increased oxidative stress and inflammation in adipose tissue, are associated with insulin resistance in morbidly obese bariatric surgery patients. In the present study, we assessed how these parameters are affected by RYGB surgery. Eleven patients (average age of 46 ± 4 years) were studied immediately prior to and 3 months post-operatively. We measured subcutaneous adipose tissue AMPK phosphorylation (Threonine 172, an index of its activation), malonyl-CoA content, protein carbonylation (a marker of oxidative stress), plasma adiponectin, and mRNA expression of several inflammatory cytokines. Following surgery, AMPK activity increased 3.5-fold and oxidative stress decreased by 50% in subcutaneous adipose tissue. In addition, malonyl-CoA levels were reduced by 80%. Furthermore, patients had improvements in their BMI, insulin sensitivity (HOMA), and increased circulating high molecular weight (HMW) adiponectin, as well as decreased fasting plasma insulin levels. In contrast, the expression of inflammatory markers in subcutaneous adipose tissue was unchanged post-operatively, although plasma CRP was diminished by 50%.
Introduction

Roux-en-Y gastric bypass (RYGB) is recognized as one of the most effective clinical interventions to achieve significant and sustainable weight loss in morbidly obese individuals. It has been reported to either cause remission or significantly improve type 2 diabetes and fatty liver disease, and it diminishes mortality from cardiovascular disease and the incidence of certain cancers [1-3]. Despite this, the molecular mechanisms underlying its effects are incompletely understood. Factors such as gut hormones, alterations in gut microbiota, and decreased food intake are thought to be at least partially responsible [4-6].

Initially known as a fuel-sensing enzyme, mounting evidence has demonstrated that AMP-activated protein kinase (AMPK) plays a much greater role in regulating cellular function. Studies in rodents and cultured cells indicate that its activation attenuates inflammation and oxidative stress, and increases mitochondrial biogenesis [7, 8]. Likewise, decreased AMPK has been observed in tissues of obese and insulin resistant rodents [7], and therapy with AMPK activators has been shown to reverse the insulin resistance in these rodents [8]. We have previously demonstrated that decreased subcutaneous and visceral adipose tissue AMPK activity is associated with insulin resistance in morbidly obese bariatric surgery patients [9, 10]. In contrast, increases in inflammatory genes were predominantly observed in visceral fat [9].

In this study, we explored the link between adipose tissue AMPK activity, insulin sensitivity and other parameters in 11 morbidly obese individuals before and 3 months after RYGB. Our data show that post-operatively, subcutaneous adipose tissue AMPK activity is increased as are insulin sensitivity and plasma adiponectin, whereas adipose tissue oxidative stress and the concentration of malonyl-CoA were diminished. In contrast, the mRNA expression of the inflammatory genes IL1β, TNFα, and IL10 in adipose tissue was unchanged despite a decrease in plasma CRP level.
Materials and Methods

Patients

Eleven patients (8 females and 3 males) with a mean age of 46.6 ± 4.3 (X±SEM) years were studied. Five of the 11 patients had type 2 diabetes at baseline. Pre- and post-operative clinical characteristics are listed in Table 1. Insulin sensitivity was assessed by homeostasis model of assessment (HOMA) with a value > 2.3 considered insulin resistant and a value < 2.3 insulin sensitive [11]. The study was approved by the Boston University Medical Center Institutional Review Board. All participants were candidates for laparoscopic RYGB and had signed informed consent forms prior to their enrollment.

Blood measurements were carried out after an overnight fast. With the exception of adiponectin, all analyses were performed by the Boston Medical Center clinical chemistry laboratory. Plasma adiponectin was measured using a commercial human high molecular weight (HMW) ELISA kit (R&D Systems, Minneapolis, MN). In humans, HMW adiponectin levels are known to reflect insulin sensitivity more accurately than do levels of low or medium molecular weight adiponectin [12].

Biopsies of abdominal subcutaneous adipose tissue (~0.5 g) were obtained either at the time of surgery (‘baseline’ group), or under local anesthesia during a study visit 3 months after the RYGB surgery (‘post-operative’ group). The tissues were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Western blot analyses

Total proteins were isolated from subcutaneous fat and their concentrations were determined using the bicinechonic acid assay (Thermo Scientific, Rockford, IL). Fifteen micrograms of protein were separated by gel electrophoresis, transferred to a PVDF membrane (Millipore, Billerica, MA), and then incubated with primary antibodies against phospho-AMPK (Thr172), total AMPK (Cell Signaling Technology), using HSP90 (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control. Proteins were visualized by enhanced chemiluminescence (Thermo Scientific), and quantified with Scion Image Software (NIH).

RNA isolation and real-time quantitative PCR

Adipose tissue was homogenized in TRIzol (Invitrogen, Frederick MD). Total RNA was extracted using the RNeasy lipid tissue mini kit (Qiagen, Valencia, CA), reverse transcribed into cDNA (Invitrogen). Real-time quantitative PCR was performed as described previously [9].

Protein carbonylation assay

Protein carbonylation was determined with an OxyBlot protein oxidation detection kit (Millipore) to provide a measure of oxidative stress, as described previously [9]. In brief, 10 µg of protein lysate was derivatized with 4-dinitrophenylhydrazine according to the manufacturer’s instructions. Total carbonylation was visualized by enhanced
chemiluminescence (Thermo Scientific), and the bands quantified with a Scion Image Software.

Malonyl CoA assay

Human subcutaneous adipose tissue was homogenized in 6% perchloric acid and then centrifuged at 14,000g for 10 min at 4°C. After that, the supernatant was neutralized with 2M KOH, 0.4 M KCl, to pH 7.0 and centrifuged at 14,000g for 10 min, 4°C. The resultant supernatant was subjected to malonyl-CoA assay, using the method by McGarry et al. [13]

Statistical analysis

Data are expressed as means ± SE. All baseline and post-operative values were compared using the Wilcoxon matched pairs test. Spearman correlation analysis was used when appropriate. Minimal level of significance was set at $p < 0.05$. GraphPad Prism software (La Jolla, CA) was used for all analyses.
Results

**Patient characterization.** The clinical characteristics of the patients at baseline and 3 months after RYGB surgery are shown in **Table 1.** Post-operatively, there were significant reductions in body weight, BMI, waist circumference, hip circumference, and fasting plasma insulin. Moreover, patients uniformly showed an improvement in insulin sensitivity based on HOMA evaluation, and a decrease in CRP. Fasting plasma glucose, HbA1c levels showed downward trends post-operatively but the differences did not reach statistical significance. Circulating HMW adiponectin was increased post-operatively. Of the 5 type 2 diabetic patients, 3 were free of diabetes 3 months post-operatively. The two patients whose diabetes was not resolved by RYGB had higher HbA1c levels pre-operatively, and a longer duration of type 2 diabetes. Also, preoperatively they were on diabetic medications other than metformin. Gender did not appear to be a factor that predicted outcome.

**Subcutaneous adipose tissue AMPK phosphorylation is uniformly improved post-operatively.** Western blot analysis was carried out to assess AMPK phosphorylation at threonine 172, an indicator of its activity [14]. As shown in **Figure 1A,** the abundance of phospho-AMPK post-operatively was ~3.5 fold higher than pre-operatively. As shown in **Supplement Table 1,** AMPK phosphorylation was increased in every subject, regardless of baseline insulin sensitivity. Since AMPK can be activated by adiponectin [15], we carried out correlation analysis to determine whether the improvement in AMPK and that of adiponectin (Table 1) were related to each other. However, such correlation was not statistically significant (**Figure 1B**).

**Malonyl-CoA and oxidative stress** Malonyl-CoA is both an intermediate in the *de novo* synthesis of long-chain fatty acids and an inhibitor of fatty acid oxidation [16]. AMPK suppresses malonyl-CoA production by phosphorylating acetyl-CoA carboxylase (ACC) [16]. In keeping with the post-operative increase in AMPK phosphorylation, we found a significantly decreased malonyl-CoA level in the post-operative compared to the baseline group (**Figure 1C**). Protein carbonylation was measured as an index of oxidative stress. As shown in Figure 2, it was significantly diminished post-operatively.

**Expression of inflammatory genes did not change in subcutaneous adipose tissue following RYGB surgery.** Results from real-time PCR indicate that the mRNA levels of pro-inflammatory cytokines TNFα and IL-1β did not differ between the baseline and post-operative groups (data not shown). The mRNA level of the anti-inflammatory cytokine IL-10 was moderately higher post-operatively (data not shown); however, the difference was not statistically significant.
Discussion

The overall objective of this study was to investigate whether there is a link between subcutaneous adipose tissue AMPK phosphorylation/activity and insulin sensitivity in a well-characterized yet small (n = 11) group of RYGB patients. We demonstrated that 3 months post-operatively, there is a substantial improvement in the patients’ metabolic profile as assessed by changes in body weight, BMI, circulating HMW adiponectin, insulin sensitivity, and increased adipose tissue AMPK phosphorylation/activation. In addition, we found decreases in oxidative stress and malonyl-CoA level in subcutaneous adipose tissue of the post-operative group. In contrast, the mRNA levels of several inflammatory genes did not change, despite a decrease in circulating CRP levels.

AMPK is an energy sensor that restores cellular energy homeostasis. Recent developments have shown that AMPK is a probable target of major antidiabetic drugs such as metformin and TZDs [7, 8]. At molecular level, evidence accumulated from cell culture and animal studies has demonstrated a role for AMPK in reversing adverse events such as oxidative stress, inflammation, and insulin resistance [8, 17, 18]. We previously showed that diminished subcutaneous and visceral adipose tissue AMPK activity is associated with insulin resistance in RYGB patients [9, 10]. In addition, Kola’s group reported decreased AMPK activity in visceral adipose tissue of patients with Cushing syndrome, many of whom are insulin resistant [19]. Findings from the present study indicate an association between improved AMPK activity and insulin sensitivity in morbidly obese individuals following RYGB weight loss surgery. To the best of our knowledge, this is the first human study that links increased adipose tissue AMPK to improvement in insulin sensitivity following RYGB surgery. Intriguingly, increased AMPK phosphorylation was observed in every patient studied, including three individuals who were identified as ‘insulin sensitive’ at baseline (Supplement Table 1). Studies with a larger number of patients will be needed to determine whether the responses to RYGB surgery are qualitatively different in patients classified as insulin sensitive and resistant.

The decrease in malonyl-CoA content in adipose tissue post-operatively is most likely caused by the activation of AMPK upstream, as AMPK phosphorylates and inhibits ACC, a rate-limiting enzyme for malonyl-CoA synthesis. To the best of our knowledge, this is the first study in which malonyl-CoA has been measured in human fat.

As for mechanism responsible for the elevated AMPK activity post-operatively, adiponectin is known to activate AMPK [15] and its plasma level increased significantly after RYGB (Table 1). Since adiponectin is produced by adipocytes exclusively, its increase could reflect a general improvement in fat cell function post-operatively. However, we did not find a significant correlation between changes in AMPK and adiponectin; the sample-to-sample variation was quite large (Figure 1B). Future studies with more participants are necessary. Other possible candidates include substantial weight loss, as AMPK is activated by energy deficit [7]. Although not
measured in this study, GLP-1 is known to increase significantly after RYGB surgery [20], and it can activate AMPK at least in endothelial cells [21].

Bariatric surgery has been demonstrated to attenuate markers of oxidative stress in liver and plasma [22, 23]. The results of the present study indicate that it has a similar effect in subcutaneous adipose tissue. Whether such changes occur in visceral adipose tissue warrants exploration. Although AMPK activation can diminish oxidative stress, oxidative stress can also suppress AMPK activity [18]. Thus, our data cannot discern whether the decrease in oxidative stress is a consequence or cause of the increased AMPK activity observed post-operatively.

Although we did not find any change in a small number of inflammatory genes, the circulating level of CRP decreased post-operatively. It is conceivable that disappearance of tissue inflammation is a slower process. However, a more likely explanation is that inflammation is more prominent in visceral than subcutaneous fat [18], and it is a change in the latter, and possibly the liver that led to the decreased CRP.

Finally, although bariatric surgery is associated with a durable remission of type 2 diabetes, about one third of the patients experience a relapse within 5 years [24]. Thus, measurements of AMPK in post-operative adipose tissue biopsies might provide insights as to why such remissions and relapses occur; and what can be done to prevent the latter. For instance, if decreased AMPK activity is found to reoccur in adipose tissue, agents that could activate AMPK such as metformin, TZDs, and GLP-1 analogs alone or in combination could prove useful.
Acknowledgements

Author Contributions: X.J.X. wrote manuscript, designed and conducted all the experiments as well as data analyses. C.A. and N.R. reviewed/edited manuscript. D.H., B.C., and C.A. did the adipose tissue biopsies. The authors would like to thank Naveed Ghani and Dr. Asish Saha for their technical assistance with the malonyl-CoA measurements. Work is supported by NIH R01 DK19514 (to N.R.). Dr. X.Julia Xu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors have no conflict of interest to report.
Figure legends:

**Figure 1.** A. Comparison of AMPK phosphorylation (Thr\(^{172}\)), a marker of its activation in the subcutaneous fat of 11 pair-matched pre- and post-bariatric surgery patients (**p < 0.001 compared to baseline group). B. Spearman correlation analysis of changes in adipose tissue AMPK phosphorylation and circulating adiponectin before and after RYGB. C. Comparison of malonyl-CoA levels in the subcutaneous adipose tissue in baseline and post-operative groups.

**Figure 2.** Comparison of the protein carbonylation (a measure of oxidative stress) in the subcutaneous fat of 11 pair-matched pre and post-bariatric surgery patients (*p < 0.05 compared to baseline group).

**Table 1.** Clinical changes in 11 morbidly obese patients before and 3 months after RYGB surgery.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Post-RYGB</th>
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<tbody>
<tr>
<td>HOMA</td>
<td>5.8 ± 1.4</td>
<td>2.6 ± 0.6</td>
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<tr>
<td>Weight, kg</td>
<td>117.9 ± 6.0</td>
<td>96.1 ± 3.8</td>
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<tr>
<td>BMI, kg/m(^2)</td>
<td>41.7 ± 1.4</td>
<td>33.9 ± 1.1</td>
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<tr>
<td>Waist circumference (cm)</td>
<td>125.7 ± 3.9</td>
<td>107.5 ± 3.4</td>
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<tr>
<td>Hip circumference (cm)</td>
<td>130.7 ± 4.3</td>
<td>115.3 ± 2.3</td>
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<tr>
<td>Plasma insulin (µIU/mL)</td>
<td>20.1 ± 4.5</td>
<td>9.6 ± 1.6</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>118.9 ± 15</td>
<td>99.9 ± 8.2</td>
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<tr>
<td>HbA1c (%)</td>
<td>6.2 ± 0.5</td>
<td>5.8 ± 0.4</td>
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<tr>
<td>HbA1c (mmol/mol)</td>
<td>44 ± 5.5</td>
<td>40 ± 4.4</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>5.4 ± 1.7</td>
<td>2.7 ± 0.7</td>
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<tr>
<td>HMW adiponectin (ng/mL)</td>
<td>5605 ± 1592</td>
<td>7538 ± 2036</td>
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<td>Type 2 diabetes</td>
<td>5</td>
<td>2</td>
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<tr>
<td>Metformin user</td>
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</table>
Data are means ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the baseline group. No significant changes were observed in plasma total LDL, HDL, cholesterol, or triglycerides, although triglycerides was decreased from 127 ± 26 to 101 ± 11 mg/dL (data not shown). Abbreviations: Hba1c, glycosylated hemoglobin A1C; hsCRP, high-sensitivity C-reactive protein.


Figure 1

A

B

C

Adiponectin fold change vs AMPK fold change (post/pre-op)

$r = 0.1515$

$p = 0.6821$

Malonyl CoA (pmol/mg tissue)

Baseline  Post-Operative

Diabetes
Figure 2
Supplement Table 1. Individual HOMA value and relative AMPK phosphorylation of the 11 study subjects.

<table>
<thead>
<tr>
<th>Study subject</th>
<th>HOMA (baseline)</th>
<th>HOMA (post-operative)</th>
<th>Fold-change of AMPK phosphorylation (post-operative/baseline)</th>
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