Amelioration of diet-induced diabetes mellitus by removal of visceral fat

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Abstract

The effect of visceral fat removal upon glucose homeostasis, insulin signal transduction, and serum adipokine levels in an animal model of diet-induced obesity and diabetes mellitus (DIO) was evaluated. Swiss mice were initially divided into two groups fed with regular rodent chow or with chow containing 24 g% saturated fat (DIO). DIO mice became obese and overly diabetic after 8 weeks. DIO mice were then divided into three groups: control, sham, and visceral (epididymal and perinephric) fat removal. All groups were submitted to evaluation of basal glucose and insulin levels and i.p. insulin tolerance test. Insulin signal transduction in muscle was evaluated by immunoprecipitation and immunoblot, and serum adipokine levels were determined by ELISA. DIO mice became diabetic (228 versus 115 mg/dl), hyperinsulinemic (7·59 versus 3·15 ng/ml) and insulin resistant (Kitt 2·88 versus 4·97%/min) as compared with control. Visceral fat removal partially reverted all parameters (147 mg/dl glucose; 3·82 ng/ml insulin; and 4·20%/min Kitt). In addition, visceral fat removal completely reversed the impairment of insulin signal transduction through insulin receptor, insulin receptor substrate (IRS)-1, IRS-2 and Akt in muscle. Finally, serum levels of the pro-inflammatory cytokines tumour necrosis factor-α, interleukin (IL)-1β and IL-6 were significantly increased, while adiponectin levels were significantly reduced in DIO mice. After visceral fat removal the levels of adipokines returned to near control levels. The present study shows that removal of visceral fat improves insulin signal transduction and glucose homeostasis in an animal model of diet-induced obesity and diabetes mellitus and these metabolic and molecular outcomes are accompanied by the restoration of adipokine levels.


Introduction

Visceral adiposity is one of the main risk factors for the development of insulin resistance, diabetes mellitus, hypertension, and cardiovascular disease (Ferrannini et al. 1997, Fujimoto et al. 1999). The mechanisms involved in these common clinical associations are not completely known, but include the impaired suppression of hepatic glucose production (O’Shaughnessy et al. 1995), increased portal release of free fatty acids (Björntorp 1990), increased visceral production of glycerol (Williamson et al. 1966), and abnormal production of adipose tissue-derived hormones and cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, leptin, adiponectin, and resistin (Hotamisligil et al. 1996, Steppan et al. 2001).

Recent studies have shown that the removal of visceral fat reverses insulin resistance in two models of obesity (Barzilai et al. 1999, Kim et al. 1999) and with reduced adipose tissue expression of pro-inflammatory cytokines (Gabriely et al. 2002). Acting on insulin sensitive tissues, pro-inflammatory cytokines can impair insulin signal transduction by promoting the serine phosphorylation of key elements of the insulin signaling pathway (Hotamisligil 2003). This inhibitory effect is dependent on the activation of serine kinases, such as JNK and I kappa kinase (IKK) (Hotamisligil 2003, Barreiro et al. 2004). Once serine-phosphorylated, proteins such as the insulin receptor, insulin receptor-substrate (IRS)-1 and IRS-2 can no longer be appropriately tyrosine-phosphorylated in response to an incoming insulin signal. As a result, the activation of downstream effectors of the insulin signaling pathway is hampered (Hotamisligil 2000, 2003).

In a recent study, we characterized an animal model of diet-induced obesity (DIO) that rapidly becomes diabetic (De Souza et al. 2005b). This animal model presents insulin resistance, visceral adiposity, hyperinsulinemia, and impaired insulin signal transduction. As an attempt to further characterize the role played by visceral fat in the development
of diabetes mellitus we decided to employ this animal model to evaluate blood glucose levels, adipokine blood levels, in vitro insulin action and insulin signal transduction in muscle.

Materials and Methods

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad. HEPES, phenylmethylsulfonyl fluoride, aprotonin, dithiothreitol, Triton X-100, Tween 20, glycerol and BSA (fraction V) were from Sigma. 125I-protein A and nitrocellulose paper (BA85, 0.2 μm) were from Amersham. Sodium thiopental and human recombinant insulin (Humulin R) were from Lilly (Indianapolis, IN, USA). The antibodies anti-insulin receptor (IR) (sc-711, rabbit polyclonal), anti-insulin receptor substrate-1 (IRS-1) (sc-559, rabbit polyclonal), anti-insulin receptor substrate-2 (IRS-2) (sc-8299, rabbit polyclonal) and anti-phosphotyrosine (sc-508, mouse monoclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody anti-phospho (Ser 473) Akl (#9271, rabbit polyclonal) was from Cell Signaling Technology (Beverly, MA, USA). The ELISA kits for determination of adipokine levels were from Pierce Biotechnology (Rockford, IL, USA).

Experimental protocols

Male, 4-week-old, inbred Swiss (SW/Uni) mice, originally imported from the Jackson Laboratory and presently bred at the State University of Campinas Breeding Center (CEMIB) were used in all experiments. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996). The animals were maintained on 12 h light:12 h darkness cycles and housed in individual cages. After the acclimatizing period (3 days), the animals were randomly divided into two experimental groups: SW/Uni fed with standard rodent chow (SW/Uni/C) and SW/Uni fed with fat-rich chow (SW/Uni/F). Fat-rich chow was composed of 24 g% protein, 41 g% carbohydrate, and 24 g% fat, whereas standard rodent chow was composed with 19 g% protein, 67 g% carbohydrate, and 4 g% fat (De Souza et al. 2005b). In the first part of the study the animals were evaluated every 4 weeks for biochemical and hormonal parameters in order to determine diet-induced modulation of glucose homeostasis. Blood samples were always collected at 11:00, after 4 h fasting. Diabetes was diagnosed in mice presenting blood glucose levels above 150 mg/dl in at least two distinct measurements. After setting 8 week of fat-rich diet feeding as the time when all SW/Uni/F mice have developed diabetes, a second part of the study was started. At this point the SW/Uni/F mice were randomly divided into three groups: non-operated (SW/Uni/F/NO), which were simply maintained on a fat-rich diet throughout the experimental period; sham-operated (SW/Uni/F/S), which were submitted to a sham procedure and maintained on a fat-rich diet throughout the experimental period; and visceral fat removal (SW/Uni/F/V), which were submitted to visceral fat removal as described below and maintained on a fat-rich diet throughout the experimental period.

Visceral fat removal

SW/Uni/F mice were anesthetized with halothane. After the loss of pedal and corneal reflexes the abdominal cavity was opened, and the epidydymal and perirenal fat pads were removed and weighed (6.1 ± 0.5 g) (SW/Uni/F/V). In the sham operation, the abdominal cavity was opened and fat was mobilized but not removed (SW/Uni/F/S). Operated mice were allowed to recover from surgery and fat-rich chow was reintroduced. Experiments were performed 8 days after the surgical procedure. During the optimization of the methodological approach groups of mice were followed up for 4, 8 and 12 days in order to determine their recovery from surgery and the regain of body mass. Daily food intake and body weight regain were the parameters evaluated to determine recovery. At 4 days the mice were still consuming 20–30% less chow than controls; at 8 days the mice were consuming about the same amount of chow as controls but no regain of body weight was detected; at 12 days, food intake was about the same as controls and body weight regain was already detectable. Therefore, we decided to employ 8 days as the optimal time point for evaluation of metabolic and hormonal parameters.

Hormone and biochemical measurements

Plasma insulin was determined by RIA, according to a previously described method (Scott et al. 1981). Serum glucose was determined by the glucose oxidase method (Trinder 1969).

I.p. glucose tolerance test (GTT)

After 4-h fast, the mice were anesthetized and after the collection of an unchallenged sample (time 0), a solution of 25% glucose (2·0 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail at 30, 60, 90, and 120 min for determination of glucose and insulin concentrations.

Insulin tolerance test (ITT)

Insulin (2·0 U/kg of weight body) was administered by i.p. injection and blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 min for serum glucose determination. The constant rate for glucose disappearance (Km) was calculated using the formula 0·693/t½. The glucose t½ was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase (Bonora et al. 1987).
**ELISA for adipokine determination**

Serum levels of TNF-α, IL-1β, IL-6, and adiponectin were determined in samples obtained at the end of the experimental period by ELISA (Pierce Biotechnology), following the recommendations of the manufacturer.

**Immunoprecipitation and immunoblotting**

At the end of the experimental period, six mice from each experimental group were employed for evaluation of the insulin signal transduction. For this, the mice were anesthetized with halothane, and after the loss of pedal and corneal reflexes the abdominal cavities were opened and the animals received an infusion of insulin (100 μl, 10^{-6} mol/l) or saline (100 μl) through the cava vein. After 10 min, fragments (3-0×3-0× 3-0 mm) of skeletal muscle (gastrocnemius) were excised and immediately homogenized in solubilization buffer at 4 °C (1% Triton X-100, 100 mmol/l Tris–HCl (pH 7-4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium orthovanadate, 2-0 mmol/l phenylmethylsulphonyl fluoride, and 0-1 mg aprotinin/ml) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Insoluble material was removed by centrifugation for 20 min at 9-000 g in a 70.Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye-binding method (Bradford, 1976). Aliquots of the resulting supernatants containing 5-0 mg total protein were used for immunoprecipitation with antibodies against IR, IRS-1, and IRS-2 at 4 °C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes and blotting with anti-phosphotyrosine (pY) antibodies. In direct immunoblot experiments, 0-2 mg protein extracts obtained from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-IR, anti-IRS-1, anti-IRS-2, and anti-phospho [Ser473]Akt antibodies. Specific bands were labeled with125I-protein A, as previously described (Gasparetti et al. 2003). Visualization of protein bands was performed by exposure of membranes to RX-films.

**Statistical analysis**

Specific protein bands present in the blots were quantified by the digital densitometry (ScionCorp, Frederick, MD, USA, Inc). Mean values ± S.E.M. obtained from densitometric scans, and values for insulin, glucose, body weight, and cytokines were compared utilizing one-way ANOVA with post-test (Tukey). A P<0.05 was accepted as statistically significant.

**Results**

**Induction of obesity and diabetes mellitus in SW/Uni mice**

Beginning at 4 weeks of age SW/Uni mice were randomly divided into two groups fed either a standard rodent chow (SW/Uni/C) or a fat rich chow (SW/Uni/F), according to a previous study (De Souza et al. 2005b). After 8 weeks of treatment, all mice in the SW/Uni/F group became obese, diabetic, hyperinsulinemic, and insulin resistant (Table 1). In addition, during a GTT, glucose clearance was impaired and blood insulin levels were significantly increased (Table 1). At this point, mice of the SW/Uni/F group were randomly divided into three groups: non-operated (SW/Uni/F/NO), sham operated (SW/Uni/F/S) and visceral fat removal (SW/Uni/F/V), and after 8 days the mice were used in the remainder of the experiments.

**Visceral fat removal partially reverts hyperglycemia and reduces insulin resistance**

The removal of the epididymal and perinephric fat pads significantly reduced body weight (Fig. 1A), and no gross regeneration of fat mass was observed at the end of the experimental period. This was accompanied by a reduction in the glucose levels (Fig. 1B), a reduction in the insulin levels (Fig. 1C), and a significant improvement in the insulin action, as determined by the K_{ITT} (Fig. 1D). The reduction in glucose levels and body weight were only partial; however, the reduction in insulin levels and the improvement in insulin action were such that insulin levels and K_{ITT} of SW/Uni/F/V mice became similar to those of SW/Uni/C.

**Visceral fat removal improves insulin signal transduction in muscle**

To evaluate the effect of visceral fat removal upon the molecular actions of insulin, the activation of key steps of the insulin signal transduction machinery were evaluated in skeletal muscle. In SW/Uni/F/NO and SW/Uni/F/S the insulin-induced tyrosine phosphorylation levels of the IR were approximately 35% lower than in SW/Uni/C mice (Fig. 2A). In addition, in SW/Uni/F/NO and SW/Uni/F/S the insulin-induced tyrosine phosphorylation levels of IRS-1 and IRS-2 were approximately 40 and 60% lower than in SW/Uni/C mice respectively (Fig. 2B and C). Finally, in SW/Uni/F/NO and SW/Uni/F/S the insulin-induced

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Table 1 Characterization of the experimental animals

<table>
<thead>
<tr>
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<th>SW/Uni/C</th>
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<tbody>
<tr>
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<td>12 weeks</td>
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<td>Body weight (g)</td>
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<td>115</td>
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<tr>
<td>AUGC (GTT)</td>
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</tr>
<tr>
<td>K_{ITT} (%/min)</td>
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*P<0.05 versus SW/Uni/C; n = 6 in the SW/Uni/C group, and 18 in the SW/Uni/F group. Blood sample collection, GTT and ITT were performed at 1100, following 4 h fasting. AUGC, area under glucose curve; AUIC, area under insulin curve; GTT, glucose tolerance test; ITT, insulin tolerance test.
Ser\textsuperscript{473} phosphorylation of Akt was approximately 40% lower than in SW/Uni/C (Fig. 2D). In SW/Uni/F/V, the insulin-induced tyrosine phosphorylation levels of IR, IRS-1, and IRS-2 and the insulin-induced Ser\textsuperscript{473} phosphorylation of Akt were all restored to levels similar to those seen in SW/Uni/C mice (Fig. 2A–D). The effects of fat diet ingestion and of visceral fat removal on insulin signal transduction were all independent of the modulation of IR, IRS-1, IRS-2 and Akt protein expression (Fig. 2E).

Visceral fat removal restores blood adipokine levels

The blood levels of the pro-inflammatory cytokines, TNF-\(\alpha\) (Fig. 3A), IL-1\(\beta\) (Fig. 3B), and IL-6 (Fig. 3C), were significantly increased, while the levels of adiponectin (Fig. 3D) were significantly reduced in SW/Uni/F/NO and SW/Uni/F/S. The removal of visceral fat exerted a major effect on blood adipokine levels. In SW/Uni/F/V mice the levels of TNF-\(\alpha\) (Fig. 3A) were approximately 40% lower than in SW/Uni/F/NO and SW/Uni/F/S, and the levels of IL-6 (Fig. 3C) were approximately 60% lower than in SW/Uni/F/NO and SW/Uni/F/S. Conversely, the blood levels of adiponectin (Fig. 3D) increased significantly in SW/Uni/F/V mice, reaching a concentration of approximately 40% higher than in SW/Uni/F/NO and SW/Uni/F/S.

Discussion

Visceral adiposity is an independent risk factor for a number of life threatening conditions that range from coronary artery disease (Ducimetiere et al. 1986), hyperlipidemia (Chan et al. 2004), hypertension (Ferrannini et al. 1997), and stroke (Carr & Brunzell 2004) to insulin resistance and diabetes mellitus (Ferrannini et al. 1997, Fujimoto et al. 1999, Carr & Brunzell 2004). The common clinical association between all these metabolic and cardiovascular conditions served as the basis for the unifying definition of the metabolic syndrome (according to the International Diabetes Federation (IDF), available at http://www.idf.org/webdata/docs/MetSyndrome_FINAL.pdf).
**Figure 2** Insulin signal transduction. Anesthetized mice were treated with saline (−) or insulin (+) through the cava vein, and after 10 min, fragments of the gastrocnemius muscle were excised and used for preparation of crude protein extracts. For direct immunoblot analysis (D and E), samples containing 0.2 mg protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies to phospho-Ser473 Akt (D), insulin receptor (IR), IRS-1, IRS-2 or Akt (E). For immunoprecipitation followed by immunoblot, samples containing 2.0 mg total protein were submitted to immunoprecipitation (IP) with antibodies to insulin receptor (A), IRS-1 (B) or IRS-2 (C); immunoprecipitates were collected with Protein A-Sepharose and separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with antibodies to phospho-tyrosine (py). Specific bands were quantified by densitometric analysis. In all experiments, n=6; *P<0.05 versus NO (+) and S (+); †P<0.05 versus C (+).
Although great advance has been achieved in the clinical characterization of this complex condition, little is known about the reasons why the increasing accumulation of fat in the visceral compartment leads to a progressive deterioration of insulin action, and ultimately, to type 2 diabetes mellitus. During the last 15 years, we have learned that fat-derived hormones and cytokines can impair insulin signal transduction in classical target tissues, such as liver, skeletal muscle and adipose tissue (Hotamisligil 2000, Carvalheira et al. 2003, Hotamisligil 2003), and also in the hypothalamic regions responsible for sensing the anorexigenic inputs carried by leptin and insulin (Bjorbaek et al. 1998, Howard et al. 2004, De Souza et al. 2005a). The mechanisms involved in these inhibitory actions are complex and include the activation of serine kinases (Hotamisligil 2003), the induction of endoplasmic reticulum stress (Ozcan et al. 2004), and the stimulation of the expression of inhibitors of signaling belonging to the suppressor of cytokine signaling family (Emanuelli et al. 2001, Calegari et al. 2005). The transduction of the insulin signal is stalled by all these mechanisms, predisposing to further weight gain and diabetes.

Recent studies have shown that surgical removal of visceral fat in animal models of insulin resistance can restore appropriate insulin action, which is associated with a reduction of expression of fat-derived pro-inflammatory cytokines (Barzilai et al. 1999, Kim et al. 1999, Gabriely et al. 2002). However, none of the animal models employed in the previous studies were overtly diabetic. Thus, in the present study we employed a mouse model that, upon hyperlipidic feeding, becomes rapidly diabetic (De Souza et al. 2005b). The SW/Uni mouse is related to the diabetes prone AKR mouse (Rossmeisl et al. 2003), and its predisposition to diabetes induced by diet was recently characterized by our group (De Souza et al. 2005b). In the present study, DIO and diabetes mellitus were achieved in 100% of the mice after 8 week of treatment. At this point, some animals were submitted to visceral fat removal and the metabolic and molecular consequences of this procedure were evaluated.

First, we observed that, 8 days after the surgical procedure, the mice were no longer diabetic. The levels of glucose were significantly reduced but did not return to the levels of control mice. However, the blood levels of insulin and the in vivo action of insulin were completely restored. In the previous studies that evaluated the effect of visceral fat removal, the experimental animals were not diabetic and the impact of the removal of visceral fat upon glucose levels was minimal.
(Barzilai et al. 1999, Kim et al. 1999, Gabriely et al. 2002). However, the effects of surgery upon blood insulin levels and insulin action in vivo were similar to ours.

The effect of visceral fat removal upon insulin signal transduction was not determined in any of these previous studies (Barzilai et al. 1999, Kim et al. 1999, Gabriely et al. 2002). Here, we observed that the surgical procedure led to a complete restoration of the transduction of the insulin signal towards the insulin receptor, IRS-1, IRS-2 and Akt in muscle, independently of the modulation of each protein's expression. Since, the levels of pro-inflammatory cytokines were also restored after surgery, we suspect that the reduction in the serine phosphorylation levels of key elements of the insulin signaling pathway may have contributed to the improvement of insulin signal transduction. In addition, the restoration of the blood levels of adiponectin, as seen previously by Gabriely and co-workers (2002), may have contributed to the improvement of insulin action in muscle, as previously shown (Pajvani et al. 2004).

Thus, we conclude that, in an animal model of DIO and diabetes mellitus, the removal of visceral fat is effective for rapidly reducing the blood levels of glucose. This is accompanied by improved in vivo and molecular actions of insulin and is paralleled by a favorable modulation of the levels of adipokines.

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