Early overnutrition impairs insulin signaling in the heart of adult Swiss mice

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Abstract

Human overnutrition has caused a rise in the prevalence of obesity in recent years. In addition to the deleterious effects of obesity during childhood, long-term effects in adulthood have been described as well. For instance, cardiovascular diseases and type 2 diabetes are among the diseases associated with a history of obesity. Altered insulin secretion and action have been described as important links between these diseases and obesity. Insulin acts as a unique anabolic hormone providing regulation of whole-body glucose homeostasis and peripheral tissue glucose uptake in tissues such as the heart. In this study, we examined insulin signaling in the heart of obese animals using an experimental model of inducing overweight adult animals by overnutrition in early life. In these animals, overfeeding during lactation was able to induce a significant increase in body weight starting at the 10th day of life, and this increased weight persisted until adulthood. Impairment in glucose tolerance, hyperinsulinemia, and an increased insulin/glucose ratio were also observed in these animals. Moreover, an increased heart weight/tibia length ratio was also observed, indicating an enlarged heart size. The overfed animals also had decreased insulin sensitivity in the heart, as confirmed by decreased insulin receptor (IR)-β and IR substrate-1 (Irs1) phosphorylation, increased phosphatase, non-receptor type 1 (Ptpn1)–IR-β association, decreased –Irs1-associated activity, and reduction in anti-phospho Akt1 phosphorylation. In conclusion, our findings showed that overnutrition during early life induces obesity and insulin resistance in the adult offspring, and further increased heart size and impaired cardiac insulin signaling, putatively due to an increase in Ptpn1 activity. Journal of Endocrinology (2008) 198, 591–598

Introduction

Recent surveys have shown that more than 1.5 billion people worldwide are thought to be overweight or obese (Deitel 2003, Park et al. 2005, Ogden et al. 2007). Additionally, it has been shown that the obesity epidemic is not confined to a specific period of life, but rather encompasses patients of all ages, including adolescents and children (Ogden et al. 2002, Miller et al. 2004). The great concern regarding childhood obesity is the similarity of symptoms to those observed in adult obesity (Dietz 1994). For instance, risk factors for coronary heart disease (CHD) such as dyslipidemia, hypertension, and impaired glucose tolerance have previously been described in overweight children (Dietz & Gortmaker 2001). Moreover, it is likely that obesity in early life will cause, among other problems, cardiovascular disease and type 2 diabetes in adulthood (Dietz 1994, Bereson et al. 1998, Weiss et al. 2004, Barker et al. 2007).

The physiological link between these diseases is not yet clear. Nevertheless, it has been suggested that insulin, an anabolic hormone that exerts a multitude of effects on metabolism and various cellular processes in the body (Watson & Pessin 2001), may be a key hormone linking the impaired metabolic pathways found in these diseases (Bogardus et al. 1985). Therefore, obesity, cardiovascular disease, and type 2 diabetes have been thought to be the result of a dysfunction in the secretion or action of insulin (Bogardus et al. 1985, Santalucia et al. 1992, Pereira et al. 2006). In addition, the insulin resistance observed in obesity is widely believed to be the first step in the development of cardiovascular diseases (Koltermann et al. 1981, Bogardus et al. 1985, Godfrey & Barker 2000) and heart failure (Morisco et al. 2006).

During development, insulin acts as a potential mediator between the intrinsic responses to nutritional state, and it coordinates important metabolic actions in almost all body tissues (Shiojima et al. 2002). Glucose uptake, glycogen synthesis, glycolysis, glucose oxidation, and inhibition of fatty acid oxidation are all insulin-dependent processes in various tissues including the cardiac muscle (Carroll et al. 2005). In particular, insulin regulates both glucose uptake and postnatal tissue growth in a peculiar manner in the heart (Weinekove & Leevers 2000). With regard to energy metabolism in early stages of life, glucose transport in the
heart occurs in an insulin-independent manner through the insulin-independent glucose transporter SLC2A1 (GLUT1), which is then gradually replaced by the insulin-dependent transporter SLC2A4 (GLUT4), later on in life (Santalucia et al. 1992, Fries et al. 2003). Furthermore, the main source of energy changes from glucose to fatty acids in the adult heart (Lopaschuk et al. 2007). During this developmental process, cellular insulin signaling is an important mechanism for cardiac development. It has been recently described that early-life nutritional changes could induce modifications in cardiac insulin signaling and metabolism (Rutter et al. 2003, Pereira et al. 2006). For instance, obesity exerts strong effects on the heart by inducing high rates of fatty acid oxidation, which in turn results in an inhibition of glucose oxidation (Mazumder et al. 2004, Carroll et al. 2005, Lopaschuk et al. 2007). It is proposed that this mechanism may also contribute to cardiac insulin resistance (Lopaschuk et al. 2007). It has also been demonstrated that cardiomyocytes from ob/ob mice are insulin resistant and show impaired insulin action in the heart (Mazumder et al. 2004).

Experimental studies have suggested that the fetus may be prone to develop obesity and cardiovascular disease in later life when the state of maternal nutrition is altered (Armitage et al. 2005). Moreover, early postnatal overnutrition may represent a risk factor for later obesity and associated metabolic and cardiovascular disturbances in addition to impaired insulin signaling in adipocytes (Rutter et al. 2003, Rodrigues et al. 2007).

In our study, mice raised in small litters to promote the development of obesity into adulthood (Aubert et al. 1980, Plagemann et al. 1992, 1999) were used to study cardiac insulin signaling. Our results showed that animals overfed during lactation later presented obesity, reduction in the insulin signaling pathway in the heart, metabolic disturbances, and diminished peripheral insulin sensitivity.

Materials and Methods

Animals and treatments

Virgin female Swiss mice were time crossed at 3 months of age. During pregnancy, they were singly housed under standard conditions. After birth, litters were adjusted to nine pups per dam. At postnatal day 3, the number of pups per dam was adjusted to three male mice per litter to form the overfed group (OG), while the control group (CG) was kept with nine newborn pups per dam. Only male Swiss mice were used in the present study in both the overfed and control groups. After weaning at postnatal day 21, the animals were housed in individual cages with free access to water and standard laboratory chow in a temperature-controlled room with a 12 h light:12 h darkness cycle. The animals were weighed periodically and the daily mean food intake of the animals was measured from 21 to 150 days of age by determining the 24-h consumption of standard pellets. The animals were killed when they reached 20 weeks of age. The animals were cared for in accordance with the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based its analysis on the principles described in the Guide for Care and Use of Laboratory Animals (Bayne 1996).

I.p. glucose tolerance test (IPGTT)

An i.p. injection of glucose (1 g/kg body weight) was administrated into 12-h fasted mice at 12 weeks of age. Blood droplets were removed from the tail vein, and blood glucose was measured with a glucometer (ACCU-CHECK Active; Roche Diagnostics GmbH) from a 3-5 μl sample of tail blood at times −30 (30 min before glucose injection), right before the injection (time 0), and at 30, 60, and 90 min following glucose administration.

Visceral fat measurement

Twenty-week-old male mice were fasted overnight, injected with heparin (5000 U/kg), and anesthetized with thiopental sodium (40 mg/kg body weight) via i.p. injection. Visceral fat (epididymal fat, retroperitoneal fat, and mesenteric fat) was excised and weighed on an analytical balance.

Blood parameters

After a 12-h fast, the 20-week-old overfed and age-matched control mice were anesthetized and killed and their trunk blood was collected to evaluate biochemical parameters. Plasma glucose concentration was determined by the glucose oxidase method (Glucose PP; Diagnostica, Belo Horizonte MG, Brazil), and plasma insulin levels were determined by RIA using specific commercial kit (125I RIA Kit; MP Biomedicals, Solon, OH, USA). Plasma levels of triglycerides (Triglycerides PP; Diagnostica) and cholesterol (Cholesterol PP; Diagnostica) were determined by enzymatic colorimetric methods.

Estimation of heart size by heart weight/tibia length ratio

Soon after killing the animals at 150 days of age, their hearts were harvested and weighed on an analytical scale. One leg was removed above the knee joint and the muscle and the skin around the tibias were dissected. The length of the tibias from the condyles to the tip of the medial malleolus was measured by micrometer calipers. The heart size was evaluated by analyzing the heart weight/tibia length ratio (Yin et al. 1982).

Preparation of cardiac muscle

Soon after harvesting from the killed animals, the hearts were excised and the left ventricles (LVs) were isolated. LVs were then minced and homogenized on ice with a Polytron for 15 s in a buffer containing 0.3 M HEPES, 0.5 M EDTA, 0.1 M HEPES, 0.5 M EDTA, 0.1 M

sodium fluoride, 1 M sodium pyrophosphate, 0.1 mM sodium orthovanadate, 2% Triton X-100, 10 mg/ml aprotinin, 5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were then centrifuged at 400 g for 15 min at 4 °C. Pellets were discarded and supernatants frozen at −20 °C.

**Immunoprecipitation**

For immunoprecipitation, homogenized LVs (0.5 mg of protein) were incubated with polyclonal rabbit anti-insulin receptor (IR)-β or polyclonal rabbit anti-IR substrate-1 (Irs1) antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h on ice and gently mixed on a shaker. Next, protein A/G agarose (20 μl/mg) was added and samples were incubated overnight at 4 °C. Immunoprecipitated complexes were collected after centrifugation at 3000 g for 5 min at 4 °C, and the supernatant was discarded. Pellets containing the immunoprecipitated complexes were denatured in Laemmli sample buffer (Laemmli 1970). Equal quantities of protein (20 μl) were measured and used for western blotting analysis.

**Gel electrophoresis and immunoblotting**

The total protein content in the cell extracts was determined by the Bradford method (Bradford 1976). Protein samples were solubilized in Laemmli sample buffer (Laemmli 1970). Equal quantities of protein (30 μl) were loaded onto 8% or 10% polyacrylamide gel in the presence of SDS, along with pre-stained molecular weight standards (Full Range Rainbow; Amersham Biosciences Ltd). After electrophoretic separation, proteins were transferred to nitrocellulose membranes (Hybond P; Amersham Biosciences Ltd). The membranes were blocked with Tween–TBS (10% Tween 20) containing 5% nonfat dry milk for 1 h and incubated with the appropriate antibodies overnight. The following primary antibodies were used: rabbit anti-Akt1/2 (H-136) Sc-8312 (1:1000; Santa Cruz Biotechnology), rabbit anti-p-Akt1/2/3 (Ser473) Sc-7985-R (1:500; Santa Cruz Biotechnology), rabbit anti-insulin R-β (C-19) Sc-711 (1:1000; Santa Cruz Biotechnology), rabbit anti-Irs1 (P-20) Sc-559 (1:1000; Santa Cruz Biotechnology), rabbit anti-PI3K p85α (Z-8) Sc-423 (1:1000; Santa Cruz Biotechnology), and goat anti-Ptpn1 (N-19) Sc-1718 (1:500; Santa Cruz Biotechnology). To detect the antigen-bound antibody, the blots were incubated with goat anti-rabbit IgG-B Sc-2040 secondary antibody conjugated with biotin for 1 h (1:10 000; Santa Cruz Biotechnology) and donkey anti-goat IgG-B Sc-2042 (1:5000; Santa Cruz Biotechnology), followed by incubation with streptavidin–horseradish peroxidase conjugate (1:10 000; Zymed, San Francisco, CA, USA) for 1 h. Immunoreactivity was visualized by enhanced chemiluminescence (ECL) using ECL and ECL-Plus (Amersham Biosciences) and subsequently quantified by densitometry using Image J Software (NIH, Bethesda, MD, USA).

**Statistical analysis**

Results are expressed as mean ± S.E.M. Statistical significance was determined by Student’s t-test for unpaired, bilaterally distributed values of equal variance. P < 0.05 was considered statistically significant.

**Results**

**Body weight and mean food intake during life**

We measured the body weight of control and overfed male Swiss mice from the day of birth to adulthood (20 weeks of age). The animals were weighed periodically, and our data demonstrated that the overfed male mice were clearly overweight when compared with the control group with a significant increase in body weight (* P < 0.05) at the 10th day of life. This difference was significant (* P < 0.05) in all measured ages until 150 days of age (Fig. 1). In addition, daily food intake was measured after the animals were weaned up until 150 days of age. No differences in daily food intake were observed between the control and overfed groups.

**Figure 1** (A) Body weight and (B) mean food intake in overfed and control mice during development. Open and closed symbols represent control and overfed mice respectively. Results are mean ± S.E.M., n=9 animals per group. * P < 0.05, when compared with the control group.

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consumption were observed in the OG during this period when compared with the CG, suggesting that the OG had increased body weight in response to the increased milk availability during the neonatal suckling period.

**Glucose tolerance test**

At 120 days of age, a glucose tolerance test was performed in both overfed mice and their age-matched controls. Basal blood glucose concentrations were similar in both control and overfed mice. However, after 30 min of glucose stimulation, the overfed mice showed an increase in the blood glucose levels compared with that of control mice. After 60 min of glucose stimulation, the blood glucose level was 47% higher in the overfed mice compared with the control group. This difference persisted until 90 min when the blood glucose levels returned to the basal levels in the control group, although the glucose levels were still 57% higher in the overfed mice (Fig. 2).

**Plasma levels of glucose, insulin, triglycerides, and cholesterol**

On the 150th day of life, glucose levels did not differ between the groups (CG: 6.78 ± 0.73 mmol/l, n = 10 vs OG: 6.74 ± 0.45 mmol/l, n = 10). Fasting plasma insulin levels were increased in overfed mice relative to control (CG: 138.39 ± 15.17 pmol/l, n = 10 vs OG: 362.69 ± 38.48 pmol/l, n = 10). Insulin/glucose ratio was approximately three times higher in overfed mice compared with that of control mice. After 60 min of glucose stimulation, the blood glucose level was 47% higher in the overfed mice compared with the control group. This difference persisted until 90 min when the blood glucose levels returned to the basal levels in the control group, although the glucose levels were still 57% higher in the overfed mice (Fig. 2).

**Effects of overnutrition during the neonatal suckling period on visceral fat weight and heart size**

In accordance with the changes observed in plasma insulin levels and total body weight, visceral fat weight in overfed mice was found to be 59% higher relative to controls at 150 days of age. Our data also showed that in the OG, the heart weight/tibia length ratios were also increased and that the hearts of overfed mice were 13.4% heavier than those of controls. These results indicated that the hearts of overfed mice were heavier, and therefore bigger, than those of their control counterparts. Moreover, overfed animals presented a clear increase in visceral fat content at 150 days of age (Table 2).

**Insulin signaling in the heart**

Finally, to investigate the effects on cardiac insulin signaling resulting from overnutrition during the neonatal suckling period, we examined the basal state phosphorylation of IR-β, Irs1, and Akt1-Ser473 in the LVs of control and overfed mice. We also investigated protein tyrosine phosphatase, non-receptor type 1 (Ptpn1) interaction with the IR-β after IR-β immunoprecipitation. Furthermore, we investigated protein tyrosine Ptpn1 and PI3K interaction with the Irs1 after Irs1 immunoprecipitation.

Overfed animals showed a 32.7% increase in IR-β content when compared with controls (Fig. 3A). Interestingly, we found that the OG demonstrated 54.8% less IR-β phosphorylation than the CG (Fig. 3B). Furthermore, Ptpn1–IR-β association was 61.8% higher in overfed animals compared with that of control mice (Fig. 3C).

With regard to Irs1 content, we did not find any difference between the groups (Fig. 4A). However, overfed animals showed 44.5% less Irs1 phosphorylation when compared with controls (Fig. 4B). Nevertheless, we did not find any difference in Ptpn1–Irs1 association between both groups (Fig. 4C). On the other hand, PI3K–Irs1 interaction was 39.6% lower in overfed groups when compared with controls (Fig. 4D).

We also analyzed Akt1 content as well as its phosphorylation in serine residues. Both the control and the overfed mice had the same Akt1 content in the heart. However, insulin signaling was markedly impaired in the overfed group since these animals presented 38.4% lower Akt1 phosphorylation when compared with control mice (Fig. 5).

**Discussion**

The present study demonstrated that the promotion of overfeeding during the neonatal suckling period of mice through a reduction in the litter size induced obesity, visceral fat accumulation, glucose intolerance, hyperinsulinemia, impairment of insulin signaling in the heart under basal conditions, and increased cardiac size. Overnutrition through
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Table 1 Fasting blood parameters. Plasma levels of glucose, triglycerides, cholesterol and insulin, and insulin/glucose ratio. Data are mean ± S.E.M., n = 10 animals per group

<table>
<thead>
<tr>
<th></th>
<th>Insulin (pmol/l)</th>
<th>Glucose (mmol/l)</th>
<th>Insulin/glucose ratio</th>
<th>Triglycerides (mmol/l)</th>
<th>Cholesterol (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>138.39 ± 15.17</td>
<td>6.78 ± 0.73</td>
<td>20.82 ± 1.86</td>
<td>0.41 ± 0.05</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>OG</td>
<td>362.69 ± 38.48*</td>
<td>6.74 ± 0.45</td>
<td>56.54 ± 8.17*</td>
<td>0.49 ± 0.06</td>
<td>1.12 ± 0.08</td>
</tr>
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*P < 0.0001 when compared with control group.

Table 2 Body weight, body length, visceral fat weight, heart weight, tibial length, and HW/TL ratio at 20 weeks of age. Effects of overnutrition during the neonatal suckling period on visceral fat weight in overfed and control mice and estimation of the cardiac size by calculating the heart weight/tibia length ratios (HW/TL). Data are mean ± S.E.M., n = 9 animals per group

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Nasoanal body length (cm)</th>
<th>Visceral fat weight (g)</th>
<th>Heart weight (g)</th>
<th>Tibial length (cm)</th>
<th>HW/TL ratio</th>
</tr>
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<tbody>
<tr>
<td>CG</td>
<td>50.4 ± 1.22</td>
<td>10.2 ± 0.10</td>
<td>0.85 ± 0.08</td>
<td>0.19 ± 0.002</td>
<td>2.09 ± 0.01</td>
<td>0.09 ± 0.001</td>
</tr>
<tr>
<td>OG</td>
<td>64.5 ± 1.00*</td>
<td>10.5 ± 0.08</td>
<td>2.07 ± 0.13*</td>
<td>0.23 ± 0.007*</td>
<td>2.12 ± 0.01</td>
<td>0.10 ± 0.003*</td>
</tr>
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*P < 0.005 when compared with the control group.
levels were found to be an independent predictor of CHD death. Similarly, fasting insulin levels were also found to be an independent risk factor for developing CHD in a case–control study of Canadian men (Pi-Sunyer 2002).

Moreover, several studies have reported a direct correlation between insulin resistance and left ventricular mass with significant ventricular hypertrophy in insulin-resistant patients (Rutter et al. 2003).

In the present study, we evaluated for possible cardiac alterations in mice raised in small litters by analyzing the heart weight/tibia length ratio and cardiac insulin signaling. Consistent with the literature, adult overfed mice presented increased heart weight/tibia length ratio, which is an indication of increased cardiac size. Moreover, overfed animals had decreased insulin sensitivity in the heart (as confirmed by decreased IR–β and Irs1 phosphorylation), increased Ptpn1–IR–β association, decreased Irs1–PI3K interaction, and decreased Akt1 phosphorylation.

It is known that cardiac insulin resistance might be an adaptive event during the progression of obesity-associated diabetes via increased lipid flux into cardiomyocytes and a compensatory decrease in glucose metabolism (Reaven et al. 2004, Park et al. 2005). As expected, cardiac insulin signaling was impaired in overfed animals. Despite decreased IR–β phosphorylation in the overfed mice, IR–β content was actually enhanced in this group. We suggested that it may be a compensatory mechanism to prevent insulin resistance in the heart. However, this adaptation was found to be inefficient to maintain normal IR phosphorylation in tyrosine (Tyr)
residues. One possible explanation for the decreased insulin Tyr phosphorylation is the increased Ptpn1–IR–β association in overfed mice, since the enzyme Ptpn1 modulates the insulin pathway by dephosphorylating phosphotyrosine residues on the IR (Asante-Appiah & Kennedy 2003).

With regard to Irs1 content, we did not find any difference between overfed and control mice. However, overfed mice displayed decreased Irs1 tyrosine phosphorylation compared with controls. With Ptpn1–Irs1 association, there was no difference when both groups were compared. Moreover, the suppressed Irs1 tyrosine phosphorylation found in overfed animals was not in response to increased Ptpn1–Irs1 association, and this may be occurring due to serine phosphorylation of Irs1 (Steinberg 2007).

Consequently, augmented IR–β tyrosine dephosphorylation by protein tyrosine phosphatases may contribute to insulin resistance (Calera et al. 2000, Asante-Appiah & Kennedy 2003, Nieto-Vazquez et al. 2007). Thus, the regulation of IR tyrosine phosphorylation is a key step in controlling insulin signaling. It is likely that the increase in Ptpn1 in the heart of overfed mice led to impaired IR phosphorylation in this tissue. Hence, our findings confirmed the importance of Ptpn1 as a negative regulator of insulin signaling in the heart.

In addition to the decreased tyrosine phosphorylation on the Irs1, the overfed animals also showed decreased PI3K–Irs1 interaction. This was an important finding since PI3K activates the phosphoinositide–dependent protein kinase that go on to phosphorylate Akt1 (Watson & Pessin 2001).

The level of Akt1 serine phosphorylation was found to be diminished in overfed animals. These data are consistent with a picture of impaired IR–β and Irs1 phosphorylation and PI3K activation, since Akt1 is a downstream molecule to IR–β, Irs1, and PI3K in the insulin signaling pathway (Watson & Pessin 2001). Accordingly, this reduction in Akt1 phosphorylation can impair cardiac glucose metabolism, since Akt1 activation is necessary for Slc2a4 (solute carrier family 2 (facilitated glucose transporter), member 4) translocation from the intracellular compartments to the cell surface, thereby allowing an adequate level of glucose uptake (Saltiel & Kahn 2003, Nieto-Vazquez et al. 2007). Thus, the quest for negative regulators of insulin action.

Declaration of Interest
This work has no conflict of interest that would prejudice its impartiality.

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