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MOLECULAR METABOLISM

A major role of insulin in promoting obesity-associated adipose tissue inflammation

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ABSTRACT

Objective: Adipose tissue (AT) inflammation is associated with systemic insulin resistance and hyperinsulinemia in obese rodents and humans. A longstanding concept is that hyperinsulinemia may promote systemic insulin resistance through downregulation of its receptor on target tissues. Here we tested the novel hypothesis that insulin also impairs systemic insulin sensitivity by specifically enhancing adipose inflammation.
Methods: Circulating insulin levels were reduced by about 50% in diet-induced and genetically obese mice by treatments with diazoxide or streptozotocin, respectively. We then examined AT crown-like structures, macrophage markers and pro-inflammatory cytokine expression in AT. AT lipogenesis and systemic insulin sensitivity was also monitored. Conversely, insulin was infused into lean mice to determine its affects on the above parameters.
Results: Lowering circulating insulin levels in obese mice by streptozotocin treatment decreased macrophage content in AT, enhancing insulin stimulated Akt phosphorylation and de novo lipogenesis (DNL). Moreover, responsiveness of blood glucose levels to injected insulin was improved by streptozotocin and diazoxide treatments of obese mice without changes in body weight. Remarkably, even in lean mice, infusion of insulin under constant euglycemic conditions stimulated expression of cytokines in AT. Consistent with these findings, insulin treatment of 3T3-L1 adipocytes caused a 10-fold increase in CCL2 mRNA levels within 6 h, which was blocked by the ERK inhibitor PD98059.
Conclusion: Taken together, these results indicate that obesity-associated hyperinsulinemia unexpectedly drives AT inflammation in obese mice, which in turn contributes to factors that suppress insulin-stimulated adipocyte DNL and systemic insulin sensitivity.

Keywords Obesity; Hyperinsulinemia; Adipose tissue; Inflammation; Insulin resistance

1. INTRODUCTION

Current evidence indicates that chronic low-grade inflammation in adipose tissue (AT) may be a contributing mechanism in obesity-induced insulin resistance and type 2 diabetes [1–10]. Many groups have observed increased pro-inflammatory cytokine and chemokine production as well as elevated macrophage and other immune cell content in the AT of obese rodents and humans [11–13]. The pro-inflammatory state of AT in obesity is considered a key event driving local and systemic metabolic dysfunction [2,4,12,14]. Consistent with this notion, deletion or silencing of pro-inflammatory genes has been demonstrated to enhance insulin action in AT and improve whole-body glucose homeostasis in obese animal models [12,15]. Evidence has also been presented that AT inflammation can be a beneficial aspect of adipose remodeling and responsiveness to obesity [16–19], suggesting that AT inflammation may have multiple roles. Nonetheless, at later stages of obesity, a predominant deleterious effect of AT inflammation on adipose insulin sensitivity and systemic glucose tolerance seems apparent [20].

Impaired adipocyte responsiveness to insulin in obesity is thought to secondarily affect whole body metabolism by limiting adipose tissue's ability to store lipid and sequester fatty acids away from peripheral tissues. It is also proposed that adipocyte de novo lipogenesis (DNL), which generates fatty acids from glucose, produces endogenous lipid ligands such as hydroxy fatty acid esters [21] and palmitoleate [22] that enhance local and systemic glucose metabolism [21–23]. These results suggest that the insulin-sensitive AT DNL pathway may play a critical role in maintaining tightly controlled whole body glucose homeostasis and high insulin sensitivity [21,22]. However, the activity of the DNL pathway and the genes associated with DNL are strongly repressed in obese, insulin-resistant AT of both humans and rodents [23–31]. The chronically elevated AT inflammatory state observed in obesity may be a key factor in this inhibition of AT-DNL. Consistent with this concept, cytokine-mediated suppression of PPARγ, SREBP1c and

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its lipogenic gene targets, such as GLUT4 and fatty acid synthase (Fasn), has been demonstrated in AT *in vivo,* as well as in cultured adipocytes *in vitro* [32–37]. Thus, AT inflammation may contribute to the suppression of glucose tolerance and systemic insulin sensitivity through its downregulation of insulin action and DNL in adipocytes.

In spite of the many studies that document the expansion of macrophages and other immune cell types within AT in obesity, the initiating factors in this process are not identified. Adipocytes express cytokines such as CCL2 and TNF-α, but at seemingly lower levels than do immune cells, and the role of these adipocyte factors is unclear. Another possible agent that might contribute to inducing AT inflammation is insulin itself. Obesity is usually associated with hyperinsulinemia and studies in humans have suggested a link between hyperinsulinemia and AT inflammation. These include reports of increased pro-inflammatory cytokine expression and macrophage infiltration into AT during a hyperinsulinemic-euglycemic clamp [38–43]. Furthermore, increased pro-inflammatory activity has also been reported in human AT from subjects following insulin therapy [44]. However, whether obesity-associated hyperinsulinemia promotes AT inflammation and the mechanism by which this occurs remains to be fully elucidated. Here we report that a reduction in circulating insulin levels in obese mice causes a marked decrease in AT macrophage expansion and attenuated pro-inflammatory AT cytokine/chemokine expression. Furthermore, this reduction in circulating insulin improved AT-DNL activity and insulin signaling in AT as well as systemic insulin sensitivity in obese mice. These results indicate that hyperinsulinemia contributes substantially to driving AT inflammation in obesity, leading to worsening whole-body glucose tolerance and insulin resistance.

2. MATERIAL AND METHODS

2.1. Subject samples and microarray study

Samples were obtained from 36 adult patients, undergoing laparoscopic Roux-en-Y gastric bypass surgery as described [13]. The demographic data including age, gender, height, weight, calculated BMI and fasting glucose and insulin levels were recorded at the time of gastric bypass surgery. All subjects provided written informed consent before taking part in the study. The study was approved by the University of Massachusetts Medical School Institutional Review Board. Adipose tissue samples were taken from lower abdominal wall (subcutaneous) and greater omentum (visceral) and snap frozen in liquid nitrogen. Total RNA was isolated from human tissues by homogenizing the frozen tissue in TRIzol (Invitrogen, Carlsbad, CA). Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays were prepared as previously described [13]. GeneChip Expression Arrays for each gene were analyzed as described [13].

2.2. Animal studies

We obtained 4-week old male C57BL/6J (WT) and B6.V-*Lepob/J (ob/ob)* mice from Jackson Laboratory. Mice were housed on a 12 h light/dark schedule and had free access to water and food, except when indicated. WT mice were fed a HFD (Research Diets) that contained 60% calories from lipids, in the absence (D12492) or presence of 1.125 mg/kg diazoxide (D12121501). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. Glucose and insulin tolerance tests were performed on ob/ob mice as indicated. Glucose (1 g/kg) and insulin (1 IU/kg) were administrated by intraperitoneal (i.p.) injection. Blood samples were withdrawn from the tail vein at the indicated times, and glycemia was determined using a Breeze 2 glucose meter (Bayer and alpha-trak). Plasma insulin and C-peptide levels were measured with Millipore insulin ELISA and ALPCO Mouse C-peptide ELISA, respectively.

2.3. Hyperinsulinemic-euglycemic clamp studies

The clamp study was performed at the UMass Mouse Metabolic Phenotyping Center. Six-week-old wild type (WT) mice were subjected to an overnight fast and a 2-h hyperinsulinemic-euglycemic clamp was conducted in awake mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 4 mU·kg−1 min−1; Humulin, Eli Lilly). During the clamp, 20% glucose was infused at variable rates to maintain euglycemia [45]. At the end of the study, mice were anesthetized, and tissues were taken for total RNA extraction and quantitative RT-PCR analysis.

2.4. Histology

Epididymal white adipose and pancreas tissues were dissected and fixed by immersion in 10% neutral buffered formalin (Sigma, St. Louis, MO) for 12 h, dehydrated, cleared, and then embedded in paraffin. Sections (7 μm) were stained with hematoxylin and eosin or with anti-F4/80 antibody to assess morphology or detect macrophage and crown-like structures in AT. Pancreatic islets were stained with insulin antibody (Cell Signaling, Danvers, MA), as indicated.

2.5. Reagents

Streptozotocin, diazoxide, bovine insulin, FA-free BSA, D-glucose, sodium pyruvate, sodium acetate, anti-tubulin and anti-beta-actin antibody were purchased from Sigma–Aldrich. PD98059 and MK2206 were from EMD Millipore and Selleckchem respectively. ^{14}C-U-glucose (250 μCi/mL) was purchased from Perkin Elmer. Antibodies against ATP-citrate lyase (Acly), fatty acid synthase (Fasn), CCL2, TNFα, Akt, phospho-Akt (ser473) and insulin receptor were from Cell Signaling Technology (Danvers, MA)

2.6. Cell culture

3T3-L1 fibroblasts were grown and differentiated into adipocytes as previously described [46]. Briefly, 3T3-L1 fibroblasts were grown to confluence in complete media (high glucose (25 mM) DMEM containing 10% fetal bovine serum, 50 units/mL penicillin and 50 μg/mL of streptomycin). Two days after confluence, differentiation media (DMEM containing 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine, and 10^{-7} M insulin) was added. Three days post-induction, differentiation media was replaced by complete media without insulin. On day 5, media was again changed with fresh complete media. On the seventh day after differentiation, media was added, adipocytes were pretreated with PD98059, MK2206 or DMSO vehicle for 30 min followed by treatment with 1 μM or 100 nM insulin, as stated in the Figure legends, for the indicated period of time.

2.7. Real-time quantitative RT-PCR

Total RNA was extracted from 3T3-L1 adipocytes or mouse tissues using TRIzol Reagent Protocol (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from 1 ug of total RNA using iScript cDNA Synthesis Kit (BioRad). Quantitative RT-PCR was performed using iQ SybrGreen supermix and analyzed as previously described [47,48]. 36B4, Hprt and Gapdh served as controls for normalization

2.8. Immunoblotting

For experiments on *ex-vivo* insulin treatment, adipose tissue explants were incubated in DMEM media supplemented with 0.5 mM glucose, 2 mM sodium pyruvate and 2 mM glutamine, in the absence or

