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# Comparison of mitochondrial and macrophage content between subcutaneous and visceral fat in *db*/*db* mice

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**Abstract**

Central (visceral) obesity is more closely associated with insulin resistance, type 2 diabetes, and cardiovascular disease than peripheral (subcutaneous) obesity, however the underlying differences in morphology and pathophysiology between subcutaneous and visceral adipose are largely unknown. To evaluate the effects of diabetes and rosiglitazone (RSG) treatment, the expression of mitochondrial Hsp60, UCP-1 and F4/80 in inguinal subcutaneous (SC) fat, composed of white and brown adipose tissues, and epididymal (EP) fat, mainly white adipose tissue, were evaluated. In diabetic *db*/*db* mice, there was significant increased number of aggregated macrophage foci compared to *db*/+ mice, especially in EP fat. On the other hand, the expression of mitochondrial Hsp60 protein was suppressed in both SC and EP fat of *db*/*db* mice compared to *db*/+ mice, and the expression level of mitochondrial Hsp60 in *db*/+ mice was lower in EP fat compared with SC. In *db*/*db* mice, RSG suppressed the number of aggregated macrophage foci in EP fat, but not in SC fat. RSG ameliorated the mitochondrial Hsp60 expression and induced the expression of UCP-1 in both SC and EP fat. Taken together, these data suggest that differences exist in mitochondrial and macrophage content, and in the response to RSG between visceral and subcutaneous adipose tissue, and adipose type and distribution may be important for obesity-linked insulin resistance.


*Keywords:* Diabetes; Macrophage; Mitochondrial biogenesis; Rosiglitazone; Subcutaneous adipose; Visceral adipose

## Introduction

Adipocytes from visceral or subcutaneous adipose tissue largely differ concerning their metabolic characteristics in the control of lipolysis, adipokine production, inflammatory cytokine production, the response to glucocorticoids and the sensitivity to insulin (Wajchenberg, 2000; Misra and Vikram, 2003; Cote et al., 2005; Bruun et al., 2005; Lundgren et al., 2004). In addition, obesity and insulin resistance are closely linked and much evidence suggests that the excessive accumulation of fat in intra-abdominal adipocytes contributes to insulin resistance (Macor et al., 1997; Schwartz and Kahn, 1999). The surgical removal of visceral adipose improves insulin's effect on hepatic glucose production in animal models of obesity (Barzilai et al., 1999). Studies showed that enlarged adipocytes were associated with insulin resistance and are an independent predictor of type 2 diabetes (Jernas et al., 2006; Skurk et al., 2006), and PPARg activation leads to improved insulin sensitivity associated with decreased adipocyte sizes (Okuno et al., 1998). Furthermore, increased cellularity in white adipocytes, resulting from increased mitochondrial content, was associated with improved insulin sensitivity in T2DM animal models (Orci et al., 2004; Um et al., 2004). Therefore, it would be important to define the regional differences of adipose in

*Abbreviations:* BAT, brown adipose tissue; EP, epididymal; MCP-1, monocyte chemotactic protein-1; RSG, rosiglitazone; SC, subcutaneous; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.

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morphology and pathophysiology for elucidating the relationship between adipose metabolic characteristics and insulin resistance.

To date, a correlative and causative relationship between inflammation and insulin resistance/T2DM has been demonstrated (Hotamisligil et al., 1994; Hotamisligil et al., 1993; Sartipy and Loskutoff, 2003b). Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), have been shown to induce insulin resistance as a result of obesity in many rodent obesity models (Hotamisligil and Spiegelman, 1994; Hotamisligil et al., 1993). In humans and rodents, TNF-α is overexpressed in WAT in obese and insulin-resistant states (Hotamisligil and Spiegelman, 1994; Hotamisligil et al., 1993; Uysal et al., 1998). The chemokine monocyte chemotactic protein-1 (MCP-1) was shown to impair adipocyte insulin sensitivity (Sartipy and Loskutoff, 2003a). Macrophages secrete numerous cytokines and chemokines, such as TNF-α, IL-1, IL-6 and MCP-1, that are known to contribute to insulin resistance in adipocytes (Amrani et al., 1996; Grimble, 2002; Hotamisligil and Spiegelman, 1994; Hrnciar et al., 1999). Recent data suggest that adipose tissue macrophages are responsible for the expression of proinflammatory cytokine, such as TNF-α (Weisberg et al., 2003). Furthermore, adipose tissue macrophages were shown to play a crucial role in the development of obesity-related insulin resistance (Xu et al., 2003).

In addition to macrophage infiltration in adipose tissue, there has been growing recognition that adipose mitochondrial dysfunction is associated with insulin resistance and obesity. Recent studies show that adipose mitochondrial gene expression is suppressed in *ob*/*ob* (Powelka et al., 2006), *db*/*db* and HFD mice (Rong et al., submitted to Diabetes), and T2DM patients (Bogacka et al., 2005), but is induced by TZD treatment, which improves the diabetic conditions in vivo. Mitochondria are indispensable in non-shivering thermogenesis, which takes place in brown adipose tissue (BAT) and relies on energy uncoupling via short-circuiting of the proton gradient across the mitochondrial inner membrane (Lin et al., 2005; Robidoux et al., 2004).

However, histopathological evidence demonstrating the effects of diabetes and insulin sensitizers, such as rosiglitazone (RSG), on mitochondria and macrophage content in subcutaneous and visceral fat has not been well investigated.

In this study, we quantitatively evaluated the expression of mitochondrial proteins (Hsp60 and UCP-1) and the number of aggregated macrophage foci in subcutaneous (SC) fat, composed mainly of white but also with the presence of brown adipose tissues (Cinti, 2005) and epididymal (EP) fat in visceral adipose tissue, generally considered as WAT, of *db*/*db* mice, and studied the effects of RSG treatment using quantitative and histopathological approaches.

## Materials and methods

### *Animals and diets*

Male C57BL/Ks *db*/*db* and *db*/+ mice were obtained from The Jackson Laboratory (Bar Harbor, MA) at 4 weeks of age. All mice were housed in groups of five in a temperature- and humidity-controlled environment (22 °C and 50%) with a 12-h light/dark cycle. *Db*/*db* and *db*/+ mice were fed a standard rodent chow (Purina 5001; TestDiet, Richmond, IN) for the duration of the experiments. All animals were allowed free access to food and water. Care and use of the animals were in accordance with protocols approved by the GlaxoSmithKline Institutional Animal Care and Use Committee.

### *RSG administration*

The study drug used in this study was RSG maleate (BRL49653C Stage 5 Drug Substance; lot no.: HW010701) and was obtained from 'Clinical Trial Supplies' GSK, Harlow, United Kingdom (UK). Treatment began in *db*/*db* and db/+ mice at 8 weeks of age. Mice ($n=10$ per treatment group) received RSG (1, 3 or 10 mg/kg) or vehicle (0.5% methylcellulose) by oral gavage twice daily for 14 days. Blood samples were taken on day 0, 7 and 14 of the study after a 6-hour fasting period for blood glucose measurements using an automated clinical chemistry analyzer (Olympus AU400; Olympus America Inc., Melville, NY). Inguinal SC fat and EP fat was collected from each mouse at necropsy.

### *Immunohistochemistry*

Adipose tissues were fixed in 10% neutral buffered formalin. Fixed samples were processed for paraffin embedding, sectioned at 5 μm and immunohistochemically stained for UCP-1, mitochondrial Hsp60 and F4/80. Adipose sections were deparaffinized with xylene and re-hydrated through graded concentrations of ethanol. For antigen retrieval, deparaffinized sections were microwaved for 10 min in Target Retrieval Solution pH 6 (DAKO, Glostrup, Denmark). Immunohistochemical staining for UCP-1, mitochondrial Hsp60 and F4/80 was performed using VECTASTAIN elite ABC kit (Vector Laboratories, Burlingame, CA). Tissue sections were quenched with Peroxidase Blocking Solution (DAKO) for 10 min and blocked with 1% normal goat or rabbit serum for 20 min. Tissue sections were incubated with rabbit anti-UCP-1 polyclonal antibody (1:2000) (Abcam, Cambridge, UK), goat anti-Hsp60 polyclonal antibody (1:200) (Santa Cruz, Santa Cruz, CA) and rat anti-F4/80 monoclonal antibody (1:1000) (BMA Biomedicals, Rheinstrasse, Switzerland). After washing with Wash Buffer (DAKO), sections were incubated with biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG or rabbit anti-rat IgG antibody (Vector Laboratories) for 30 min and then incubated with ABC reagent of VECTASTAIN (Vector Laboratories) for 30 min. Reaction product was visualized by incubation with DAKO Liquid DAB Substrate Chromogen (DAKO) for 2 min and immersed in distilled water for 3 min. Tissue sections were counterstained with hematoxylin for 2 min and rinsed with distilled water, dehydrated by sequential immersion in gradient ethanol and xylene then mounted with Entellan New (MERCK) and cover slipped.

### *Image analysis*

Average sizes of adipocytes were quantitatively analyzed using an innovative object-based quantitative image analysis system, eCognition (Definiens AG, Germany). For measurement of the average adipocyte size, five images from one H&E stained section (total 2.86 $mm^2$ area per section; 5 sections per animal) were randomly acquired at ×100 magnification using light microscope with DP70 system (Olympus, Tokyo, Japan). Average adipocyte sizes were determined from 400 to 1000 adipocytes in WAT. For object (adipocyte) extraction and measurement, an image was segmented by using the algorithm of multiresolution segmentation according to the following criteria: scale parameter (40), composition of color homogeneity (0.8), composition of shape homogeneity (0.2), composition of smoothness homogeneity (0.1) and composition of compactness homogeneity (0.9). After the segmentation, adipocytes were classified by morphological features according to the following criteria: compactness $\leq 3.1$, elliptic fit $\geq 0.375$, mean of green image layer value $\geq 179$. Then, the size and number of the classified adipocytes were measured. Quantification of UCP-1 and mitochondrial Hsp60 positive area was performed using eCognition. For quantitative analysis of UCP-1 and mitochondrial Hsp60 expression, five images from one section