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Basal lipolysis, not the degree of insulin resistance, differentiates large from small isolated adipocytes in high-fat fed mice

S. Wueest · R. A. Rapold · J. M. Rytka · E. J. Schoenle · D. Konrad

Abstract

Aims/hypothesis Adipocytes in obesity are characterised by increased cell size and insulin resistance compared with adipocytes isolated from lean patients. However, it is not clear at present whether hypertrophy actually does drive adipocyte insulin resistance. Thus, the aim of the present study was to metabolically characterise small and large adipocytes isolated from epididymal fat pads of mice fed a high-fat diet (HFD).

Methods C57BL/6J mice were fed normal chow or HFD for 8 weeks. Adipocytes from epididymal fat pads were isolated by collagenase digestion and, in HFD-fed mice, separated into two fractions according to their size by filtration through a nylon mesh. Viability was assessed by lactate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays. Basal and insulin-stimulated d-[U-^{14}C]glucose incorporation and lipolysis were measured. Protein levels and mRNA expression were determined by western blot and real-time RT-PCR, respectively.

Results Insulin-stimulated D-[U-^{14}C]glucose incorporation into adipocytes isolated from HFD-fed mice was reduced by 50% compared with adipocytes from chow-fed mice. However, it was similar between small (average diameter 60.9±3.1 μm) and large (average diameter 83.0±6.6 μm) adipocytes. Similarly, insulin-stimulated phosphorylation of protein kinase B and AS160 were reduced to the same extent in small and large adipocytes isolated from HFD-mice. In addition, insulin failed to inhibit lipolysis in both adipocyte fractions, whereas it decreased lipolysis by 30% in adipocytes of chow-fed mice. In contrast, large and small adipocytes differed in basal lipolysis rate, which was twofold higher in the larger cells. The latter finding was associated with higher mRNA expression levels of *Atgl* (also known as *Pnpla2*) and *Hsl* (also known as *Lipe*) in larger adipocytes. Viability was not different between small and large adipocytes.

Conclusions/interpretation Rate of basal lipolysis but not insulin responsiveness is different between small and large adipocytes isolated from epididymal fat pads of HFD-fed mice.

Abbreviations

Akt	protein kinase B
ATGL	adipose triacylglycerol lipase
HFD	high-fat diet
HSL	hormone-sensitive lipase
ipGTT	intraperitoneal glucose tolerance test
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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Introduction

Adipocyte hypertrophy has been proposed to induce adipose tissue dysfunction in obesity, while cell death of

hypertrophic adipocytes is thought to be a driving force for macrophage infiltration into adipose tissue [1] and large adipocytes may secrete higher levels of pro-inflammatory cytokines [2]. At a more metabolic level, hypertrophic adipocytes may exhibit a reduced capacity to store and retain NEFA, leading to elevated levels of circulating NEFA [3]. In contrast, hyperplastic adipocytes may favour the deposition of NEFA and thus protect other tissues such as liver and skeletal muscle from lipotoxicity by sequestering NEFA away from them [3]. In this regard, treatment with thiazolidinediones has been shown to increase cellularity in adipose tissue by promoting pre-adipocyte differentiation and inducing apoptosis of large adipocytes, resulting in smaller and more insulin-sensitive adipocytes [4]. Yet, most studies comparing large and small adipocytes isolated them from different participants/animals (small adipocytes from lean, large adipocytes from obese) [5, 6]. Thus, differences between large and small adipocytes could have resulted from other factors that differ between lean and obese sources [5, 6] and not from genuine biological differences between large and small fat cells within the same biological context.

Thus, the aim of the present study was to metabolically characterise large versus small adipocytes isolated from the same depot of mice fed a high-fat diet (HFD) for 8 weeks. Such feeding is associated with whole-body insulin resistance, but with minimal adipocyte cell death and macrophage infiltration [1]. We hypothesised that insulin responsiveness in large adipocytes is reduced compared with small adipocytes isolated from the same fat pad.

Methods

Animals We used 6- to 8-week-old male C57BL6JOlaHsd mice, which had free access to standard rodent diet or HFD (D12331; Research Diets, New Brunswick, NJ, USA) for 8 weeks. HFD consisted of 56% of energy derived from fat, 28% from carbohydrate and 16% from protein. In total, 30 mice were used for this study. All protocols conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

Intraperitoneal glucose tolerance test For the intraperitoneal glucose tolerance test (ipGTT), mice were injected intraperitoneally with 2 mg/g body weight glucose after overnight fasting. Blood glucose concentration was measured with a glucometer (Accu-Check Aviva; Roche Diagnostics, Rotkreuz, Switzerland) with blood from tail-tip bleedings [7].

Cell separation and viability assessment Adipocytes isolated from HFD-fed mice were separated into two fractions according to their size by filtration through a 60 μm nylon mesh (Millipore, Zug, Switzerland). Cells caught on the mesh (large adipocytes) were gently washed away into a separate vial. After separation, adipocyte viability was assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma, Buchs, Switzerland) as follows. Small and large cells were incubated with 0.5 mg/ml MTT for 1 h and salt was extracted from them with DMSO. The amount of yellow MTT reduced to purple formazan was measured spectrophotometrically (550–655 nm) with an ELISA reader. A lactate dehydrogenase (LDH) release assay using a nonradioactive cytotoxicity assay (CytoTox 96; Promega, Dübendorf, Switzerland) was also performed. LDH released into supernatant fraction was measured and normalised to cell number in incubation medium.

Glucose incorporation into isolated white adipocytes Adipocyte isolation and glucose incorporation were performed as described previously [8] with the following adaptations. Adipocytes (4-8% suspension [9]) were incubated for 60 min with D-[U-^{14}C]glucose. In this setting, most of the labelled glucose is incorporated into lipids and is therefore a readout for lipid synthesis [10]. Where indicated, cells were thereafter separated into two fractions according to their size by filtration through a 60 μm nylon net filter. Glucose incorporation was stopped by separating cells from the medium by centrifugation (about 2000 *g*) through phthalic acid dinonyl ester. Cells were then subjected to liquid scintillation counting. Cell number was determined with a Neubauer haematocytometer (Brand, Wertheim, Germany) under the light microscope.

Cell size determination Aliquots of all adipocyte fractions of each experiment were used to determine mean cell diameters. Photographs of isolated adipocytes in the haematocytometer were taken and images were analysed using ImageJ software for quantification (National Institutes of Health, Bethesda, MD, USA). At least 100 adipocytes per fraction of four independent experiments were analysed.

Lipolysis assays To assess lipolysis, cells were separated as mentioned above and incubated in the absence or presence of 100 nmol/l insulin or 1 μmol/l isoproterenol (Sigma) for 1 h. NEFA levels were measured using a method (ACS-ACOD-MEHA) from Wako Chemicals (Neuss, Germany). Glycerol content of the incubation medium was determined using a colorimetric assay, as described [11].

RNA extraction and quantitative RT-PCR Total RNA was extracted using a kit (RNeasy Lipid Tissue Mini Kit; Qiagen, Basel, Switzerland). RNA (0.5 μg) was reverse-transcribed with Superscript III Reverse Transcriptase

(Invitrogen, Basel, Switzerland) using random hexamer primer. Taqman was used for real-time PCR amplification. The following PCR primers (Applied Biosystems, Rorkreuz, Switzerland) were used: *Hsl* (also known as *Lipe*) Mm00495359_m1, *Atgl* (also known as *Pnpla2*) Mm00503040_m1. Relative gene expression was obtained after normalisation to *36B4* (also known as *Rplp0*) RNA (Applied Biosystems), using the formula $2^{-\Delta\Delta\text{cp}}$.
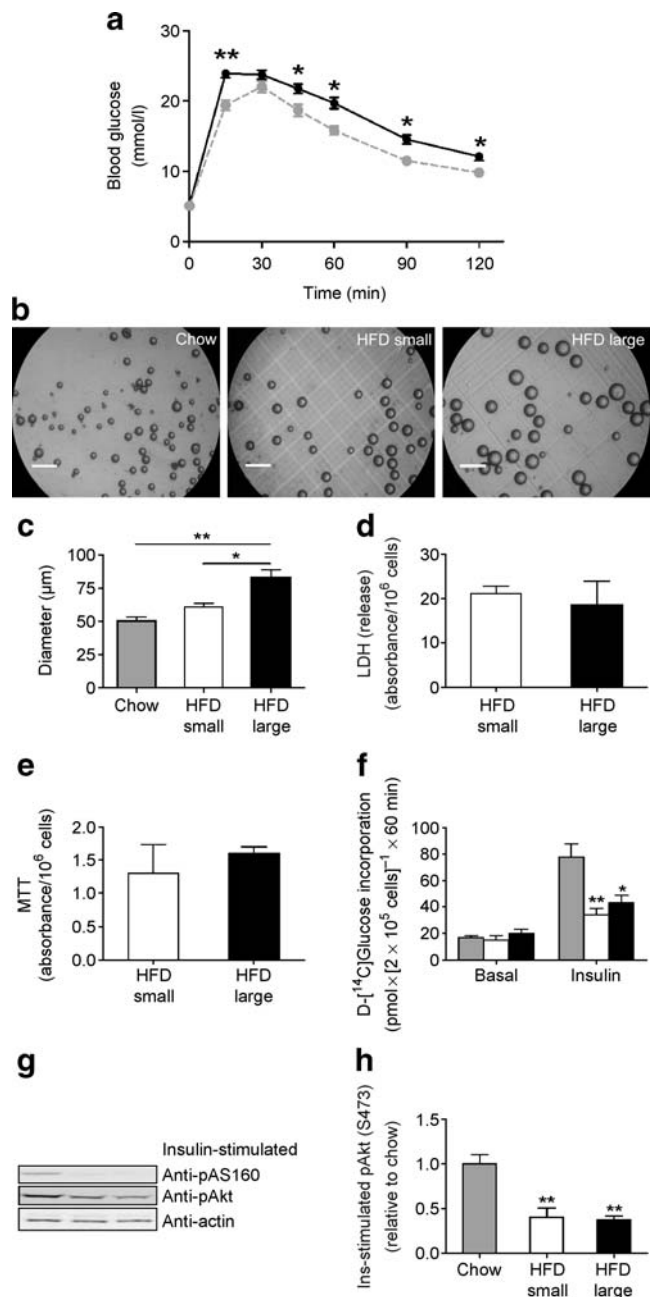
Western blot Isolated and separated adipocytes were homogenised in a buffer containing 150 mmol/l NaCl, 50 mmol/l Tris–HCl (pH 7.5), 1 mmol/l EGTA, 1% (vol./vol.) NP-40, 0.25% (vol./vol.) sodium deoxycholate, 1 mmol/l sodium vanadate, 1 mmol/l NaF, 10 mmol/l sodium β-glycerophosphate, 100 nmol/l okadaic acid, 0.2 mmol/l phenylmethylsulphonyl fluoride (PMSF) and a 1:1000 dilution of protease inhibitor cocktail (Sigma). Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and equivalent amounts of protein (40 μg) were resolved by LDS-PAGE (4–12% gel; NuPAGE; Invitrogen). Proteins were electro-transferred on to nitrocellulose membranes (0.2 μm; BioRad, Reinach, Switzerland) and immunoblotted for phosphoS473-protein kinase B (Akt), phosphoT308-Akt, phosphoAS160 (Cell Signalling, Beverly, MA, USA), anti-GLUT4 (gift from A. Klip, The Hospital for Sick Children, Toronto, ON, Canada) or anti-actin (Millipore, Zug, Switzerland). Membranes were exposed in an Image Reader and analysed with an Image Analyzer (FujiFilm, Dielsdorf, Switzerland).

Data analysis Statistical analyses were performed using either Student's *t* test or ANOVA test (Tukey's multiple comparisons test).

Results

Separation of adipocytes isolated from HFD-fed mice HFD for 8 weeks significantly impaired glucose tolerance in male C57BL6JOlaHsd mice compared with chow-fed animals as assessed by an ipGTT (Fig. 1a). Adipocytes isolated from HFD-fed mice were separated into two

Fig. 1 Reduced insulin responsiveness in small and hypertrophic adipocytes isolated from HFD-fed mice. **a** Intraperitoneal glucose tolerance test was performed in chow-fed (grey symbols) and HFD-fed (black symbols) mice. Glucose (2 g/kg body weight) was injected intraperitoneally and blood glucose levels measured at indicated time points. Results are means±SEM of seven (chow-fed) to 24 (HFD-fed) animals per group. *$p<0.05$, **$p<0.01$ (Student's *t* test). **b** Representative photographs of adipocytes isolated from epididymal fat pads of chow-fed or HFD-fed mice (scale bar, 250 μm), with graph (**c**) showing mean adipocyte diameter of each fraction. Results represent the mean±SEM of three to four independent experiments. *$p<0.05$, **$p<0.01$ (ANOVA). **d** Adipocyte viability was assessed by an LDH and (**e**) an MTT assay. Results represent the mean±SEM of four independent experiments. **f** D-[^{14}C]Glucose incorporation into isolated adipocytes of chow-fed (grey bars), HFD-fed small (white bars) and HFD-fed large (black bars) adipocyte fractions. Results represent the mean±SEM of three to five independent experiments. *$p<0.05$, **$p<0.01$ (ANOVA) compared with insulin-stimulated glucose incorporation into adipocytes of chow-fed mice. **g** Total cell lysates were prepared from isolated adipocytes treated for 10 min with 100 nmol/l human insulin (Ins). Lysates (40 μg) were resolved by LDS-PAGE and immunoblotted with anti-phospho (p)S473-Akt, anti-pAS160 or anti-actin antibody. Representative immunoblots are shown. Membranes were exposed in an Image Reader and quantified (**h**) with Image Analyzer. Results represent the mean±SEM of three independent experiments. **$p<0.01$ (ANOVA) compared with chow-fed mice

fractions by filtration through a 60 μm nylon mesh. For each experiment, epididymal fat pads of two mice were pooled. Small adipocytes (mean diameter 60.9±3.1 μm) were significantly smaller than large fat cells (mean diameter 83.0±6.6 μm; $p<0.05$) (Fig. 1b, c). For comparison, mean diameter of adipocytes isolated from chow-fed animals was 50.7±3.3 μm, significantly ($p<0.01$) smaller only than the large adipocytes from high-fat fed mice (Fig. 1b, c). To rule out the possibility of higher occurrence of cell rupture/cell damage in one cell fraction compared with the other after filtration through the nylon mesh, cell viability was assessed by an MTT and LDH assay. As depicted in Fig. 1d and e, viability of the two adipocyte cell fractions was comparable.

No difference in insulin-stimulated glucose incorporation between small and large adipocytes isolated from HFD-fed mice In order to assess insulin responsiveness, D-[U-14C] glucose incorporation into adipocytes was determined next. The fold increase in insulin-stimulated glucose incorporation into both adipocyte fractions prepared from HFD-fed mice was greatly reduced compared with adipocytes isolated from chow-fed animals (Fig. 1f), but surprisingly there was no difference in glucose incorporation between large and small adipocytes in HFD-fed mice in response to insulin (Fig. 1f). This observation held true when the fold-increase in insulin-stimulated glucose incorporation was calculated per cell surface or cell volume (data not shown). Accordingly, the insulin-stimulated increase in phosphorylation of Akt on S473 and on T308 residues as well as of AS160 was similar in both fractions, but clearly reduced compared with adipocytes isolated from chow-fed mice (Fig. 1g, h). Similarly, protein levels of GLUT4 were not different between small and large adipocytes, but was decreased by approximately 35% compared with adipocytes from chow-fed mice (data not shown).

Increased NEFA release from large epididymal adipocytes isolated from HFD-fed mice In order to evaluate lipolysis, concentrations of NEFA and glycerol were determined in the incubation medium. Basal NEFA release per cell was significantly higher in large than in small adipocytes (Fig. 2a), a difference that was not significant when controlled for surface area. This finding suggests that the elevated lipolytic activity of the large adipocytes is a direct effect of cell hypertrophy. Similar results for basal lipolysis were obtained when release of glycerol was measured (Fig. 2b). Isoproterenol-stimulated lipolysis was significantly increased compared with basal NEFA release, but not different between small and large adipocytes from HFD-fed mice (Fig. 2a). Remarkably, the ability of insulin to inhibit lipolysis was fully blunted in large and small adipocytes compared with adipocytes from chow-fed mice (in which lipolysis was inhibited by ~30%) (Fig. 2c), a finding that is
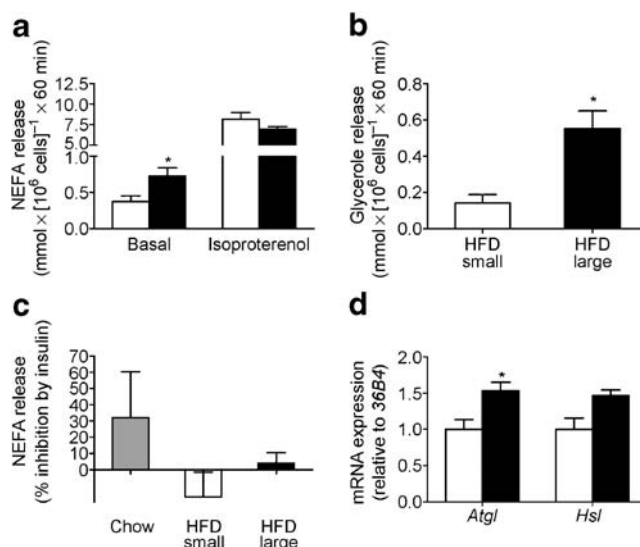

Fig. 2 Increased basal lipolysis in large adipocytes. **a** Basal and isoproterenol-stimulated NEFA release from HFD small (white bars) and HFD large (black bars) adipocytes. Results are means±SEM of five (isoproterenol) to seven (basal) independent experiments. *$p<0.05$ (Student's *t* test). **b** Basal glycerol release from HFD small and HFD large adipocytes. Results are means±SEM of three independent experiments. *$p<0.05$ (Student's *t* test). **c** Per cent inhibition of NEFA release by insulin in chow-fed, HFD-fed small and HFD-fed large adipocytes. Results represent the mean±SEM of three to five independent experiments. **d** Quantitative RT-PCR detection of *Atgl* and *Hsl* mRNA expression. The level of mRNA expression was normalised to *36B4* RNA. Results represent the mean±SEM of three independent experiments and are expressed relative to expression in the HFD-small fraction. *$p<0.05$ (Student's *t* test)

consistent with the similar degree of insulin resistance observed in signalling and glucose incorporation (Fig. 1d).

Adipose triacylglycerol lipase (ATGL) and hormone-sensitive lipase (HSL) are key players in adipocyte lipolysis. It has been suggested that ATGL and HSL may act in coordination such that ATGL is the primary lipase responsible for hydrolysing the first fatty acid from triacylglycerol and that HSL is the primary diacylglycerol lipase [12]. Alternatively, it has been proposed that HSL is the primary triacylglycerol lipase responsible for catecholamine-stimulated lipolysis from adipocytes and ATGL is the primary triacylglycerol lipase for basal lipolysis [13]. Given the differences in basal lipolysis rate between large and small adipocytes, mRNA expression levels of these two lipases were determined. There was a slight but significant increase in *Atgl* mRNA levels in large adipocytes (Fig. 2d). Similarly, *Hsl* expression tended to be higher in large adipocytes; however, this increase did not reach statistical significance.

Discussion

Adipocytes from obese persons or animals compared with those from lean persons or animals are characterised by

increased cell size and decreased insulin-stimulated glucose uptake [14, 15]. It has therefore been speculated that larger adipocytes are more insulin resistant. Although this conclusion is plausible, it is difficult to analyse the sole contribution of cell size to insulin resistance independently of other factors since cells are isolated from different individuals. It is possible that these factors, along with or independently of cell size, causatively contribute to the insulin resistance observed in adipocytes derived from the obese.

To address this point, we have characterised larger versus smaller adipocytes from the same fat depot of mice fed HFD for 8 weeks. The time span of 8 weeks of HFD was chosen to examine an early phase of obesity-induced changes in adipose tissue. Macrophage infiltration of adipose tissue and occurrence of adipocyte death may have just started at this time point [1]. Yet, 8 weeks of fat-enriched diet was already sufficient to induce whole-body insulin resistance as demonstrated by a significantly impaired ipGTT (Fig. 1a), by a reduction in insulin-stimulated glucose incorporation (Fig. 1f) and by a blunted capacity of insulin to inhibit lipolysis (Fig. 2c). Nevertheless, when comparing large to small adipocytes isolated from HFD-fed mice, we did not detect any differences in insulin responsiveness (Figs 1f and 2c). This observation was true both for insulin-mediated stimulation of glucose incorporation, as well as for the inhibition of lipolysis. It is still possible that the severity of insulin resistance differs between the extremes of adipocyte size. Yet for the cell size range of most adipocytes composing the adipose tissue of HFD mice, the data would suggest that the degree of cellular hypertrophy is not a direct determinant of severity of adipocyte insulin resistance. More probably, insulin resistance in adipocytes of HFD-fed mice could be a secondary phenomenon to alterations in the intra-fat depot environment, such as increased release of pro-inflammatory cytokines and/or NEFA. These would seem to similarly affect insulin sensitivity in larger versus smaller adipocytes within the same fat depot.

One of the main findings in the present study is the significant increase in basal lipolysis in large compared with small adipocytes (Fig. 2a, b). This finding is in accordance with a previous study showing a correlation of adipocyte size with basal rates of lipolysis [16]. The latter finding would imply increased circulating NEFA levels in patients with adipocyte hypertrophy. Indeed, enlarged adipocytes were found more frequently in patients with obesity-related metabolic disorders [17], suggesting that increased basal lipolysis of large adipocytes may directly contribute to obesity-induced metabolic alterations, potentially via deposition of lipids in liver and skeletal muscle (lipotoxicity). It would be intriguing to assess whether, by increasing local NEFA concentrations in adipose tissue, large adipocytes contribute to the insulin resistance that similarly develops in large versus small adipocytes.

Intriguingly, whereas NEFA-induced insulin resistance is a well established phenomenon in skeletal muscle and liver, results in adipocytes are somewhat controversial [18, 19], leaving this putative mechanism unsettled.

In conclusion, adipocyte hypertrophy associated with diet-induced obesity does not seem to be a direct determinant of the severity of adipocyte insulin resistance. Instead, larger adipocytes have a higher basal lipolysis rate than smaller adipocytes. Hence, of the various alterations to adipose tissue in obesity, adipocyte hypertrophy may predominantly, albeit indirectly, contribute to whole-body insulin resistance, e.g. by affecting the release of secreted products like adipokines and NEFAs.

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