Published in final edited form as:

Obesity (Silver Spring). 2011 November ; 19(11): 2121–2129. doi:10.1038/oby.2011.254.

Large Size Cells in the Visceral Adipose Depot Predict Insulin Resistance in the Canine Model

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Abstract

Adipocyte size plays a key role in the development of insulin resistance. We examined longitudinal changes in adipocyte size and distribution in visceral (VIS) and subcutaneous (SQ) fat during obesity-induced insulin resistance and after treatment with CB-1 receptor antagonist,rimonabant (RIM) in canines. We also examined whether adipocyte size and/or distribution is predictive of insulin resistance. Adipocyte morphology was assessed by direct microscopy and analysis of digital images in previously studied animals 6 weeks after high-fat diet (HFD) and 16 weeks of HFD + placebo (PL; $n = 8$) or HFD + RIM (1.25 mg/kg/day; $n = 11$). At 6 weeks, mean adipocyte diameter increased in both depots with a bimodal pattern only in VIS. Sixteen weeks of HFD+PL resulted in four normally distributed cell populations in VIS and a bimodal pattern in SQ. Multilevel mixed-effects linear regression with random-effects model of repeated measures showed that size combined with share of adipocytes $>75 \mu\text{m}$ in VIS only was related to hepatic insulin resistance. VIS adipocytes $>75 \mu\text{m}$ were predictive of whole body and hepatic insulin resistance. In contrast, there was no predictive power of SQ adipocytes $>75 \mu\text{m}$ regarding insulin resistance. RIM prevented the formation of large cells, normalizing to pre-fat status in both depots. The appearance of hypertrophic adipocytes in VIS is a critical predictor of insulin resistance, supporting the deleterious effects of increased VIS adiposity in the pathogenesis of insulin resistance.

Introduction

Obesity is characterized by an increase of fat mass, which includes morphological changes at the level of the adipocyte, such as increased cell size (hypertrophy) and/or increased number of cells (hyperplasia) (1). There is an abundance of evidence demonstrating the association of obesity, in particular central obesity, with a cluster of metabolic disorders, such as type 2 diabetes, dyslipidemia, hypertension, and atherosclerosis (2–4). However, the link between adipocyte morphology and disease risk remains unclear. Moreover, the studies

that have examined this topic appear to support contradictory conclusions. In a cross-sectional study between insulinsensitive and insulin-resistant subjects, the insulin-resistant group presented with a predominance of small adipose cells (5). Thus the authors concluded that insulin resistance is not attributable to larger hypertrophic adipocytes but the inability to store additional lipids in the face of excess energy, resulting in a population of small adipocytes. In contrast, several other studies demonstrate that hypertrophic adipocytes are associated with adverse changes in lipid metabolism and metabolic function (6,7). Independent of increased cell number, increased adipocyte size has been associated with reduced insulin sensitivity in obese animals and subjects (8,9), which could be due in part to enhanced expression and secretion of proinflammatory cytokines from hypertrophic adipocytes (10). For example, Skurk *et al.* (11) show that when human subcutaneous (SQ) adipocytes are separated according to cell size, very large hypertrophic adipocytes exhibit markedly impaired adipokine secretion, promoting inflammation. Albeit, evidence exists supporting a role for small and large adipocytes being linked to obesity-related insulin resistance.

The discrepancy in these findings may in part be due to most studies being cross-sectional, rendering it difficult to establish time relationships and causality between cell size and insulin resistance. In addition, for obvious reasons, most human studies examine adipocyte cell size in SQ tissue but not in the visceral (VIS) depot (12). In our canine model, we can easily harvest tissue from both SQ and VIS fat depots.

Here, exploiting our canine model of obesity, we have the opportunity to longitudinally examine adipocytes during the induction of obesity-related insulin resistance and after pharmacologic reversal of obesity and insulin resistance with drug treatment (12).

We have previously shown in the canine model that 6 weeks of fat feeding increases both VIS and SQ fat deposition with a concomitant reduction in insulin sensitivity (13). Moreover, we demonstrated that rimonabant (RIM), a selective type 1 cannabinoid receptor antagonist reduced both body fat and weight (14) along with improving insulin sensitivity (15). The present study extends our earlier findings by examining the role of adipocytes during high-fat feeding and CB-1 antagonism on a cellular level. Moreover, given that adipocyte cell morphology may play a role in the development of insulin resistance, we set out to elucidate whether adipocyte cell size and/or distribution may be predictive of insulin resistance.

Methods and Procedures

Animals and diet

Data depicting metabolic changes have been previously published (14). Briefly, 20 male mongrel dogs (body weight 29 ± 0.9 kg) were housed in the Keck School of Medicine, University of Southern California (Los Angeles, CA) vivarium under controlled kennel conditions (12:12-h light-dark cycle). Animals were surgically outfitted with chronic indwelling catheters for blood sampling and infusions. Experimental protocols were approved by the University Institutional Animal Care and Use Committee.

Upon arrival, dogs underwent a period of acclimation for 3 weeks, during which they were fed a standard diet. The standard diet consisted of one can of Hill's Prescription Diet (Hill's Pet Nutrition, Topeka, KS) and 825 g of dry chow (Wayne Dog Food; Alfred Mills, Chicago, IL). This standard diet was given as a 3-week acclimatization, weight maintenance diet and consisted of (~3,885 kcal/day, 39% carbohydrates, 33% lipids, and 27% proteins). Meals were presented daily for 3 h, from 9:00 am to 12:00 pm.

After 3 weeks of standard diet we fed the dogs high-fat diet (HFD) which consisted of ~5,527 kcal/day (28% carbohydrates, 53% lipids, and 19% proteins), representing an ~60% increase in calories from fat. The 3-h daily feeding period was maintained. Food intake measurements were recorded daily and body weight measurements were recorded weekly. Previously we have shown that 6 weeks of fat feeding promotes a consistent increase of fat in the abdominal trunk and SQ depot rendering the dog insulin resistant (13). Thus, the HFD was maintained for a minimum of 6 weeks (average: 9 ± 2 weeks) period before any treatment.

Treatment

After the initial establishment of obesity with the HFD, dogs were randomly divided into two treatment groups while still being maintained on the HFD for an additional 16 weeks: RIM, HFD+RIM ($n = 11$), or placebo (PL), HFD+PL ($n = 9$). Animals were matched for body weight (31.7 ± 1.3 and 31.8 ± 1.5 kg, $P = \text{NS}$; HFD+RIM (W16) vs. HFD+PL (W16) group, respectively). RIM (Sanofi-Aventis, Paris, France) was encapsulated (AMC Pharmacy, Burbank, CA) and administered orally at 1.25 mg/kg/day. PL-treated animals received inert capsules. The dose of RIM was chosen based on a pilot study carried out in a small group of dogs ($n = 5$), testing different doses ranging from 5 to 1.25 mg/kg. We chose 1.25 mg/kg, as this dose did not produce any observable clinical adverse effects (14).

Body weight and metabolic parameters

As previously reported (14), we performed daily recordings of food intake throughout the study as well as weekly recordings of body weight. All animals underwent various metabolic assessments in random order, prior to fat feeding (week -6); after an initial fat feeding period to establish obesity (week 0) and following the continuation of fat feeding for an additional 16 weeks with +RIM or without (PL) treatment (week 16). At each of the three designated time points (weeks -6, 0, and 16) biopsies were taken from SQ and VIS fat depots, excising ~2 g of adipose tissue. Biopsies were conducted under general anesthesia with appropriate monitoring. Metabolic assessment included the euglycemic hyperinsulinemic clamp that allowed for quantification of insulin sensitivity. The clamp studies were performed in 18 h fasted dogs that were fully conscious and resting comfortably in a Pavlov sling. All studies and plasma measurements were performed after an overnight fast (~12 h).

Abdominal trunk composition

The amount of fat and nonfat tissues in the abdominal trunk were quantified by magnetic resonance imaging (MRI) using a 1.5 T Gemsow Scanner (General Electric, Milwaukee, WI), as previously published (14).

Adipocyte isolation

VIS and SQ adipocytes were isolated and examined during the pre-fat (week 6 (W6)) period, after establishment of increased body weight and adiposity and before initiation of drug with HFD (W0), and thereafter at weeks 16 of treatment (W16). Adipocytes were isolated according to the method performed by Rodbell (16), in a Krebs–Ringer bicarbonate buffer (pH 7.4) containing 3.5% bovine serum albumin, plus 1 mg/ml collagenase (type II; Sigma-Aldrich, St Louis, MO). Digestion took place in a shaking water bath for 40 min at 37 °C. The suspension was then filtered and the cellular filtrate obtained was washed three times with 15 ml of Krebs buffer, the isolated cells were not centrifuged. Approximately 1 ml of pure fat cell suspension was obtained and resuspended in 6 ml Krebs buffer (solution A). Three drops, 10 µl aliquots each of solution A were placed on a glass slide and 300–1,000 cells were photographed and measured using the Olympus CKX41 inverted microscope. Cell diameters were counted using Image-Pro Express software (Media Cybernetics, Bethesda, MD), lipids in a 500 µl aliquot of solution A were extracted according to the method of Dole and Meinertz for cell number determination (17) and the lipid content was determined gravimetrically. The number of fat cells incubated was obtained by dividing the mean lipid content of the incubation tubes by the mean fat cell weight, assuming that lipids constitute >95% of the fat cell weight. Additionally, we estimated the number of cells by the following formula: cell number = volume of the depot (SQ or VIS)/weighted volume per cell. Volume of adipose tissue was obtained from MRI analysis and average volume per cell was calculated as a weighed volume based on the relative share per bin from the cell size histogram generated by the univariate normal mixture decomposition method (18).

Euglycemic–hyperinsulinemic clamps

The euglycemic–hyperinsulinemic clamps were performed as previously described (15) during weeks –6, 0, and 16 of the study.

Rates of glucose disposal (Rd) and endogenous glucose production (EGP) were calculated using Steele's equation as modified for use with labeled glucose infusion. Insulin sensitivity was calculated as previously described (19). Whole body insulin sensitivity (combined actions of insulin to stimulate Rd and suppress EGP) was defined as (SI_{GINF}) and expressed as:

$$SI_{GINF} = \frac{GINF_{ss}}{\Delta Insulin \times Glucose_{ss}},$$

where Insulin_{plasma} represents the increment in steady-state plasma insulin concentration above basal and Glucose_{ss} is the steady-state glucose concentration. Similarly, SI_{EGP} (insulin's action to suppress EGP) and SI_{Rd} (insulin's action on peripheral (muscle) glucose uptake) are expressed as:

$$SI_{EGP} = \frac{\Delta EGP_{ss}}{\Delta Insulin \times Glucose_{ss}} \quad SI_{Rd} = \frac{\Delta Rd_{ss}}{\Delta Insulin \times Glucose_{ss}},$$

Where EGP and Rd reflect change from basal ($t = -30$ min before insulin infusion) to steady state (last 30 min of clamp).

Data and statistical analyses

Metabolic parameters—For body weight, MRI, and insulin sensitivity, nonpaired t -tests were used to compare means between groups. All the analyses were done using Statistica (StatSoft, Tulsa, OK). Differences were assumed to be significant if the P value was <0.05 .

Adipocyte cell number and distribution—We used the generalized least squares random effects of repeated measures linear regression to compare cell numbers. Prior to this study there was very limited data available in regards to the adipocyte cell size and distribution in the canine model. Additionally, the longitudinal changes in adipocyte cell size and distribution in this model were virtually unknown. To establish average trends in cell size and distribution we pooled all the data per week and employed the univariate normal mixture decomposition method developed by Stanislav Kolenikov for STATA 10, (StataCorp, College Station, TX) to quantify the general trends in adipocyte cell size and distribution. The multiple normal distribution model that best explained the data histogram was chosen based on the lowest P value of the χ^2 -test. After establishment of trends from the initial analysis, the univariate normal mixture decomposition method was applied again using a maximum of four normal distributions to cells grouped by four criteria: (i) animal, (ii) type of adipose tissue (VIS or SQ), (iii) time (weeks), and (iv) treatment (PL or RIM). Thus, we were able to obtain unique multiple normal distributions for each animal at each week and following treatment with RIM (selective CB-1 antagonist) which were used in the subsequent statistical analysis.

Adipose depot volume calculation normalized by volume of adipocytes—

Specific adipose depot (SQ or VIS) volume as measured by MRI methodology is equivalent to the total volume of all adipocytes within that particular depot. However, partitioning of adipocyte sizes (small, normal, large, and very large) within a depot cannot be discerned from the MRI. Thus, we calculated the partitioning by cell size within each depot according to the following formulas.

$$n_{\text{aliquots}} = \frac{V_{\text{depot_MRI}}}{V_{\text{aliquot}}} \quad (1)$$

$$V_{\text{depot}} = \sum_i n_{\text{aliquots}} \cdot n_{\text{cells}} \cdot \text{share}_i \cdot \frac{4}{3} \cdot \pi \cdot \left(\frac{\mu_i}{2} \right)^3, i = \text{small, normal, large, X-large} \quad (2)$$

Partitioning was based on MRI depot volume ($V_{\text{depot_MRI}}$) relative to the measured volume of the 10 μl aliquot (V_{aliquot}) isolated from that specific fat depot (Equation 1). Each adipocyte size partition volume was calculated as the product of the total number of aliquots (n_{aliquots}), total number of cells within one aliquot (n_{cells}), the share of specific adipocyte cell size partition, share_i , and the volume for the average adipocyte for the given partition.

The sum of all partition volumes equaled the total volume (V_{depot}) per fat depot (Equation 2).

Estimation of insulin resistance prediction indices

We used multilevel mixed model linear regression longitudinal model with random-effects model of repeated measures per week to examine the relationship between each adipose-cell population and morphological characteristics (i.e., share, family of adipocyte cell and size) and the degree of insulin resistance. To predict changes in SI_{GINF} , SI_{EGP} , and SI_{Rd} , we considered the following independent variables: (i) each adipose depot (VIS or SQ), (ii) number of weeks on diet, (iii) number of normal distributions of adipocytes in each depot, (iv) mean of the normal distribution with largest cells, and (v) share (or percentage) of normal distribution with large cells. The animal's identification number was the clustering variable for the random-effects model. Mean normal distribution and share of the small population of cells were not considered due to insufficient number of observations.

Results

Body weight, adiposity, and insulin sensitivity

The general effects of fat feeding and treatments on body weight, adiposity, and insulin sensitivity are presented in Table 1. Consistent with previous work (20), 6 weeks of fat feeding was sufficient to observe increased body weight and adiposity in both VIS and SQ fat depots. As expected, insulin sensitivity (SI_{GINF} , SI_{Rd} , and SI_{EGP}) decreased significantly with fat feeding.

Animals in HFD+PL (W16) group increased their body weight by 1.78 ± 0.43 kg ($P < 0.001$) whereas HFD+RIM (W16) treated dogs lost ~ 0.90 kg. In addition, HFD+RIM (W16) decreased their VIS depot by 14% ($P < 0.01$), whereas the volumetric decrease in the SQ depot was more pronounced 21% ($P < 0.001$) compared to HFD+PL (W16) group. RIM increased SI_{GINF} by 18% compared to week 0 ($P = 0.056$). Similarly, treatment with RIM increased SI_{EGP} by 38% ($P < 0.05$). However, RIM treatment did not change peripheral insulin resistance (SI_{Rd}). Insulin sensitivity remained low in untreated animals (HFD+PL (W16)) with continuation of fat feeding and did not decrease any further. As previously published (14), caloric intake was shown to be similar between HFD+PL and HFD+RIM groups by week 16, at the time of euthanasia and adipocyte analysis.

Adipocyte cell number and distribution

Cell number—The fat cell number/mg lipid in VIS and SQ depots was measured utilizing Dole extraction. Cell number remained relatively constant throughout fat feeding and treatment phases in the SQ depot. However, we observed a modest increase in the VIS depot in HFD+RIM group ($P < 0.05$) (Table 2). Average cell diameter was used to calculate cell number per mg of lipid. In addition, we estimated the cell number per depot by dividing the volume of the depot by weighted average volume per cell (18). Since we identified multiple normal distributions for adipocytes in both depots, we decided that the best average cell volume would be the average of the means for each bin of adipocytes normalized by their share (Table 2). This calculation demonstrated an increase of average cell volume in both

SQ and VIS depots in HFD+PL and a decrease in HFD+RIM groups. However, this calculation masks the dynamic remodeling of the adipocytes by ignoring the importance of the share, therefore this calculation was used only to estimate the cell number per depot. The cell number per depot remained unchanged with diet or drug treatments at week 16 compared to pre-fat diet. However, there was a small increase in SQ fat after 6 weeks of fat diet ($P < 0.05$) (Table 2).

Adipocyte distribution

VIS fat: Utilizing the univariate normal mixture decomposition method we characterized adipocyte size distribution for the grouped observations of cell size per week and type of adipose tissue as illustrated in Figures 1 and 2. Each identifiable significant normal distribution of cells is characterized by the mean diameter = μ , the s.d. of the distribution (sigma) = σ , and the fraction of the cells accounted for by the specific distribution, percentage (%) = share. We arbitrarily assigned each identifiable normal distribution as a family or population of small, normal, large, or very large (or X-large) size cells. In the VIS fat depot, during the pre-fat period (W6), we identified one major population of normal size cells that accounted for 91% of the total number of cells: (Figure 1a) ($\mu_1 = 71.85$, $\sigma_1 = 11.75$, share 1 = 0.91). Three additional families were observed, consisting primarily of small, large, and very large cells, but these populations were very small; when combined, they contributed <10% of total cells ($\mu_2 = 98.59$, $\sigma_2 = 14.12$, share 2 = 0.05, $\mu_3 = 33.54$, $\sigma_3 = 7.42$, share 3 = 0.035, $\mu_4 = 127.01$, $\sigma_4 = 23.60$, share 4 = 0.003). After 6 weeks of fat feeding (W0), the four distinct families of cells were still evident but instead of one major family of normal size cells accounting for over 90% of total cells, two major distribution (bimodal) patterns existed, one consisting primarily of normal cells accounting for 53% of total and the other of large cells, now representing 45% of the total ($\mu_1 = 64.65$, $\sigma_1 = 11.08$, share 1 = 0.53; $\mu_2 = 90.46$, $\sigma_2 = 14.09$, share 2 = 0.45). The other two minor families, characterized as small and very large cells, represented <1% of the total cell population ($\mu_3 = 27.11$, $\sigma_3 = 4.22$, share 3 = 0.009, $\mu_4 = 170$, $\sigma_4 = 31.42$, share 4 = 0.005) (Figure 1b). After an additional 16 weeks of fat feeding (HFD+PL) (Figure 1c), the four families were still evident, but with higher mean sizes compared to the initial 6 weeks of fat feeding (W0); there are two major ($\mu_1 = 75.49$, $\sigma_1 = 11.63$, share 1 = 0.62, $\mu_2 = 96.02$, $\sigma_2 = 22.14$, share 2 = 0.32) and two minor distributions ($\mu_3 = 29.61$, $\sigma_3 = 6.55$, share 3 = 0.04; $\mu_4 = 184.41$, $\sigma_4 = 21.63$, share 4 = 0.003). Impressively, despite the high-fat diet, the CB-1 receptor inhibitor RIM (Figure 1d) restored the distribution pattern to pre-fat conditions, eliminating the multiple families of cells. The shape and mean of the distribution of cells after RIM were virtually identical to the pattern of adipocyte distribution observed before switching to the high-fat diet.

Following 16 weeks of HFD+RIM treatment, the sizes of cells were again represented by a single major family ($\mu_1 = 75.75$, $\sigma_1 = 15.54$, share 1 = 100). Thus, CB-1 inhibition with RIM totally prevented the appearance of large cells despite the continuation of the high-fat diet.

SQ fat: Figure 2 shows the adipocyte distribution in the SQ fat over a period of 16 weeks on fat diet with or without RIM. Recapitulating VIS adipose, during the pre-fat period (Figure

2a) there was one normal size family that best explained the distribution of VIS adipocytes, with a slightly lower mean size compared to the VIS depot ($\mu_1 = 65.53$, $\sigma_1 = 44.48$, share 1 = 100). After 6 weeks of HFD, the model identified two populations, the major family representing 99% of the total adipocytes and a minor population of very large cells representing <1% ($\mu_2 = 145.72$, $\sigma_2 = 44.48$, share 2 = 0.007) (Figure 2b). After 16 weeks of HFD+PL (Figure 2c), the major family of average-normal size cells explained 72% of the total distribution ($\mu_1 = 69.92$, $\sigma_1 = 10.24$, share 1 = 0.72) and the population of larger cell types accounted for 28% of the total cell population ($\mu_2 = 90.50$, $\sigma_2 = 24.12$, share 2 = 0.28). Similar to VIS fat, HFD+RIM (W16) treatment (Figure 2d) restored all the cells to a single population, preventing the appearance of multiple families in the SQ tissue ($\mu_1 = 66.59$, $\sigma_1 = 12.78$, share 1 = 100).

Adipose depot volume partitioning by families of adipocytes cell size

We estimated the partitioning of the total volume of VIS and SQ depots using adipocyte volume as described above (Figure 3). Before fat feeding, the total volume of large size cells represented only 10% of the total cells in the VIS depot (Figure 3a). But, after 6 weeks of fat feeding, the volume of normal-average size cells decreased by more than half, whereas the volume of large cells increased sevenfold. In addition, the volume of very large cells increased by fourfold after 6 weeks of fat feeding. Sixteen additional weeks of HFD+PL (W16) resulted in doubling the size of VIS adipose depots. The twofold increase in the VIS depot consisted of normal size adipocytes, similar to pre-fat (W6) sizes, while the fraction of large and very large size cells remained the same as week 0. In the SQ depot, the normal cell volume remained virtually the same, however, the large size cell volume increased by 13-fold after 16 weeks of HFD+PL (W16). Interestingly, in the HFD+RIM (W16) group there was complete elimination of large and very large adipocytes in both VIS and SQ depots.

Insulin resistance prediction indices

It was of interest to ask whether cell size could predict insulin resistance. Table 3 shows the estimated prediction indices. Of the morphological traits considered only the share of the VIS depot cells larger than 75 μm was related to insulin resistance per given week on high-fat diet period. Share of adipocytes >75 μm was able to predict hepatic insulin resistance (SI_{EGP}) per week of high-fat diet. In contrast to large VIS adipocytes, none of the cell populations in the SQ adipose depot had any predictive power of insulin resistance. Furthermore, there was no significant relationship between the morphological traits in the group that was treated with RIM, as very few dogs expressed fractions that had a mean size >75 μm . Thus, fat feeding caused the appearance of large cells in the VIS and SQ fat depots, but only VIS fat correlated with the degree of hepatic insulin resistance in a given week of high-fat feeding.

Discussion

In the present longitudinal study, we demonstrate in the canine model that adipocytes from the VIS depot are larger and their distributions are strikingly different from SQ adipocytes. Additionally, the partitioning of adipocytes in regards to their size varied each week and with respect to the type of adipose depot. Moreover, a critical finding in the present study is

that we found morphological traits of adipocytes larger than 75 μm in only the VIS fat depot to be independent predictors of whole body as well as hepatic insulin resistance. Hence, our data suggest that VIS hypertrophic adipocytes may play a key role in the development or impairment of insulin sensitivity in the canine model and lend itself to the understanding of the induction of insulin resistance in obese humans (8).

Increased adiposity is a well-characterized abnormality that negatively impacts insulin sensitivity, yet little is known regarding the morphological traits of the adipose depot that lead to impairment of insulin sensitivity. Previous studies demonstrate that the mean adipocyte size is inversely related to insulin sensitivity (8). However, to date most studies examining the role of adipocyte morphology and insulin resistance have been crosssectional and primarily limited to SQ fat tissue. These findings have yielded a wide range of interpretations, from large hypertrophic (21) to numerous small adipocytes (5) being associated with obesity-related insulin resistance.

Our MRI analyses shown in Table 1 demonstrate that fat feeding increases fat volume in both VIS and SQ depots. However, there were qualitative cellular differences in these depots that could not be detected by MRI. Based on our calculation of cell number using Dole extraction (cell number per mg of lipid) we found that cell number was unchanged in the SQ group. We demonstrated a modest increase in cell number in the HFD+RIM group. Given that we identified multiple normal distributions for adipocytes in both depots, our estimation of fat cell number/mg lipid was based on average adipocytes diameter. Cell number estimation per depot was based on the average of the means for each bin of adipocytes normalized by their share. Hence, this estimation of cell number is limited in scope because the share of each subfamily cannot be discerned and therefore fails to convey a crucial aspect of our study, which is dynamic remodeling of adipocyte distribution with fat feeding. Therefore, we limited the use of these numbers only to the estimation of cell number. The estimation of cell number by dividing volume of the depot (SQ or VIS) by weighted volume per cell, showed a modest increase of cell number after 6 weeks of fat diet in SQ. By week 16, the cell number returned to pre-fat values.

In the VIS depot we observed cells $>90\ \mu\text{m}$ and no hyperplasia at week 0. Thus, our data suggest that during the initial weeks of fat feeding, adipose tissue expansion in the VIS depot may be attributed to adipocyte enlargement or hypertrophy. In contrast, we did not observe adipocytes $>90\ \mu\text{m}$ in SQ fat during the early stages of fat feeding but a modest increase in mean cell size and number, these two factors could account for the increase in fat volume. The cause of cell loss by week 16 in the SQ depot is unknown, but we speculate that it could be attributed to apoptosis. Recently, Alkhouri *et al.* (22) demonstrated that feeding mice either a high-fat diet or a high-sucrose diet, which typically results in obesity, insulin resistance, were associated with a marked increase in caspase 3 activation in adipocytes and apoptotic cell death. Nevertheless, the statistically significant increase of volume and mass of the SQ depot after 16 weeks of fat feeding in the PL group as identified by the MRI methodology is best explained by hypertrophy and not hyperplasia since the number of cells returned back to the pre-fat diet level.

The adipocyte distribution patterns were shown to be different between VIS and SQ depots. At week 0, the volume increase in the VIS depot could have been driven by expansion of cells >90 μm . The emergence of large cells >90 μm in SQ was observed by week 16 and not during the earlier weeks of fat feeding. Hence, we are led to believe that during overfeeding, fat accumulation or adipocyte enlargement begins in the VIS compartment. This observation gives credence to our previously described “overflow hypothesis” (23), which states that during fat accumulation, filling occurs first in the VIS depot and then “overflows” into the SQ depot. It can therefore be postulated that once adipocytes in the VIS depot reach a limit to store lipids, excess fat is stored in the SQ depot. In contrast, other investigators contend that filling occurs first in the SQ depot and then spills over into VIS and ectopic areas such as liver and muscle (24). It is possible that the time course of filling depots can best be observed in longitudinal, as opposed to cross-sectional studies. On the other hand, there could be a difference between species in the time course of filling of VIS vs. SQ depots. This latter notion is supported by the apparent relative differences in VIS and SQ adipose tissue volumes in dogs when compared to humans.

Noteworthy is that cell number and mean size were both shown to be increased in SQ at week 0 with both being normalized by week 16 in the RIM group. At week 16, insulin sensitivity was also improved with RIM, presumably due to the elimination of hypertrophic cells. In addition, we observed a modest increase of adipocyte number with RIM treatment as measured by mg of lipid in the VIS depot. However when we estimated the cell number per depot there was no difference in cell number at week 16. This observation was supported by the MRI results that showed no increase of fat mass by RIM treatment. Therefore, we consider the slight increase in cell number as a potential artefact of the Dole methodology for calculation of cell number, which depends on the average adipocyte volume and not the weighted average of adipocyte. Although data is limited regarding CB-1 antagonism on adipocyte proliferation, several studies have shown that RIM actually decreases adipocyte proliferation (25,26). To our knowledge the molecular mechanism that may have resulted in normal size adipocyte proliferation with RIM treatment remains unknown. Thus, presently we conclude that the major effect of RIM was to eliminate hypertrophic dysfunctional fat cells. However, in the PL group, cell number was normalized while hypertrophic cells were still evident along with reduced insulin sensitivity. Together, these data suggest that cell size is associated and perhaps influences insulin sensitivity.

In addition to differential fat deposition between the two studied depots (VIS and SQ), we also observed variation in adipocyte distribution patterns. As evidenced in Figure 1, the VIS depot displayed greater heterogeneity, possibly underscoring the relative importance of increased metabolic activity associated with VIS compared to SQ (27). In the VIS depot, a bimodal pattern was observed after 6 weeks of fat feeding and by week 16, there were 4 distinct patterns. In contrast, SQ consisted of one major normal distribution pattern after the initial 6 weeks of fat feeding and displayed a bimodal pattern by week 16, due to the appearance of a small population of very large cells (>90 μm). Remarkably, RIM reversed the effects of the high-fat diet in both depots, returning distribution patterns to pre-fat states by elimination of large adipocyte populations >75 μm . Consistent with our observations in dog, similar findings have been noted with weight loss. Pasarcia *et al.* also identified four

adipocyte subfractions (small, medium, large, and very-large) in SQ adipose tissue from patients with type 2 diabetes (24). Moreover, with weight loss treatment, they demonstrated a reduction in size of the large adipocytes subfraction, which was associated with improvements in insulin sensitivity (21).

The mechanism by which RIM prevents fat accumulation and maintains or decreases cell size is unknown. RIM did not change basal lipolysis in the fat-fed dog (data not shown). Alternatively, lipogenesis, re-esterification, and oxidation pathways need to be investigated. In addition, RIM may alter lipoprotein lipase activity, whereas CB-1 activation has been shown to increase lipoprotein lipase activity in primary mouse adipocytes (28). Another proposed mechanism may be mediated by adiponectin; we have shown in a separate study that plasma adiponectin as well as adiponectin gene expression in the VIS fat were significantly increased by RIM treatment in the fat-fed dog (29). Adiponectin, being a known insulin sensitizer and potent anti-inflammatory molecule may prevent inflammation and cell death, thereby maintaining normal size cells (30). More importantly, given that RIM eliminated large cell types along with a concurrent improvement in insulin sensitivity, suggest that hypertrophic adipocytes especially in the VIS depot hold an important implication in obesity.

To our knowledge, we are the first to demonstrate that adipocytes larger than 75 μm and those found only in the VIS depot are associated with whole body and hepatic insulin resistance. Moreover, we show that partitioning of the adipose depot into subfractions of morphologically distinct adipocytes to be a dynamic longitudinal process. Our findings are consistent with several studies (31,32) that suggest excess fat accumulation in the abdominal region (VIS) is a better predictor of insulin resistance than excess fat in lower body (SQ). These latter reports have recently been substantiated by improvement in insulin sensitivity with removal of VIS or omental fat in rats (33), humans (34), and dogs (35). VIS adipocytes in the RIM group did not demonstrate any significant predictive power, because the population of cells with $>75 \mu\text{m}$ were virtually eliminated by treatment, which further supports the notion that large cell types play a negative role. In addition, none of the traits of the SQ adipocyte depot showed any predictive power though there was a clear correlation between the size of SQ adipocyte and peripheral insulin sensitivity.

The present findings provide additional support for the portal hypothesis, whereas large adipocytes from the VIS depot may lead to increased portal vein delivery of free fatty acids to the liver, which would contribute to hepatic insulin resistance (23,36). Although we did not determine metabolic function and secretory patterns in the observed four populations of cells (small, average, large, and very large), it may be expected that VIS hypertrophic cells are dysfunctional and release inflammatory adipokines and thereby contribute to hepatic insulin resistance, supported by the improvement of sensitivity with elimination of hypertrophic cells.

This notion has not been totally clarified due to most studies being conducted in SQ rather than the VIS depot. It has been demonstrated that hypertrophic adipocytes in SQ release inflammatory adipokines (37). Hence, it would be of interest to study how cell populations

in the VIS depot differ in their metabolic function and secretory patterns of adipokines and consequently, the development of obesity-related insulin resistance.

Our findings are at odds with others reporting that smaller adipocytes are crucial determinants in insulin sensitivity. We found only minute amounts of small adipocyte cells regardless of the adipose depot (Figures 1 and 2). These differences may be attributed to the adipocyte size determination technique, such as the Coulter counter vs. collagenase and light microscopy. Limitations with collagenase digestion include using fresh tissue, making the method inconvenient for large-scale tissue handling, potential for the collagenase to eliminate very large cells and limited visualization of very small adipocytes ($<10\text{ }\mu\text{m}$). Alternatively, the Coulter counter technique is an advantageous and very efficient method, utilizing very small amounts of tissue with the ability to count thousands of cells. More importantly, this technique can detect very small cells. For example, 30–40% of the adipocytes detected with the Coulter counter are very small cells which are not commonly seen with light microscopy or electron microscopic methods (38).

In summary, we demonstrate that during diet-induced insulin resistance in the canine model, VIS adipocytes become significantly larger and present a different distribution pattern compared to the SQ depot. Morphological traits large adipocytes, defined as $>75\text{ }\mu\text{m}$ in the VIS depot were found to be predictors of insulin resistance, providing additional information regarding the role of VIS fat and metabolic risk. CB-1 receptor antagonism was effective in reducing adipocyte cell size and subsequently normalizing distribution, a potential mechanism to improve insulin sensitivity. Thus, the appearance of hypertrophic adipocytes in the VIS depot provides a potential link to the development of insulin resistance.

Acknowledgments

We express our extreme gratitude to Dr Erlinda Kirkman for her excellent veterinarian expertise in facilitating these studies. We are very grateful to radiologist Linda Needham for her help with the MRI scans and to Rita Thomas for her excellent assistance with the MRI images. We also thank our lead laboratory animal technician Edward Zuñiga and his assistant Edgardo Paredes. This work has been supported by Sanofi-aventis and grant from the National Institutes of Health (NIH DK029867).

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Visceral adipocyte distribution

Figure 1.

Visceral adipocyte distribution in **(a)** pre-fat dogs at week -6, **(b)** after 6 weeks of fat diet (HFD (W0)), and **(c)** after 16 weeks of HFD+PL (W16) or HFD+RIM (W16) treatments. HFD, high-fat diet; PL, placebo; RIM, rimonabant.

Subcutaneous adipocyte distribution

Figure 2. Subcutaneous adipocyte distribution in (a) pre-fat dogs at week -6, (b) after 6 weeks of fat diet (HFD (W0)), and (c) after 16 weeks of HFD+PL (W16) or HFD+RIM (W16) treatments. HFD, high-fat diet; PL, placebo; RIM, rimonabant.

Figure 3.

Depot volume partitioning for (a) visceral and (b) subcutaneous depots. Based on previously identified mean adipocyte volume and share of the four adipocyte families (small, normal, large, and very large adipocytes) for grouped adipocytes, for all dogs and average MRI per week and treatment. Adipose depot partitioning in pre-fat (W6), after 6 weeks of HFD (W0) and after 16 weeks of treatments (HFD+PL (W16)) or (HFD+RIM (W16)). HFD, high-fat diet; PL, placebo; RIM, rimonabant.

Table 1

Body weight, abdominal trunk composition, and insulin sensitivity (SI_{GINF} , SI_{Rd} and SI_{EGP}), in pre-fat (W-6), high-fat (HFD (W0)), HFD + placebo (W16), and HFD + rimonabant (W16) ($n = 20$)

	Pre-fat (W6)	HFD (W0)	HFD+PL (W16)	HFD+RIM (W16)
Body weight (kg)	29.6 $\pm$ 0.8	31.8 $\pm$ 0.9*	33.9 $\pm$ 1.9	30.8 $\pm$ 1.1 [†]
VIS (cm ³)	447 $\pm$ 35	506 $\pm$ 70*	641.0 $\pm$ 103.8	555.9 $\pm$ 63.9 ^{††}
SQ (cm ³)	285 $\pm$ 27	374 $\pm$ 75**	480.8 $\pm$ 97.5	379.0 $\pm$ 48.9 [‡]
SI_{GINF} (dl/kg/min/pmol/l)	6.03 $\pm$ 0.47	3.59 $\pm$ 0.37***	4.05 $\pm$ 0.54	4.36 $\pm$ 0.38
SI_{Rd} (dl/kg/min/pmol/l)	3.98 $\pm$ 0.32	2.70 $\pm$ 0.42**	3.03 $\pm$ 0.55	3.25 $\pm$ 0.38
SI_{EGP} (dl/kg/min/pmol/l)	1.97 $\pm$ 0.30	0.72 $\pm$ 0.18*	1.01 $\pm$ 0.20	1.17 $\pm$ 0.20 [‡]

Values are means $\pm$ s.e.m.

SQ, subcutaneous adipose tissue; VIS, visceral adipose tissue.

* $P < 0.001$ vs. pre-fat;

** $P < 0.05$ vs. pre-fat;

*** $P < 0.0001$ vs. pre-fat;

[†] $P < 0.001$ vs. post-fat;

^{††} $P < 0.01$ vs. post-fat;

[‡] $P < 0.05$ vs. post-fat.

Assessments of fat cell number in visceral and subcutaneous depots throughout the experimental protocol

Table 2

	Subcutaneous					Visceral			
	Pre-fat (W6)	HFD (W0)	HFD+PL (W16)	HFD+RIM (W16)	Pre-fat (W6)	HFD (W0)	HFD+PL (W16)	HFD+RIM (W16)	
Fat cell number / mg of lipid	7,070 ± 787	7,172 ± 1,041	5,645 ± 1,314	7,466 ± 1,454	4,783 ± 501	5,076 ± 577	3,757 ± 735	5,815 ± 856*	
Weighted average volume of single adipocyte (cm ³)	$8.13 \times 10^{-7} \pm 9.23 \times 10^{-8}$	$8.38 \times 10^{-7} \pm 1.77 \times 10^{-7}$	$1.13 \times 10^{-6} \pm 2.08 \times 10^{-7}$ *	$6.92 \times 10^{-7} \pm 3.40 \times 10^{-7}$ *	$1.14 \times 10^{-6} \pm 1.12 \times 10^{-7}$	$1.25 \times 10^{-6} \pm 2.07 \times 10^{-7}$	$1.54 \times 10^{-6} \pm 2.42 \times 10^{-7}$ *	$8.26 \times 10^{-7} \pm 3.9 \times 10^{-7}$ *	
Fat cell number/ depot	$3.82 \times 10^8 \pm 4.31 \times 10^7$	$5.24 \times 10^8 \pm 1.04 \times 10^8$ *	$3.87 \times 10^8 \pm 1.25 \times 10^8$	$8.82 \times 10^8 \pm 2.56 \times 10^8$	$4.49 \times 10^8 \pm 4.46 \times 10^7$	$5.25 \times 10^8 \pm 1.05 \times 10^8$	$4.85 \times 10^8 \pm 1.27 \times 10^8$	$1.00 \times 10^9 \pm 2.63 \times 10^8$	

Values are means ± s.e.m. Cell number per mg of lipid, necessary additional indices for cell number calculation per depot (weighted average volume of single adipocyte) and cell number based on depot volume per average adipocyte volume ($n = 20$).

* $P < 0.05$ vs. pre-fat.

Table 3

Longitudinal predictions of SI_{GINF} and SI_{EGP} using the specific diameter and share of adipocytes >75 μm per animal per depot as independent variables

	Regression coefficients	P	CI 95%
<i>Predictors of SI_{GINF}</i>			
Week 0	-2.02 ± 0.54	0.000	-3.09 to -0.94
Week 16	-2.22 ± 0.79	0.005	-3.79 to -0.65
VIS large adipo.diameter (μm)	-0.08 ± 0.04	0.032	-0.16 to -0.00
VIS share	-1.84 ± 0.88	0.037	-3.58 to -0.11
<i>Predictors of SI_{EGP}</i>			
Week 0	-0.85 ± 0.21	0.000	-1.27 to -0.42
Week 16	-0.64 ± 0.26	0.014	-1.15 to -0.13
VIS share	-0.96 ± 0.41	0.021	-1.78 to -0.14

Only the multilevel mixed-effects linear regressions with statistically significant coefficients of regression are presented. Analysis was performed using multiple linear regressions with random-effects model of repeated $\times$ measures. Regression coefficients are presented as means $\pm$ s.e.m. Statistical model predicting SI_{GINF} = week-6 + week 0 $\times$ (0.1) + week 16 $\times$ (0.1) + VISLrgAdipocyteDiameter $\times$ diameter + VIS Share $\times$ share + $b_i + \epsilon_i$. Statistical model predicting SI_{EGP} = week-6 + week 0 $\times$ (0.1) + week 16 $\times$ (0.1) + VISShare $\times$ share + $b_i + \epsilon_i$.