Subcutaneous adipose tissue imaging of human obesity reveals two types of adipocyte membranes: Insulin-responsive and -nonresponsive

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In adipose tissue, resistance to insulin’s ability to increase glucose uptake can be induced by multiple factors, including obesity. Impaired insulin action may take place at different spatial loci at the cellular or subcellular level. To begin to understand the spatial response to insulin in human subcutaneous adipose tissue (hSAT), we developed a quantitative imaging method for activation of a major signaling node in the glucoregulatory insulin signaling pathway. After treatment with insulin or control media, biopsied tissues were immunostained for Akt phosphorylation at Thr-308/9 (pAkt) and then imaged by confocal fluorescence microscopy automated to collect a large grid of high resolution fields. In hSAT from 40 men and women with obesity, substantial heterogeneity of pAkt densities in adipocyte membranes were quantified in each image mosaic using a spatial unit of at least twice the size of the point spread function. Statistical analysis of the distribution of pAkt spatial units was best fit as the weighted sum of two separate distributions, corresponding to either a low or high pAkt density. A “high pAkt fraction” metric was calculated from the fraction of high pAkt distributed units over the total units. Importantly, upon insulin stimulation, tissues from the same biopsy showed either a minimal or a substantial change in the high pAkt fraction. Further supporting a two-state response to insulin stimulation, subjects with similar insulin sensitivity indices are also segregated into either of two clusters identified by the amount of membrane-localized pAkt.

Obesity among adults in the United States, as defined by a BMI\(^3\) ≥30 kg/m\(^2\), has increased from 30.5 to 39.8% between 1999 and 2016 (1). Obesity is strongly linked with resistance to the ability of insulin to act as a glucoregulatory and lipolysis-regulating hormone. In adipose tissue, insulin resistance encompasses deficiencies in the ability to respond to extracellular insulin to stimulate glucose uptake and to suppress lipolysis (2). The San Antonio Metabolism Study highlighted that a progressive decline in pancreatic function leading to type 2 diabetes (T2D) begins with a progressive increase in circulating free fatty acids and an increase in adipose insulin resistance, identifying a potentially important role for adipose tissue in the transition from the prediabetic to diabetic state (3). Adipose can therefore be interpreted as an endocrine and paracrine signaling hub worthy of investigation both in vitro (4) and ex vivo (5). Moreover, a loss of just 5% of weight (primarily fat) leads to profound effects in health outcomes including prevention or delay in onset of T2D (6). Weight-related changes in adipose may explain this finding, in part, but the mechanisms through which a small change in weight leads to dramatic health benefits remain unclear.

There exists a commonality in the mechanisms of insulin signaling for acute induction of glucose uptake among different peripheral tissues that includes three separate critical nodes: 1) the insulin receptor and its associated substrates, 2) phosphoinositide 3-kinase (PI3K) and its subunits, and 3) Akt/protein kinase B and its isoforms (7–9). The result of the addition of insulin to the extracellular milieu bathing an adipocyte is activation of the three nodes leading to the translocation of glucose transporter type 4 (GLUT4), the primary insulin-dependent glucose transporter, to the plasma membrane and subsequent glucose uptake with the concentration of active forms of the nodes reaching a steady state in ~20 min (10, 11).

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This article contains Figs. S1 and S2.

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3. The abbreviations used are: BMI, body mass index; T2D, type 2 diabetes; pAkt, phospho–Thr-308/9 Akt; hSAT, human subcutaneous adipose tissue; SI, insulin sensitivity; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; hsCRP, high-sensitivity C-reactive protein; CI, confidence interval; AIC, Akaike information criterion; FLIM, fluorescence-lifetime imaging microscopy; wAIC, weight of Akaike information criterion; AICc, corrected Akaike information criterion for small sample size; CV, coefficient of variation; KRBH, Krebs-Ringer bicarbonate HEPES; CDF, cumulative distribution function; PDF, probability density function.
Of these nodes, the most proximal to GLUT4 is Akt, a kinase which is plasma membrane-bound when phosphorylated at Thr-308/9 (pAkt) by phosphoinositide-dependent kinase 1 and at Ser-473 by mTOR complex 2. Once activated, pAkt translocates to the cytosol and nucleus where it regulates numerous target proteins and transcription factors and is dephosphorylated by protein phosphatase 2A and pleckstrin homology domain and leucine-rich repeat phosphatase 1/2, respectively (12). Specifically, in 3T3-L1 cells, a fibroblast model system differentiated to have adipocyte-like properties, it has been shown that phosphorylation of Akt at Thr-309 is required for GLUT4 translocation (13), and impaired Akt kinase activity has been associated with insulin resistance in mouse models (14). However, few studies have investigated pAkt and GLUT4 dynamics directly in human adipose tissue.

Several mechanisms have been proposed to describe how insulin binding leads to active GLUT4 on the membrane (15–17). In human adipocytes, insulin resistance is associated with impaired tethering and fusion of GLUT4 storage vesicles to the plasma membrane (15). Individual human adipocytes likely do not exist along a spectrum of GLUT4 translocation activity, but rather exist in one of two classes: refractory or responsive to insulin (4). However, it is not known if two populations of cells or if subcellular populations of cellular membranes exist in ex vivo tissue samples based on the intracellular signaling pathway between insulin binding and GLUT4 translocation.

Here we report on the imaging by large-scale immunofluorescence microscopy of explants obtained from subcutaneous adipose tissue (hSAT) of humans with obesity for the response to insulin of that GLUT4–proximal node–pAkt. Finding a heterogeneous pAkt response to insulin in explant culture, we developed tools and concepts to test whether the activation of adipocytes in human tissue is best described using a single distribution, as was originally proposed (18, 19), or if in the native human adipose tissue environment of explants a binary (two-state) mixture model is more appropriate. At the cell membrane level, there existed two types of Akt response to insulin in adipose samples from each study participant, high or low pAkt density. Upon insulin stimulation, there existed two types of pAkt tissue response, a minimal or substantial change in the high pAkt density designated as nonresponsive or responsive to insulin. Finally, subjects having similar insulin sensitivity indices could be members of either two clusters identified by the amount of high pAkt density observed in insulin-stimulated tissue.

Results

Forty subjects with obesity were recruited to explore their systemic and adipose insulin resistances. Characteristics and systemic clinical parameters of subjects are reported in Table 1. The cohort was disproportionately female and ranged in age from early twenties to mid-sixties. All subjects recruited were obese with a range of BMI from 31 kg/m² to 54 kg/m². Twenty-seven subjects (67.5%) qualified as having metabolic syndrome based on having any three of the five following criteria: increased waist circumference, reduced HDL, high triglycerides, hypertension, and impaired fasting glucose (20). None of the subjects had T2D. On average, the cohort had a total body fat percentage of 47%, meaning nearly half their body mass came from fat and implies an important role of this tissue for their metabolic state. Most subjects were systemically insulin resistant and prediabetic based on their low SI as measured by an insulin-modified, frequently sampled, intravenous glucose tolerance test (less than 2.1 × 10⁻⁴ min⁻¹ per milliunit/liter), high HOMA-IR (over 2.6 milliunits/liter) (21), and impaired fasting glucose measurements. The hsCRP average of 4.7 mg/liter was greater than 3 mg/liter, indicative of increased inflammation and increased risk of cardiovascular events (22).

**Table 1**

### Characteristics and clinical parameters of volunteers

| Characteristic                  | Mean (S.D.) |
|--------------------------------|-------------|
| **Subjects**                   | 40          |
| **Male**                       | 11          |
| **Female**                     | 29          |
| **Age, mean (S.D.) range**     | 47 (11) [23–66] |

### Clinical parameters, mean (S.D.) range

| Parameter                | Mean (S.D.) |
|--------------------------|-------------|
| **Height (cm)**          | 167.1 (9.5) [152.2–191.4] |
| **Weight (kg)**          | 106.1 (12.1) [77.0–180.1] |
| **BMI (kg/m²)**          | 37.9 (5.7) [31.0–54.4] |
| **Waist circumference (cm)** | 113.4 (13.8) [90.9–158.1] |
| **Total body fat percentage by DXA (%)** | 47.4 (5.9) [33.0–58.2] |
| **Total cholesterol (mg/dL)** | 190 (31) [120–259] |
| **LDL**                  | 52 (14) [27–81] |
| **Triglycerides (mg/dL)** | 114 (47) [35–250] |
| **Systolic blood pressure (mm Hg)** | 129 (12) [103–153] |
| **Diastolic blood pressure (mm Hg)** | 75 (11) [53–100] |
| **SI (× 10⁻⁴) (min⁻¹ × μm⁻¹ × ml⁻¹)** | 1.34 (0.59) [0.51–2.83] |
| **Fasting glucose (mg/dL)** | 100 (8) [82–117] |
| **Fasting insulin (μu/mL)** | 17.3 (9.4) [5.0–41.3] |
| **HOMA-IR (μu/liter × mmol/liter)** | 4.42 (2.96) [0.60–11.73] |
| **HbA1c (%)**            | 5.4 (0.5) [4.4–6.5] |
| **hsCRP (mg/liter)**     | 4.7 (3.9) [0.6–14.5] |

### GLUT4 translocation correlates with phosphorylation of Akt in human subcutaneous adipose tissue

Development of an immunofluorescence method for GLUT4 translocation and its upstream activators in hSAT could be used to determine correlations in the insulin signaling pathway in native tissue. Specifically, the relationship of pAkt and GLUT4 was directly tested. Visualization of native plasma membrane GLUT4 in ex vivo tissue samples was punctate, indicative of clusters and possibly single transporters, which is consistent with previous isolated adipocyte cell measurements (5). Puncta are most striking when an adipocyte plasma membrane is coincident with the focal plane (Fig. 1, asterisk). The relationship between GLUT4 translocation to the plasma membrane and pAkt was tested in insulin-stimulated hSAT and showed a high degree of co-localization at low magnification (Fig. 1, Merge, yellow, top panel; Spearman’s ρ = 0.47 ± 0.02, p = 1.10 × 10⁻⁸³ ± 1.06 × 10⁻⁸³; mean ± 95% bootstrap CI). A zoomed-in view (Fig. 1, bottom panels) shows that co-localization is variable from cell to cell as well as in different contiguous membrane regions of the same adipocyte. Some cells had no appreciable GLUT4 plasma membrane residence (Fig. 1, white arrow) whereas other cells showed stop-start regions of overlap of GLUT4 and pAkt (Fig. 1, green and red arrows). The weighted mean Spearman’s correlation coefficient between translocated GLUT4 and pAkt (Thr-308/9) among
Figure 1. Phosphorylation of the signaling protein Akt (pAkt [Thr-308/9]) is positively correlated with plasma membrane–resident GLUT4. Average of three contiguous immunofluorescent confocal z-sections of human subcutaneous adipose tissue from an insulin-responsive control subject post treatment with insulin is shown. Left panels, tissue probed with antibody to the exofacial loop of glucose transporter GLUT4 labels translocation of GLUT4 in tissue upon insulin addition. Membrane labeling coincident with the z-slices appear as puncta (asterisk). Bottom panels, the boxed region is enlarged 3-fold. Middle panels, the same tissue probed with antibody to pAkt (Thr-308/9). Right panels, merged image shows GLUT4 and pAkt co-localization in contiguous membrane regions with significant pixel correlation (Spearman’s correlation coefficient is 0.47 ± 0.02; p < 0.0001 in this example). Bottom panels, zoomed image shows variability in membrane co-localization at both the intercellular (white arrow, absence of GLUT4 signal) and intracellular (green arrow, co-localization adjacent to red arrow without GLUT4 signal) level. Scale bars are 250 μm.

Adipose tissues was 0.27 (95% CI −0.15, +0.16; n = 11 tissues; p < 0.0001 for each tissue) indicative of a positive correlation between GLUT4 and pAkt in hSAT as visualized by immunofluorescence of fixed tissue. GLUT4-alone control experiments had a weighted mean correlation with background of 0.05 (95% CI −0.11, +0.11; n = 5 tissues; p > 0.05 in four out of five tissues) consistent with a minimal background.

To further test the hypothesis that GLUT4 and pAkt co-localize in heterogeneous membrane regions, a spatial permutation of pAkt intensities was performed and the respective Spearman’s correlation coefficients were tabulated. Upon randomizing the pAkt signal in silico there was no longer a correlation between the two molecules (0.00, 95% CI −0.00, +0.00, n = 10 scrambled datasets; p = 0.5 in all cases). The scrambled, control, and experimental groups all were significantly different from each other (p < 0.05 among Fisher-transformed Spearman’s coefficients). The finding of pAkt puncta and their heterogeneous distribution may indicate that in human adipocytes the coupling between pAkt and GLUT4 is affected by other molecules, which may require multiple immunoimaging studies to decipher (23). Nonetheless, these results confirm that pAkt can function as a major node in the cell surface expression of GLUT4 in human subcutaneous adipose tissue.

Two distributions of Akt activation exist in contiguous membranous regions of human SAT: High and low pAkt

Akt phosphorylation can be used to monitor the insulin response in ex vivo tissue of individuals with obesity. An example of an increase in phosphorylation upon insulin addition is shown in Fig. 2, A and B. Following insulin stimulation, the distribution of pAkt intensities in identified regions of adipocyte plasma membrane increases at the higher intensities with consequent decreases at the lower intensities (Fig. 2C). A mixture model of two beta distributions describes the combined distributions better than a single beta distribution (Fig. 2D) according to the Akaike information criteria (AIC), which has a lower value for the mixture model (ΔAIC = 0.0; AIC weight, w_AIC = 100.00) than for a single distribution (ΔAIC = 1.5 × 10^4; w_AIC = 0.00), indicating that the mixture model is preferred and the single distribution model has a ~0% relative likelihood of being the preferred model. In paired basal and insulin-stimulated pAkt immunofluorescence of large mosaics of adipose tissues from all 40 subjects, the Akaike information criterion consistently supported a mixture model with two pAkt distributions rather than one distribution. Thus, it appears that the response of hSAT to maximal insulin stimulation is best described as a mixture of two distributions, and the fraction of each component of the mixture model is an important biological variable.

Determining the spatial features of the pAkt intensities in identified regions of adipocyte plasma membrane required separation of the total intensity distribution into its two distributional components. The two components of the model were termed “low pAkt” and “high pAkt.” As an example, data from one participant organized in this fashion are shown in Fig. 3. The fraction of high pAkt increased from 14 to 52% upon insulin stimulation. For consistency, we report the high pAkt fraction as the tissue metric. Each intensity value was mapped to its probability of being a member of the high pAkt distribution. Fig. 3B inset shows how the transformed pAkt value corresponds to its probability of being a member of the high pAkt distribution. When applied to the basal adipose data (Fig. 3C), there exists a majority of contiguous adipocyte cell membranes with a low probability of high pAkt. In contrast, when applied to the insulin-stimulated data (Fig. 3D), the density of adipocyte membranes with a high probability of high pAkt is noted by the increase in the values color coded in red. In all subjects, the likelihood of pAkt activation in intact adipose tissue appeared visually to occur at the level of adjacent pixels or neighboring adipocyte membranes, not necessarily over the entire adipocyte
membrane of a single cell. The methods developed here to monitor changes in pAkt activation following insulin stimulation can be used to study larger spatial dimensions such as contiguous regions of adipocyte cell membranes.

Two populations of subjects exist post insulin challenge: Responsive and nonresponsive

Not every subject responded with a robust increase in the high pAkt fraction upon insulin administration. Given the high variability of high pAkt response to insulin addition among subjects, a \(k\)-means clustering algorithm was used to separate subjects into two clusters designated by the magnitude of their change as responsive or nonresponsive. The distribution of the fraction of high pAkt of individual tissues was color coded according to their \(k\)-means cluster grouping. Responders’ tissue samples demonstrated significant pAkt change (fraction change cluster mean \(= 0.35\)) after insulin challenge (Kolmogorov–Smirnov type 2, \(p = 0.0001\)), whereas pAkt in tissue from nonresponders did not significantly change (fraction change cluster mean \(= 0.01\)) after insulin (Kolmogorov–Smirnov type 2, \(p = 0.14\)) (Fig. 4).

Additionally, there was an increase in the fraction of high pAkt in nonresponsive individuals in the absence of insulin (dotted lines; Kolmogorov–Smirnov type 2, \(p = 0.03\)). Taken together, these results are consistent with the hypothesis that there exist two types of adipose tissues: Those that substantially respond to insulin and those that minimally respond.

Given the variability in the basal state of subjects, it is often preferable to look at just the data post insulin challenge, rather than as a -fold change over basal (24). When \(k\)-means clustering was applied to insulin-stimulated tissues from 40 subjects, the tissues’ response to insulin was separated into two groups: A large fraction of high pAkt clustered around 0.57 termed Cluster 1 or a small fraction of high pAkt clustered around 0.26 termed Cluster 2. Tissues in Cluster 1 in Fig. 5A are black and those within Cluster 2 are gray. Furthermore, to quantify the distributions, the cumulative distribution function of the 40 tissues was better fit using a mixture model than a single distribution model (Fig. 5B; \(\Delta\text{AIC} = 110.10\); Akaike weight, \(w_{\text{AIC}} = 0.00\); \(\Delta\text{AIC}\) of mixture is 0.00; and \(w_{\text{AIC}} = 100.00\)). Therefore,
Two-state adipose insulin response

using the fractions of the high pAkt distribution component, the data were best described by two types of subjects: those associated with Cluster 1 or Cluster 2.

To test whether peripheral insulin resistance of adipose, as monitored by the fraction of the high pAkt post-insulin challenge, relates to systemic insulin resistance, we examined the correlation between the fraction of high pAkt stimulated with insulin and the insulin sensitivity index from matching subjects (Fig. 5C). We again tested two models: A continuous linear model versus a model containing two separate populations of subjects. Linear regression represents the continuous model, whereas the two-populations model is represented by the means of the two groups identified using k-means clustering. The data are best described by two parallel lines representing the mean values of each group. A feature of this model is the overlap at SI values between 0.5 and $2.3 \times 10^{-4}$ in this cohort. Further supporting the two-population model is the reduction in the residuals, $\sigma_{res}$ (Fig. 5D; $0.09 \pm 0.01$ for two populations compared with $0.18 \pm 0.02$ for one; $\sigma_{res} \pm $ S.E.). Additionally, the continuous model has a change in Akaike information criterion corrected for small population ($\Delta$AICc; $n = 38$; Akaike weight, $w_{AIC} = 0.00$) of 49.75 as opposed to the two-population model of 0.00 ($w_{AIC} = 100.00$), again supporting a two-population model. A person with obesity, irrespective of their systemic insulin sensitivity, can therefore have biopsied adipose tissue in Cluster 1 ($0.57 \pm 0.004$; mean $\pm 95\%$ confidence interval) or have biopsied adipose tissue in Cluster 2 ($0.26 \pm 0.002$) (Fig. 6).

Among clinical parameters, arc sine square root–transformed basal high pAkt fraction was significantly correlated linearly at the 95% confidence level with HOMA-IR ($p = 0.44$ (0.15/0.66), correlation (lower/upper 95% CI); $p = 0.005$) and fasting insulin ($p = 0.39$ (0.08/0.62), correlation (lower/upper 95% CI); $p = 0.014$) but not with age ($p = -0.02$ ($-0.33/0.29$), correlation (lower/upper 95% CI); $p = 0.886$), BMI ($p = 0.23$ ($-0.09/0.50$), correlation (lower/upper 95% CI); $p = 0.154$), or fasting glucose ($p = 0.25$ ($-0.06/0.52$), correlation (lower/up-

**Figure 3. Low and high pAkt pixels localize to contiguous regions of adipocyte plasma membrane.** A, mixture model distributions of PDF intensities observed in Fig. 2A, basal tissue; low (dotted black line) and high (dotted green line) distributions of intensity. Percentages correspond to the fraction of each distribution present in the mixture model: 86% low (black) or 14% high (green) lines. The 95% confidence interval of the fitted fraction is $10^{-4}$. B, mixture model distributions of PDF intensities observed in Fig. 2B, insulin stimulated tissue. B, inset, relationship between the transformed pAkt intensity to the probability that a transformed value is a member of the high distribution component of the model. C and D, conversion of transformed pAkt intensity of Fig. 2, A and B (basal and insulin-stimulated tissues with regions of high autofluorescence removed as in “Experimental Procedures”) to the probability that a transformed value is a member of the high distribution component of the model. Pixels dark green to red represent high pAkt with higher probability scores compared with pixels light green to blue and black with low probability scores. Probability values mapped to colors using the Fiji Rainbow RGB look-up table. Scale bar is 500 μm.
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Figure 4. Two types of subcutaneous adipose tissue response to insulin challenge were observed in this cohort of people with obesity. Subjects were divided into two groups following k-means clustering according to the magnitude of their change in pAkt and were termed responsive and nonresponsive. Cumulative distribution functions (CDF) of responsive (blue) and nonresponsive (red) subjects are shown. Basal tissue values are represented as dotted lines, and insulin-stimulated tissues are represented as solid lines. Kolmogorov-Smirnov type 2 tests indicate that at 95% confidence the blue responsive lines are separate distributions (p = 0.0001) whereas the red nonresponsive lines are the same distribution (p = 0.14). Additionally, the same test confirms that basal distributions are not the same (p = 0.03), with nonresponsive subjects having a higher basal high pAkt fraction. 

Discussion

In this study, we examined abdominal (25, 26) subcutaneous adipose tissue samples from 40 subjects with obesity and varying degrees of systemic insulin resistance to differentiate between two hypotheses about peripheral insulin resistance in ex vivo adipose. The response to insulin stimulation is 1) graded or 2) not graded, with at least two responding types. Large-scale mosaic confocal immunofluorescence images of paired tissues with and without insulin stimulation enabled monitoring of more cells and cell membranes than many of the previous studies and focused on the tissue in its native, ex vivo, environment. The paired tissues were best described using a mixture model of two types of Akt phosphorylation density and termed low pAkt and high pAkt. The density analysis does not include normalization by the total amount of pAkt in the cells because we are not measuring absolute amounts of pAkt, but rather fractions of the membrane that are detected to have bound pAkt. This fraction of membrane area is expected to be independent of the total amount of Akt. A potentially better (but more difficult to implement) alternative to this technique is combined FRET/FLIM imaging of both rabbit pAkt (Thr-308) and mouse pan-Akt antibodies as a pAkt activity sensor (27) which has also isolated molecular heterogeneity within various tissue samples. The spatially heterogeneous pAkt response to insulin stimulation can best be described by a change in the fraction of two statistically defined types of pAkt membranous regions, not a graded shift in the response of already existing high pAkt regions. Insulin signaling has been suggested to occur in microdomains of the plasma membrane (28). We find continuous membrane regions of high pAkt in each cell. These regions are hypothesized to be regions of high PIP3 density and are consistent with a nonuniform, spatially intermittent PIP3 membrane concentration. These regions are also consistent with the complex signaling properties present in the tissue environment. The heterogeneity found in the context of the intact tissue may be lost in dissociated cells. Additionally, each subject’s tissue exhibited either a small or a large fraction of high pAkt regions. Following insulin simulation, two groups of subjects were revealed: those with a nominal change in the high pAkt fraction and those with a substantial change in the high pAkt fraction, indicative of two groups of subjects with obesity, classical insulin-responsive and -nonresponsive.

Multiple adipose tissue depots have been associated with insulin resistance, including visceral adipose tissue and intrahepatic fat (29–32). However, subcutaneous fat, being the major adipose depot in the body, has also previously been shown to be associated with insulin resistance (25, 26). As we biopsied only abdominal subcutaneous adipose in this study, our results cannot explain any differences between hepatic, intra-abdominal, and subcutaneous fat as each may play distinct or overlapping roles in insulin resistance.

Multiple cellular types have been observed in other signaling paradigms. Classically, the Escherichia coli lac operon shows two types as a result of two subsequent repressors acting as a feedback activator (as reviewed in Ref. 33). The MAPK/ERK pathway has also been shown to lead to a binary output from graded Ras activation with positive feedback in lymphoid cells (34). Similarly, it has been suggested that the graded insulin signal for the PI3K/Akt pathway with a combination of positive and negative feedbacks yields two states with increased sensitivity of downstream components like Akt and GLUT4 (10, 35) in support of our current ex vivo dataset. This conceptualization is contrary to the classical paradigm that insulin signaling yields a graded response (18, 19). However, our analysis of the primary data from these early publications also supports a two-state mixture model better than a graded model (Fig. S1).

There have been other model systems where two distributions of insulin response were not seen at a cellular or continuous membrane level. Most notably, 3T3-L1 cells are notoriously heterogeneous (36, 37) which can be explained, at least in part, by the variability of the differentiation process in preparing the cultures (38). Nonetheless, our human tissue system is able to recapitulate some of the findings of the 3T3-L1 model system, namely the correlation of GLUT4 translocation with phosphorylation of Akt at Thr-308/9 (13). Recapitulation of this phenomenon in a 3T3-L1 differentiated model system and in ex vivo human adipose is indicative of a robust biological strategy that manifests in many different model organisms. Additionally, using human tissue we were able to distinguish two types of adipose, ex vivo, insulin-responsive and -nonresponsive. Interestingly, the nonresponsive tissues showed a higher level of basahigh pAkt than their responsive counterparts which is consistent with a diet-induced obesity mouse model (39, 40). Change in basal high pAkt could provide a useful metric for determining disease progression or regression because our analysis of basal high pAkt fraction correlation with HOMA-IR predicts that with increasing insulin resistance, subjects have an increasing basal high pAkt fraction (41).

Following insulin stimulation, subjects that have tissue with a small change in the fraction of high pAkt would be considered nonresponsive, whereas a large change would be considered...
responsive, yet the biological cause for this change in phenotype remains poorly understood. It is likely that nonresponsive and responsive adipose depots have differences in their extracellular milieu which causes variable expression at the gene level that could desensitize an adipocyte to insulin. One candidate, \textit{TBX15}, differentially regulates metabolism in white adipose. Tbx15\textsuperscript{Hi} pre-adipocytes and adipocytes are highly glycolytic whereas Tbx15\textsuperscript{Low} are more oxidative and less glycolytic. This gene is down-regulated with obesity in both humans and mice and is one possible mechanism for the observations of two functional depots of fat tissue: dysfunctional depots derived from Tbx15\textsuperscript{Low} pre-adipocytes and functional depots derived from Tbx15\textsuperscript{Hi} pre-adipocytes (42). Plurality in the genotype and expression profiles of subtypes of tissues within obese subjects likely leads to two phenotypes: responsive or nonresponsive with regard to Akt response. The biological cause for the nonresponsive and responsive subsets of tissue may be in the differential expression levels of key cellular regulators in adipose.

In conclusion, a cohort of 40 subjects with obesity exhibited adipocytes whose membranes were highly responsive (high pAkt) or minimally responsive (low pAkt) to insulin challenge, much like the response of GLUT4, a downstream pathway component (4). This study is unable to distinguish at which step in the insulin signaling pathway a graded signal becomes binary, but confirms that a two-state output remains after Akt phosphorylation and at GLUT4 presentation. The responsive element in the tissue is not the entire adipocyte, but rather regions of plasma membrane, which supports a need for imaging studies in the native tissue environment, as many cellular properties that are dependent on tissue architecture may be preserved better in tissue, \textit{ex vivo}, compared with findings from individual adipocytes studied in culture after extracellular matrix digestion. Subjects’ tissues could be further separated into responding and nonresponding populations. For every subject, a mixture model of two distributions and not a model of a single distribution was a better description of the data. A two-state

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**Figure 5.** This cohort is a mixture of two populations: Either a small or large fraction in the high pAkt component of the mixture model is observed. A, Swarm plot of fraction of SAT with high pAkt in the presence of 360 nM insulin. Each dot represents a tissue sample from one individual (n = 40 subjects). The k-means cluster means for two groups of the insulin-stimulated dataset were at 0.72 (termed Cluster 1) for the large pAkt high fraction and 0.26 (termed Cluster 2) for the small pAkt high fraction. Tissues in the small pAkt high fraction are color coded gray; those with a large pAkt high fraction are black. B, empirical CDF of high pAkt fractions of +Insulin tissues in A. Magenta line represents model of a single distribution of a truncated Gaussian. Cyan line represents mixture model of two truncated Gaussians. Residuals follow color scheme. C, insulin-stimulated high pAkt fraction of cohort as a function of SI (n = 38 subjects). Solid black line is the mean of Cluster 1. Solid gray line is the mean of Cluster 2. D, PDF of residuals for two models describing the relationship between high pAkt fraction and SI observed in C demonstrates that the dependence on SI is best described by two populations: Red line, a single linear relationship with S.D. of residuals (σ\textsubscript{res} ± S.E.) = 0.18 ± 0.02, difference in Akaike information criteria corrected for small sample size (ΔAICc) = 49.75; w\textit{AIC} = 0.00 versus (black line) two population distributions (σ\textsubscript{res} = 0.09 ± 0.01; ΔAICc = −176.17; w\textit{AIC} = 100.00).
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[Image: Diagram showing adipose tissue with SI ≤ 2.3* and SI > 2.3* with corresponding membrane states]

Figure 6. Illustration summarizing the major findings of this study. Subjects with obesity typically had systemic insulin sensitivity less than $2.3 \times 10^{-4} \text{min}^{-1} \times \text{milliunit}^{-1} \times \text{liter}$ (red symbols), and their adipose tissue has either a low fraction of high density pAkt (green arcs) or a high fraction of high density pAkt on adipocyte membranes (top and bottom row, respectively). Subjects with insulin sensitivity greater than $2.3 \times 10^{-4} \text{min}^{-1} \times \text{milliunit}^{-1} \times \text{liter}$ (blue symbols) have a high fraction of high density pAkt on adipocyte membranes. Membranes have contiguous regions of either high density pAkt (green) or low density pAkt (blue).

output in response to insulin may contribute to the mechanism by which a modest change in weight of ≥5% improves many health complications including delay of T2D (6).

Experimental procedures

Participants

Biopsies of subcutaneous abdominal fat were obtained using a mini-liposuction technique (43) from subjects with varying degrees of obesity, enrolled in a clinical study registered at [https://clinicaltrials.gov/](https://clinicaltrials.gov/) (NCT02153983). The study was approved by the Institutional Review Board of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. All volunteer subjects provided written consent to participate and supplied blood and adipose tissue samples before undergoing any medical intervention. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Clinical assays and testing

Body fat percent was determined by dual energy X-ray absorptiometry using a total body scanner (Lunar iDXA; GE Healthcare). Insulin was measured with Roche Diagnostics reagents on a Roche Cobas 6000 instrument (model e601; Roche Diagnostics) via electrochemiluminescence immunoassay. For insulin, the analytical sensitivity was 0.2 microunit/ml, cross-reactivity with proinsulin was 0.05%, average intra-assay coefficient of variation (CV) was 1.1%, and average inter-assay CV was 4.3%. Plasma was collected and kept on ice in tubes with powdered sodium fluoride until centrifugation and glucose concentration was determined using a Roche/Hitachi instrument (model c502; Roche Diagnostics). An insulin sensitivity index was calculated from an insulin-modified frequently sampled intravenous glucose tolerance test as described previously (44) using the Simulation Analysis and Modeling II (SAAMII) software tool (The Epsilon Group, Charlottesville, VA). Briefly, subjects came to the NIH Clinical Center after a 10-h overnight fast. After placing intravenous catheters in both arms, a bolus injection of 50% dextrose (0.3 g/kg, maximum dose 30 g) was administered over 2 min at time 0, and a bolus of insulin (0.05 unit/kg) was injected at 20 min. Blood was obtained for insulin and glucose analysis at the following time points: −15, −10, −5, −1, 2, 3, 4, 5, 6, 8, 10, 14, 19, 22, 25, 30, 40, 50, 70, 100, 140, and 180 min. Fasting insulin resistance was calculated using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) from the baseline blood draw (45). High-sensitivity C-reactive protein (hsCRP) and other laboratory measurements from human serum samples reported in Table 1 were analyzed by the NIH Clinical Research Center Department of Laboratory Medicine, using commercial reagents (Roche Diagnostics).

Reagents

Fatty acid–free bovine serum albumin (BSA) and Krebs-Ringer bicarbonate HEPES buffer (KRHB) were obtained from Sigma-Aldrich. Human recombinant insulin was obtained from Thermo Fisher Scientific. Insulin was prepared as a stock (250 IU/ml) and diluted into KRHB buffer with 1% BSA to a final concentration of 360 nM. 16% paraformaldehyde was diluted to a working concentration of 4% in phosphate-buffered saline (PBS) purchased from Electron Microscopy Sciences (Hatfield, PA) and Thermo Fisher Scientific, respectively.

The primary antibodies used were mouse anti-human phospho-Akt (Thr-308/9) (D25E6) XP rabbit mAb (13038S; Lot 5; 1:1600 dilution) from Cell Signaling Technologies (Danvers, MA) and goat anti-human GLUT4 (N-20) polyclonal antibody (sc-1606; Lot C0415; 1:5 dilution) from Santa Cruz Biotechnology (Dallas, TX). Secondary antibodies were all purchased from Thermo Fisher Scientific: Alexa Fluor 488 goat anti-rabbit IgG (H+L) cross-adsorbed (A-11008; Lot 1797971; 1:150 dilution) and Alexa Fluor 647 donkey anti-goat IgG (H+L) cross-adsorbed (A-21447; Lot 1661244; 1:150 dilution). For fixed-tissue imaging SensoPlateTM 24-Well Glass-Bottom Plates from Greiner Bio-One (Monroe, NC) were used.

Tissue preparation

Biopsies were initially washed three times in PBS and regions containing obvious blood vessels were removed prior to transporting tissue. 2 g of adipose tissue was placed in KRHB buffer with 1% BSA (5) and then transported to the imaging laboratory. The time from tissue isolation to fixation was routinely less than 1 h. Tissues were washed at least twice more using 20 ml KRHB with 1% BSA and additional tissues with large numbers of blood vessels were removed, including any tissues unable to float in the buffer. 50–100 mg of adipose tissue were placed into a separate well of a 24-well plate and incubated for 20 min with or without 360 nm insulin. For GLUT4 experiments, tissues were co-incubated with GLUT4 (N-20) antibody during insulin incubation (46). Tissues were fixed for 1 h at...
37 °C in 4% paraformaldehyde diluted in PBS and then placed in KRBH with 1% BSA prior to standard immunofluorescence preparations.

**Microscopy**

Single tissues were placed into individual imaging wells of the SensoPlateTM covered with a 15-mm circular coverslip and metal washer to keep the fixed sample adjacent to the coverslip, and then 1 ml KRBH with 1% BSA was added to the well. Tiled, confocal, immunofluorescence z-stacks were acquired starting from the first layer of adipose tissue cells nearest the coverslip surface. Typical imaging dimensions were 2.5 mm × 2.5 mm × 30 μm with a typical pixel size of around 1 μm corresponding to a spatial unit of at least twice the size of the point spread function. Images were acquired at room temperature using a Nikon Eclipse Ti equipped with a CSU-X1 and Andor 897 Plus camera (Nikon Instruments, Inc., Melville, PA) and a CFI Plan Apo Lambda 20X 0.75 NA objective or a Zeiss LSM 880 (Carl Zeiss Microscopy, Thornwood, NY) with a Plan-Apochromat 63 × 1.4 NA objective. Images were overlapped by 10% and stitched using software native to the imaging platform (N!S Elements or ZEN Black, respectively) before export to Fiji using software native to the imaging platform (NIS Elements or 1.4 NA objective. Images were overlapped by 10% and stitched using software native to the imaging platform (NIS Elements or ZEN Black, respectively) before export to Fiji for processing. For Nikon spinning disk confocal microscopy, autofluorescence was imaged using 405 nm excitation with a bandpass filter of 438 to 473 nm and fluorescence in the 488 nm channel was collected from 500 to 550 nm. For GLUT4 experiments, a 641 nm laser was used for excitation and emission was collected from 665 to 735 nm by frame sequentially. For Zeiss point-scanning confocal microscopy, autofluorescence was imaged using 405 nm excitation with a PMT detector set to acquire emission wavelengths from 405 nm to 488 nm and fluorescence in the 488 nm channel was directed to a GaAsP detector with an emission range of 488 to 561 nm. GLUT4 experiments also used a 633 nm HeNe laser for excitation and emission was collected from 633 to 758 nm using a PMT by frame sequentially.

**Image analysis**

Multichannel datasets consisted of an average projection of three central slices of the first layer of adipocytes which were derived from Perfect Focus (Nikon) or Definite Focus.2 (Zeiss) using automated refocusing parameters which were 10 ± 3 μm from the surface, indicative of flattened tissue. The projection average was performed on multiple channels from the tiled montage with either 405 nm excitation (for autofluorescence), 488 nm (for imaging of Akt), or 633/641 nm (for imaging GLUT4). Images with and without insulin were paired and combined into a larger montage. Images were segmented to remove the low signal noise floor, out-of-focus background, and highly autofluorescent regions such as blood vessels, collagen, and muscle fibers using a custom Fiji macro allowing semi-automated analysis with user feedback. Typically, a “Default” IsoData threshold was applied to the autofluorescence data. The immunofluorescence intensity images were treated in a separate macro typically using a Li threshold to isolate cells. The two images were combined to form an adipocyte mask that was applied to a background-subtracted image of the raw montaged data and typically isolated adipocyte cell membranes.

Raw pixel intensities were adjusted to span the range 0<Intensity<1 by setting the brightest intensity of the paired, masked, basal, and insulin-stimulated data to 1 using MATLAB (MathWorks, Natick, MA). Adjusting the dataset to these fractional values allowed for implementation of a probability scale and normalization of tissue datasets using individuals as their own control. Adjusted intensities having values of 0 or 1 were truncated from the dataset. This adjusted intensity dataset became the starting point for all subsequent analyses for each subject.

**Statistical analysis**

Custom MATLAB codes were used for all statistical analyses. For GLUT4/pAkt co-localization experiments, the Spearman’s correlation coefficient was reported on the masked dataset truncating the brightest 5% of the data which persisted because of incomplete masking. To understand the strength of the correlation, a bootstrap algorithm was applied to the matrix of transformed pixel intensities. Briefly, the dataset was randomized extracting 10% of the total pixels and calculating the new Spearman’s correlation coefficient. This was repeated 10,000 times for each GLUT4/pAkt dataset to derive a 95% confidence interval for the bootstrapped data.

Additionally, to further determine whether the co-localization was robust, the normalized intensities from the pAkt channel were completely randomized and the Spearman’s correlation coefficient with bootstrap analysis was repeated. These datasets were compared with samples without pAkt primary antibody as a bleed-through control.

For pAkt datasets, intensities from combined basal and insulin-stimulated tissues can be viewed as probability density functions using an Epanechnikov kernel smoothing technique or converted to an empirical cumulative distribution function (CDF). Raw intensity distributions were clearly not Gaussian, with or without the use of an EMCCD camera. Beta distributions were used to describe the transformed intensity values, that spanned the domain 0<Intensity<1. The CDF in transformed intensity space was fit using either a single beta cumulative distribution function (betacdf) with two shape parameters or the sum of two betacdfs (mixture model) with four total shape parameters and a parameter for the fraction each individual betacdf contributed to the mixture model.

Empirical cumulative distributions of combined basal and stimulated tissue data were fit in MATLAB using nonlinear least squares analysis implementing the Trust–Region algorithm. To compare models, the sum of squared errors was determined and the Akaike Information Criterion or the AIC corrected for small populations was used to evaluate the relative quality of each model to select between the two models. The generalized form of AIC is approximated by Equation 1:

\[
\text{AIC} = n \times \ln(\text{sum of square error})/n + 2 \times K \tag{Eq. 1}
\]

where \(n\) is the number of data points and \(K\) is the number of fit parameters.

Specifically, the \(K\) value for the single distribution model is three: shape parameters alpha and beta, and fitting error...
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whereas for the mixture model the K value is six: shape parameters alpha1 and beta1, alpha2 and beta2, mixture fraction, and fitting error. Given the large pixel counts for all combined images, these simplifying assumptions hold and the model with a smaller AIC value is preferred.

Once the mixture model was determined by AIC to better represent the transformed intensities of combined basal and insulin-stimulated tissues, each separate tissue was individually fit using the same mixture model, fixing the shape parameters, and only allowing the fraction of each distribution to vary. Decomposing the mixture model into its separate additive distributions revealed a component with a smaller median value (designated low pAkt) and one with a higher median value (designated high pAkt). The fraction parameter for the high pAkt was reported as the high pAkt fraction.

To determine whether the distribution of pixels identified using the mixture model was not spatial random, each unmasked pixel was converted from the transformed intensity value to a probability of being a member of the high pAkt distribution. To perform this conversion, the parameters representing the higher median distribution, the high pAkt fit, was applied without a fractional factor to each transformed intensity value resulting in a new image whose values correspond to the probabilities of high pAkt.

Using the change in high pAkt fraction upon insulin stimulation, k-means clustering (53) separated clusters of minimal change and substantial change in tissues from different subjects. Additionally, k-means clustering was used to separate the 40 insulin-stimulated tissues assayed into two groups with high pAkt fraction means of 0.57 (Cluster 1) and 0.26 (Cluster 2). To further validate this separation, two models were tested: A truncated normal distribution and the sum of two truncated normal distributions (both truncated at 0 and 1). Again, the Akaike Information Criterion was applied, but given the smaller sample size the corrected generalized form AICc was approximated as in Equation 2 (52):

\[ \text{AICc} = n \times \ln\left(\frac{\text{sum of square error}}{n}\right) + 2 \times K \]

\[ + \left(2 \times K \times (K - 1)/\left(n - K - 1\right)\right) \quad \text{(Eq. 2)} \]

To distinguish between a linear relationship between the fraction of high pAkt as a function of systemic insulin sensitivity parameters and an alternate model consisting of two populations characterized by their respective cluster means, the two models were compared using AICc.

To calculate AICc, the value of \( n \) was 38 tissues with associated SI measurements and the value for \( K \) is 3 for the single distribution (slope, offset, error) and 4 for the mixture model (cluster means 0.57 and 0.26, and errors of the means).

To determine model preference, the \( \Delta \text{AIC} \) was calculated as the difference of the AIC of the given model from the model with the lowest AIC. If the difference is greater than 10, there is essentially no empirical support for the model in question. The normalized probability that one model is preferred over another was evaluated using Akaike weights \( w_{\text{AIC}} \) and calculated using the differences in AIC, \( \Delta \text{AIC} \) as derived in Ref. 54 by the formula in Equation 3:

\[ w_{\text{AIC}} = \exp\left(-1/2\Delta \text{AIC}\right) \]

\[ \quad \text{(Eq. 3)} \]

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