Reduced Adipose Tissue Oxygenation in Human Obesity - Evidence for Rarefaction, Macrophage Chemotaxis and Inflammation without an Angiogenic Response

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Clinicaltrials.gov ID: NCT00704197

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ABSTRACT

Objective: Based on rodent studies, we examined the hypothesis that increased adipose tissue mass in obesity without an adequate support of vascularization might lead to hypoxia, macrophage infiltration and inflammation.

Research Design and Methods: Oxygen partial pressure [AT pO2] and temperature [AT temperature] in abdominal adipose tissue [9 lean, 12 overweight/obese men and women] was measured by direct insertion of a polarographic Clark electrode. Body composition was measured by DEXA, insulin sensitivity by hyperinsulinemic euglycemic clamp. Abdominal subcutaneous tissue was used for staining, qRT-PCR and chemokine secretion assay.

Results: AT pO2 was lower in overweight/obese as compared to lean [47 ± 10.6 vs. 55 ± 9.1 mm Hg], however this level of pO2 did not activate the classic hypoxia targets [PDK1, VEGF]. AT pO2 was negatively correlated with % fat [R=-0.50, p<0.05]. Compared to lean, overweight/obese subjects had 44% lower capillary density and 58% lower Vascular Endothelial Growth Factor [VEGF] suggesting adipose tissue rarefaction [capillary dropout]. This might be due to lower PPARγ1 and higher collagenVI mRNA expression, which correlated with AT pO2 [p<0.05]. Of clinical importance, AT pO2 negatively correlated with CD68 mRNA and MIP1α secretion [R=-0.58, R=-0.79, p<0.05], suggesting that lower AT pO2 could drive AT inflammation in obesity.

Conclusions: Adipose tissue rarefaction might lie upstream of both low AT pO2 and inflammation in obesity. These results suggest novel approaches to treat the dysfunctional adipose tissue found in obesity.
Both insulin resistance and β cell failure are present in persons with type 2 diabetes. Insulin resistance is closely linked to adiposity with a central or visceral pattern providing a greater risk of insulin resistance and metabolic dysfunction. Adipose tissue (AT) serves as an endocrine organ secreting a variety of autocrine, paracrine and endocrine factors that can produce or prevent insulin resistance (1). The failure of AT to adequately proliferate and/or differentiate to sequester lipid away from liver, skeletal muscle and the pancreatic β cell has been proposed as a precursor to Type 2 diabetes, broadening the number of potential mechanisms linking obesity to insulin resistance (2).

The increase in body fat stored in obese should be accompanied by an increase in vascularization, in order to provide adequate oxygen and nutrients (3). In contrast to expectations, obese mice have lower AT capillary density [rarefaction, also known as capillary dropout] and decreased vascular endothelial growth factor [VEGF], the most potent angiogenic factor, (4; 5). Consistent with this model, preclinical studies suggest that obese AT is hypoxic (6), however, the hypothesis that AT rarefaction might lead to hypoxia remained untested.

In humans, short term whole body hypoxia decreases insulin sensitivity (7) and short term whole body hyper oxygenation increases insulin sensitivity (8). In mice, obesity is associated with lower oxygen partial pressure in subcutaneous and visceral AT (6; 9). Studies in post-surgical patients support the idea that AT oxygen partial pressure is lower in obesity (10).

In vitro hypoxic adipocytes secrete inflammatory molecules such as: TNFα, IL1, IL6, MIF and PAI-1 (6; 11). Increased AT inflammation is a feature of obesity and Type 2 diabetes (12). Hypoxic cells secrete chemokines which attract macrophages, presumably to clear out necrotic cells and tissue (13). This suggests the hypothesis that the increase in AT macrophage content seen in human obesity (12) might be due to AT hypoxia.

These pre-clinical and cell culture experiments suggest that hypoxia might play a role in the inflammation and insulin resistance observed in human obesity. To test this hypothesis, we measured subcutaneous abdominal AT oxygenation [AT pO2] in lean and overweight/obese human subjects and related AT pO2 to the structure and function of adipose tissue.

**RESEARCH DESIGN AND METHODS**

**Population and study design:** Twenty-one subjects were recruited and screened based on their BMI: lean [20-25 kg/m²] or overweight/obese [27-35 kg/m²]. Recruiting was conducted via newsprint, postcards and the Pennington Biomedical Research Center [PBRC] webpage. Subjects were excluded if they had significant renal, cardiac, liver, lung, or neurological disease. Hypertension was acceptable if blood pressure was less than 140/90 mmHg on medications. Subjects were excluded for prior use of thiazolidinediones or injectable antihyperglycemic medication, drugs known to affect lipid metabolism, energy metabolism or body weight, alcohol or other drug abuse, smoking. The protocol was approved by the Institutional Review Board at the PBRC and all volunteers gave written informed consent.

Body composition was measured by DEXA on a Hologic Dual Energy X-Ray Absorptiometer in the fan beam mode (QDR 4500, Hologic, Inc. Waltham, MA). The coefficient of variation for the measurement of percentage of body fat is 1.7%.

Two days prior and during an in-patient stay participants were fed a standardized diet [50% carbohydrate, 15% protein and 35% fat]. The number of calories to be provided
Rarefaction and Inflammation in Obese Human Adipose Tissue

(and consumed) was calculated as 1.3 X basal energy expenditure [BEE] using Harris-Benedict equation [665.10 + (9.56 x Weight in kg) + (1.85 x Height in cm) – (4.68 X Age in years for females and 66.47 + (13.75 x Weight in Kg) + (5.0 x Height in cm) – (6.76 x Age in years) for males].

**Measurement of subcutaneous abdominal adipose tissue oxygen partial pressure [ATpO2] and temperature [AT temperature]:** ATpO2 was measured using a polarographic micro Clark type electrode and AT temperature was measured using a thermocouple concomitantly during two distinct procedures. The measurements were done with a single oxygen probe [cat# C1], a single temperature probe [cat # C8] or a combined oxygen and temperature probe [cat# CC1.P1, Integra Lifesciences Corporation, NJ, USA]. The probes were connected to an electronic unit [LICOX CMP, brain oxygen monitoring unit]. This unit displays the AT temperature in °C and the ATpO2 in mmHg [after controlling for the contribution of temperature on AT pO2]. After insertion, the system was allowed to equilibrate for 30 minutes. Recording was stopped when difference between measurements done at 5 minutes interval was less than 1 mm Hg [steady state]; values from the last 10 minutes were averaged [Supplementary Figure 3A]. All measurements were made supine, on the left side of the abdomen, at 1/3 distance between the umbilicus and the superior iliac crest [Supplemental figure 1] and with the skin uncovered at an ambient room temperature of approximately 25°C.

First, we measured ATpO2 [mmHg] with the probes inserted into a gas permeable silastic tubing implanted in the subdermal space as described by Hopf (14) and optimized in our laboratory [indirect method-supplemental figure 2A]. Second, ATpO2 was measured using a direct insertion method developed by our laboratory [Supplemental figure 2B]. After cleaning the skin with povidone-iodine solution and removal of the dried iodine with sterile saline on gauze, a skin wheal was raised with 1% lidocaine. A combined oxygen and temperature probe was inserted through a 3.2 cm long 14 Ga IV catheter [Medex Inc] to a depth of 1 cm. The values obtained with the two methods were highly correlated (R = 0.64; p < 0.01, N = 13). However, the second method was less invasive and provided less discomfort to the volunteers. Therefore after 13 subjects we only used the second method to measure ATpO2 and AT temperature. All values in this manuscript are from the ‘direct’ measurement technique.

**Euglycemic-hyperinsulinemic clamp:** The clamp was performed as previously described (15). Briefly, intravenous catheters were inserted in an antecubital vein for infusions and in a vein on the dorsum of the contralateral hand for sampling of arterialized blood. After baseline sampling, a primed-continuous insulin infusion [80 mU/m²/min] was continued for 3-4 hours. Insulin was infused for at least one hour after reaching a concentration of glucose ~90 mg/dl. Plasma glucose was measured every 5 minutes and maintained by a variable 20% glucose infusion. The mean rate of exogenous glucose infusion during steady-state [last 30 min] was corrected for changes in glycemia and divided by fat free mass [FFM] to assess insulin sensitivity.

**Laboratory measures:** The following assays were done on blood drawn after an overnight fast. Glucose was analyzed using a Beckman Coulter DXC 600 Pro (Brea, CA) and insulin via immunoassay on the Siemens Immulite 2000 (Siemens, Los Angeles, CA).

**AT biopsy:** The skin was anesthetized with a mixture of lidocaine [2%] and bupivocaine [0.025%]. AT was obtained using a blunt-ended needle designed for liposuction (3-4 mm diameter “mercedes” liposuction needle, M.D. Resource, Hayward, CA) and processed
at the bedside by washing in 37°C PBS and snap frozen or preserved in 10% formalin for paraffin blocking. The biopsies obtained from the first completed 14 subjects [8 females and 6 males] were used for the following procedures.

**Short-term AT release of cytokines:** As previously described (16), fresh AT was collected into pre-gassed 37°C Media 199, and minced into 2-5 mg fragments and washed over a nylon mesh. Aliquots were incubated for 3 h in Medium 199 containing 1% BSA under a 95% O2-5% CO2 atmosphere in a shaking water bath (60 cycles/min, 37°C). MIP1α [CCL3], VEGF, TNFα, leptin, IL-1α, MCP1 and MIP1α were measured in the condition media using the Luminex system (cat# HCYT060K03, Millipore). The conditioned media was assayed for LDH and confirmed the absence of cell lysis. From the 14 subjects, 1 subject had no biopsy tissue for the assay [because of technical limitations] and 1 subject had the assay compromised during processing and therefore could not be used. Subsequently, cytokine release was measured in 12 subjects [5 lean and 7 overweight/obese, from which 5 were males and 7 females]. Conditioned media concentrations of VEGF, TNFα and leptin were below the detection level of the assay [data not shown].

**Capillary density:** The tissue was cleaned in PBS, fixed in 10% formalin, embedded in paraffin. Before staining, 3 μm sections were de-paraffinized and dehydrated by incubation in Xylene [cat# 247642, Sigma-Aldrich, MO, USA ] for 20 minutes followed by incubation with 100%, 90%, 80%, 70% and 60% ethanol. Slides were rinsed with PBS (20 min). The section were incubated for 30 minutes in a dark moist container with the staining solution containing Lectin FITC conjugate (from *Griffonia simplicifolia*; GS) 25ug/ml [cat# L2895, Sigma-Aldrich], Lectin TRITC conjugate (from *Ulex europaeus*; UEA) 10ug/ml [cat# L4889, Sigma-Aldrich] and DAPI 0.3 μ [cat# D9564, Sigma-Aldrich]. The GS lectin stains plasmalemma, UEA stains capillaries (17) and DAPI stains nuclei (18). In a preliminary experiment the AT sections were stained with DAPI in addition to GS lectin and UEA lectin and showed that UEA lectin does not stain nuclei [supplemental figure 4]. The sections were rinsed with PBS (40 min) and then mounted on microscope slides with a water soluble mounting medium [cat#M7644-1, CardinalHealth]. Images of the stained sections were taken with a Zeiss Axioplan 2 upright microscope [Intelligent Imaging Innovations] using Zeiss Axioplan 2 with a Photometrics CoolSnap HQ CCD camera and a Sutter Lambda LS 175W Xenon arc lamp. A planar Apochromat 20x/0.75 objective lens, 3 filter sets [DAPI -EX 360/40, FITC - EX HQ487/25, CY3 - EX HQ535/50] and Slidebook Software v2.0 were used for image capture. Microvessels were counted using MBF ImageJ Bundle software. Microvessel density was expressed as number of microvessels/mm of section area, averaged across 6-10 images acquired from each section.

**Quantitative real time-polymerase chain reaction [qRT-PCR]:** Human total RNA from ~100mg AT was isolated by column purification (Qiagen). All primers and probes were designed using Primer Express version 2.1 (Applied Biosystems). Sequences of primers and probes are shown in supplementary table. Leptin and GLUT1 were from ABI [cat# Hs00174877_m1, Hs00197884_m1]. GLUT1 mRNA was not detectable in the AT samples. Real-time qRT-PCR reactions (19)were performed as one-step reactions in ABI PRISM 7900 (Applied, Biosystems) using the following parameters: one cycle of 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The relative standard curve method was used to calculate the quantity of the target gene for each tissue.
extract with an internal control. The “housekeeping gene” Cyclophilin B was previously demonstrated to be “stable” across lean and obese subjects (20-22). Therefore, each sample value was divided by the quantity of Cyclophilin B as previously described (20-23).

**Adipocyte size:** Mean adipocyte size was measured as previously described (24). Tissue was fixed in osmium tetroxide. Dissociation and digestion of proteins was performed with 8 M urea/NaCl. Cells were filtered over a 10 μm nylon screen then recollected in a Triton X-100 solution. Approximately 2500 cells from each sample were analyzed on a Coulter Counter using a 400 μm aperture.

**Statistical methods:** Comparison between the lean and overweight/obese was performed using an unpaired t-test. Statistical significance was defined relative to a nominal two-sided 5% type I error rate. Values are presented as Mean ± Standard deviation. All analyses were performed in JMP (v 5.0.1, SAS, Cary NC).

**RESULTS**

**Subjects characteristics.** Subject characteristics are listed in Table 1. The subjects were men [N=11] and women [N=10] with diverse ethnicity: Caucasian [N=10], African American [N=10] or Chinese [N=1]. The lean subjects were matched for age with the overweight/obese without diabetes, however both grops were younger than the overweight/obese with diabetes [p<0.05]. Waist circumference was significantly larger in the overweight/obese vs. lean group [p<0.05]. The range of BMI was 20.4 to 23.8 kg/m² in the lean group and 28.9 to 34.7 kg/m² in the overweight/obese group. By design, the overweight/obese group had greater BMI compared with the lean group [p<0.05] and greater % fat [p<0.05]. As expected, overweight/obese subjects had lower insulin sensitivity as shown by the glucose disposal rates compared to lean [p<0.05].

**Overweight/obese subjects have lower ATpO2 and AT temperature without activation of hypoxia target genes.** We observed lower ATpO2 in overweight/obese as compared to lean subjects [Range: 29.1 - 62.8 vs. 40.5-73.8 mmHg, p<0.05; Figure 1A; Table1]. In addition, ATpO2 was negatively correlated with % fat [R = -0.50, p<0.05; Figure 1B] and fat mass [R = -0.48, p<0.05]. AT temperature was lower in overweight/obese as compared to lean [p<0.05; Table 1] and was negatively correlated with % body fat [R = -0.62, p<0.01, Figure 1C]. Race, sex and age were not significant contributors to ATpO2 or AT temperature [p=NS]. There was no relationship between the use of antihypertensive medication and ATpO2 [p=NS].

The differences in ATpO2 cannot be explained by differences in AT temperature. A 6°C difference in temperature, leads to a 1 mmHg difference in pO2 at atmospheric pressure [suplemental figure 3B]; a value that is trivial as compared to the range of values for ATpO2 (~35 mmHg).

Next, we measured the expression of known hypoxia targets: PDK1 and VEGF. We found that PDK1 and VEGF were not upregulated in overweight/obese subjects [Table 2], suggesting that the decrease in ATpO2 is not sufficient to activate the hypoxia pathway that would increase ATpO2 to normal values.

Within the overweight/obese group, there were 6 overweight/obese without type 2 diabetes and 6 overweight/obese with type 2 diabetes. The groups had similar BMI, insulin sensitivity and we found similar ATpO2 and AT temperature.

Data in humans suggests that whole body hypoxia might cause insulin resistance (7 ; 8), however the relationship with ATpO2 has not been explored. We found that ATpO2 did not
correlate with GDR, a gold standard measure of skeletal muscle insulin resistance \([R=0.21, p=NS]\).

**Evidence for rarefaction in overweight/obese AT.** ATPo2 could be reduced via several potential mechanisms: increased demand for oxygen, decreased blood flow due to vasoconstriction, or decreased blood flow due to rarefaction. To explore the latter possibility, we measured capillary density in AT samples collected from the contra lateral abdomen. Capillary density in AT was reduced by 44% in overweight/obese compared to lean subjects \([172\pm 60 \text{ vs. } 308\pm 135, p<0.05, \text{ Figure 2C}]\). We found that VEGF mRNA was 58% lower in overweight/obese vs. lean subjects \([p<0.05; \text{ Figure 2D}]\). Capillary density and VEGF expression were strongly correlated \([R = 0.81, p<0.01, \text{ Figure 2E}]\). ATPo2 was also positively correlated with VEGF mRNA \([R = 0.54, p<0.05]\). Total body fat [%] was negatively correlated with capillary density \([R = -0.69, p<0.01]\) and VEGF mRNA \([R = -0.78, p=0.01, \text{ Figure 2F}]\).

In order to determine the effect of sex, we used capillary density as the dependent variable and % fat and sex as the independent variables. The effect of sex on capillary density was not significant [data not shown].

Classically, PPARγ is known as a nuclear hormone receptor that increases adipogenesis and turns on lipogenesis \((25)\). However, recent evidence strongly suggests that PPARγ also drives angiogenesis \((26-28)\). We found that PPARγ1 was strongly correlated with VEGF \([R = 0.94, p<0.01, \text{ Figure 2G}]\) and capillary density \([R = 0.72, p<0.01]\). Ultimately, PPARγ1 was correlated with ATPo2 \([R = 0.60, p<0.05]\).

Angiopoietin 1 [ANG1] is involved in vascular remodeling, and is negatively correlated with the rate of body weight change in animals \((4; 29)\). We found that ANG1 negatively correlated with ATPo2 \([R = -0.57, p<0.05]\) and positively with % fat \([R=0.73, p<0.05]\). These suggest that overweight/obese subjects with lower ATPo2 have less vascular remodeling compared to lean subjects. Overweight/obese subjects also had a 66% greater expression of collagen VI (COL6), a extracellular matrix collagen \([p<0.05, \text{ Table 2}]\). Expression of COL6 was positively correlated with % body fat \([R = 0.55, p<0.05]\) and inversely correlated with ATPo2 \([R = -0.81, p<0.01; \text{ Figure 2H}]\).

In addition to increased adipose tissue mass, overweight/obese subjects had greater mean adipocyte size compared to lean subjects \([0.86 \pm 0.2 \text{ vs. } 0.43 \pm 0.13, \mu l, p<0.05]\). Mean adipocyte size was negatively correlated with capillary density \([R = -0.66, p<0.01]\) and VEGF \([R = -0.69, p<0.01]\).

**ATPo2 and AT inflammation.** Based on *in vitro* studies in cultured adipocytes \((6; 11)\), we hypothesized that lower ATPo2 might lead to macrophage recruitment and the secretion of inflammatory cytokines. We have previously validated the use of MAC2/CD163 and CD68 as markers of AT macrophage infiltration \([R^2 = 0.77, P <0.001]\), unpublished data by B Kozak, Gimble J, Smith SR. Both MAC2/CD163 and CD68 were inversely correlated with ATPo2 \([R = -0.66, p<0.05 \text{ and } R = -0.51, p=NS]\) consistent with accumulation of macrophages in AT with reduced oxygenation. Furthermore, MIP1α was higher in the overweight/obese subjects [Table 2] and ATPo2 was inversely correlated with MIP1α expression \([R = -0.41, p=NS, \text{ Figure 3C}]\) and MIP1α secretion in the media \([R= -0.79, p<0.05; \text{ Figure 3D}]\). There was a trend towards higher MCP1 and IL-1α in obesity and negative correlations with ATPo2 but these did not reach statistical significance. However, IL1α secretion was negatively correlated with VEGF mRNA \([R= -0.70, p<0.001]\) and capillary density \([R= -0.61, p<0.05]\).
DISCUSSION

Trayhurn first hypothesized that hypoxia of AT might play a role in insulin resistance (30). This hypothesis was based on experiments demonstrating that surrogate markers of hypoxia were increased in the AT of obese animals (30). We found that reduced ATpO2 in overweight/obese subjects and ATpO2 strongly correlates with % body fat. We also found a significant reduction in AT temperature in overweight/obese subjects and a strong inverse correlation with % total fat. This is in accordance with previous findings showing a lower skin temperature in obesity (31). The presence of capillary rarefaction suggests that decreased AT perfusion might also play a role. Indeed there is evidence to suggest that obese subjects have lower blood flow in abdominal adipose subcutaneous tissue (32). Further work using direct measures of both ATpO2 and direct measures of AT blood flow such as xenon washout are needed to formally address this hypothesis. There is additional evidence that obese mice have decreased oxygenation in epididymal and retroperitoneal AT (6; 9) and that weight loss increases oxygenation (6). Choban et al. observed an increase incidence of post-surgical wound infections in obese patients (33). This same group, measuring ATpO2 in the upper arm, subsequently showed that decreased oxygenation of AT contributes to the increased risk of infection (34). AT mass and adipocyte hypertrophy are closely related to the metabolic complications of obesity (35). In vivo data suggested a role for hypoxia in insulin resistance even though ATpO2 has never been measured in human subjects (7; 8). We were unable to demonstrate a correlation between insulin sensitivity and AT pO2. A lack of correlation of ATpO2 and insulin sensitivity may be due to lack of power because this ATpO2 and inflammation are just 2 of the many factors that induce insulin resistance or perhaps because there is simply no meaningful biological relationship. The correlation between macrophage content and insulin resistance is modest (36). One limitation of our study is that only one region of the body was measured and only at one depth.

Most hypoxic tissues develop strong transcriptional, metabolic and secretory responses to reduced oxygenation in order to increase capillary density and to correct the hypoxia. Hypoxia turns on genes that act to increase oxygen availability by decreasing oxygen consumption (switching ‘on’ anaerobic glycolysis) and stimulating angiogenesis. Hypoxia target genes expressed in AT include pyruvate dehydrogenase kinase [PDK1] and VEGF (37). Consistent with this data, VEGF and PDK1 are upregulated in adipocytes cultured in 1% oxygen [a hypoxic environment] (6; 9; 11). In contrast to our expectations, we found that lower ATpO2 in overweight/obese subjects did not induce an increase in hypoxia targets [PDK1, VEGF]. Also, capillary density and VEGF were decreased in overweight/obese AT along with a lower AT pO2. This suggests that the transcriptional counter-regulatory system was not activated. One should note, however, that the lowest value of ATpO2 in the overweight/obese group was 29 mmHg; this corresponds to 3.8% oxygen as compared to the 1% oxygen used in the cell culture experiments. This suggests overweight/obese subjects have low ATpO2 but not low enough to mount a counter regulatory response driven during a response to hypoxia. Consistent to our results, Lijnen et al. found that obese mice have both lower VEGF and lower vascular density in AT (5). In addition, it is known that spleen, thymus and retina have low pO2 in normal rats (38), suggesting that the angiogenesis is activated at different levels of oxygenation for different types of tissue or that oxygenation could influence angiogenesis. One limitation of our study is that we did not measure VEGF protein and
this should be addressed in future experiments.

Recent data suggests that PPARγ1 might be required for angiogenesis in AT (39). PPARγ drives VEGF [and angiogenesis] (26-28). We found a strong positive correlation between PPARγ1 and VEGF and between VEGF and AT pO2. One way to interpret this data is that PPARγ1 drives angiogenesis in human AT and therefore is a key controller of AT pO2. More work is needed to test this hypothesis.

AT expansion [adipogenesis] during development is preceded by a wave of neo-vascularization (40). Vascular plasticity may play a role in the ability of AT to increase or decrease in size (29; 41). Our data suggests that reduced capillary density might restrict the growth of AT. Our finding of reduced capillary density in subcutaneous adipose tissue in obesity suggests the hypothesis that the failure of the vasculature to expand with increasing subcutaneous obesity might limit adipogenesis in subcutaneous depot. If visceral adipose tissue were not similarly restricted this might allow for the growth of visceral AT. Further work, measuring ATpO2 and capillary density in omental and mesenteric adipose tissue is needed to test this hypothesis.

Previous studies have shown that COL6 is abundantly expressed by adipocytes (42) and obese mice have increased COL6 expression in the extracellular matrix (43). We found that O/O subjects with low ATpO2 have greater expression of COL6 and COL6 expression increased with increased body fat and fat cell size. Scherer et al. (44) suggests that proteolytic fragments of COL6 promote tumor growth through pro survival and proliferation signaling pathways such as Akt and β catenin. This suggests that new blood vessel formation is restricted by increased extracellular matrix or that a reduction in angiogenesis leads to increases in the formation of the extracellular matrix as exemplified by COL6. Further work is needed to separate these 2 possibilities.

AT inflammation has received much attention as an important factor in insulin resistance and type 2 diabetes (6; 12; 45). Previous in vitro and in vivo pre-clinical studies showed that hypoxia induces inflammation which might contribute to insulin resistance (6; 11). We found that in humans ATpO2 correlates with macrophage markers [CD68 and MAC2/CD163]. More than this, AT secretion of MIP1α a potent macrophage chemokine (46; 47) increased as ATpO2 decreased. This is consistent with recent data showing up-regulation of MIP1α in obesity (48). This is supportive of the hypothesis that lower oxygenation drives inflammation by up regulating adipocyte chemokine secretion, but, as discussed previously, not by activating the classic hypoxia pathway and VEGF. However, it is possible that inflammation could drive hypoxia. Given that MIP1α has been implicated in angiogenesis it is unclear why MIP1α is up when capillary density is down. Further work is needed to understand the factors regulating angiogenesis in human adipose tissue.

In summary, we provide direct evidence of lower ATpO2 in overweight/obese subjects, and that the most likely cause are decreased capillary density and reduced expression of the angiogenic factors like VEGF and PPARγ1 and increased expression of COL6. This suggests that low ATpO2 in overweight/obese does not results in a complete counter regulatory response to reduced ATpO2 and that neo-vascularization is not activated. Decreased ATpO2 was paralleled by an increase in the expression and secretion of the chemokine and markers of macrophage infiltration. These data suggests that pro-angiogenic therapies might reduce AT inflammation, improve insulin action and reduce cardiovascular disease risk in obesity and type 2 diabetes.
ACKNOWLEDGEMENTS

This work was funded by a grant from the Health and Performance Enhancement Division of PBRC and in part by an unrestricted grant from the Organization for the Study of Sex Differences. Research Support was provided by the Clinical Nutrition Research Unit grant # P30 – DK072476. We also want to acknowledge Drs. George Bray and Eric Ravussin and especially Dr. Harriet Hopf for their support, advice and counsel.
REFERENCES

Table 1: Clinical characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>O/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>22.6 ± 3.3</td>
<td>38.9 ± 15.8*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64.8 ± 7.7</td>
<td>92.0 ± 12.8*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.1 ± 1.0</td>
<td>31.7 ± 1.9</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>73 ± 4.4</td>
<td>100 ± 10.4 *</td>
</tr>
<tr>
<td>% Fat (DEXA)</td>
<td>20.9 ± 7.6</td>
<td>34.2 ± 8.2</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>89 ± 3</td>
<td>110 ± 27</td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>4.9 ± 2.0</td>
<td>14.4 ± 9.6 *</td>
</tr>
<tr>
<td>GDR (mg/min x kg FFM)</td>
<td>11.2 ± 3.4</td>
<td>6.0 ± 2.2</td>
</tr>
</tbody>
</table>

Abdominal subcutaneous adipose tissue

| AT pO₂ (mm Hg) | 55.4 ± 9.1 | 46.8 ± 10.6 * |
| AT temperature (°C) | 34.0 ± 1.0 | 32.1 ± 1.4  * |

Values are mean ± SD. * p< 0.05

O/O: overweight/obese group; BMI; body mass index; % body fat was measured by DEXA and represents mass as the % of total body weight; GDR: glucose disposal rate measured during euglycemic hyperinsulinemic clamp. The lean group included 2 African Americans and 6 Caucasians and 1 Asian. The overweight/obese group included 8 African Americans and 4 Caucasians.
### Table 2: AT characteristics of lean vs. O/O volunteers

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Lean (N=6)</th>
<th>O/O (N=8)</th>
<th>Correlation with % fat</th>
</tr>
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<tbody>
<tr>
<td>Leptin mRNA</td>
<td>0.19 ± 0.11</td>
<td>0.57 ± 0.12</td>
<td>* 0.74 &lt;0.05</td>
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<tr>
<td>PDK1 mRNA</td>
<td>0.63 ± 0.24</td>
<td>0.45 ± 0.21</td>
<td>-0.10</td>
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<tr>
<td>VEGF mRNA</td>
<td>2.46 ± 1.11</td>
<td>1.04 ± 0.34</td>
<td>* -0.78 &lt;0.05</td>
</tr>
<tr>
<td>ANG1 mRNA</td>
<td>0.41 ± 0.13</td>
<td>0.69 ± 0.11</td>
<td>0.73 &lt;0.05</td>
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<td>PPARγ mRNA</td>
<td>1.33 ± 0.36</td>
<td>0.74 ± 0.21</td>
<td>* -0.73 &lt;0.05</td>
</tr>
<tr>
<td>COL6 mRNA</td>
<td>0.32 ± 0.17</td>
<td>0.53 ± 0.16</td>
<td>* 0.55 &lt;0.05</td>
</tr>
<tr>
<td>CD68 mRNA</td>
<td>0.23 ± 0.05</td>
<td>0.62 ± 0.27</td>
<td>* 0.64 &lt;0.05</td>
</tr>
<tr>
<td>MAC2/CD163 mRNA</td>
<td>0.56 ± 0.22</td>
<td>1.49 ± 0.63</td>
<td>* 0.72 &lt;0.05</td>
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<tr>
<td>MIP1α mRNA</td>
<td>0.02 ± 0.01</td>
<td>0.21 ± 0.18</td>
<td>* 0.60 &lt;0.05</td>
</tr>
<tr>
<td>MCP1 mRNA</td>
<td>0.12 ± 0.05</td>
<td>0.25 ± 0.11</td>
<td>* 0.43</td>
</tr>
</tbody>
</table>

### Cytokine release (pg/mg tissue/h)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Lean (N=6)</th>
<th>O/O (N=8)</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1α</td>
<td>0.44 ± 0.38</td>
<td>0.96 ± 0.69</td>
<td>0.60 &lt;0.05</td>
</tr>
<tr>
<td>MCP1</td>
<td>2.26 ± 0.83</td>
<td>2.36 ± 1.18</td>
<td>0.12</td>
</tr>
<tr>
<td>IL1α</td>
<td>0.09 ± 0.08</td>
<td>0.20 ± 0.07</td>
<td>* 0.67 &lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SD. * p< 0.05

O/O: overweight/obese group; PDK1: pyruvate dehydrogenase kinase; VEGF, vascular endothelial growth factor; ANG1, Angiopoietin-1; PPARγ1, peroxisome proliferative activated receptor, gamma 1; COL6, collagen VI alpha 3 subunit, CD68, CD68 antigen; MAC-2, macrophage-associated antigen [CD163]; MIP1α, macrophage inflammatory protein 1, alpha subunit [CCL3 by the latest nomenclature], MCP1, macrophage chemoattractant protein 1; IL1α, interleukin 1 alpha.
Figure 1: AT pO₂ and AT temperature are inversely correlated with % body fat
AT pO₂, measured by direct insertion of a micro Clark type electrode into abdominal subcutaneous AT, was lower in overweight/obese group [O/O] compared to lean subjects [A] and inversely correlated with % body fat [B]. AT temperature measured by a thermocouple inserted into the abdominal AT was inversely correlated with % body fat [C]. Males are represented by squares and females by circles, filled with different colors as follows: white for lean, grey for O/O without type 2 diabetes and black for O/O with type 2 diabetes.
**Figure 2: Vascularization of AT**

Representative AT sections from lean [A] and O/O [B] subjects stained with UEA lectin (orange) to label capillaries and with GS lectin (green) to label the adipocyte plasmalemma. Capillary density [C] was measured and averaged across 6-10 histological sections for each subject and VEGF mRNA expression measured by qRT-PCR [D]; both were lower in O/O vs. lean subjects. VEGF mRNA was positively correlated with capillary density [E] and PPARγ1 mRNA [G] and inversely with % body fat [F]. CollagenVI [Col6] mRNA was negatively correlated with AT pO₂ [H]. Males are represented by squares and females by circles, filled with different colors as follows: white for lean, grey for O/O without type 2 diabetes and black for O/O with type 2 diabetes.
Rarefaction and Inflammation in Obese Human Adipose Tissue

**E**

E

**F**

F

**G**

G

**H**

H

R = 0.81
p < 0.05

R = 0.94
p < 0.05

R = 0.78
p < 0.05

R = -0.81
p < 0.05
Figure 3: Hypoxia and AT inflammation
AT pO$_2$ was inversely correlated with the inflammation markers CD68 mRNA [A] and MAC2/CD163 mRNA [B], with the chemokine MIP1$\alpha$ mRNA expression [C] and MIP1$\alpha$ secretion into culture media ex vivo [D]. Previous studies in our lab demonstrated a strong correlation between MAC2/CD163 and CD68 mRNA and macrophage infiltration by macrophage staining in AT sections By immuno histochemistry [$R^2 = 0.77$, $P <0.001$], unpublished data by B Kozak, Gimble J, Smith SR. Males are represented by squares and females by circles, filled with different colors as follows: white for lean, grey for O/O without type 2 diabetes and black for O/O with type 2 diabetes.