Chapter 7

Insulin-Induced Changes in Skeletal Muscle Microvascular Perfusion are Regulated by Perivascular Adipose Tissue in Women

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Abstract

Rationale Obesity increases the risk of cardiovascular disease and type 2 diabetes, at least partly through its association with microvascular dysfunction and insulin resistance. In the skeletal muscle microcirculation of obese subjects, insulin's vasodilator effects are impaired, reducing insulin-induced glucose delivery and uptake. Here, we studied whether perivascular adipose tissue (PVAT) controls insulin-induced vasodilation in human muscle, and whether altered properties of PVAT relate to reduced perfusion and insulin resistance in obesity.

Methods & Results We studied 15 lean and 18 obese women (18-55 years), and determined insulin-induced microvascular recruitment using contrast-enhanced ultrasound (CEU) before and during a hyperinsulinemic euglycemic clamp. Obese women had lower microvascular recruitment and metabolic insulin sensitivity compared to lean women. Microvascular recruitment was identified as a significant mediator of the association between obesity and metabolic insulin sensitivity. The difference in microvascular recruitment between obese and lean women was statistically explained by increased size of perivascular adipocytes in skeletal muscle biopsies. To separate intrinsic endothelial dysfunction from changes induced by PVAT, we examined the modulation of insulin-induced vasomotor responses by PVAT in microvessels dissected from human skeletal muscle biopsies in a pressure-myograph. In the biopsies of tissue from lean women PVAT enhanced insulin-induced vasodilation, while in those from obese women PVAT caused insulin-induced vasoconstriction.

Conclusions These results strongly suggest that PVAT enhances insulin-induced microvascular recruitment and, thereby, insulin-induced glucose uptake in skeletal muscle of lean women, this effect being abolished in obesity. Microvascular perivascular adipose tissue may therefore be an important factor in insulin resistance and cardiovascular disease.
**Introduction**

Arterioles, capillaries and venules together make up the microcirculation. Important functions of the microcirculation are to optimize nutrient and oxygen supply to tissue in response to variations in demand, and to regulate peripheral resistance.\(^1\,^2\) Arterioles regulate flow toward different sites according to demand by increasing or decreasing their tone.\(^1\) Arteriolar tone is generally increased in obesity, and thereby contributes to hypertension and insulin resistance.\(^3\,^4\)

Insulin has vasoactive properties in the microcirculation, leading to vasodilation in insulin sensitive subjects, thereby increasing muscle perfusion through the recruitment of additional capillaries, so-called ‘microvascular recruitment’. This augments delivery of insulin and glucose to skeletal muscle, the organ most important for insulin sensitivity, thus enhancing glucose uptake.\(^4\,^6\) Insulin-induced microvascular recruitment is blunted in insulin-resistant states such as obesity and hypertension, and in turn, impaired microvascular recruitment contributes to insulin resistance,\(^5\,^7\,^8\) the underlying mechanisms of which are unclear.

We recently identified perivascular adipose tissue (PVAT) around microvascular structures in skeletal muscle as a depot of ectopic adipose tissue, and proposed a regulatory role of PVAT in muscle perfusion and insulin sensitivity.\(^9\,^{10}\) In mice, we demonstrated that ex-vivo, PVAT exerts paracrine effects on the vessels it surrounds.\(^{11}\) These paracrine effects enhance insulin-induced vasodilation in lean mice, are mediated through the secretion of adipokines, and are abolished in extreme obesity.\(^{11}\) This anticontractile effect of healthy PVAT extends previous findings that PVAT controls vascular smooth muscle function.\(^{11}\,^{14}\) Inflammation and adiponectin receptor-1 agonists both have major effects on the vasoactive effects of PVAT.\(^{11}\) Changes in PVAT function in obesity may be caused by inflammation, which is partly mediated by macrophage infiltration and/or macrophage polarization.\(^{13}\,^{16}\) Whether PVAT in the muscle microcirculation enhances insulin-induced vasodilation, and therefore microvascular recruitment, in insulin sensitive humans is unknown.
In this study, we hypothesized that PVAT in the human skeletal muscle microcirculation regulates insulin-induced microvascular recruitment in-vivo, and that this regulation is altered in obesity.

**Research design and methods**

*Participants*

A total of 15 lean (BMI 18-25 kg/m²) and 18 obese (BMI >30 kg/m²) female volunteers participated in this study. Physical health was determined by medical history, physical examination and screening blood tests. Inclusion criteria were female gender, 18-55 years old, and Caucasian descent. Exclusion criteria consisted of current illness, a history of cardiovascular disease, hypertension, diabetes mellitus or impaired kidney function, use of medication known to affect endothelial function or glucose metabolism, excessive physical exercise (i.e. more than three times a week), recent changes in body weight, pregnancy, alcohol abuse, and smoking. All volunteers were recruited through advertisements. The study protocol was approved by the medical ethics committee of the VU University Medical Center, and was conducted in accordance with the Declaration of Helsinki. All volunteers provided written informed consent before enrollment in the study.

*Study design*

The study was a case-control study. Participants visited a quiet, temperature controlled room at the Clinical Research Unit on three separate days within two months, the first time for a screening visit, the second time for the hyperinsulinemic euglycemic clamp and contrast enhanced ultrasound (CEU) measurements, and the third time for the skeletal muscle biopsy. Participants were fasted overnight for the screening and the euglycemic clamp visits. Volunteers were asked to refrain from physical exercise on the day before the euglycemic clamp as well as the day before and two days after the skeletal muscle biopsy. In the three cases where the skeletal muscle biopsy was taken before the hyperinsulinemic euglycemic clamp, CEU measurements were performed in the contralateral thigh to avoid residual effects of wound healing.

After arrival at the Clinical Research Unit, the participants had 30 minutes of acclimatization after which the first measurements started.
Anthropometry was determined at the screening and at the morning of the hyperinsulinemic euglycemic clamp, and fat percentage was assessed by bioelectrical impedance analysis (BF906, Maltron, Rayleigh, UK).5

**Hyperinsulinemic euglycemic clamp**

Insulin sensitivity was determined with the hyperinsulinemic euglycemic clamp technique, as described previously and depicted in figure 1.17 Insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), was infused in a primed (4 mU/m²), continuous manner, at a rate of 40 mU/m²/min for 120 minutes. Euglycemia was maintained at 5 mmol/l by varying the infusion rate of glucose 20%, based on whole-blood venous samples (YSI 2300 STAT Plus analyzer, Yellow Springs, OH), at an interval of 5 minutes. Whole-body glucose uptake or M-value, was determined from the glucose infusion rate during the last 60 minutes of the clamp, and expressed as mg/kg/min.

**Microvascular Blood Volume - Contrast Enhanced Ultrasound**

Contrast enhanced ultrasound measurements were performed with a Siemens-Acuson Sequoia 512, equipped with a 17L5 transducer as described.5 The vastus lateralis muscle was imaged approximately 15 cm proximal to the knee, with the volunteer in the supine position. During the baseline measurement, the location of the probe on the skin was carefully outlined, as well as landmark structures in the ultrasound screen. Microbubbles (SonoVue; Bracco, Milan, Italy) were infused undiluted, at a constant rate of 2.5 ml/min for 4 minutes in both lean and obese participants, as the exact volume of the total intravascular space is unknown for individual participants. After steady-state concentration of the microbubbles was achieved (2 minutes), 3 real-time inflow curves with a duration of >25 seconds were performed at a mechanical index of 0.28, after destruction of the microbubbles with a mechanical index of 1.7. Postprocessing was set to linear. Microbubbles were freshly prepared approximately 2 minutes prior to infusion for both the baseline and the hyperinsulinemia measurement. After preparation, the microbubble solutions were constantly agitated until completion of the infusion. Video-intensities of the CEU clips were analyzed using the Image Processing toolbox in MATLAB, version
R2011a (Mathworks, Natick, MA, USA). Mean video-intensity during the first 0.5 seconds was subtracted from the signal to correct for large vessels and background noise. Real-time curves from the region of interest in skeletal muscle were then fitted to the exponential function \( V_I = MBV(1-e^{-MFV(t-0.5)}) \), \( V_I \) is the video intensity at \( t \), where \( t \) represents the time (sec) after microbubble destruction, MBV is Microvascular Blood Volume, MFV is Microvascular Flow Velocity and \( e \) is the natural logarithm. CEU does not provide an absolute measure of volume flow (ml/min/gram tissue), but is a relative measure used as a paired measurement within subjects, and we therefore report only the percentage change in MBV.\(^{18}\)

Skeletal muscle biopsy

Surgical skeletal muscle biopsies were taken in the non-fasting state. The open surgical muscle biopsy was taken at a similar location as the CEU measurement, and obtained from the vastus lateralis muscle at approximately 15 cm proximal to the knee. Lidocaïne 2% was used as a local anesthetic and subsequently the skin and fascia were incised. A skeletal muscle biopsy of approximately 7mm x 7mm x 7mm was taken, exercising due care to minimize damage to the microvessels. The biopsy was immediately stored in ice-cold (0-5°C) MOPS-buffer (in mM: 145 NaCl, 4.7 KCl, 3.3 CaCl\(_2\), 2.0 MgSO\(_4\), 1.4 NaH\(_2\)PO\(_4\), 2.5 pyruvate, 0.02 EDTA, 3.0 MOPS (3-(N-morpholino) propanesulfonic acid), 5.6 glucose, and quickly transferred to the laboratory to harvest microvessels for testing.
Pressure myography

To independently investigate the effect of PVAT on insulin-induced vasoreactivity, microvessels were isolated on ice from one half of the skeletal muscle biopsy, and separated from the surrounding PVAT. The vessels were then mounted on glass cannulae in both ends, and randomly assigned to be co-incubated without PVAT or with PVAT. The PVAT from alongside the microvessel was then fastened to one of the cannulae. Ex vivo vasoreactivity of isolated microvessels was studied in the pressure myograph at 80 mmHg and 37°C in K-MOPS buffer (in mM: 125 NaCl, 26 KCl, 3.3 CaCl₂, 2.0 MgSO₄, 1.4 NaH₂PO₄, 2.5 pyruvate, 0.02 EDTA, 3.0 MOPS, 5.6 glucose, and 0.1% Bovine Serum Albumin). Microvessels were preconstricted by the potassium in the buffer, and inner diameters were recorded to determine baseline diameter. Diameter changes induced by four cumulative concentrations of insulin (0.02, 0.2, 2.0 and 20 nM) were examined for 30 minutes each. To ascertain having isolated an arteriole or resistance artery instead of a venule, as well as to check endothelial integrity, the endothelium-dependent vasodilator acetylcholine (ACh) 1*10⁻⁷ M and ACh 1*10⁻⁶ M was tested at the end of each experiment, if a microvessel failed to achieve at least 10% vasodilation to ACh 1*10⁻⁶ M, it was excluded from analysis entirely. Maximum diameter was assessed after administration of papaverine 0.1 mmol/l.

(Immunohistology)

The second half of the skeletal muscle biopsy was used for histology. This half was stored in buffered formaldehyde (4%) and paraffin embedded the next morning. For histochemical analysis, slices with a thickness of 5 μm were dewaxed, rehydrated and stained with hematoxylin and eosin. Adipocyte cross-sectional areas were analyzed with Image J in a blinded fashion. Only adipocytes at a distance no greater than three adipocytes from the microvessel were included to prevent confounding by e.g. subcutaneous adipose tissue. Total macrophage count in PVAT was quantified after CD68 staining and presented as the fraction of the number of adipocytes. For CD68 immunohistochemical analysis, 5 um sections were dewaxed, rehydrated, and incubated in methanol/H₂O₂ (0.3%) for 30 minutes to block endogenous peroxidases. Next, antigen retrieval was performed.
by heat inactivation in citrate buffer (pH 6.0). Followed by incubation with mouse anti-human CD68 (1:400, Dako Cytomation, Denmark) for 1 hour at room temperature. Sections were then incubated with Envision (undiluted, anti-mouse and rabbit, Dako Cytomation, Denmark) for 30 minutes at room temperature. Staining was visualized using 3,3'-diaminobenzidine (DAB 0.1 mg/ml, 0.02% H2O2). Sections were then counterstained with hematoxylin, dehydrated, and covered.

Statistical analysis

Data were analyzed with paired (within group) and unpaired (between groups) tests. Normally distributed data are reported as mean ± SD. Non-normally distributed data were log-transformed to obtain normal distribution, or reported as median and range and analyzed with non-parametric tests. Pressure myography experiments were analyzed using a two-way ANOVA with Bonferroni post hoc test. A p-value smaller than 0.05 was considered statistically significant. Linear regression analyses were performed to examine the relations between two variables, controlling for age, and standardized betas (B) are reported. Bias corrected bootstrapping according to Preacher and Hayes was used to assess mediation effects. In short, this analysis performs multiple regression analyses consecutively on multiple random subsets of the dataset, thereby estimating significance of the change in the beta, and therefore the statistical mediation. Analyses were performed using IBM SPSS Statistics version 21 and Graphpad Prism 5.01.

Results

Baseline characteristics

The baseline characteristics of the participants involved are presented in table 1. Two women in the lean group, and three women in the obese group retracted their informed consent after the hyperinsulinemic clamp but before the skeletal muscle biopsy. In three women in the lean, and six in the obese group, the skeletal muscle biopsy yielded insufficient tissue for histology, although pressure-myography was successful.
### Table 1 baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42 (24-55)</td>
<td>41 (19-53)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>64±7</td>
<td>96±17</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.72±0.06</td>
<td>1.70±0.07</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.4 (18.7-25.0)</td>
<td>33.0 (30.0-49.3)</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.82±0.07</td>
<td>0.89±0.06</td>
</tr>
<tr>
<td>Fat percentage</td>
<td>25.0 (15.9-31.7)</td>
<td>44.6 (28.3-54.9)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>114±10</td>
<td>127±10</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71±7</td>
<td>74±9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>85±8</td>
<td>92±8</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.9±0.8</td>
<td>4.8±0.9</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.6±0.7</td>
<td>2.7±0.7</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.9 (1.2-2.5)</td>
<td>1.6 (1.2-2.1)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8 (0.5-1.4)</td>
<td>1.3 (0.6-1.8)</td>
</tr>
<tr>
<td>eGFR (MDRD)</td>
<td>87±13</td>
<td>93±20</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>32 (18-61)</td>
<td>64 (23-122)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.6±0.4</td>
<td>5.4±1.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.83 (0.60-1.93)</td>
<td>2.12 (0.84-5.93)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3±0.2</td>
<td>5.6±0.5</td>
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**Microvascular recruitment partly explains the difference in metabolic insulin sensitivity between lean and obese women**

To assess insulin-induced microvascular recruitment, as well as metabolic insulin sensitivity, contrast enhanced ultrasound was performed before and at the end of a hyperinsulinemic euglycemic clamp. Metabolic insulin sensitivity (M-value) was higher in lean compared with the obese women (p<0.001) (table 2). During hyperinsulinemia, microvascular blood volume (MBV) increased in the lean, but not in the obese women; (Figure 2A). Microvascular recruitment was significantly different between the two groups. In paired analyses, Microvascular Flow Velocity did not change in either lean or obese women, from baseline to hyperinsulinemia.
Table 2 Hyperinsulinemic euglycemic clamp parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lean</th>
<th>Obese</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean glucose (mmol/l)</td>
<td>5.0±0.3</td>
<td>4.9±0.3</td>
<td>0.29</td>
</tr>
<tr>
<td>M-value</td>
<td>10.9 (4.4-14.9)</td>
<td>6.0 (2.2-15.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MBV baseline (a.u.)</td>
<td>0.62 (0.13-2.04)</td>
<td>1.15 (0.47-2.34)</td>
<td>n/a</td>
</tr>
<tr>
<td>MBV hyperinsulinemia (a.u.)</td>
<td>0.82 (0.10-2.67)</td>
<td>1.00 (0.20-2.87)</td>
<td>n/a</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td>551±103</td>
<td>647±97</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 2. The difference in insulin-induced microvascular recruitment between lean and obese women, and the contribution thereof to metabolic insulin sensitivity

   In lean women, a larger MBV is recruited by insulin, (26% (-44 to +210), *=p<0.05). In the obese women, insulin did not significantly recruit additional MBV (10% (-57 to +62), p=0.97). When these data were log transformed and tested parametrically, the p-value for the change in MBV was (* p<0.05).

B. Indirect effects of microvascular recruitment on metabolic insulin sensitivity (M-value). BMI group was related with M-value with a beta of -0.76, p<0.001, corrected for age. The beta for BMI group to microvascular recruitment was -0.40, p<0.05, and the beta for microvascular recruitment to M-value 0.45, p<0.05. Mediation analysis confirmed the mediating role of microvascular recruitment in the relation between BMI group and M-value (B=-0.34, CI (confidence interval) -0.82 to -0.08. Indeed, the beta to from BMI group to M-value was attenuated to -0.60, p<0.05. *p < 0.05, ***p < 0.001
Using mediation analyses, microvascular recruitment was identified as a significant mediator in the relation between the group (lean or obese) and metabolic insulin sensitivity (Figure 2B).

*Increased PVAT adipocyte size mediates the relation between obesity and disturbed microvascular recruitment*

In obese women, the median perivascular adipocyte cross-sectional area was larger than in lean women (figures 3A and 3B), although perivascular adipocyte size and BMI did not correlate within these groups (B=0.259, p=0.42 for the lean, and B=-367, p=0.22 for the obese).

Macrophage (CD68+) count per number of adipocytes was not significantly different between the lean and obese group (respectively 0.43 macrophage/adipocyte in PVAT of lean women vs. 0.25 macrophage/adipocyte in PVAT from obese women, p=0.19). When expressed as macrophages per calculated adipocyte surface area, the amount of macrophages was non-significantly higher in the obese (2.3 (1.0-3.5) vs 1.4 (0.3-5.0), p=0.36).

We went on to explore whether PVAT adipocyte size in PVAT explained the relation between the study-group and microvascular recruitment using mediation-analyses. This revealed that PVAT adipocyte size was indeed a significant statistical mediator in the relation between the study-group (lean or obese) and microvascular recruitment (Figure 3C). Furthermore, perivascular adipocyte size was also related to metabolic insulin sensitivity (B=-0.58, p=0.003).
Figure 3. Perivascular adipocyte size partly explains the difference in microvascular recruitment between lean and obese women.

A. Typical example of a HE-staining with an arteriole and microvascular muscle PVAT. Black arrow: resistance artery, yellow arrow: PVAT, red arrow: skeletal muscle.

B. In lean women, the size of individual adipocytes in PVAT was smaller than in obese women (530 (152-1002) vs. 1637 (195-3751), p<0.01).

C. Indirect effects of perivascular adipocyte size on microvascular recruitment. BMI group was related with microvascular recruitment with a beta of -0.40, p<0.05, corrected for age. The beta for BMI group to PVAT adipocyte size was 1.08, p<0.05, and the beta for PVAT adipocyte size to microvascular recruitment -0.40, p=0.065. Mediation analysis confirmed the mediating role of PVAT adipocyte size in the relation between BMI group and microvascular recruitment (B=-0.29, CI (confidence interval) -0.96 to -0.01. Indeed, the beta to from BMI group to M-value was attenuated to 0.01, and was no longer significant, this shows that perivascular adipocyte size is a significant mediator in the relationship between BMI group and microvascular recruitment. *p < 0.05.

PVAT from lean women potentiates insulin-induced vasodilation and PVAT from obese women enhances insulin-induced vasoconstriction ex vivo

To study direct effects of PVAT on insulin-induced vasoreactivity, instead of statistical relations, we performed ex-vivo pressure-myography with microvessels harvested from the skeletal muscle biopsies, see figure 4A for an example of a mounted microvessel. Intact
vasoreactivity, as determined by at least 10% vasodilation induced by acetylcholine $10^{-6}$ at the end of the experiment is indicated in table 3. Failing this test resulted in exclusion from the vasoreactivity analyses and occurred in 50% and 56% of the experiments with lean, and obese microvessels respectively.

Microvessels obtained from lean women and incubated without PVAT showed no insulin-induced changes in diameter ex-vivo, comparable to previous murine results. In contrast, microvessels incubated with PVAT from the same subject showed insulin-induced vasodilation (Figure 4B), supporting that PVAT secretes factors contributing to insulin-induced microvascular vasodilation. Microvessels from obese women without PVAT showed no insulin-induced responses in diameter, similar to the lean microvessels. However, when incubated with their own PVAT these microvessels constricted in response to increasing doses of insulin (Figure 4B), which was both different from the obese microvessels without PVAT as well as the lean microvessels with PVAT. These results show that in lean, healthy women, PVAT secretes adipokines that permit insulin-induced vasodilation. In contrast, PVAT from obese women has an altered adipokine secretion-profile, leading to insulin-induced vasoconstriction.

**Figure 4. PVAT regulates insulin-induced vasoreactivity ex-vivo**

A. Example of a cannulated microvessel free from PVAT.

B. Without PVAT, microvessels from both lean (n=6) and obese women (n=8) do not exhibit changes in vascular diameter to increasing doses of insulin in the pressure myograph, but PVAT from lean women (n=8) potentiates insulin-induced vasodilation at the highest concentration, whereas PVAT from obese women (n=8) enhances insulin-induced vasoconstriction.
Table 3 Ex-vivo microvessel characteristics

<table>
<thead>
<tr>
<th></th>
<th>Lean, no PVAT (n=15)</th>
<th>Lean + PVAT (n=15)</th>
<th>Obese, no PVAT (n=18)</th>
<th>Obese + PVAT (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of successful ex-vivo experiments of all biopsies performed (%)</td>
<td>6 (40)</td>
<td>9 (60)</td>
<td>8 (44)</td>
<td>8 (44)</td>
</tr>
<tr>
<td>Maximum diameter; papaverine 0.1 mmol/l (μm)</td>
<td>172 (96-325)</td>
<td>145 (102-246)</td>
<td>138 (94-188)</td>
<td>164 (53-294)</td>
</tr>
<tr>
<td>Acetylcholine mediated dilation (%)</td>
<td>57 (11-117)</td>
<td>48 (14-100)</td>
<td>21 (10-42)*</td>
<td>32 (10-57)</td>
</tr>
<tr>
<td>Tone (% of maximum)</td>
<td>20 (10-58)</td>
<td>42 (10-67)</td>
<td>42 (19-77)</td>
<td>29 (16-78)</td>
</tr>
</tbody>
</table>

*p<0.05 compared with lean microvessels without PVAT

Discussion

The relation between microvascular PVAT and microvascular vasomotor responses in vivo was hitherto unknown. This study is the first to directly demonstrate a relation between PVAT characteristics and microvascular function in muscle. More specifically, perivascular adipocyte size mediates the difference in insulin-induced microvascular recruitment between lean and obese women. These results were confirmed by the ex-vivo experiments, which directly show that PVAT from lean women potentiates the vasodilator effect of insulin, whereas it causes insulin-induced vasoconstriction with PVAT from obese women. These findings suggest that, in humans, PVAT regulates insulin-induced vasodilation, and insulin-induced microvascular recruitment.
The relations between PVAT and microvascular recruitment are in line with a study relating PVAT around the brachial artery to (non-insulin induced) microvascular vasodilator capacity.\textsuperscript{20} However, in contrast to that study, we studied PVAT which abuts the microcirculation itself, and provide more direct ex-vivo experimental evidence for a role of PVAT. In PVAT and vessels obtained from subcutaneous adipose tissue of lean humans, PVAT also shows an ex-vivo anticontractile effect, which is lost in obese individuals.\textsuperscript{12} The lost anticontractile effect of PVAT in obesity can be restored by bariatric surgery, a process independent of endothelial function.\textsuperscript{21} The latter study also found a reduced macrophage count in obese PVAT after bariatric surgery. We did not find a difference in PVAT macrophage content between lean and obese women, possibly because our obese participants were less extremely obese or acute weight reduction has specific effects of its own. A recent study also did not find a difference in macrophage content in PVAT after a high fat diet in wild-type mice and mice with reduced inflammatory responses in PVAT, despite functional differences of the PVAT.\textsuperscript{22} Another issue to be considered is that the macrophage count may not adequately reflect the pro-inflammatory potential of PVAT, as macrophages may differ in anti-/pro-inflammatory (M1/M2) polarization and activation status.\textsuperscript{13, 23} Despite the lack of clear evidence of inflammation, we could demonstrate functional changes in PVAT.

Our analyses suggest that the degree of insulin-induced microvascular recruitment is a significant mediator in the relationship between obesity and metabolic insulin sensitivity (Fig2B). Also, PVAT adipocyte cross-sectional area seems to be a mediator in the relationship between obesity and microvascular recruitment, even though one of the component analyses was of borderline significance (p=0.065) (Fig 3C). It appears that perivascular adipocyte size is not just a marker for generalized adiposity, as evidenced by the lack of a correlation with BMI. Perivascular adipocyte size itself seems to be an unlikely cause of the altered PVAT functional phenotype. Probably, larger adipocyte size was a proxy for altered PVAT characteristics (e.g. hypoxia, inflammation) and therefore an altered secretory adipokine profile.\textsuperscript{24}
Recently, we described an important role for JNK in murine PVAT, suggesting that inflammation contributes to the deterioration of PVAT’s anticontractile effects in obesity.\textsuperscript{11} This is supported by data from others, who found that reactive oxygen species and TNFα mediate this deterioration.\textsuperscript{12} In addition, adiponectin R1 agonists have been shown to propagate the vasodilator effects of lean PVAT through signaling via AMPKα2.\textsuperscript{11} The role of globular adiponectin is particularly well investigated in this context.\textsuperscript{11, 12, 25} Further support for a local effect of PVAT comes from the observation that venous concentrations of adiponectin are higher than arterial concentrations, indicating that there must be local production of adiponectin by PVAT in the microcirculation,\textsuperscript{26} and PVAT has been shown to secrete a different adipokine profile from both subcutaneous and visceral adipose tissue.\textsuperscript{24, 27} In the current study, we did not examine adipokine secretion by PVAT or protein-signaling in the microvessels. Although adiponectin signaling from PVAT provides an attractive explanation for our results, there are other candidate components signaling from PVAT to the vessel wall, such as leptin, hydrogen peroxide and angiopoietin-like protein 2.\textsuperscript{28-30} We also cannot exclude the possibility that in these human microvessels PVAT determines smooth muscle cell relaxation and contraction more than endothelial signaling, as others have used de-endothelialized resistance arteries and also found anticontractile effects.\textsuperscript{21} However, insulin’s microvascular vasoreactivity is a endothelium-dependent response.\textsuperscript{3} The vasodilator response uncovered by PVAT from lean women forms an elegant explanation for the increased insulin-induced microvascular perfusion in lean women. Furthermore, the enhancement of the insulin-induced vasoconstriction with PVAT from obese women ex-vivo is in line with the lack of microvascular response in-vivo to hyperinsulinemia.

To the best of our knowledge, this is the first study using human skeletal muscle microvessels in a vascular function ex-vivo experiment. The experimental success rate of 47% was lower than in comparable murine studies, despite a somewhat more lenient quality control of >10% dilation at ACh 10\textsuperscript{-6} instead of ACh 10\textsuperscript{-7}.\textsuperscript{11} It is critically important to obtain sufficiently large surgical biopsies to increase the chance of finding a large enough muscular microvessel. In these experiments, in-
vivo conditions are simulated as well as possible, although adjustments need to be made. For example, preconstriction is established through 25 mM potassium, which is high compared to interstitial concentrations in-vivo, but low compared with other studies examining ex-vivo vasoreactivity.\textsuperscript{12, 21} Despite our best efforts, there may be some degree of selection bias (of the microvessels) inherent to these experiments. We manually selected microvessels that appeared suitable for cannulation, but no formal diameter measurement is possible before cannulation, probably favoring larger microvessels. PVAT was separated from the microvessels to prevent concerns about mechanical effects of PVAT on ex-vivo vasoreactivity. Furthermore, as most obese participants had long-standing obesity, they might exhibit long-standing endothelial dysfunction, so that failing the quality control of minimal 10% vasodilation on acetylcholine $10^{-6}$ may be due either to experimental circumstances, or to established endothelial dysfunction. We performed only co-incubation experiments with microvessels and their own abutting PVAT. Ideally, we would have also performed cross-incubation experiments with PVAT from the other BMI-group (i.e. a microvessel from a lean participant with PVAT from an obese participant, and vice versa), but this would have required two parallel biopsies, which was not feasible.

The results described in figure 4 do show that PVAT from obese subjects still possesses vasoactive properties. It is tempting to speculate that PVAT is involved in the pathogenesis of obesity-associated endothelial insulin resistance. Perivascular adipose tissue helps explain the differences in microvascular recruitment between lean and obese which has been shown in this and other studies.\textsuperscript{7, 8}

These results support the hypothesis that PVAT is a new mechanistic determinant for impaired microvascular recruitment in skeletal muscle, and therefore for insulin resistance and cardiovascular disease.

Summarizing, we found that PVAT characteristics partly explain the relationship between obesity and blunted insulin-induced microvascular recruitment. PVAT may be an important target to alleviate obesity-associated microvascular dysfunction.
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