Local Inflammation and Hypoxia Abolish the Protective Anticontractile Properties of Perivascular Fat in Obese Patients

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Background—Inflammation in adipose tissue has been implicated in vascular dysfunction, but the local mechanisms by which this occurs are unknown.

Methods and Results—Small arteries with and without perivascular adipose tissue were taken from subcutaneous gluteal fat biopsy samples and studied with wire myography and immunohistochemistry. We established that healthy adipose tissue around human small arteries secretes factors that influence vasodilation by increasing nitric oxide bioavailability. However, in perivascular fat from obese subjects with metabolic syndrome (waist circumference 111 ± 2.8 versus 91.1 ± 3.5 cm in control subjects, P < 0.001; insulin sensitivity 41 ± 5.9% versus 121 ± 18.6% in control subjects, P < 0.001), the loss of this dilator effect was accompanied by an increase in adipocyte area (1786 ± 346 versus 673 ± 60 μm², P < 0.01) and immunohistochemical evidence of inflammation (tumor necrosis factor receptor 1 12.4 ± 1.1% versus 6.7 ± 1%, P < 0.001). Application of the cytokines tumor necrosis factor receptor-α and interleukin-6 to perivascular fat around healthy blood vessels reduced dilator activity, resulting in the obese phenotype. These effects could be reversed with free radical scavengers or cytokine antagonists. Similarly, induction of hypoxia stimulated inflammation and resulted in loss of anticontractile capacity, which could be rescued by catalase and superoxide dismutase or cytokine antagonists. Incubation with a soluble fragment of adiponectin type 1 receptor or inhibition of nitric oxide synthase blocked the vasodilator effect of healthy perivascular adipose tissue.

Conclusions—We conclude that adipocytes secrete adiponectin and provide the first functional evidence that it is a physiological modulator of local vascular tone by increasing nitric oxide bioavailability. This capacity is lost in obesity which this occurs are unknown.

Key Words: hypoxia ■ inflammation ■ obesity ■ microcirculation ■ nitric oxide synthase

Metabolic syndrome is a precursor to type 2 diabetes mellitus and cardiovascular disease, with a prevalence of almost 40% in the adult population. Central obesity is believed to be the main cause of metabolic syndrome, and this is reflected in newer definitions of the condition with large waist circumference as a prerequisite. Although associations of obesity with hypertension, insulin resistance, and cardiovascular disease are well described, the underlying mechanisms are poorly understood. Two areas of research that may provide insight into these are the vasoactive properties of perivascular adipose tissue (PVAT) and the inflammatory changes that occur in fat as obesity develops. Demonstrated in 1991, it is now accepted that healthy PVAT has an anticontractile effect. The mechanism appears to be both endothelium dependent via release of nitric oxide and endothelium-independent via generation of hydrogen peroxide. In obesity and metabolic syndrome, there is also a conformational change in adipose tissue: Infiltration and activation of circulating macrophages leads to inflammation characterized by upregulation of tumor necrosis factor alpha (TNF-α). Until recently, the primary consequences of this inflammation were thought to be a reduction in insulin sensitivity and an increase in nonesterified free fatty acids. However, given the profound effects of cytokines on arterial...
function, it has been suggested that the changes seen in adipose tissue in obesity are likely to have detrimental effects on the ability of PVAT to regulate vascular tone. Furthermore, a reduction in downstream flow would limit nutritive delivery contributing to insulin resistance, whereas reduced exposure of triglycerides to endothelial lipoprotein lipase would contribute to dyslipidemia. Therefore, the present series of studies was designed to test the hypothesis that in obesity and the metabolic syndrome, local adipocyte inflammation affects the anticontractile properties of PVAT.

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Methods

Study Population
Ten patients with obesity and at least 3 additional characteristics of the metabolic syndrome and 10 healthy lean subjects gave full written informed consent and participated in the study, which was approved by the local research ethics committee. Metabolic syndrome was defined according to the 2001 National Cholesterol Education Program Adult Treatment Panel guidelines. Fasting venous blood samples were taken to assess glycemic and lipid profiles and inflammatory markers. Blood pressure was measured with subjects seated, after 15 minutes of rest, by a semiautomatic machine (OMRON 705CP, White Medical, Clifton-Upon-Dunsmore, United Kingdom) with a mean of 3 readings recorded. Anthropometric measurements and bioimpedance were also recorded.

Biochemical Analysis
High-sensitivity C-reactive protein, leptin, interleukin (IL)-6, TNF, and adiponectin were assayed by an in-house ELISA technique (hs-CRP, Abcam, Cambridge, Mass; remainder, R&D Systems, Minneapolis, Minn). Insulin levels were measured by a radio labeling technique described previously. The homoeostasis model assessment (HOMA) was used to estimate B-cell function (HOMA-B) and peripheral insulin sensitivity (HOMA-S).

Pressure Myography
A subcutaneous gluteal fat biopsy sample was obtained from each subject under local anesthesia, which allowed tissue (2 x 1.5 x 1.5 cm) to be harvested and placed immediately in physiological saline solution (PSS). Small arteries, 100 to 150 μm in diameter, were dissected from the fat under a dissecting microscope, transferred to an arteriographic bath chamber, and cannulated.

The chamber was placed on the stage of an inverted microscope and superfused with PSS, gassed with 5% CO2/95% air (pH 7.4) at 37°C, at a superfusion rate of 20 mL/min. PSS composition was (in mmol/L) 139 NaCl, 4.7 KCl, 25 NaHCO3, 1.17 KH2PO4, 1.17 MgSO4, 0.026 EDTA, 1.6 CaCl2, and 5.5 glucose. Lumen diameter was recorded with the use of a Video Dimension Analyzer (Living Systems Instrumentations, Burlington, Vt) connected to a chart recorder. Vessels were pressurized to 60 mm Hg with a pressure servo system and equilibrated at 37°C for 1 hour before challenge with 60 mmol/L potassium-enriched PSS (KPSS).

Pressure Myography: Pharmacological Assessment
Each vessel was stimulated as follows: (1) Cumulative addition of norepinephrine (Sigma-Aldrich, Dorset, UK): 10⁻⁹, 3 x 10⁻⁹, 10⁻⁸, 3 x 10⁻⁸, 10⁻⁷, 3 x 10⁻⁷, 10⁻⁶, 3 x 10⁻⁶, and 10⁻⁵ mol/L, with 5 minutes’ incubation per concentration. (2) Cumulative response to acetylcholine (Sigma, in mol/L): 10⁻⁹, 3 x 10⁻⁹, 10⁻⁸, 3 x 10⁻⁸, 10⁻⁷, 3 x 10⁻⁷, 10⁻⁶, 3 x 10⁻⁶, and 10⁻⁵ to a vessel preconstricted with 10⁻⁵ mol/L norepinephrine. (3) After 1 hour of incubation with 5 x 10⁻⁷ mol/L N⁶-monomethyl-L-arginine (L-NMMA; Sigma), a nitric oxide synthase inhibitor, the responses to acetylcholine were repeated as in step 2.

Wire Myography
From the same biopsy, a larger arterial segment (diameter 250 to 350 μm) was dissected. One segment of this artery was cleaned of PVAT, whereas on the adjacent segment, PVAT was left intact. Both arteries were mounted on 40-μm wires in a wire myograph (Danish Myotech, Aarhus, Denmark). After an initial 30-minute incubation, vessel wall tension and diameter were normalized in a standardized procedure and stabilized for 1 hour.

Arteries were first challenged with KPSS 60 mmol/L to establish a baseline contractile response. A cumulative dose response to norepinephrine was then constructed (as with the pressure myography protocol). Contractile responses to norepinephrine are expressed as a percentage of KPSS contraction, consistent with other studies.

Bioassay experiments studied the release of relaxing factors from PVAT. We used arteries with PVAT intact (PVAT+), as donors and arteries stripped of PVAT as recipients (PVAT−). Arterial segments came from the same branch of the artery from the biopsy. Both arteries were preconstricted with norepinephrine 1 x 10⁻⁶ mol/L. We then removed solutions from both baths and transferred solution from the bath that contained the donor artery (PVAT+) to the bath containing the recipient artery (PVAT−). The subsequent change in response to tension in the recipient artery (PVAT−) was then recorded. This was performed in both healthy control subjects (n = 3) and obese patients (n = 3).

Pharmacological Assessment
We used pharmacological protocols to investigate whether reproduced aspects of the inflammatory obese environment were able to attenuate the anticontractile effects of PVAT. Thus, from healthy participants, arterial segments both with and without PVAT were incubated with an adiponectin type 1 receptor-blocking fragment, the cytokine TNF, and the inhibitor of nitric oxide synthase, L-NMMA. We also investigated whether PVAT function, which is damaged in patients with obesity and metabolic syndrome, could be restored by incubation with the anti-TNF antibody infliximab. The incubation time was 45 minutes for TNF (Sigma; 0.001 μg/mL, 5.9 x 10⁻⁶ mol/L). For adiponectin blockade, an incubation time of 45 minutes was used (5 μg/mL, 1.6 x 10⁻⁴ mol/L Acrp-30 [N-20]-P adiponectin receptor blocking peptide; Santa Cruz Biotechnology, Santa Cruz, Calif). One hour of incubation was used with L-NMMA (Sigma; 5 x 10⁻⁵ mol/L). For anti-TNF, an incubation time of 10 minutes was used (50 μg/mL infliximab, 3.4 x 10⁻⁵ mol/L; Schering-Plough Ltd, Hertfordshire, UK).

Animal Experiments
Male Wistar rats aged 12 to 15 weeks were killed by stunning, followed by cervical dislocation according to Home Office regulations. Mesenteric arteries (diameter 300 μm) were exposed and dissected. Part of the artery was cleaned of fat, whereas part of the artery was left with fat intact. Arteries were mounted and normalized on the wire myograph, and a cumulative concentration-response curve to norepinephrine was constructed (described above).

Protocols with healthy animal tissue were designed to investigate mechanisms of both inflammatory damage to PVAT and subsequent restoration of function. Incubations performed for arteries with and without PVAT included the following: (1) Adiponectin blocking peptide; (2) single cytokines (TNF, IL-6, or IL-1); (3) TNF and anti-TNF blocking antibody; (4) IL-6 and IL-6 blocking antibody; (5) IL-6 and TNF, followed by incubation with superoxide dismutase and catalase, followed by incubation with adiponectin blocking peptide; (6) hypoxia; and (7) hypoxia followed by incubation with anti-TNF blocking antibody (infliximab) or anti-IL-6 blocking peptide.
Protocols were as follows: TNF incubation, 0.001 μg/mL (5.9×10⁻⁹ mol/L; Sigma) for 45 minutes. TNF antagonism, incubation for 30 minutes with TNF-α followed by addition of 50 μg/mL infliximab (3.4×10⁻⁷ mol/L; Schering-Plough Ltd) for 10 minutes. IL-6, 0.01 μg/mL (Sigma) for 45 minutes. IL-6 was blocked by an IL-6 antagonist (Sigma) at a concentration of 0.2 μg/mL for 20 minutes. IL-1, 0.02 μg/mL for 45 minutes. Adiponectin blockade, incubation with 5 μg/mL (1.6×10⁻⁴ mol/L) Acrp-30 (N-20)-P adiponectin blocking peptide (Santa Cruz Biotechnology) for 10 minutes. Superoxide dismutase and catalase (Sigma), 80 and 120 U/mL, respectively, with 45 minutes’ incubation. The effect of hypoxia on PVAT was investigated by gassing mounted arteries with 95% nitrogen and 5% CO₂ for 2.5 hours. Norepinephrine dose-response curves were constructed both before and after hypoxic incubation.

Vasodilating Effects of Exogenous Adiponectin
Pressure myography was used to investigate the ability of adiponectin, secreted outside of the artery by PVAT, to exert a vasodilatory effect by moving transluminally to stimulate endothelial nitric oxide production. A dose-response curve to norepinephrine was constructed with rat mesenteric arteries. The artery was then preconstricted at an ED50 concentration for norepinephrine before the addition of adiponectin (BioVendor, Heidelberg, Germany; 10 μg/mL) to the bath solution. Because the vessels are tied off firmly at both ends, adiponectin-mediated effects must be via adventitia or the smooth muscle of the vessel wall.

Immunohistochemistry
Sagittal sections from the biopsy that incorporated skin and subcutaneous fat were placed in 4% paraformaldehyde in phosphate-buffered saline for 24 hours and then processed on an 18-hour cycle in a Shandon automatic tissue processor. The sample was embedded in paraffin wax and serially sectioned at 5 μm. Dewaxed and rehydrated sections were immunostained for type 1 and type 2 TNF receptors (TNFRc1 and TNFRc2; rabbit polyclonal antibodies, Abcam). Tissue sections were incubated for 18 hours with primary antibodies, followed by biotinylated secondary antibodies and streptavidin-horseradish peroxidase–conjugated complexes (DAKO, Glostrup, Denmark) and developed with SG chromogen (Vector

Figure 1. Bioassay experiments. A, Illustration of bioassay protocol. B, Percentage change in resting tension after solution transfer. There was significantly greater relaxation of arteries from control participants than from patients with obesity and metabolic syndrome. (50.4±8.9% vs 12.8±6.5%, P<0.05, n=3, unpaired t test). C and D, Wire myograph recordings of tension generated by arteries without PVAT from control participants (C) and obese patients with metabolic syndrome (D). Upward deflections indicate an increase in tension (ie, constriction). Downward deflections indicate a decrease in tension (ie, dilation). Step 1 indicates artery without PVAT constricted with norepinephrine (10⁻⁶ mol/L); 2, solution transfer from donor artery (+ PVAT) constricted with norepinephrine (10⁻⁶ mol/L); and 3, replacement of original solution (from step 1).
Laboratories, Peterborough, UK). Immunostaining for TNFRc1 required heat-induced antigen retrieval in an autoclave (at 125 psi) in 10 mmol/L citrate buffer (pH 6.0). Color images were captured with a Go-3 QImaging camera (QImaging Corp, Vancouver, Canada) mounted on a Leitz Diaplan microscope and converted to grayscale images. Quantitative analysis of immunostaining was obtained with a macro subroutine in an Image-Pro version 6.2 image-analysis program. The extent of staining was expressed as a percentage of the entire area photographed. Adipocyte size was quantified by manually tracing the margins of 100 consecutive adipocytes on immunostained sections to avoid selection bias (total: 2000 cells).

Statistical Analysis
Data are presented as mean±SEM. For the cumulative dose-response experiments on human tissue (Figures 2A, 2C, 3D, and 3E), differences between dose-diameter relations (pressure myography) and dose-tension relations (wire myography) were analyzed as repeated-measures ANOVA. For the pharmacological assays (Figures 3A through 3C and Figures 4 through 6), differences in tension at specific points on the dose-response curve were analyzed with Student’s t test. A probability value <0.05 was considered statistically significant. Analyses were performed with SPSS software (SPSS, Inc, Chicago, Ill).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Study Design and Participants
Compared with healthy subjects, obese patients with metabolic syndrome had significantly greater waist circumference, body mass index, total body fat content, fasting glucose, pancreatic β-cell workload (HOMA-B), and leptin levels and lower bioimpedance, peripheral insulin sensitivity (HOMA-S), and high-density lipoprotein cholesterol (P<0.05). Blood pressure, markers of systemic inflammation, serum adiponectin levels, age (control group 49.6±3.65 years versus metabolic syndrome group 54.5±2.2 years), and sex (8 males and 2 females in the control group versus 7 males and 3 females

Figure 2. Effect of obesity and the metabolic syndrome on anticontractile capacity of PVAT in small arteries from subcutaneous gluteal fat. *P<0.01. A, In healthy control participants, PVAT exerted a significant anticontractile effect (P=0.009, multiple ANOVA) compared with contractility of arteries without PVAT (n=10). B and D, The presence of PVAT had no effect on contractility in response to KPSS 60 mmol/L either in health or disease (n=10; t test for paired samples). C, In patients with obesity and metabolic syndrome, the presence of PVAT had no effect on contractility (n=10).
in the metabolic syndrome group) were not significantly different (Table).

**Anticontractile Capacity and Release of Relaxing Factor From PVAT Is Reduced in Metabolic Syndrome**

In healthy volunteers, the presence of intact PVAT exerted a significant anticontractile effect on small arteries ($P<0.05$; Figure 2A). The effect was independent of endothelium-derived hyperpolarizing factor, because there was no difference in contraction to KPSS 60 mmol/L when vessels were studied in the presence or absence of PVAT (Figure 2B). In obese patients with metabolic syndrome, the anticontractile capacity of PVAT was abolished completely, with no difference in contractility to norepinephrine, whether PVAT was intact or removed (Figure 2C). The presence of intact PVAT in this group had no influence on contractility to KPSS 60 mmol/L (Figure 2D).

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Figure 3. Pharmacological manipulation of PVAT on small arteries from subcutaneous gluteal fat biopsy samples from healthy participants (*$P<0.05$, paired $t$ test for individual points: PVAT vs PVAT plus intervention). A, Incubation with adiponectin blocking peptide reduced the anticontractile effect of PVAT to a level equivalent to that seen in arteries without PVAT ($P=0.034$; $n=3$). B, Inhibition of nitric oxide synthase by incubation with L-NMMA reduced the anticontractile effect of PVAT ($P=0.025$; $n=4$). C, Incubation with TNF reduced the anticontractile effect of PVAT ($P=0.026$; $n=3$). D, Pressure myography: Effect of acetylcholine dilation on preconstricted arteries. Indicates control subjects; †, obese patients with metabolic syndrome. †$P<0.001$, multiple ANOVA. E, Pressure myography: Preincubation of small arteries with L-NMMA ($5 \times 10^{-5}$ mol/L) followed by norepinephrine/acetylcholine protocol ($P=0.115$, multiple ANOVA).
Adipose inflammation and adipocyte hypertrophy in metabolic syndrome

Quantitative image analysis demonstrated a significant increase in expression of TNF-α receptor 1 in adipose tissue from obese patients with metabolic syndrome (12.4 ± 1.1%) compared with control participants (6.7 ± 1%; P < 0.001). There were no significant differences in TNF-α receptor 2 expression (control participants 4.6 ± 0.9% versus obese patients 8.7 ± 3.5%). Adipocyte cross-sectional area measured in 2000 consecutive cells was increased significantly in obese patients with metabolic syndrome compared with healthy participants (control 673 ± 60 versus obese 1786 ± 346 μm²; P = 0.006).

Pharmacological Assays

Factors damaging the anticontractile pathway in healthy control subjects

Application of a fragment of the human type 1 adiponectin receptor to arteries with intact PVAT from healthy subjects completely abolished the anticontractile capacity of PVAT, restoring the contractile profile to that of a healthy artery devoid of PVAT (Figure 3A). Also, in healthy participants, incubation of arteries with intact PVAT with L-NMMA (Figure 3B) or the cytokine TNF (Figure 3C) caused an attenuation of the anticontractile response, mimicking the obese phenotype. Using pressure myography, we confirmed a defect in small-artery endothelial function in patients with obesity and metabolic syndrome (Figure 3D), assessed as a surrogate measure by the ability of a preconstricted artery to dilate in response to increasing doses of acetylcholine. Subsequent incubation with L-NMMA showed the defect in endothelial function in obesity to be caused by downregulation of nitric oxide synthase (Figure 3E).

Restoration of PVAT anticontractile function in unhealthy obese patients

We attempted to restore the anticontractile effect of PVAT in patients with obesity and metabolic syndrome by incubation with the anti-TNF antibody infliximab, but this had no effect on the capacity of PVAT to reduce contractility of human arteries (supplemental Figure I).

Mechanisms of damage to and restoration of PVAT function in an animal model

Intact PVAT exerted an anticontractile effect on small mesenteric arteries from healthy Wistar rats (Figure 4A). In agreement with our studies in small human arteries, a soluble fragment of the rodent adiponectin type 1 receptor almost completely abolished the anticontractile capacity of healthy rat PVAT (Figure 4B). We found a selective effect of inflammatory cytokines on the anticontractile effect of PVAT: Incubation with TNF-α (Figure 5A) and IL-6 (supplemental Figure II) but not IL-1 (Figure 5B) attenuated the anticontractile effect of healthy PVAT. The effects of TNF and IL-6 were fully reversible by subsequent incubation with their antagonists, the anti-TNF antibody infliximab and a bovine IL-6 antibody, respectively (supplemental Figure III). After incubation with TNF and IL-6 together, we were able to restore the anticontractile effect of PVAT using the free radical scavengers superoxide dismutase and catalase (Figure 5C). Further application of the soluble fragment of the adiponectin receptor 1 reduced the anticontractile effect (Figure 5D). Cytokine application had no effect on the contractility of arteries without PVAT (data not shown).

Effects of hypoxia on PVAT function in an animal model

The physiological effects of hypoxia on vascular contractility and PVAT function were investigated by incubation of...
arterial segments for 2.5 hours with 95% N₂/5% CO₂. Hypoxia significantly attenuated the anticontractile effect of PVAT (*P* < 0.05; Figure 6A). We assessed whether the effect of hypoxia on PVAT was mediated by inflammation. After a period of hypoxia, arteries both with and without PVAT were incubated with an anti-TNF-α antibody (Figure 6B) or anti-IL-6 antibody (supplemental Figure II). These incubations restored the anticontractile capacity of the PVAT in both cases. In arteries without fat, neither hypoxic incubation nor subsequent incubation with anti-TNF or anti-IL-6 after hypoxia had significant effects on contractility (supplemental Figure III).

**Bioactivity of Exogenous Adiponectin**

The ability of adiponectin to act as a paracrine factor by diffusion through the vascular smooth muscle of the media was examined with pressure myography. Rat mesenteric arteries, tied firmly at both ends and pressurized to 60 mm Hg, were preconstricted with norepinephrine to an ED50 concentration. Adiponectin applied directly to the bath solution, but not the intraluminal compartment, elicited a vasodilation (71.5 ± 7.8%, *n* = 3) within 2 minutes of exposure to the artery (supplemental Figure IV, representative tracing).

**Discussion**

Our data provide the first evidence in humans to support the hypothesis that obesity-related changes in adipose tissue have direct effects on the vasoactive properties of PVAT. We report abnormalities in adiponectin-mediated anticontractile properties of PVAT, endothelium-mediated dilation of the artery, and changes to the inflammatory and hypoxic profile of subcutaneous adipose tissue in obesity and metabolic syndrome. Additionally, using in vitro animal and human small-artery studies, hypoxia and inflammation were shown to attenuate the local vasoactive properties of PVAT by oxidative stress.

In healthy individuals, PVAT mediated an anticontractile effect. Although observed previously in medium-sized arteries, this is the first demonstration of this effect in human small arteries, and we are also the first to demonstrate a complete loss of the anticontractile effect in patients with obesity and metabolic syndrome. Subsequently, in vitro, we
were able to replicate the biological changes that occur in adipose tissue in obesity and have delineated the pathway by which obesity-related damage to PVAT function occurs. Although it is accepted that adipose inflammation has a central role in insulin resistance, there are no data investigating the mechanisms or effects that this inflammation will have on the vasoactive properties of PVAT. When human arteries with intact PVAT were exposed to TNF, there was a reduction in the anticontractile function of PVAT, which produced the unhealthy obese phenotype. Exogenous TNF damages small-artery endothelial function by downregulation of nitric oxide synthase, which is responsible for the endothelium-dependent mechanism by which PVAT mediates an anticontractile effect in healthy rat adipose tissue.

In an animal model, exposure to cytokines had a selective anticontractile effect in healthy rat adipose tissue.8 The endothelium-dependent mechanism by which PVAT mediates the anticontractile capacity of healthy human PVAT, as with TNF, thus negating the vasoactive function of the fat. Our study of arteries with pressure myography confirmed that in obese patients with metabolic syndrome, the defect in endothelial function was caused by downregulation of nitric oxide synthase.

In an animal model, exposure to cytokines had a selective effect on PVAT, with reduction in function after incubation with TNF and IL-6 but not IL-1. The damage to the anticontractile function caused by these cytokines was completely reversible by their blocking antibodies but also by superoxide dismutase and catalase, which suggests the inflammatory damage is mediated by oxidative stress. The animal model also permitted investigation into the effects of hypoxia on PVAT function. It is thought that adipocyte hypertrophy, quantified in patients in the present study and other studies, causes cellular hypoxia, which stimulates adipocyte and macrophage production of inflammatory cytokines, including TNF and IL-6.25-28 We observed complete loss of the anticontractile effect after 2.5 hours under hypoxic conditions, but we were able to rescue this function of the fat physiologically by subsequent incubation with antibodies to either TNF or IL-6. Although the data appear robust, we acknowledge that the sequential nature of these pharmacological protocols increases the risk of a type 1 statistical error. In arteries without PVAT, neither hypoxia nor incubation with anti-TNF or anti-IL-6 after hypoxia had any effect on contractility, which suggests that the ability of anti-TNF or anti-IL-6 to restore the anticontractile capacity of PVAT was mediated through actions on adipose tissue. In obese patients, however, incubation with anti-TNF was unable to restore anticontractile function in PVAT. This may be due to the prolonged period of inflammation associated with disease, but this negative finding mirrors observations seen when anti-TNF is used to treat other cardiovascular disorders.30,31

Adiponectin displays many characteristics observed of the relaxing factor released from human PVAT. It is released exclusively by adipocytes and increases nitric oxide synthase activity through endothelial receptors.32 Furthermore, both hypoxia33 and inflammation34 reduce its production. Adiponectin was recently discounted as a potential candidate for this role on the grounds that PVAT surrounding mesenteric...
arteries from the adiponectin receptor 1 knockout mouse remains able to elicit an anticontractive effect. However, when we incubated arteries with intact PVAT from healthy individuals with an adiponectin type 1 receptor–blocking fragment, the anticontractive properties of the fat were abolished entirely, which suggests that in human subcutaneous adipose tissue, adiponectin is the predominant mediator of the relaxation. This was reinforced by a similar finding in our animal model. We have also shown that adiponectin, if released adjacent to a small artery by perivascular adipocytes, is able to effect a vasodilation.

The concept that adiponectin is central to the function of healthy PVAT is not incompatible with the findings from the study with the adiponectin type 1 receptor knockout mouse. Two pathways have been delineated by which PVAT mediates an effect on rat aorta: An endothelium-dependent pathway that involves nitric oxide and an endothelium-independent pathway that involves hydrogen peroxide. Given its association with nitric oxide synthase, adiponectin clearly would occupy a position in the former of these categories. In the adiponectin type 1 receptor knockout mouse, it is likely that the endothelium-independent pathway is upregulated in response to the genetic defect and thus is responsible for the anticontractive properties of the PVAT observed. Alternatively, in this model, adiponectin may act through either the adiponectin type 2 receptor or T-cadherin, a glycosylphosphatidylinositol-linked cell surface molecule that has been suggested as a potential receptor for adiponectin and is highly expressed on vascular endothelium.

The predominant physiological mechanism by which perivascular fat modulates contraction in human subcutaneous small arteries has been identified. Adipocytes release adiponectin, which maintains endothelial nitric oxide production and thereby reduces vascular tone. That blocking the effect of adiponectin completely abolishes this response implies that in humans, this is the major component of the anticontractive pathway. Further investigation is clearly indicated to validate this and to examine whether this is the case in other circulatory beds. In obesity and metabolic syndrome, however, the anticontractive capacity of PVAT is lost, because individual adipocyte size is greater, and consequently, there is local tissue hypoxia and inflammation. Such an environment provides the stimulus for adipocyte and macrophage release of inflammatory cytokines. Both hypoxia and inflammation are known to reduce adiponectin production from adipocytes, and in human and animal preparations, we have demonstrated that these stimuli abrogate the vasorelaxant properties of PVAT through increased oxidative stress. The reduction in adiponectin generation or function is likely to be responsible for the observed downregulation of nitric oxide synthase that contributes to endothelial dysfunction. However, given that cytokines are known to have a direct effect on endothelium, there is likely to be an additive effect also. The relative in vivo contributions of reduced adiponectin bioaction, increased local production of reactive oxygen species, and upregulation of tissue cytokines that impair local PVAT function is not entirely clear and will also require further investigation.

The present findings are also consistent with in vivo clinical studies in obesity that have shown reductions in flow-mediated dilation and both visceral and cutaneous capillary recruitment consistent with an enhanced vasoconstriction and impaired endothelial function. However, although the data provide evidence to support mechanisms by which obesity damages the influence of local adipose tissue on small arteries, the physiological consequences of this functional damage remain unknown. It has already been suggested that loss of PVAT function would result in increased peripheral vascular resistance and blood pressure. Furthermore, as proposed by Yudkin et al, the loss of a vasodilatory paracrine effect from adipose tissue may limit downstream microcirculatory nutritive flow, contributing to insulin resistance that is already compromised by the direct effects of TNF-α on adipocytes. A similar hypothesis has been applied to triglyceride metabolism. Whether this is the case remains to be established, but experiments using in vivo correlates are now clearly indicated to further our understanding of adipose tissue pathophysiology in obesity.

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**Disclosures**

None.

**References**

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SUPPLEMENTARY MATERIALS

Supplementary methods

Biochemical analysis

Highly sensitive-CRP was assayed by an in-house, antibody sandwich ELISA technique; rabbit anti-human CRP antibodies (unlabelled and horse-radish peroxidase-labelled), calibrators and controls were obtained from Abcam (Cambridge, UK), o-phenylenediamine (Sigma-Aldrich, Poole, Dorset) being used to detect the amount of bound HRP. Leptin, IL-6, TNFα and adiponectin were measured by in-house ELISA methods using DuoSet development kits from R&D Systems (Abingdon, UK)

To measure serum insulin, aliquots (100μl) of sample sera were reacted with 100μl of a working solution of antibody to insulin (Polyclonal anti-porcine insulin, raised in guinea-pig, Diagnostics Scotland, Carluke, Scotland) and incubated overnight at 4°C. After 24 hours 100μl of a working solution of radiolabelled insulin (c.3000counts/tube, \(^{125}\text{I}-\text{Insulin, DSL-1620, 185kBq, DSL Ltd, Oxford Bio-Innovation Ltd, Oxon, UK}\) was added to each assay tube and the mixture incubated for a further 24 hours. Bound and free antigen was then separated using a charcoal separation technique. Once separated the radioactivity in the precipitate was counted on a gamma counter (Wallac, LKB Instruments, Surrey UK) set up to detect \(^{125}\text{I}.\) The amount of insulin in the sample was calculated by comparison to a standard curve of human insulin (WHO 1\(^{st}\) international reference preparation 66/304) made up in confirmed hormone free plasma.\(^{1}\)
Supplementary Results

Supplementary Figure 1 (human tissue):

A: Removal of PVAT from arteries taken from healthy participants results in a non-significant increase in contractility (n=3, p=ns)

B: Removal of PVAT from arteries taken from unhealthy participants results in a non-significant reduction in contractility (n=3, p=ns)

C: Incubation with anti-TNF has no effect on PVAT from obese patients with metabolic syndrome.
Supplementary Figure 2 (animal tissue)

A. Incubation with IL-6 reverses anticontractile effect of PVAT (□=no PVAT (n=23), ■=PVAT (n=23), ●=PVAT+IL-6 (1ng/ml)(n=6)p=0.008). *denotes difference between ● vs ■

B: Incubation with Anti-IL-6 restores anticontractile capacity of PVAT which is lost after hypoxia (■=PVAT (n=23), ●=PVAT + hypoxia (n=9), ▲=PVAT + hypoxia + Anti-IL-6 (n=3) p<0.001). *denotes difference between ● vs ▲
Supplementary Figure 3 (animal tissue):

A. Anti-TNF antibody blocks detrimental effect of TNF ((□ = no fat (n=23), ■ = fat (n=23), ● = fat + TNF + Anti-TNF (n=3)).

B. Anti-IL-6 antibody blocks detrimental effect of IL-6 (□ = no fat (n=23), ■ = fat (n=23), ● = fat + IL-6 + Anti-IL-6 (n=5)).

C. Hypoxia has small non-significant effect on contraction of artery with no PVAT (□ = no fat (n=23), ■ = fat (n=23), ● = no fat + hypoxia (n=9)).

D. Incubation with anti-TNF has no effect on contractility of arteries without PVAT after hypoxia. PVAT (□ = no fat (n=23), ■ = fat (n=23), ● = no fat + hypoxia + anti-TNF antibody (n=3).

E. Incubation with anti-IL-6 antibody has no effect on the contractility of arteries without PVAT after hypoxia (□ = no fat (n=23), ■ = fat (n=23), ● = no fat + hypoxia + anti-IL-6 antibody (n=3)).
Supplementary Figure 4 (animal tissue)

A: Exogenous adiponectin causes a dilation of rat mesenteric arteries preconstricted with Noradrenaline on a pressure myograph. Representative trace shown.