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DOI: 10.1161/ATVBAHA.116.307210

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):

Published in:
Arteriosclerosis, thrombosis, and vascular biology

Citing this paper
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Obesity-Related Perivascular Adipose Tissue Damage Is Reversed by Sustained Weight Loss in the Rat

Charlotte E. Bussey, Sarah B. Withers, Robert G. Aldous, Gillian Edwards, Anthony M. Heagerty

Objective—Perivascular adipose tissue (PVAT) exerts an anticontractile effect in response to various vasoconstrictor agonists, and this is lost in obesity. A recent study reported that bariatric surgery reverses the damaging effects of obesity on PVAT function. However, PVAT function has not been characterized after weight loss induced by caloric restriction, which is often the first line treatment for obesity.

Approach and Results—Contractility studies were performed using wire myography on small mesenteric arteries with and without PVAT from control, diet-induced obese, calorie restricted and sustained weight loss rats. Changes in the PVAT environment were assessed using immunohistochemistry. PVAT from healthy animals elicited an anticontractile effect in response to norepinephrine. This was abolished in diet-induced obesity through a mechanism involving increased local tumor necrosis factor-α and reduced nitric oxide bioavailability within PVAT. Sustained weight loss led to improvement in PVAT function associated with restoration of adipocyte size, reduced tumor necrosis factor-α, and increased nitric oxide synthase function. This was associated with reversal of obesity-induced hypertension and normalization of plasma adipokine levels, including leptin and insulin.

Conclusions—We have shown that diet-induced weight loss reverses obesity-induced PVAT damage through a mechanism involving reduced inflammation and increased nitric oxide synthase activity within PVAT. These data reaveal inflammation and nitric oxide synthase, particularly endothelial nitric oxide synthase, as potential targets for the treatment of PVAT dysfunction associated with obesity and metabolic syndrome. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.116.307210.)

Key Words: adipose tissue ■ bariatric surgery ■ nitric oxide ■ obesity ■ weight loss

Obesity is one of the major causes of illness and death in the world, and it is a significant public health burden currently affecting an estimated half a billion adults and 40 million children globally. It is associated with many comorbidities, and overwhelming evidence supports the importance of obesity in the pathogenesis and progression of cardiovascular disease. The rapidly increasing gap between the availability of medical therapies and the steadily rising rates of obesity emphasize the need for investigation into novel therapies to prevent the devastating effects of being overweight.

Healthy perivascular adipose tissue (PVAT) exerts an anticontractile effect on adjacent arteries,3–5 that is lost in both rodent models6–9 and human obesity and the metabolic syndrome,4,10 suggesting that changes in PVAT function and morphology may contribute to vascular dysfunction associated with increased body weight5 and diabetes mellitus.11,12

The most obvious treatment for obesity is weight loss (WL), which can be achieved through lifestyle changes and surgical methods. Bariatric surgery is an established method of reducing obesity-associated morbidity, and the cardiovascular benefits have been clearly demonstrated.13,14 The mechanisms that underlie these improvements are unclear but are likely to be a consequence of improvements in inflammatory and adipokine profiles.15–17 Our studies demonstrate that bariatric surgery can reverse obesity-induced PVAT damage 6 months after surgery through reduction of adipose inflammation and increased local adiponectin and nitric oxide (NO) bioavailability.18 This correlated with reduced blood pressure and improvements in lipid profiles and blood glucose levels, suggesting that the restoration of PVAT function could contribute to the cardiovascular benefits of losing weight.

Bariatric surgery is not suitable for all obese patients meaning that simple lifestyle measures such as caloric restriction and increased exercise should not be ignored and are often the first line in treatment for obesity.19 Few studies have been specially designed to determine the effects of WL produced by dietary intervention in obesity; however, the majority of clinical trials and animal studies have reported a beneficial effect of diet-induced WL on blood pressure13,18,20 and adipokine balance,20–22 along with improvements in the inflammatory profile.9,22
Investigation into the mechanisms involved in any improvement in PVAT function could lead to identification of much needed novel therapeutic targets for the treatment of obesity-related hypertension and cardiovascular disease. Therefore, this study was designed to explore changes in the PVAT environment that occur in obesity and to investigate the effects of diet-induced WL on PVAT anticontractile function. This study tested the hypothesis that diet-induced WL would lead to the restoration of PVAT function through changes in local inflammation and NO bioavailability. We report that diet-induced obesity perturbs PVAT function through a mechanism involving inflammation and nitric oxide synthase (NOS) and the PVAT damage can be reversed by sustained WL and associated reduction in adipose inflammation and increased NOS availability.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

WL Reversed Cardiometabolic Damage Induced by High-Fat Diet

There were no significant differences in body weight of the rats before starting their respective diets (P=0.39). After 16 weeks of consuming the 45% fat diet (high fat diet [HFD]), animals were significantly heavier than controls (P<0.01, Figure 1). Two groups of obese animals (WL and weight maintenance [WM]) were then subjected to caloric restriction for 4 weeks to induce WL. Caloric restriction produced a gradual reduction in body weight with animals losing an average of 130 g by week 20, at which time the average body weight was no longer different from control (P=0.57, Figure 1). Animals consuming the HFD continued to gain weight and were heavier than controls at week 20. To assess whether the vascular effects of WL were altered after the maintenance of body weight, rats in one of the groups (WM) were provided with 70 kcal/d for an additional 4 weeks and were able to maintain their body weight in comparison with control (P=0.89, Figure 1). Animals fed the HFD consumed significantly more calories than animals provided with a control diet (Figure I in the online-only Data Supplement, P<0.001), indicating that the animals did not compensate for the increased caloric content of the HFD by eating less.

The cardiometabolic profile of animals with dietary interventions is shown in Table. HFD produced insulin resistance without overt diabetes mellitus as shown by the increase in fasting plasma insulin levels (P<0.0001) without a change in blood glucose levels (P=0.83). Hyperinsulinemia was reversed after caloric restriction (P<0.0001), and this was sustained after the 4-week WM period (P<0.01) with no effect on blood glucose levels (P=0.85). Body weight did not have an effect on plasma total adiponectin levels (P=0.13). Plasma leptin levels were significantly increased compared with those of controls after 8 weeks of high-fat feeding (P<0.01, data not shown) and were 142% greater than control by week 20 (P<0.0001). Hyperleptinemia was reversed after caloric restriction (P<0.001), and this reduction was sustained after the 4-week WM period (P<0.001).

Systolic and diastolic blood pressure did not change during the 20-week period in animals fed control diet. High-fat feeding induced significant increases in both systolic (P<0.0001) and diastolic (P<0.0001) blood pressure. Increases in systolic blood pressure were reversed after 4-week WL (control versus WL: P=0.06), and this was maintained during the WM period (control versus WM: P=0.09). Diastolic blood pressure was reduced after WL; however, it remained significantly elevated compared with control (P<0.01). Maintenance of body weight led to complete reversal of the hypertensive phenotype as diastolic blood pressure decreased to control levels (control versus WM: P=0.37). Significant tachycardia was observed in diet-induced obesity (control: 299±5.0 bpm versus obese: 335±6.9 bpm; P<0.0001). Caloric restriction induced transient bradycardia (WL: 246±10.9 bpm, P<0.0001); however, heart rate returned to control levels in following WM (294±8.1 bpm, P=0.56) suggesting that the reduced heart rate was a consequence of extreme WL.

Anticontractile Capacity of PVAT Is Restored After Sustained WL

The presence of PVAT did not alter the contractile response evoked by stimulation with high-potassium physiological salt solution in any of the animal models used (Figure II in the online-only Data Supplement). Moreover, responses to high-potassium physiological salt solution were not altered by body...
weight (P=0.45) allowing contractile responses to be expressed as percentage high-potassium physiological salt solution.

The presence of PVAT reduced the vasoconstrictor response to norepinephrine in lean controls (P<0.0001, Figure 2A). However, the presence of PVAT did not alter the contractile response in vessels isolated from animals provided HFD (P=0.21, Figure 2B) and the contractile response of vessels with PVAT to norepinephrine was increased compared with controls (P=0.03, Figure IIIA in the online-only Data Supplement), indicating that diet-induced obesity diminished the anticontractile capacity of PVAT. WL induced by 50% caloric restriction did not reverse obesity-induced loss of PVAT anticontractile effect because responses were unaltered by the presence of PVAT (P=0.14, Figure 2C) and the vasoconstrictor response to norepinephrine was increased compared with controls (P=0.03, Figure IIIA in the online-only Data Supplement). Changes in body weight had no effect on the vasoconstrictor response to norepinephrine in endothelium intact vessels lacking PVAT (P=0.14, Figure IIIB in the online-only Data Supplement).

Adipocyte Hypertrophy and PVAT Inflammation Are Reduced by WL

Obesity produced an increase in adipocyte cross-sectional area (P<0.0001), which was reduced but not completely reversed after caloric restriction (obese versus WL: P<0.0001, control versus WL: P<0.0001). However, complete restoration of adipocyte size was observed after a 4-week WM period (control versus WM: P=0.67, Figure 3A and 3B). The change in adipocyte area positively correlated with change in body weight (r=0.73, P<0.0001).

Eosinophil number within PVAT was reduced in diet-induced obesity (P<0.0001). Eosinophil number increased

Table. Diet-Induced Changes in Cardiometabolic Parameters can be Reversed by Caloric Restriction

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Obese</th>
<th>Weight Loss</th>
<th>Weight Maintenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP, mmHg</td>
<td>119.9±1.2*</td>
<td>142.4±2.1†</td>
<td>124.1±2.03*</td>
<td>116.0±1.9‡</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>82.6±1.2*</td>
<td>99.04±1.6†</td>
<td>91.02±1.7†</td>
<td>84.40±1.6‡</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>6.65±0.27</td>
<td>6.91±0.26</td>
<td>6.66±0.23</td>
<td>6.86±0.24</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>1.31±0.18*</td>
<td>2.80±0.35†</td>
<td>1.03±0.19*</td>
<td>1.17±0.08*</td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>3.46±0.53*</td>
<td>8.40±0.91†</td>
<td>4.17±0.59*</td>
<td>2.62±0.33*</td>
</tr>
<tr>
<td>Adiponectin, μg/mL</td>
<td>3.84±0.27</td>
<td>4.24±0.22</td>
<td>3.43±0.38</td>
<td>4.58±0.55</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.

*P<0.05 versus obese; †P<0.05 versus control; ‡P<0.05 versus weight loss 1-way ANOVA with Tukey post hoc test, n=8 to 12.
after caloric restriction but levels were not significantly different to either control or obese \( (P>0.05) \). However, eosinophil levels within PVAT were restored by WM \( (P=0.67, \text{Figure } 3C) \). Moreover, the number of eosinophils within PVAT negatively correlated with body weight \( (r=-0.41, P=0.04) \).

Tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) staining intensity was significantly increased in PVAT from obese animals compared with control \( (P<0.0001) \) and staining remained high after 4-week caloric restriction \( (\text{control versus WL: } P=0.0019 \text{ and obese versus WL: } P=0.07) \). However, the increased TNF-\( \alpha \) immunostaining was reversed by sustained WL \( (\text{control versus WM: } P=0.11 \text{ and obese versus WM: } P<0.0001, \text{Figure } 4) \).

Immunostaining for CD68\(^+\) cells was used to detect the total number of macrophages within PVAT (Figure 5). Macrophage number was reduced in diet-induced obesity \( (\text{control versus obese: } P<0.0001) \). This was increased after WL, and eosinophil number was not significantly different to either control or obese. The additional 4-week WM period reversed obesity-induced adipocyte hypertrophy \( (\text{control versus WM: } P>0.05) \) and adipocyte number was not significantly different to either control or obese. The additional 4-week WM period reversed eosinophil number to control levels \( (\text{controls versus WM: } P=0.67) \). Data are expressed as mean±SE \(* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, 1\text{-way ANOVA with Tukey post hoc test. Control: } n=5, \text{ obese: } n=6, \text{ WL: } n=5, \text{ WM: } n=9 \text{ animals. Hundred consecutive adipocytes were analyzed from 1 slide per animal.}"

**Figure 3.** Adipocyte hypertrophy is reversed by sustained weight loss (WL). A, Mesenteric peri-vascular adipose tissue (PVAT) from (i) control, (ii) obese, (iii) WL, and (iv) weight maintenance (WM) animals were stained with hematoxylin and eosin to determine adipocyte area and number of eosinophils. Representative images were obtained at x40 magnification, scale bar represents 50 \( \mu \)m and arrows highlight eosinophils. B, Average adipocyte areas were increased in diet-induced obesity (control versus obese: \( P<0.0001 \)). Adipocyte hypertrophy was reversed after WL (obese versus WL: \( P<0.0001 \)) but not completely restored to control size (control versus WL: \( P<0.001 \)). The additional 4-week WM period reversed obesity-induced adipocyte hypertrophy (control versus WM: \( P=0.05 \)). C, The number of eosinophils in mesenteric PVAT was reduced in diet-induced obesity (control versus obese: \( P<0.0001 \)) and staining remained high after WL (obese versus WL: \( P<0.0001 \)) but not completely restored to control size (control versus WL: \( P>0.05 \)). Data are expressed as mean±SE \(* P<0.05, ** P<0.001, *** P<0.0001, 1\text{-way ANOVA with Tukey post hoc test. Control: } n=5, \text{ obese: } n=6, \text{ WL: } n=5, \text{ WM: } n=9 \text{ animals.}

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**Figure 4.** Inflammation within perivascular adipose tissue (PVAT) is reversed by sustained weight loss (WL). A, Immunostaining for tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) in mesenteric PVAT from (i) control, (ii) obese, (iii) WL, and (iv) weight maintenance (WM) animals. Representative image of a negative control, where samples were incubated with mouse IgG is shown (v). Representative images were obtained at x40 magnification, and scale bar represents 50 \( \mu \)m. B, Adipocytes were positive for TNF-\( \alpha \) to a higher extent in obese samples than in control \( (P<0.0001) \), and staining remained high after WL \( (\text{control versus WL: } P=0.0019 \text{ and obese versus WL: } P=0.07) \). Increased TNF-\( \alpha \) was reversed after the 4-week WM period \( (\text{control versus WM: } P=0.11 \text{ and obese versus WM: } P<0.0001, \text{Figure } 4) \). Immunostaining for CD68\(^+\) cells was used to detect the total number of macrophages within PVAT (Figure 5). Macrophage number was significantly increased in PVAT from obese animals compared with control \( (P<0.0001) \) and staining remained high after 4-week caloric restriction \( (\text{control versus WL: } P=0.0019 \text{ and obese versus WL: } P=0.07) \). However, the increased TNF-\( \alpha \) immunostaining was reversed by sustained WL \( (\text{control versus WM: } P=0.11 \text{ and obese versus WM: } P<0.0001, \text{Figure } 4) \). Percentage staining intensity is expressed as mean±SE \(* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, 1\text{-way ANOVA with Tukey post hoc test. Control: } n=5, \text{ obese: } n=6, \text{ WL: } n=5, \text{ WM: } n=9 \text{ animals. Five fields of view per slide and 1 slide per animal.}"

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**Figure 5.** Immunostaining for CD68\(^+\) cells was used to detect the total number of macrophages within PVAT (Figure 5). Macrophage number was significantly increased in PVAT from obese animals compared with control \( (P<0.0001) \) and staining remained high after 4-week caloric restriction \( (\text{control versus WL: } P=0.0019 \text{ and obese versus WL: } P=0.07) \). Increased TNF-\( \alpha \) was reversed after the 4-week WM period \( (\text{control versus WM: } P=0.11 \text{ and obese versus WM: } P<0.0001, \text{Figure } 4) \). Percentage staining intensity is expressed as mean±SE \(* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, 1\text{-way ANOVA with Tukey post hoc test. Control: } n=5, \text{ obese: } n=6, \text{ WL: } n=5, \text{ WM: } n=9 \text{ animals. Five fields of view per slide and 1 slide per animal.}"

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infiltration occurred in diet-induced obesity (control versus obese: *P*<0.0001) and was decreased after caloric restriction (control versus WL: *P*>0.05 and obese versus WL: *P*=0.02). However, macrophage number was further reduced to control levels after 4-week WM (control versus WM: *P*=0.80 and WL versus WM: *P*=0.0062).

**PVAT-Derived NO Contributes to Restoration of PVAT Anticontractility After Maintenance of Body Weight**

Total endothelial nitric oxide synthase (eNOS) expression within PVAT (Figure 6A and 6B) was reduced by diet-induced obesity (*P*=0.029) and remained reduced after four-week caloric restriction (*P*=0.04). WM increased eNOS expression although levels were not significantly different to either control (*P*=0.94) or obese (*P*=0.34).

Incubation of arteries with intact endothelium but lacking PVAT with the NOS inhibitor, NG-nitro-L-arginine did not alter the vasoconstrictor response to norepinephrine (*P*=0.86, data not shown). However, the presence of NG-nitro-L-arginine increased the vasoconstrictor response to norepinephrine in PVAT intact vessels taken from control animals (*P*<0.0001, Figure 6C). Incubation of PVAT intact vessels with NG-nitro-L-arginine reduced the contractile response to norepinephrine in obesity (*P*=0.0067, Figure 6D) but had no effect after WL induced by caloric restriction (*P*=0.06, Figure 6E). However, the effects of NOS inhibition were restored after WM as an increase in the contractile response was observed in vessels with PVAT in the presence of NG-nitro-L-arginine (*P*=0.0012, Figure 6F). Moreover, the presence of PVAT did not alter vasodilation in response to carbachol (*P*=0.81, Figure IV in the online-only Data Supplement).

**Discussion**

This study investigated the effects of obesity and diet-induced WL on the anticontractile properties of PVAT. The main findings were (1) diet-induced obesity perturbs PVAT function through a mechanism involving downregulation of NOS and increased inflammation, (2) the impaired anticontractile effect of PVAT associated with obesity can be ameliorated by sustained WL, and (3) sustained WL improves PVAT anticontractile function by reducing adipose inflammation and increasing NOS availability. These observations advance our understanding of how changes in the PVAT environment contribute to vascular dysfunction associated with obesity and how these may be reversed to restore normal PVAT function.

Animals consuming the HFD rapidly gained weight becoming significantly heavier than those fed control diet indicative of the development of obesity. In agreement with human obesity and rat models of diet-induced obesity, high-fat feeding was associated with hypertension and hyperleptinemia. In contrast to the human phenotype, blood glucose levels were unaltered in obesity; however, overt hyperglycaemia has rarely been reported in studies using Sprague–Dawley rats and in agreement with our data, insulin resistance is common and its severity seems to be dependent on the fat content of the diet and duration of feeding.

The initial caloric restriction to 40 kcal/d was chosen to mimic human dieting and successfully produced a significant reduction in body weight during the 4-week period, consistent with that observed in other rodent models. Consistent with previous studies reporting the beneficial effects of WL on cardiovascular risk factors, we also found circulating leptin and insulin levels were reduced to normal at the end of the 4-week caloric restriction period. The increases in...
Figure 6. Adipocyte-derived nitric oxide plays a key role in the restoration of the perivascular adipose tissue (PVAT) anticontractile capacity. A, Expression of total endothelial nitric oxide synthase (eNOS) within PVAT was reduced by diet-induced obesity (P<0.03, n=4) and remained reduced after 4-week caloric restriction (P=0.04, n=4). Sustained weight loss increased eNOS expression although levels were not significantly different to either control (P=0.94, n=4) or obese (P=0.34, n=4). Vessels with endothelium were incubated with 100 μmol/L N\(^{\text{-}}\)monomethyl-L-arginine (-NMMa), an NOS inhibitor, for 30 minutes. B, Healthy control animals: incubation of vessels with -NMMa potentiated the vasoconstrictor response in the presence of PVAT (P<0.0001, n=12). C, Diet-induced obesity: -NMMa reduced the contractile response to norepinephrine in vessels with PVAT (P=0.0067, n=14). D, Diet-induced weight loss: NOS inhibition had no effect on the contractile response (P=0.06, n=8). E, Weight maintenance: NOS inhibition increased the contractile response to norepinephrine (P=0.0012, n=7). Data are expressed mean±SE *P<0.05, **P<0.01, 2-way ANOVA with Bonferroni post hoc test.

systolic blood pressure associated with obesity were reversed after caloric restriction; however, diastolic blood pressure was only restored to control levels at the end of the 4-week WM period, suggesting that reversal of obesity-induced hypertension required sustained WL. The cause of the difference in the effect of caloric restriction on systolic versus diastolic blood pressure is unclear but may be a consequence of changes in peripheral resistance because this directly influences diastolic blood pressure. This is supported by the data presented in this study, as obesity-associated changes in the PVAT environment were still present after 4-week caloric restriction, so increased diastolic blood pressure in WL animals might reflect the reduced PVAT anticontractile capacity.

In line with studies of small arteries taken from obese patients\(^{5,10}\) and rodent models of obesity,\(^{7-9,31}\) we found that the anticontractile effect of PVAT was lost in the rat model of diet-induced obesity. Obesity has been described as the perfect storm as the storage of excess energy in adipocytes leads to hypertrophy and subsequent hypoxia, resulting in chronic inflammation and abnormalities in adipocyte function.\(^{3}\) Adipocyte hypertrophy is one of the major hallmarks of obesity and similar to previous studies,\(^{5,9,10}\) we found that the cross-sectional area of adipocytes from mesenteric PVAT of obese animals was greatly increased. It has been shown that blood supply to adipocytes does not increase to compensate for enlarged adipocyte size,\(^{32}\) and hypertrophied adipocytes are larger than the normal diffusion distance of oxygen within tissues,\(^{33}\) suggesting that obese adipocytes exist in a state of hypoxia.\(^{3}\) This results in the development of a chronic inflammatory state within the adipose tissue with increased production of proinflammatory cytokines, such as TNF-α,\(^{5,10,36}\) which was observed in PVAT from obese animals.

Increased adipocyte size and subsequent inflammation within PVAT contribute to the obesity-induced loss of PVAT anticontractile effect. This is supported by previous work within our laboratory showing that experimental hypoxia, produced by gassing arteries with 95% nitrogen and 5% CO\(_2\) for 2.5 hours, significantly attenuated the anticontractile effect of PVAT in both rat\(^{5,36}\) and mouse\(^{37}\) mesenteric arteries through a mechanism that involved increased TNFα and interleukin-6. However, the damaging effects of obesity on PVAT function could not be reversed by incubation of arteries from obese patients with an anti-TNFα antibody,\(^{3}\) suggesting that chronic inflammation produces changes in adipocyte function and subsequent release of adipokines.

The origins of proinflammatory cytokines within PVAT have not been explored; however, previous studies have suggested increased levels of TNFα are a consequence of both increased adipokine secretion from the adipocytes and increased macrophage infiltration into the adipose tissue.\(^{38,39}\) Our data support a role for increased macrophage infiltration as the number of CD68\(^{+}\) cells within PVAT was increased in obesity. We accept that CD68\(^{+}\) may not be entirely macrophage specific as discussed previously by Kunisch et al.\(^{40}\)
and it is feasible that increased adipose fibrosis may contribute to the changes observed.\textsuperscript{41} However, studies using a mouse model of macrophage ablation showed a key role for macrophage activation in the loss of anticontractile effect when healthy PVAT is subjected to inflammatory insults,\textsuperscript{36} suggesting that the observed infiltration of proinflammatory macrophages contribute to the loss of PVAT anticontractile capacity in our animal model of diet-induced obesity. Moreover, a recent study reported increased macrophage infiltration in the aortic PVAT of obese mice when identified using either CD68 or F4/80.\textsuperscript{42}

Eosinophils play a role in sustaining anti-inflammatory M2 macrophages within the adipose tissue,\textsuperscript{43} and recent studies within our laboratory have shown a loss of PVAT anticontractile effect in ΔdbGATA-F2 mice, which are deficient in eosinophils.\textsuperscript{44} We show a reduction in the number of eosinophils within PVAT in diet-induced obesity and an inverse correlation with body weight, consistent with reports in perigonal adipose tissue from obese mice.\textsuperscript{45} This suggests that loss of eosinophils may contribute to the obesity-induced loss of PVAT anticontractile function, although the mechanism is currently unclear.

Consistent with our data showing reduced eNOS levels in PVAT in obesity, several studies have reported decreased eNOS expression in white adipose tissue taken from animal models of obesity,\textsuperscript{30,46} suggesting that this may contribute to the observed reduction in NO activity. Moreover, TNF\textsubscript{α} activity has been shown to induce eNOS downregulation in rodent white adipose tissue\textsuperscript{45} and contribute to endothelial dysfunction via downregulation of NOS in small mesenteric arteries,\textsuperscript{47} suggesting that changes in eNOS activity could be the link between increased inflammation in PVAT and reduced function.

Previous studies in human subcutaneous small arteries reported NOS inhibition had no effect on PVAT function in human obesity.\textsuperscript{30,48} We found that NOS inhibition produced a reduction in vascular contractility in obesity, suggesting that changes in NOS activity and subsequent reduced NO bioavailability contribute to the attenuation of PVAT function. The reasons for this are unclear, but increased levels of reactive oxygen species have been reported to lead to the uncoupling of eNOS resulting in the formation of peroxynitrite rather than NO.\textsuperscript{31,48} This is supported by previous data from our laboratory showing free radical scavengers can rescue PVAT anticontractile function in human obesity\textsuperscript{49} and the recent work of Xia et al\textsuperscript{42} showing eNOS uncoupling within aortic PVAT from high-fat fed mice.

A previous study reported that bariatric surgery could restore PVAT function by a reduction in adipose inflammation and increasing NO bioavailability;\textsuperscript{10} therefore, we investigated whether WL induced by dietary restriction could produce similar effects. To separate the effects of reduced energy intake and the physiological effects of WL, PVAT function was explored after a 4-week caloric restriction period and after a WM period, in which animals were maintained on a healthy caloric intake for a further 4 weeks leading to sustained WL. We found that the anticontractile effect of PVAT was restored after sustained WL, and this was associated with reversal of hypertension and reduction in markers of the metabolic syndrome. However, the magnitude of the PVAT effect was not as pronounced as that observed in control animals providing evidence for the existence of parallel signaling pathways and PVAT-derived relaxing factors mediating PVAT function.

The improvement in PVAT function was associated with the restoration of adipocyte size to control levels, infiltration of eosinophils, decreased macrophage infiltration, reduction in PVAT TNF\textsubscript{α}, and restoration of eNOS expression suggesting that reduced inflammation facilitates restoration of PVAT function. The effects of diet-induced WL on adipose inflammation have not been widely studied; however, our findings are consistent with previous studies showing significant reductions in TNF\textsubscript{α} expression within mesenteric white adipose tissue after caloric restriction.\textsuperscript{49} The increased eosinophil number within PVAT and their link to M2 macrophage stabilization,\textsuperscript{48} along with the reduced CD68\textsuperscript{+} cell staining indicate that reduction in macrophage infiltration may contribute to the restoration of PVAT function. Moreover, a recent study within our laboratory also supports the role of eosinophil infiltration in reversal of obesity-induced PVAT damage as reconstitution of eosinophils in ΔdbGATA-F2 mice restored the PVAT anticontractile capacity.\textsuperscript{44}

Along with the reduction in TNF\textsubscript{α}, we found that eNOS levels were restored after WM. This may be a consequence of the decreased macrophage numbers because this would reduce the high level of NO associated with uncoupling and also reduce TNF\textsubscript{α} production allowing restoration of normal eNOS expression. Moreover, inhibition of NOS increased the contractile response to norepinephrine, suggesting NOS function within PVAT was improved with a subsequent increase in NO bioavailability. This is supported by observations of increased plasma nitrite levels in obese patients after 12-week dietary intervention.\textsuperscript{50} Moreover, enhanced NO bioavailability mediated increased endothelium-dependent relaxation after 10% WL in obese patients.\textsuperscript{51} Improvements in vascular responses to L-arginine were also reported after WL and correlated with decline in serum TNF\textsubscript{α},\textsuperscript{52} suggesting a role for reduced TNF\textsubscript{α} in the restoration of NO.

We have shown that obesity is associated with a reversible reduction in NO bioavailability, and restoration of adipose eNOS levels may also contribute to reversal of the metabolic syndrome. Overexpression of eNOS in mice was found to prevent HFD-induced weight gain and hyperinsulinemia and attenuate diet-induced adipocyte hypertrophy through increased metabolic rate.\textsuperscript{46} Moreover, eNOS has been suggested to be a key regulator of metabolic homeostasis after observations that deletion of the eNOS gene induced insulin resistance through its effects on vasodilation and insulin signaling within the skeletal muscle.\textsuperscript{53,54} This suggests that the observed improvement in insulin sensitivity may be a consequence of improved eNOS function and a defect in NO synthesis may represent a mechanism linking metabolic syndrome and cardiovascular disease. Taken together, these data reveal eNOS as a new target for the treatment of PVAT dysfunction associated with obesity and the metabolic syndrome.

Four-week caloric restriction and its associated WL did not restore the anticontractile capacity of PVAT, even though...
systolic blood pressure returned to normal. Adipocyte hypertrophy and local inflammation were reduced within PVAT but not restored to control levels and NOS activity remained perturbed after WL. Moreover, diastolic blood pressure remained elevated suggesting that the damaging effects of obesity on PVAT function were not immediately reversed after return to control body weight and providing additional evidence for the role of PVAT in modulation of vascular tone.

Bariatric surgery led to complete restoration of the PVAT anticontractile capacity within 6 months that was associated with reduced systolic blood pressure even though patients were still obese and had enlarged adipocytes within PVAT. Similar to our results after sustained WL, where the anticontractile effect was restored, a reduction in TNFα staining associated with increased local NO bioavailability was reported within PVAT after bariatric surgery, suggesting that reductions in local inflammation are key to restoration of the PVAT anticontractile capacity.

The results presented here have shown that diet-induced obesity impairs PVAT anticontractile function through a mechanism involving increased TNFα and downregulation of NOS and that PVAT anticontractile function can be restored by sustained WL through reduction in local TNFα and increased NO availability. The findings support the targeting of inflammation and NOS for the treatment of PVAT dysfunction associated with obesity and the metabolic syndrome.

Acknowledgments

We thank the Histology Core Facility at the University of Manchester for assistance with histology.

Sources of Funding

This work was funded by a grant from the British Heart Foundation (FS/11/81/29331).

Disclosures

None.

References


This study identifies inflammation and nitric oxide synthase as potential targets for the treatment of obesity-related hypertension.

Sustained diet-induced weight loss led to an improvement in perivascular adipose tissue function through reduced inflammation and restored nitric oxide synthase activity. This study identifies inflammation and nitric oxide synthase as potential targets for the treatment of obesity-related hypertension.
Obesity-Related Perivascular Adipose Tissue Damage Is Reversed by Sustained Weight Loss in the Rat

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Arterioscler Thromb Vasc Biol. published online May 12, 2016;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Detailed Materials and Methods

Animal models.
Experiments involving the use of animals were performed in accord with the UK Animals (Scientific Procedures) Act 1986 under the authority of a valid project licence and were approved by The University of Manchester Ethical Review Process.

Male Sprague Dawley rats (8 weeks old, Charles River, UK) were fed 45% fat diet (HFD, 824018, SDS Diets, Witham, UK) ad libitum for 16 weeks to induce obesity. They were then singly housed and divided into two groups; obese rats maintained on the diet and weight loss rats that were subjected to caloric restriction (40 kcal/day) for a further four weeks. A control group was provided with 10% fat diet (824050, SDS diets, Witham UK) ad libitum for the 20-week period. In order to determine if weight loss induced functionally significant changes, a weight maintenance group was also established where an additional group of weight loss animals were provided with 70 kcal/day, equivalent to normal dietary intake, for a further 4 weeks at the end of the caloric restriction period. During the study period, body weight was recorded weekly and food consumption measured daily. At the end of the study period, animals were fasted overnight and euthanized by CO₂ inhalation with death confirmed by permanent cessation of the circulation by severing the diaphragm. The mesenteric bed was immediately removed and placed in ice-cold physiological salt solution (PSS in mM: 119 NaCl, 4.7 KCl, 1.17 MgSO₄, 25 NaHCO₃, 1.17 KH₂PO₄, 0.03 K₂EDTA, 5.5 glucose and 1.6 CaCl₂).

Biochemical analysis.
Fasting blood samples were taken from conscious, restrained rats by lateral tail vein blood sampling for measurement of various metabolic markers. Blood glucose concentration was immediately measured using an automatic blood glucose system (Accu-Chek Aviva Nano, Roche Diagnostics, Indianapolis, USA). Plasma was separated by centrifugation at 3000 x g for 10 minutes and samples stored at -80°C prior to use. Concentrations of total adiponectin (R&D Systems, Minneapolis, USA), insulin (Alpco, Salem, USA) and leptin (R&D systems, Minneapolis, USA) within plasma samples were quantified using commercially available ELISA kits according to manufacturers’ instructions.

Measurement of cardiovascular variables.
Systolic blood pressure, diastolic blood pressure and heart rate were measured in conscious, restrained animals prior to feeding and at the end of the study period using a CODA tail-volume cuff blood pressure monitoring system (Kent Scientific, Torrington, USA).

Wire myography.
Second-order mesenteric arteries both with and without PVAT were mounted in a wire myograph (Danish Myo Technology, Aarhus, Denmark) in order to assess changes in vascular contractility. Vessels were gassed (95% air/ 5% CO₂) and maintained at 37°C for 30 minutes before vessel wall tension and diameter were normalised using a standardised procedure. Isometric tension was continuously recorded (Chart 5, v5.5, AD Instruments, UK). Vessels were challenged with 60 mM high potassium PSS (KPSS in mM: 63.7 NaCl, 60 KCl, 1.17 MgSO₄, 25 NaHCO₃, 1.17 KH₂PO₄, 0.03 K₂EDTA, 5.5 glucose and1.6 CaCl₂) to establish viability and functional endothelial integrity was assessed by relaxation to 10 µM carbachol.

The contribution of nitric oxide to PVAT function was assessed by 30 minute incubation with the NOS inhibitor, L-NMMA (R&D systems, Minneapolis, USA) prior to construction of concentration-responses through the cumulative addition of norepinephrine (1 x 10⁻⁷ – 3.5 x 10⁻⁵ M) to the myograph bath.
**Immunohistochemistry.**

Immediately after dissection, PVAT was placed in 4% paraformaldehyde in PBS for 18 hours and subsequently processed to paraffin wax blocks prior to serial sectioning at 5 µm. Samples were dewaxed with xylene and rehydrated with ethanol prior to haematoxylin and eosin staining or immunostaining.

PVAT was immunostained for TNFα (ab1793, Abcam, Cambridge, UK, 1 µg/ml) and CD68 (ab31630, Abcam, Cambridge, UK, 10 µg/ml), a macrophage marker. Heat-induced antigen retrieval was performed followed by blocking of endogenous peroxidase using 3% H₂O₂ and block of non-specific binding sites using goat serum (R&D Systems, Minneapolis, USA). Sections were incubated with primary antibodies overnight, followed by incubation with Biotin-SP conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Westgrove, USA, 1.5 µg/ml) followed by detection using Vectastain ABC complex (Vector Laboratories, Peterborough, UK) and 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Peterborough, UK) to allow visualisation of antibody binding. Negative controls were treated according to the above protocol but incubated with mouse IgG at the same concentration as the primary antibody prior to addition of the secondary antibody.

Images were visualised using a light microscope (DM5000, Leica, Wetzlar, Germany) and photomicrographs were taken of each slide using a colour camera (DFC450 Leica, Wetzlar, Germany). Adipocyte size was quantified following H&E staining using ImageJ (v1.47, NIH, New York City, USA) on photomicrographs taken at 20x magnification by manually tracing the margins of 100 consecutive cells per animal to avoid selection bias. To enable the number of eosinophils to be measured, five fields of view were randomly selected per PVAT sample and 40x images obtained. The eosinophils were then counted and data presented as eosinophils/mm². For assessment of TNFα, images were taken at 40x magnification from five fields of view per slide. Images were white balanced to remove excess background and using ImageJ software, the percentage area of tissue stained was quantified using set threshold values. Macrophages stained with CD68 present in adipose tissue were counted, and the results expressed as cells/mm².

**Statistical Analyses.**

All data are presented as mean ± standard error (S.E). Differences in rat body weight and cardiometabolic parameters were tested using a one-way ANOVA followed by a Tukey post hoc test to compare individual groups. Changes in protein expression determined by western blotting or immunohistochemistry were analysed using one-way ANOVA with Tukey post hoc test. The Pearson’s correlation coefficient was calculated as a measure of linear correlation between adipocyte size and body weight.

Contractile responses obtained using wire myography experiments are presented as mean percentage KPSS constriction ± S.E. consistent with previously published data and curves fitted using non-linear regression analysis. These were analysed using two-way ANOVA followed by Bonferroni post hoc test. GraphPad Prism (v6, GraphPad Software, La Jolla, USA) was used for all statistical analyses and P values < 0.05 were considered statistically significant.

**References**


Supplementary Figure I. Caloric intake. Food intake was measured daily, animals on the HFD consumed significantly more calories than those on the control diet ($P < 0.001$). Data are expressed as mean ± S.E, control: $n = 6$, obese: $n = 5$, weight loss: $n = 6$. 
Supplementary Figure II. The presence of PVAT had no effect on the contractile response to KPSS in health or disease ($P = 0.4511$). Data are expressed mean ± S.E.M. * $P < 0.05$, one-way ANOVA with Tukey post hoc test, control: $n = 12$, obese: $n = 14$, weight loss (WL): $n = 12$, weight maintain (WM): $n = 8$. 
Supplementary Figure III. Diet does not alter vasoconstriction in the absence of PVAT. A, The contractile response of vessels with PVAT taken from obese animals was increased compared to controls ($P = 0.0332$). The vasoconstrictor response remained elevated compared to controls in vessels taken from weight loss animals ($P = 0.0148$). However, weight maintenance led to improvement in the PVAT anticontractile capacity as the response was no longer different to controls ($P = 0.35$). B, Body weight had no effect on contractile response to norepinephrine in endothelium intact vessels lacking PVAT ($P = 0.1353$). Data are expressed as mean ± S.E. * $P < 0.05$, two-way ANOVA with Bonferroni post hoc test. control: $n = 12$, obese: $n = 14$, weight loss: $n = 12$, weight maintain: $n = 8$. 
Supplementary Figure IV. PVAT has no effect on endothelium-dependent dilation. The presence of PVAT did not alter the dilation response to carbachol in vessels taken from control rats following constriction with 10 µmol/L norepinephrine (P = 0.8057, n = 12). Data are expressed as mean ± S.E.M. * P < 0.05, two-way ANOVA with Bonferroni post hoc test.
Diet-induced obesity:

- Insulin resistance
- Hypertension
- Reduced adipocyte size
- Increased eosinophil number
- Increased macrophage infiltration (CD68 staining)
- Reduced NO production
- Diminished PVAT anticontractile capacity
- Increased inflammation (TNFα)

Sustained weight loss:

- Restored insulin sensitivity
- Restored adipocyte size
- Restored eosinophil number
- Reduced macrophage numbers (CD68 staining)
- Increased NO bioavailability (L-NMMA)
- Reduced blood pressure
- Restored PVAT anticontractile function
- Reduced inflammation (TNFα)