Obesity-related perivascular adipose tissue damage is reversed by sustained weight loss in the rat

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Abstract

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 characterised following 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 TNFα and reduced nitric oxide bioavailability within PVAT. Sustained weight loss led to improvement in PVAT function associated with restoration of adipocyte size, reduced TNFα and increased nitric oxide synthase function. This was associated with reversal of obesity-induced hypertension and normalisation 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 reveal inflammation and nitric oxide synthase, particularly eNOS, as potential targets for the treatment of PVAT dysfunction associated with obesity and the metabolic syndrome.

Abbreviations:
eNOS, *endothelial nitric oxide synthase*; HFD, *high fat diet*; KPSS, *high potassium physiological salt solution*; L-NMMA, N^G*-monomethyl-L-arginine*; NO, nitric oxide; NOS, *nitric oxide synthase*; PSS, *physiological salt solution*; PVAT, *perivascular adipose tissue*; TNFα, *tumour-necrosis factor-alpha;* WL*, weight loss;* WM, *weight maintenance.*

Introduction

Obesity is one of the major causes of illness and death in the world, and is a significant public health burden currently affecting an estimated half a billion adults and 40 million children globally[1]. It is associated with many co-morbidities and overwhelming evidence supports the importance of obesity in the pathogenesis and progression of cardiovascular disease[2]. The rapidly increasing gap between the availability of medical therapies and the steadily rising rates of obesity emphasise the need for investigation into novel therapies to prevent the devastating effects of being overweight.

Healthy perivascular adipose tissue exerts an anticontractile effect on adjacent arteries[3-5]. that is lost in both rodent models[6-9] and human obesity and the metabolic syndrome[5, 10] suggesting that changes in PVAT function and morphology may contribute to vascular dysfunction associated with increased body weight[5] and diabetes[11, 12].

The most obvious treatment for obesity is weight loss, 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 six months after surgery through reduction of adipose inflammation and increasing local adiponectin and nitric oxide (NO) bioavailability[10]. 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[18]. Few studies have been specially designed to determine the effects of weight loss produced by dietary intervention in obesity, however, the majority of clinical trials and animal studies have reported a beneficial effect of diet-induced weight loss on blood pressure[13, 19, 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, the present study was designed to explore changes in the PVAT environment that occur in obesity and to investigate the effects of diet-induced weight loss on PVAT anticontractile function. The present study tested the hypothesis that diet-induced weight loss 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 weight loss 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

Weight loss reversed cardiometabolic damage induced by high fat diet.
There were no significant differences in body weight of the rats prior to starting their respective diets (P=0.39). After 16 weeks of of consuming the 45% fat diet (HFD), animals were significantly heavier than controls (P< 0.01, Figure 1). Two groups of obese animals (WL and WM) were then subjected to caloric restriction for four weeks in order to induce weight loss. Caloric restriction produced a gradual reduction in body weight with animals losing an average of 130g 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 weight loss were altered following maintenance of body weight, rats in one of the groups (WM) were provided with 70 kcal/day for an additional four 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 rats provided with a control diet (Supplementary Figure I, P<0.001), indicating that the animals did not compensate for the increased caloric content of the high fat diet by eating less.

The cardiometabolic profile of animals with dietary interventions is shown in Table 1. HFD produced insulin resistance without overt diabetes 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 following caloric restriction (P<0.0001) and this was sustained following the four-week weight maintenance 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 to those of controls after eight 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 following caloric restriction (P<0.001) and this reduction was sustained following the four-week weight maintenance period (P<0.001).

Systolic and diastolic blood pressure did not change over 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 following four-week weight loss (control vs. WL: P=0.06) and this was maintained during the weight maintenance period (control vs. WM: P=0.09). Diastolic blood pressure was reduced following weight loss; 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 vs. WM: P=0.37). Significant tachycardia was observed in diet-induced obesity (control: 299±5.0 vs. obese: 335±6.9bpm, P<0.0001). Caloric restriction induced transient bradycardia (WL: 246±10.9bpm, P<0.0001), however heart rate returned to control levels in following weight maintenance (WM: 294±8.1bpm, P=0.56) suggesting that the reduced heart rate was a consequence of extreme weight loss.

The anticontractile capacity of PVAT is restored following sustained weight loss
The presence of PVAT did not alter the contractile response evoked by stimulation with KPSS in any of the animal models used (Supplementary Figure II). Moreover, responses to KPSS were not altered by body weight (P=0.45) allowing contractile responses to be expressed as percentage KPSS.

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 to controls (P= 0.03, Supplementary Figure IIIA), indicating that diet-induced obesity diminished the anticontractile capacity of PVAT. Weight loss induced by 50% caloric restriction did not reverse obesity-induced loss of PVAT anticontractile effect as responses were unaltered by the presence of PVAT (P=0.14, Figure 2C) and the vasoconstrictor response to norepinephrine remained elevated compared to controls (P=0.01, Supplementary Figure IIIA). However, maintenance of body weight for four weeks following caloric restriction led to restoration of the PVAT anticontractile function as the presence of PVAT reduced the contractile response to norepinephrine (P<0.0001, Figure 2D) and the response of vessels with PVAT was no longer different to controls (P=0.35, Supplementary Figure IIIA). Changes in body weight had no effect on the vasoconstrictor response to norepinephrine in endothelium intact vessels lacking PVAT (P=0.14, Supplementary Figure IIIB).

Adipocyte hypertrophy and PVAT inflammation are reduced by weight loss

Obesity produced an increase in adipocyte cross-sectional area (P<0.0001), which was reduced but not completely reversed following caloric restriction (obese vs. WL: P<0.0001, control vs. WL: P<0.0001). However, complete restoration of adipocyte size was observed following a four-week weight maintenance period (control vs. WM: P=0.67, Figure 3A and B). 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 following caloric restriction but levels were not significantly different to either control or obese (P>0.05). However, eosinophil levels within PVAT were restored by weight maintenance (P=0.67, Figure 3C). Moreover, the number of eosinophils within PVAT negatively correlated with body weight (r=-0.41, P=0.04)

TNFα staining intensity was significantly increased in PVAT from obese animals compared to control (P<0.0001) and staining remained high following four-week caloric restriction (control vs WL: P=0.0019, obese vs WL: P=0.07). However, the increased TNFα immunostaining was reversed by sustained weight loss (control vs WM: P=0.11, obese vs WM: P<0.0001, Figure 4).

Immunostaining for $CD68^+$ cells was used to detect the total number of macrophages within PVAT (Figure 5). Macrophage infiltration occurred in diet-induced obesity (control vs obese: P<0.0001) and was decreased following caloric restriction (control vs WL: P>0.05, obese vs WL: P=0.02). However, macrophage number was further reduced to control levels following four-week weight maintenance (control vs WM: P=0.80, WL vs WM: P=0.0062).

PVAT-derived NO contributes to restoration of PVAT anticontractility following maintenance of body weight

Total eNOS expression within PVAT (Figure 6A and B) was reduced by diet-induced obesity (P=0.029) and remained reduced following four-week caloric restriction (P=0.04). Weight maintenance 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, L-NMMA did not alter the vasoconstrictor response to norepinephrine (P=0.86, data not shown). However, the presence of L-NMMA 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 L-NMMA reduced the contractile response to

norepinephrine in obesity (P=0.0067, Figure 6D) but had no effect following weight loss induced by caloric restriction (P=0.06, Figure 6E). However, the effects of NOS inhibition were restored following weight maintenance as an increase in the contractile response was observed in vessels with PVAT in the presence of L-NMMA (P=0.0012, Figure 6F). Moreover, the presence of PVAT did not alter vasodilation in response to carbachol (P=0.81, Supplementary Figure IV).

Discussion

The present study investigated the effects of obesity and diet-induced weight loss 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 weight loss; and 3) sustained weight loss 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[23] and rat models of diet-induced obesity[21, 24, 25], high fat feeding was associated with hypertension and hyperleptinaemia. 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[26] and in agreement with our data, insulin resistance is common and its severity appears to be dependent on the fat content of the diet and duration of feeding[27].

The initial caloric restriction to 40 kcal/day was chosen to mimic human dieting[28] and successfully produced a significant reduction in body weight over the four-week period, consistent with that observed in other rodent models[21, 22, 29]. Consistent with previous studies reporting the beneficial effects of weight loss on cardiovascular risk factors[13, 21, 30], we also found circulating leptin and insulin levels were reduced to normal at the end of the four-week caloric restriction period. The increases in systolic blood pressure associated with obesity were reversed following caloric restriction, however diastolic blood pressure was only restored to control levels at the end of the four-week weight maintenance period suggesting that reversal of obesity-induced hypertension required sustained weight loss. 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 as 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 following four-week caloric restriction, so increased diastolic blood pressure in weight loss 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[2]. 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[34]. This results in the development of a chronic inflammatory state within the adipose tissue with increased production of pro-inflammatory cytokines, such as TNFα[5, 10, 35], 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 IL-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[5] suggesting that chronic inflammation produces changes in adipocyte function and subsequent release of adipokines.

The origins of pro-inflammatory 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[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[36] suggesting that the observed infiltration of pro-inflammatory 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[42].

Eosinophils play a role in sustaining anti-inflammatory M2 macrophages within the adipose tissue[43] and recent studies within our laboratory have shown a loss of PVAT anticontractile effect in ΔdbGATA-F2 mice, which are deficient in eosinophils[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 perigonadal adipose tissue from obese mice[43]. 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[45, 46] suggesting that this may contribute to the observed reduction in NO activity. Moreover, TNFα activity has been shown to induce eNOS downregulation in rodent white adipose tissue[45] and contribute to endothelial dysfunction via downregulation of NOS in small mesenteric arteries[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[5, 10]. 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 perioxynitrite rather than NO[31, 48]. This is supported by previous data from our laboratory showing free radical scavengers can rescue PVAT anticontractile function in human obesity[10] and the recent work of Xia *et al.* showing eNOS uncoupling within aortic PVAT from high fat fed mice[42].

A previous study reported that bariatric surgery could restore PVAT function by a reduction in adipose inflammation and increasing NO bioavailability[10], therefore we investigated whether weight loss induced by dietary restriction could produce similar effects. In order to separate the effects of reduced energy intake and the physiological effects of weight loss, PVAT function was explored after a four-week caloric restriction period and following a weight maintenance period, in which animals were maintained on a healthy caloric intake for a further four-weeks leading to sustained weight loss. We found that the anticontractile effect of PVAT was restored following sustained weight loss 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 signalling 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 TNFα and restoration of eNOS expression suggesting that reduced inflammation facilitates restoration of PVAT function. The effects of diet-induced weight loss on adipose inflammation have not been widely studied, however our findings are consistent with previous studies showing significant reductions in TNFα expression within mesenteric white adipose tissue following caloric restriction[49]. The increased eosinophil number within PVAT and their link to M2 macrophage stabilisation[43]; along with the reduced $CD68^+$ 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[44].

Along with the reduction in TNFα, we found that eNOS levels were restored following weight maintenance. This may be a consequence of the decreased macrophage numbers as this would reduce the high level of NO associated with uncoupling and also reduce TNFα 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 following 12-week dietary intervention[50]. Moreover, enhanced NO bioavailability mediated increased endothelium-dependent relaxation following 10% weight loss in obese patients[51]. Improvements in vascular responses to L-arginine were also reported following weight loss and correlated with decline in serum TNFα[52] suggesting a role for reduced TNFα 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 high fat diet-induced weight gain and hyperinsulinaeima and attenuate diet-induced adipocyte hypertrophy through increased metabolic rate[46]. Moreover, eNOS has been suggested to be a key regulator of metabolic homeostasis following observations that deletion of the eNOS gene induced insulin resistance through its effects on vasodilation and insulin signalling within the skeletal muscle[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 weight loss 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 following weight loss. Moreover, diastolic blood pressure remained elevated suggesting that the damaging effects of obesity on PVAT function were not immediately reversed following 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 six months that was associated with reduced systolic blood pressure even though patients were still obese and had enlarged adipocytes within PVAT[10]. Similar to our results following sustained weight loss, where the anticontractile effect was restored, a reduction in TNFα staining associated with increased local NO bioavailability was reported within PVAT

following bariatric surgery suggesting that reductions in local inflammation are key to restoration of the PVAT anticontractile capacity.

The results presented 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 weight loss through reduction in local TNFα and increased NO availability. The findings support the targeting of inflammation and nitric oxide synthase for the treatment of PVAT dysfunction associated with obesity and the metabolic syndrome.

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Highlights

- The anticontractile effect of PVAT on adjacent arteries was abolished in diet-induced obesity.
- Sustained diet-induced weight loss led to an improvement in PVAT function through reduced inflammation and restored NOS activity.
- This study identifies inflammation and NOS as potential targets for the treatment of obesity-related hypertension.

Figure legends

Figure 1. The effect of caloric intake on body weight. HFD increased body weight and this could be reversed by caloric restriction to 40 kcal/day (control vs obese: $P < 0.0001$, weight loss vs obese: $P < 0.01$). Data are expressed as mean ± S.E. **** indicates $P < 0.0001$, two-way ANOVA with Bonferroni *post hoc* test, control: $n = 12$, obese: $n = 14$, weight loss: $n = 12$, weight maintain: $n = 8$. CR: caloric restriction; WM: weight maintenance period.

Figure 2. Effect of diet-induced weight loss on PVAT anticontractile capacity. *A*, Healthy control animals: the presence of PVAT reduced the vasoconstrictor response to norepinephrine in vessels ($P < 0.0001$, $n = 12$). *B*, Diet-induced obesity: the presence of PVAT did not alter the contractile response to norepinephrine ($P = 0.21$, $n = 14$). *C*, Diet-induced weight loss: the anticontractile effect of PVAT was not restored ($P = 0.14$, $n = 12$) but D, Weight maintenance: PVAT anticontractile capacity was improved after an additional 4-weeks ($P < 0.0001$, $n = 8$). Data are expressed mean ± S.E. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, two-way ANOVA with Bonferroni *post hoc* test.

Figure 3. Adipocyte hypertrophy is reversed by sustained weight loss. ***A*,** Mesenteric PVAT from control (i), obese (ii), weight loss (WL) (iii) and weight maintenance (WM) (iv) animals were stained with H&E to determine adipocyte area and number of eosinophils. Representative images were obtained at 40x magnification, scale bar represents 50 µm and arrows highlight eosinophils. *B*, Average adipocyte areas were increased in diet-induced obesity (control vs obese: $P < 0.0001$). Adipocyte hypertrophy was reduced following weight loss (obese vs weight loss: $P < 0.0001$) but not completely restored to control size (control vs weight loss: $P < 0.001$). The additional 4-week weight maintenance period reversed obesity-induced adipocyte hypertrophy (control vs weight maintenance: $P > 0.05$). *C*, The number of eosinophils in mesenteric PVAT was reduced in diet-induced obesity (control vs obese: $P < 0.0001$), this was increased following weight loss and eosinophil number was not significantly different to either control or obese. The additional 4-week weight maintenance period restored eosinophil number to control levels (controls vs weight maintain: $P = 0.67$). Data are expressed as mean ± S.E. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, one-way ANOVA with Tukey *post hoc* test. Control: $n = 5$, obese: $n = 6$, weight loss (WL): $n = 5$, weight maintain (WM): $n = 9$ animals. 100 consecutive adipocytes were analysed from 1 slide per animal.

Figure 4. Inflammation within PVAT is reversed by sustained weight loss. ***A*,** Immunostaining for TNFα in mesenteric PVAT from control (i), obese (ii), weight loss (iii) and weight maintenance (iv) animals. Representative image of a negative control where samples were incubated with mouse IgG is shown (v). Representative images were obtained at 40x magnification and scale bar represents 50 µm. *B*, Adipocytes were positive for TNFα to a higher extent in obese samples than control ($P < 0.0001$) and staining remained high following weight loss (control vs weight loss: $P = 0.0019$, obese vs weight loss: $P = 0.07$). Increased TNFα was reversed following the four-week weight maintenance period (control vs weight maintain: $P = 0.11$, obese vs weight maintain: $P < 0.0001$). Percentage staining intensity is expressed as mean ± S.E. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, one-way ANOVA with Tukey *post hoc* test. Control: $n = 5$, obese: $n = 6$, weight loss (WL): $n = 5$, weight maintain (WM): $n = 9$ animals. 5 fields of view per slide and 1 slide per animal.

Figure 5. Macrophage infiltration is reversed by diet-induced weight loss. *A*, Immunostaining for CD68+ cells in mesenteric PVAT from control (i), obese (ii), weight loss (iii) and weight maintenance (iv) animals. Representative image of a negative control where samples were incubated with mouse IgG is shown (v). Representative images were obtained at 40x magnification, scale bars represent 75 µm and arrows highlight CD68+ cells. *B*, The number of CD68+ cells within PVAT was increased in diet-induced obesity (control vs obese: $P < 0.0001$). This was reduced following caloric restriction (obese vs weight loss: $P = 0.02$) although further reductions were observed at the end of the 4-week weight maintenance period (weight loss vs weight maintain: $P = 0.0062$, control vs weight maintain: $P = 0.80$). Data are expressed mean ± S.E. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, one-way ANOVA with Tukey *post hoc* test. Control: $n = 5$, obese: $n = 6$, weight loss (WL): $n = 5$, weight maintain (WM): $n = 9$ animals. 5 fields of view per slide and 1 slide per animal.

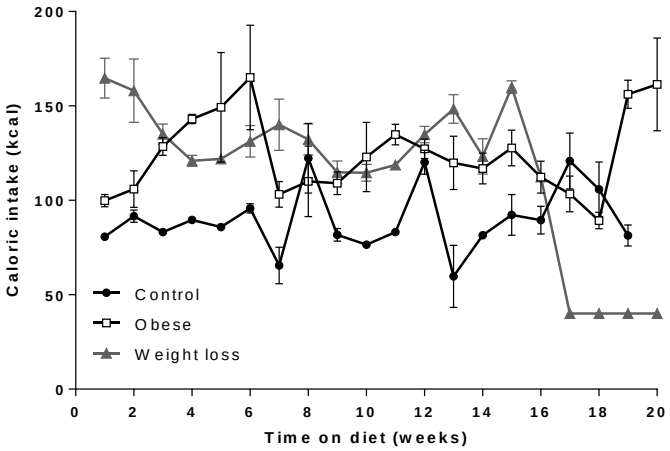
Figure 6. Adipocyte-derived nitric oxide plays a key role in the restoration of the PVAT anticontractile capacity. *A*, Expression of total eNOS within PVAT was reduced by diet-induced obesity ($P = 0.03$, $n = 4$) and remained reduced following 4-week caloric restriction ($P = 0.04$, $n = 4$). Sustained 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 µM L-NMMA, a NOS inhibitor, for 30 minutes. *B*, Healthy control animals: Incubation of vessels with L-NMMA potentiated the vasoconstrictor response in the presence of PVAT ($P < 0.0001$, $n = 12$) *C*, Diet-induced obesity: L-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 ± S.E. * $P < 0.05$, ** $P < 0.01$, two-way ANOVA with Bonferroni *post hoc* test.

Tables

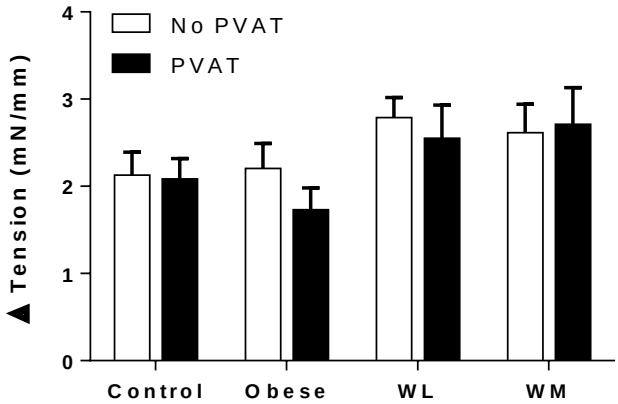
Table 1. Diet-induced changes in cardiometabolic parameters can be reversed by caloric restriction.

	Control	Obese	Weight loss	Weight maintain
Systolic BP (mmHg)	119.9 ± 1.2†	142.4 ± 2.1*	124.1 ± 2.03†	116.0 ± 1.9†§
Diastolic BP (mmHg)	82.6 ± 1.2†	99.04 ± 1.6*	91.03 ± 1.7*†	84.40 ± 1.6†§
Blood glucose (mmol/L)	6.65 ± 0.27	6.91 ± 0.26	6.66 ± 0.23	6.86 ± 0.24
Insulin (ng/ml)	1.31 ± 0.18†	2.80 ± 0.35*	1.03 ± 0.19†	1.17 ± 0.08†
Leptin (ng/ml)	3.46 ± 0.53†	8.40 ± 0.91*	4.17 ± 0.59†	2.62 ± 0.33†
Adiponectin (µg/ml)	3.84 ± 0.27	4.24 ± 0.22	3.43 ± 0.38	4.58 ± 0.55

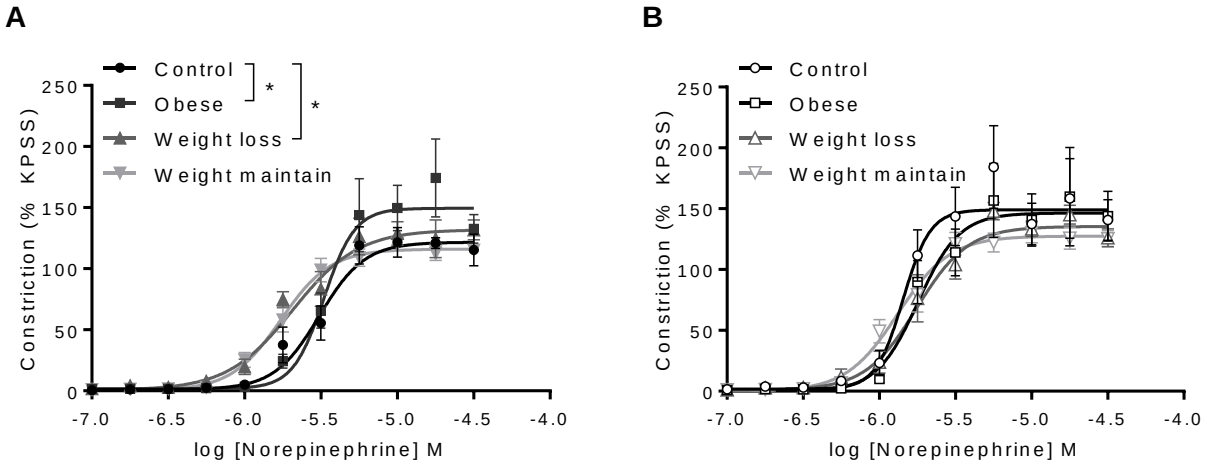
Values are expressed as mean ± SE. $^{*}P<0.05$ versus control; $^{\dagger}P<0.05$ versus obese, $^{\S}P<0.05$ versus weight loss one-way ANOVA with Tukey *post hoc* test, $n = 8 – 12$.



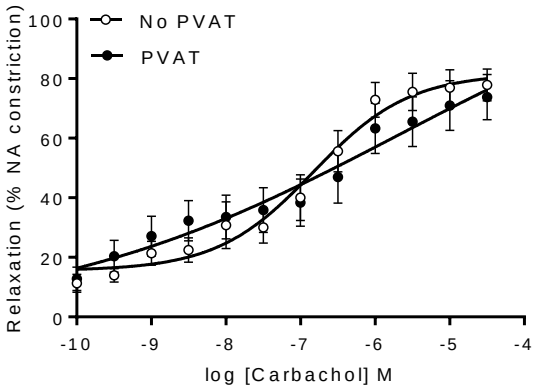
Supplementary Figure I. Caloric intake. Food intake was measured daily, animals on the HFD consumed significantly more calories than those on the control diet ($P < 0.001$). Data are expressed as mean ± S.E, control: $n = 6$, obese: $n = 5$, weight loss: $n = 6$.



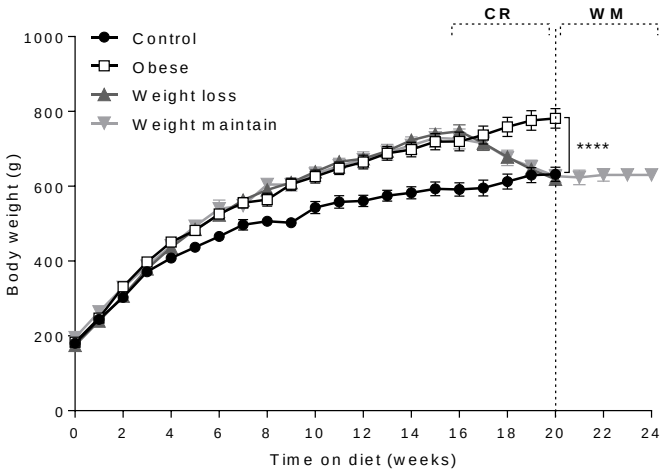
Supplementary Figure II. The presence of PVAT had no effect on the contractile response to KPSS in health or disease ($P = 0.4511$). Data are expressed mean ± S.E.M * $P < 0.05$, one-way ANOVA with Tukey *post hoc* test, control: $n = 12$, obese: $n = 14$, weight loss (WL): $n = 12$, weight maintain (WM): $n = 8$.

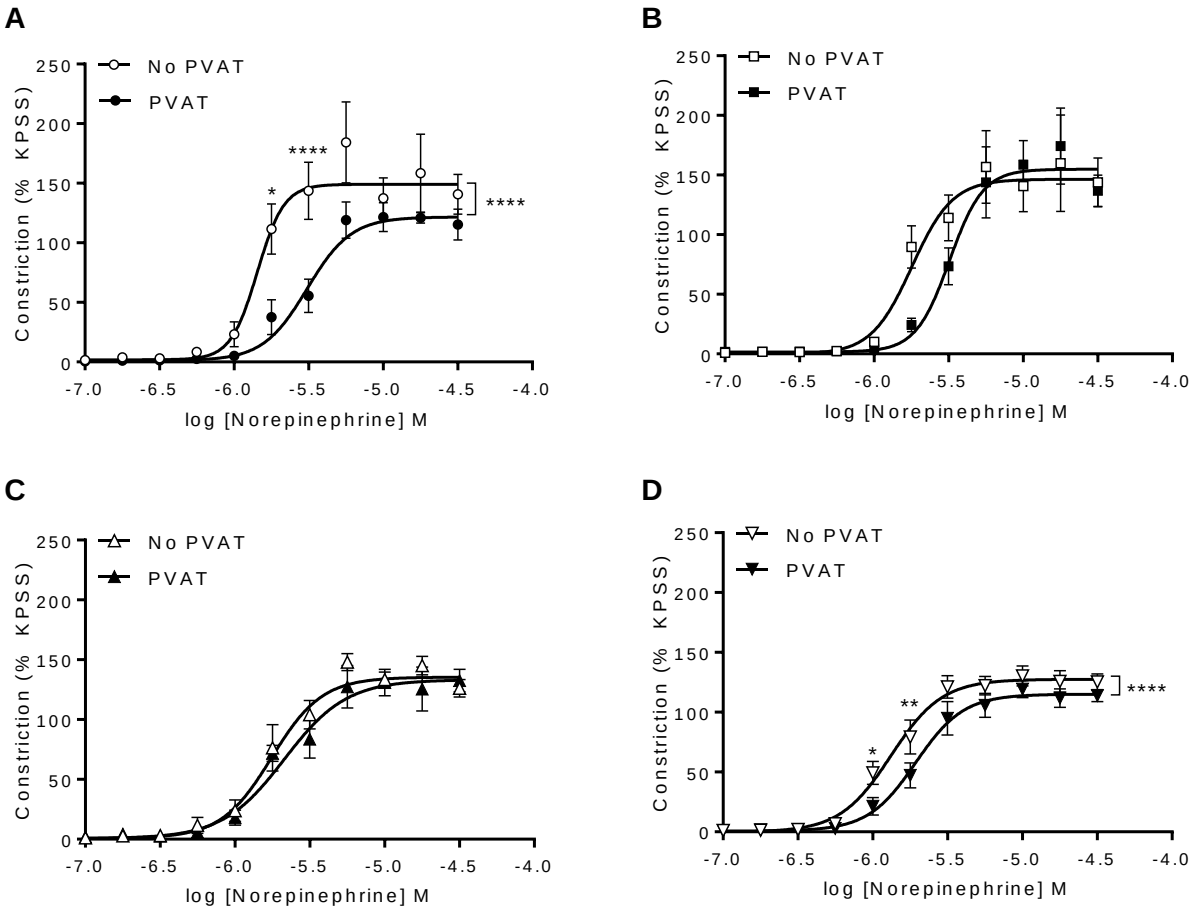


Supplementary Figure III. Diet does not alter vasoconstriction in the absence of PVAT. *A,* The contractile response of vessels with PVAT taken from obese animals was increased compared to controls (P = 0.0332). The vasoconstrictor response remained elevated compared to controls in vessels taken from 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.

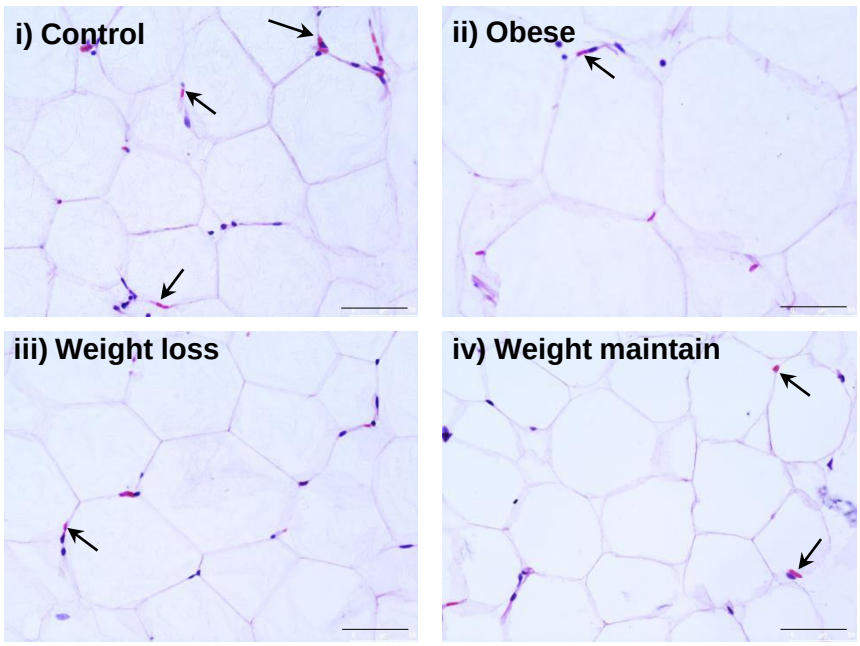


Supplementary Figure IV. PVAT has no effect on endothelium-dependent dilation. The presence of PVAT did not alter the dilation response to carbachol in vessels taken from control rats following constriction with 10 µmol/L norepinephrine (P = 0.8057, n = 12). Data are expressed as mean ± S.E.M. * P < 0.05, two-way ANOVA with Bonferroni *post hoc* test.

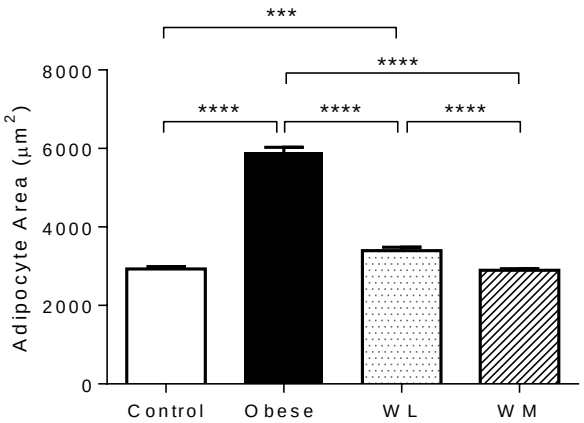

CR
WM
Control
Obese
Weight loss
Weight maintain
****
Body weight (g)
Time on diet (weeks)
0
200
400
600
800
1000
0
2
4
6
8
10
12
14
16
18
20
22
24


A
No PVAT
PVAT
Constriction (% KPSS)
log [Norepinephrine] M
*
****
****
B
No PVAT
PVAT
Constriction (% KPSS)
log [Norepinephrine] M
C
No PVAT
PVAT
Constriction (% KPSS)
log [Norepinephrine] M
D
No PVAT
PVAT
Constriction (% KPSS)
log [Norepinephrine] M
*
**
****

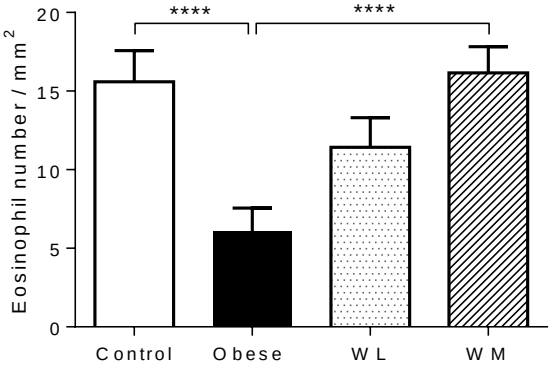
A


i) Control
ii) Obese
iii) Weight loss
iv) Weight maintain

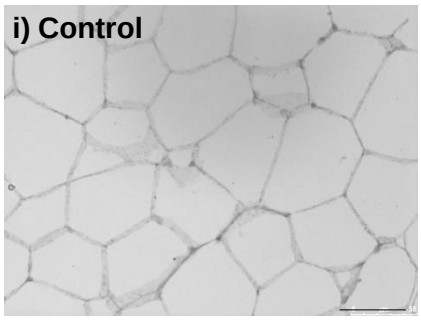
B

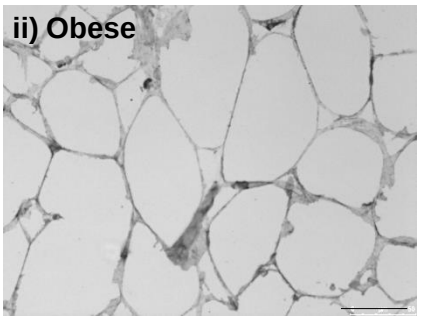

***
****
****
****
****
8000
6000
4000
2000
0
Adipocyte Area (μm2)
Control
Obese
WL
WM

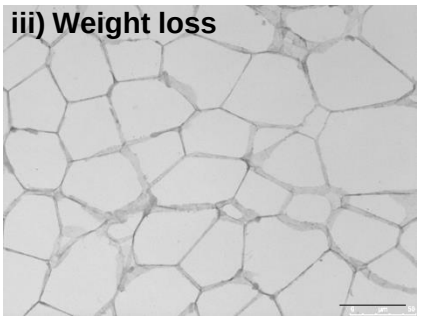
C

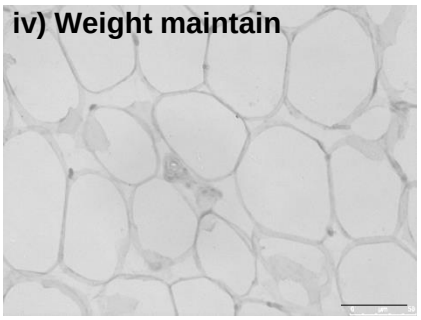

****
****
20
15
10
5
0
Eosinophil number / mm2
Control
Obese
WL
WM

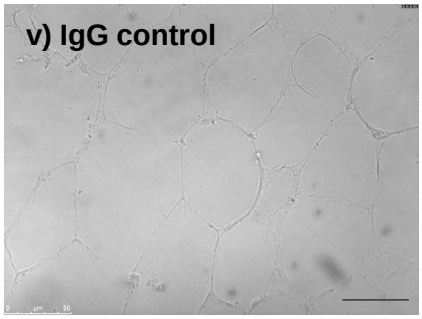
A


i) Control

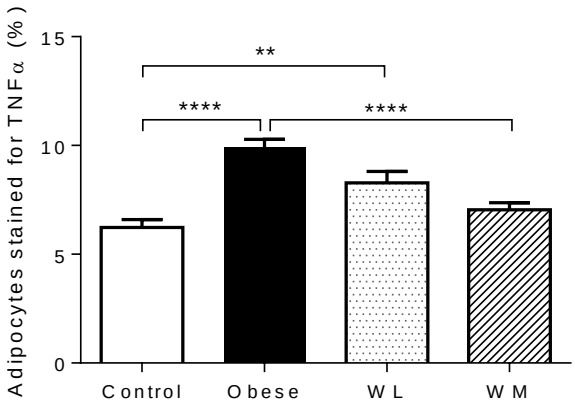

ii) Obese

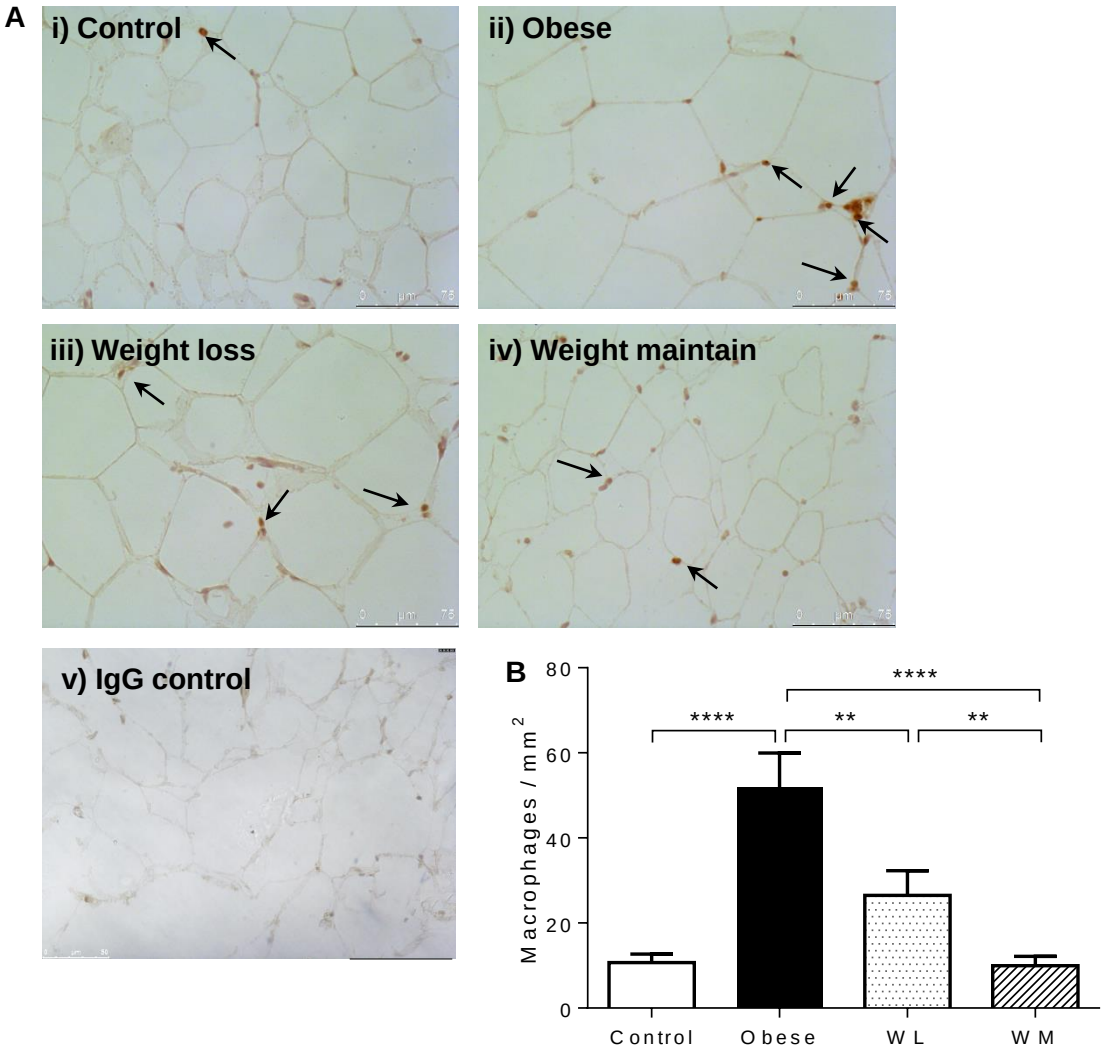

iii) Weight loss

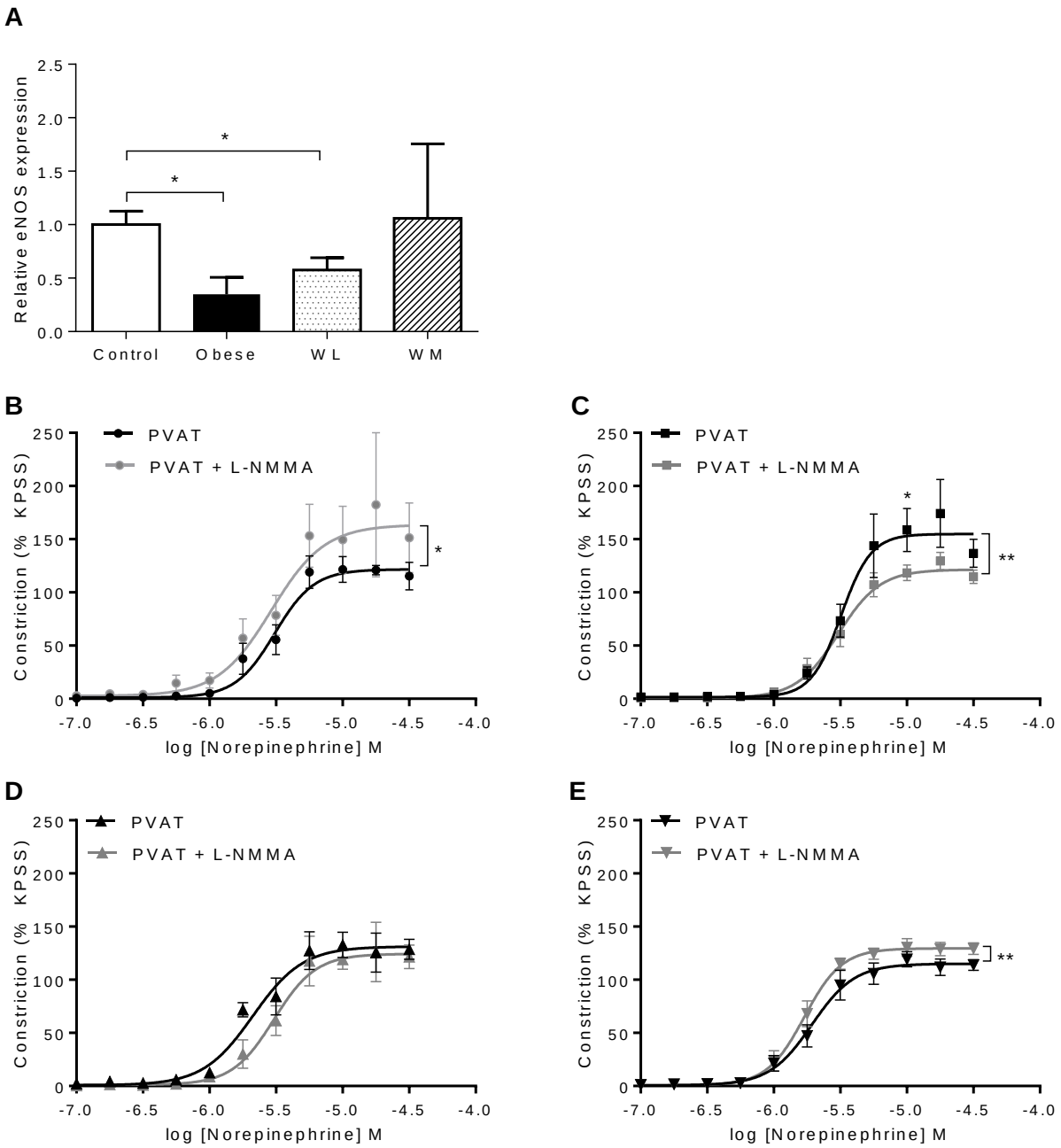

iv) Weight maintain


v) IgG control

B


Adipocytes stained for TNFα (%)
15
10
5
0
**
****
****
Control
Obese
WL
WM


A
i) Control
ii) Obese
iii) Weight loss
iv) Weight maintain
v) IgG control
B
Macrophages / mm2
80
60
40
20
0
****
****
**
**
Control
Obese
W L
W M


A
Relative eNOS expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
Obese
WL
WM
B
PVAT
PVAT + L-NMMA
Constriction (% KPSS)
250
200
150
100
50
0
*
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
log [Norepinephrine] M
C
PVAT
PVAT + L-NMMA
Constriction (% KPSS)
*
**
log [Norepinephrine] M
D
PVAT
PVAT + L-NMMA
Constriction (% KPSS)
log [Norepinephrine] M
E
PVAT
PVAT + L-NMMA
Constriction (% KPSS)
**
log [Norepinephrine] M