

Perivascular Adipose Tissue Control of Insulin-Induced Vasoreactivity in Muscle Is Impaired in *db/db* Mice

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Microvascular recruitment in muscle is a determinant of insulin sensitivity. Whether perivascular adipose tissue (PVAT) is involved in disturbed insulin-induced vasoreactivity is unknown, as are the underlying mechanisms. This study investigates whether PVAT regulates insulin-induced vasodilation in muscle, the underlying mechanisms, and how obesity disturbs this vasodilation. Insulin-induced vasoreactivity of resistance arteries was studied with PVAT from C57BL/6 or *db/db* mice. PVAT weight in muscle was higher in *db/db* mice compared with C57BL/6 mice. PVAT from C57BL/6 mice uncovered insulin-induced vasodilation; this vasodilation was abrogated with PVAT from *db/db* mice. Blocking adiponectin abolished the vasodilator effect of insulin in the presence of C57BL/6 PVAT, and adiponectin secretion was lower in *db/db* PVAT. To investigate this interaction further, resistance arteries of *AMPKα2*^{+/+} and *AMPKα2*^{-/-} were studied. In *AMPKα2*^{-/-} resistance arteries, insulin caused vasoconstriction in the presence of PVAT, and *AMPKα2*^{+/+} resistance arteries showed a neutral response. On the other hand, inhibition of the inflammatory kinase Jun NH₂-terminal kinase (JNK) in *db/db* PVAT restored insulin-induced vasodilation in an adiponectin-dependent manner. In conclusion, PVAT controls insulin-induced vasoreactivity in the muscle microcirculation through secretion of adiponectin and subsequent AMPKα2 signaling. PVAT from obese mice inhibits insulin-induced vasodilation, which can be restored by inhibition of JNK. *Diabetes* 62:590–598, 2013

Obesity is associated with insulin resistance in all organs, including skeletal muscle and the vasculature. The increase of adipose tissue mass in obesity is associated with an altered adipokine secretion profile (1). However, the mechanisms through which this increase in adipose tissue mass leads to insulin resistance are not fully understood. One of these mechanisms may be modulation of insulin-induced vasoreactivity. Indeed, the ability of insulin to induce vasodilation in the muscle microcirculation is an important determinant for whole-body insulin sensitivity (2–5). Nevertheless, whether and how microvascular recruitment is controlled by adipose tissue are unclear.

Insulin's vasoactive properties are affected through pathways shared with insulin's metabolic effects (6). Insulin initiates both vasodilator and vasoconstrictor responses, the former through activation of insulin receptor substrate-1, leading to phosphorylation of Akt and endothelial nitric oxide synthase (eNOS) and subsequent NO production. Vasoconstriction is achieved through extracellular signal-related kinase 1/2 (ERK1/2), which increases endothelin-1 activity (7). In lean, insulin-sensitive subjects, the net result of the balance between insulin-stimulated vasodilation and vasoconstriction is usually toward vasodilation (8). In obesity, the net result is shifted toward less vasodilation or even vasoconstriction, but how obesity leads to a blunted insulin-induced vasodilation has not been elucidated.

One of the mechanisms involved may be that obesity is associated with adipose tissue accumulation in ectopic locations, which may disturb microvascular function (9). The location of adipose tissue is one critical determinant for its function; another is its adipokine secretion profile. Adiponectin is a major adipokine of which secretion is altered in obesity (10,11), and it is related to insulin sensitivity and vascular function (12,13). Adiponectin has been proposed to mediate cross-talk between perivascular adipose tissue (PVAT) and vascular endothelium and to contribute to the regulation of muscle perfusion (14). An important effector of adiponectin is 5'-AMP-activated protein kinase (AMPK), a regulator of metabolic homeostasis. AMPK is activated by adiponectin and, when activated by AICAR, enhances vasodilation and muscle perfusion (15). In obesity, the secretion of adiponectin changes, as well as numerous other adipokines, a major part of which are cytokines (1,7). Inflammatory cytokines can blunt insulin-stimulated vasoreactivity (7), but it is unknown whether inhibition of inflammation can restore this effect.

Recently, interest in the role of deposits of PVAT in the regulation of microvascular function has increased. Adipokines affect insulin signaling in the vasculature, leading to a disturbed microvascular function (7,9,16–18). Nevertheless, the disturbance of microvascular function in obesity cannot be fully explained by circulating adipokine concentrations, which are mainly determined by the large subcutaneous and visceral adipose tissue depots (19). The concentrations of circulating adipokines are too low for biological effects on insulin-stimulated vasoreactivity (9). An alternative source of adipokines is PVAT (9). PVAT may have a direct paracrine, and possibly vasocrine (i.e., from one vessel to downstream vessels), effect on the vasculature due to the inherent proximity to the vasculature (9,20). In humans, PVAT around the brachial artery is independently associated with insulin sensitivity (21). Additionally, PVAT exhibits an altered

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adipokine profile compared with other adipose depots in obesity (10,11,22). PVAT can therefore be considered as a distinct adipose tissue depot. It is unknown whether PVAT affects insulin-induced vasoreactivity in the muscle microcirculation through secretion of adipokines.

Isolated adipokines are known to affect insulin-induced vasoreactivity, but few studies have investigated directly whether PVAT affects microvascular function. Previous studies have been targeted at the modulating effect of PVAT on the response to norepinephrine and acetylcholine in the aorta and larger arteries; PVAT has anticontractile effects in these vessels (23–25). Microvessels were investigated in only one study; however, as the microvessels were derived from subcutaneous adipose tissue, they do not control nutrient exchange (11). In muscle, the microcirculation controls nutrient exchange, but the effects of PVAT on muscle resistance arteries have never been studied. The direct effect of PVAT, as well as the potentially associated role of adiponectin and AMPK, on insulin-induced vasoreactivity in the lean and obese state remains unknown. Likewise, the role of inflammation in PVAT in insulin-induced vasoreactivity is not fully elucidated.

We hypothesized that PVAT affects insulin-induced vasoreactivity in isolated muscle resistance arteries, that it does so through adiponectin secretion, and that this effect of PVAT is blunted in obesity. Moreover, the underlying mechanisms, focusing particularly on the potential involvement of AMPK α 2, and the signaling pathway molecule Akt, which is used as a marker of NO production, were examined. To assess whether inflammation impairs PVAT function, we studied the involvement of Jun NH₂-terminal kinase (JNK), a mediator of inflammation.

RESEARCH DESIGN AND METHODS

Animals. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85-23, revised 1996).

All animal work was approved by the local ethics committee for animal experiments of the VU University Amsterdam and complied with Dutch government guidelines. Male C57BL/6NCRl (further indicated as C57BL/6) (Charles River, Amsterdam, the Netherlands), male *db/db* (Harlan, Zeist, the Netherlands), and AMPK α 2^{-/-} and AMPK α 2^{+/+} mice 10 weeks of age were killed by isoflurane overdose. To study the role of AMPK in insulin-induced vasoreactivity, male C57BL/6N mice (back-crossed for >10 generations; Taconic, Ejby, Denmark) with a deletion of the AMPK α 2 (AMPK α 2^{-/-}) catalytic subunit gene, together with their AMPK α 2^{+/+} littermates, were generated as previously described (26).

Weight of PVAT. PVAT was dissected in one piece, free of surrounding connective tissue, from C57BL/6 (*n* = 6) and *db/db* (*n* = 6) mice and was quantified on a microbalance. Figure 1A gives an impression of the stretch of the dissected PVAT.

Vasoreactivity experiments. To study the insulin-induced vasoreactivity of resistance arteries in the absence of PVAT, first-order resistance arteries from the gracilis muscle were isolated from lean C57BL/6 mice. After dissection, resistance arteries were placed in a pressure myograph, cannulated, and studied at a pressure of 80 mmHg and a temperature of 37°C in K-MOPS buffer, as described previously (16). In addition to the resistance artery itself, PVAT that surrounds the resistance artery was isolated from the origin of the gracilis resistance artery at the femoral artery to its first major side branch (Fig. 1A). PVAT derived from the same stretch of the resistance artery in C57BL/6 mice was also isolated from *db/db* mice. To study the interaction between PVAT and insulin-induced vasoreactivity of isolated resistance arteries, PVAT was incubated with resistance arteries in the pressure myograph for 45 min (Fig. 1B). Resistance arteries were randomly assigned to incubation without PVAT (*n* = 9), C57BL/6 PVAT (*n* = 10), or *db/db* PVAT (*n* = 8) of approximately equal amounts. After incubation, the inner diameter of resistance arteries was recorded to determine the baseline diameter after precontraction to ~50% with KCl (25 mmol/L), and diameter changes induced by four concentrations of insulin (0.02, 0.2, 2.0, and 20 nmol/L; Actrapid; Novo Nordisk) were examined for 30 min (16). The three lowest concentrations of insulin are within the physiological range, with

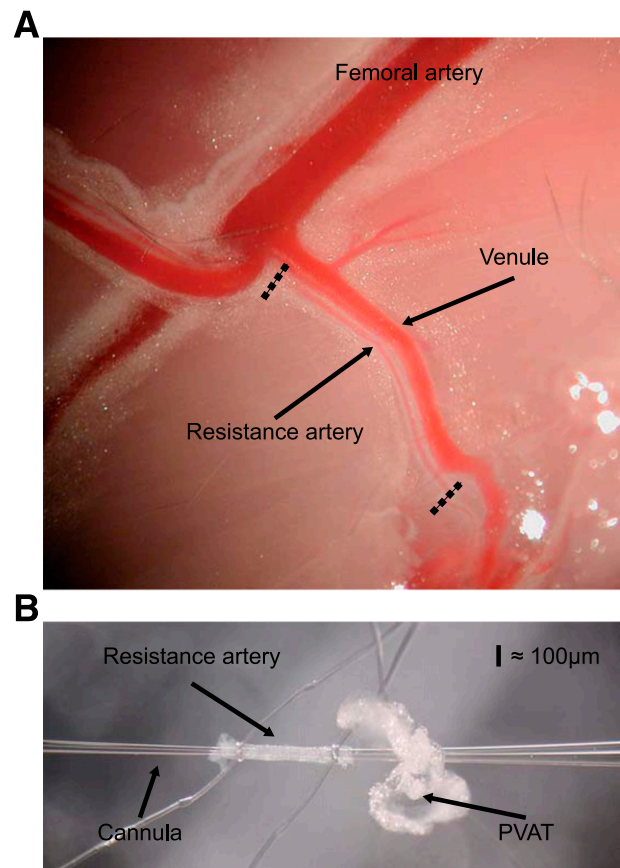


FIG. 1. A: Picture of the gracilis muscle in the mouse hind leg. All the relevant structures are indicated. The dotted lines indicate the length of the PVAT isolated in each experiment. **B:** Picture of the resistance artery mounted on two glass cannulae with the PVAT adjacent to it. The resistance artery and PVAT are surrounded by buffer, and the cannulae are also filled with buffer and pressurized at 80 mmHg. (A high-quality digital representation of this figure is available in the online issue.)

the third concentration (2.0 nmol/L) corresponding to postprandial levels and the fourth concentration to pharmacological levels. Endothelial integrity was determined by measuring responses to the endothelium-dependent vasodilator acetylcholine (0.1 μ mol/L) after each experiment, with a minimum dilation of 10% used as proof of endothelial integrity.

Moreover, it was investigated whether reducing concentrations of adiponectin would affect insulin-induced vasoreactivity in resistance arteries from C57BL/6 mice with PVAT from C57BL/6 mice (*n* = 6). A soluble receptor fragment (Acrp-30 [N-20]-P adiponectin receptor blocking peptide; Santa Cruz Biotechnology) (0.4 μ g/mL), which binds adiponectin, was preincubated in the pressure myograph for 30 min. Additionally, globular adiponectin (Enzo Life Sciences, Antwerpen, Belgium) was preincubated for 45 min in the vessel bath of the pressure myograph at a concentration of 1 μ g/mL with C57BL/6 resistance arteries without PVAT (*n* = 5) to investigate whether adiponectin can affect insulin-induced vasoreactivity when added from outside the resistance artery. Globular adiponectin was also incubated in the presence of *db/db* PVAT (*n* = 7) to study whether it could restore insulin-induced vasodilation. As a model to inhibit downstream adiponectin signaling, the effects of the AMPK inhibitor Compound C (1 μ mol/L; product number 171260; Calbiochem) on insulin-induced vasoreactivity were examined in C57BL/6 resistance arteries in the presence of C57BL/6 PVAT (*n* = 7).

Vasoreactivity in resistance arteries of AMPK α 2^{-/-} mice. To further study the role of AMPK α 2 in the interaction between PVAT and insulin-induced vasoreactivity, resistance arteries from AMPK α 2^{+/+} and AMPK α 2^{-/-} mice were collected using the same techniques. Resistance arteries of AMPK α 2^{-/-} (*n* = 8) and AMPK α 2^{+/+} (*n* = 6) were mounted in the pressure myograph and incubated with PVAT from AMPK α 2^{+/+} mice for 45 min. After incubation, inner diameter and responses to insulin and acetylcholine were studied as described above.

Vasoreactivity experiments studying inflammatory properties of PVAT. To study whether inflammatory adipokines are involved in the regulation of insulin-induced vasoreactivity by PVAT, we tested whether one phenotype of

PVAT would prevail during dual incubation with both C57BL/6 and *db/db* PVAT ($n = 6$).

Pressure myography was used to test whether acute inhibition of inflammation using L-JNKi (L-JNKi 1 trifluoroacetate salt, 5 $\mu\text{mol/L}$; Sigma Aldrich, Zwijndrecht, the Netherlands) could restore vasodilator properties of *db/db* PVAT ($n = 6$). Furthermore, the soluble adiponectin receptor fragment (Acrp-30 [N-20]-P adiponectin receptor-blocking peptide, 0.4 $\mu\text{g/mL}$; Santa Cruz Biotechnology) was coincubated with L-JNKi in experiments with *db/db* PVAT to test whether the restored vasodilation by L-JNKi was mediated by adiponectin ($n = 6$).

Western blot. In order to investigate the effects of C57BL/6 and *db/db* PVAT on downstream insulin signaling in resistance arteries, Western blot analysis was performed as previously described (16). After 45 min incubation in the presence of PVAT from C57BL/6 or *db/db* mice, segments of gracilis arteries from C57BL/6 mice were exposed to solvent or insulin (2 nmol/L) for 30 min at 37°C. To investigate whether JNK inhibition restored insulin-mediated Akt phosphorylation in gracilis arteries exposed to *db/db* PVAT, other arterial segments, exposed to C57BL/6 or *db/db* PVAT, were exposed to insulin or solvent after pretreatment with L-JNKi. The protein lysates of resistance artery segments were stained with a specific primary antibody against Ser 473-phosphorylated Akt and threonine 172-phosphorylated AMPK α (antibodies from Cell Signaling Technology, Boston, MA) and were visualized with a chemiluminescence kit (GE Healthcare, Diegem, Belgium). As protein yields from gracilis muscle resistance arteries are low (<2 μg), and we were not able to stain AMPK α after stripping, we avoided stripping and reprobing blots. To obviate this, we used ERK1 as a loading control instead of total Akt or AMPK α . ERK1 correlates well with total Akt levels (27). As all experiments were paired and the duration of experiments was 1.5 h in total, differences in protein levels of Akt and AMPK α between experimental conditions are unlikely.

Adiponectin secretion (ELISA). To study whether PVAT from lean C57BL/6 mice secretes more adiponectin than PVAT from *db/db* mice, the amount of secreted adiponectin was quantified. PVAT isolated from lean ($n = 7$) and *db/db* ($n = 7$) mice, in amounts of comparable size, was incubated with 2 nmol/L insulin for 45 min at 37°C in order to study the release of adiponectin. Adiponectin concentration in conditioned medium was measured with a mouse adiponectin ELISA kit (Millipore, Amsterdam, the Netherlands) and detected using the Luminex system.

Statistics. Steady-state responses are reported as mean change from baseline (percentages) \pm SEM. Baseline diameter was determined as the diameter just prior to the addition of the first concentration of insulin. Statistical analyses for differences in insulin-induced vasoreactivity were performed using one-way ANOVA with Bonferroni post hoc tests, or Mann-Whitney *U* test where appropriate. Differences in protein phosphorylation, as found by Western blot, were determined using the Wilcoxon signed rank test. Phosphorylation was expressed as the fold increase over the unstimulated controls, assigning a value of 1 to the control. Differences with *P* values <0.05 were considered statistically significant.

RESULTS

Intramuscular PVAT weight is increased in *db/db* mice. PVAT was located around the resistance arteries, as well as A1 and A2 arterioles, in the muscle microcirculation (Fig. 1A). The weight of PVAT was higher in the *db/db* mice ($1,365 \pm 451 \mu\text{g}$) than in lean C57BL/6 mice ($33.3 \pm 6.6 \mu\text{g}$; $P < 0.01$) (Fig. 2B).

Intramuscular PVAT from lean C57BL/6 mice, but not *db/db* mice, uncovers insulin-induced vasodilation. Baseline vessel tone was not affected in resistance arteries by either C57BL/6 PVAT or *db/db* PVAT, nor was acetylcholine-induced vasodilation (Fig. 3A and B). Resistance arteries from C57BL/6 mice in the absence of PVAT did not show vasodilation or vasoconstriction in response to insulin (2 nmol/L; $0 \pm 9\%$). After incubation with PVAT from C57BL/6 mice, insulin induced significant vasodilation (2 nmol/L; $52 \pm 21\%$; $P < 0.02$ compared with baseline and $P < 0.05$ vs. resistance arteries without PVAT) (Fig. 3C).

The vasodilation to insulin observed in the presence of C57BL/6 PVAT was completely abrogated when PVAT from *db/db* mice was used (2 nmol/L; $-5 \pm 3\%$; $P < 0.02$ compared with C57BL/6 PVAT) (Fig. 3D). To further study the insulin-induced vasodilator pathway, which is

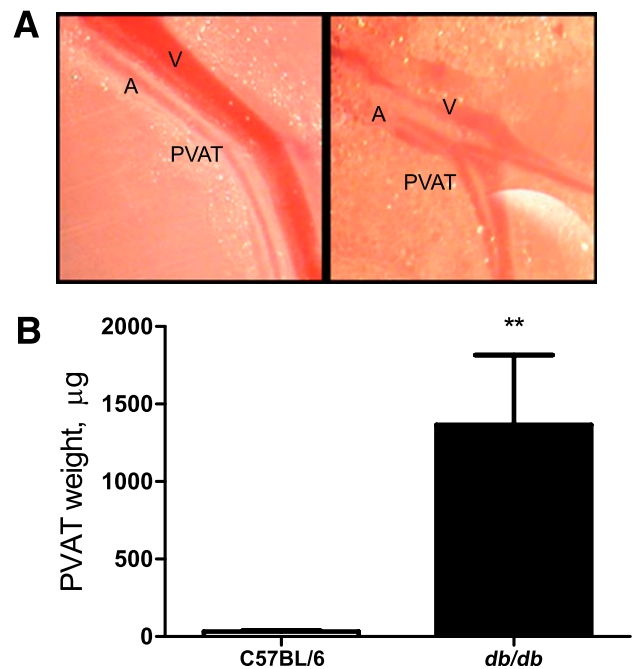


FIG. 2. Morphological aspects of PVAT. **A:** Picture of the gracilis resistance artery and venule with the adjacent PVAT of a control mouse (left) and a *db/db* mouse (right). A, resistance artery; V, venule. **B:** The weight of PVAT is increased in *db/db* mice (white bar, weight of PVAT from C57BL/6 mice; black bar, weight of PVAT from *db/db* mice). ** $P < 0.01$. (A high-quality digital representation of this figure is available in the online issue.)

mediated through Akt and eNOS phosphorylation, phosphorylation of Akt was studied in the presence of C57BL/6 or *db/db* PVAT. The phosphorylation of Akt was significantly increased after insulin stimulation in resistance arteries of C57BL/6 mice in the presence of C57BL/6 as well as *db/db* PVAT. In the presence of C57BL/6 PVAT, the increase in Akt phosphorylation was significantly higher ($P < 0.01$ vs. *db/db* PVAT) (Fig. 3E).

Adiponectin from intramuscular PVAT mediates PVAT regulation of insulin-induced vasodilation and is reduced in *db/db* mice. To elucidate the mechanisms involved in the interaction of intramuscular PVAT with insulin-induced vasoreactivity, we examined the role of adiponectin in this interaction. Adiponectin was secreted more abundantly by PVAT from C57BL/6 mice ($502 \pm 115 \text{ pg/mL}$ medium) than by PVAT from *db/db* mice ($178 \pm 33 \text{ pg/mL}$; $P = 0.02$) (Fig. 4A). When adiponectin was scavenged with a soluble adiponectin receptor fragment, Acrp-30 [N-20]-P, so that the *db/db* PVAT situation was mimicked, insulin-induced vasodilation was abolished (2 nmol/L ; $-19 \pm 10\%$; $P < 0.01$ vs. C57BL/6 resistance arteries in the presence of PVAT). This indicates the importance of adiponectin from PVAT in insulin-induced vasodilation (Fig. 4B). Adiponectin, in the absence of PVAT, stimulated insulin-induced vasodilation in C57BL/6 resistance arteries (2 nmol/L; $21 \pm 4\%$; $P = 0.02$ vs. C57BL/6 resistance arteries without PVAT) (Fig. 4C). Moreover, we incubated globular adiponectin in the presence of *db/db* PVAT ($n = 7$) to study whether supplementation of adiponectin could restore insulin-induced vasoreactivity. Adiponectin, in the presence of *db/db* PVAT, stimulated insulin-induced vasodilation $17 \pm 4\%$ ($P < 0.01$ compared with *db/db* PVAT alone) (Fig. 4D).

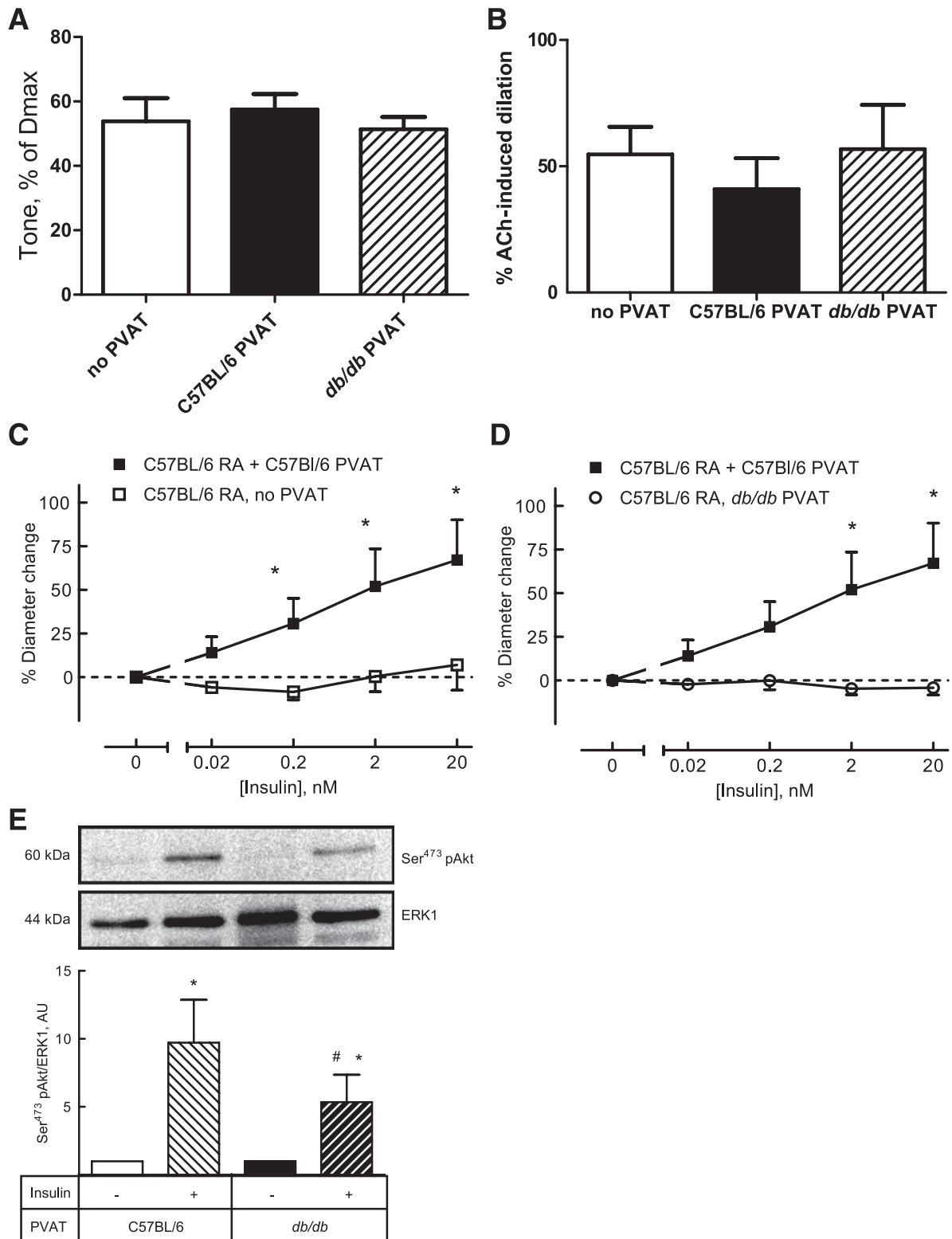


FIG. 3. The effects of C57BL/6 and *db/db* PVAT on basal tone, acetylcholine-induced vasoreactivity, insulin-induced vasoreactivity, and the insulin-signaling pathway, as well as the amount of adiponectin secretion. **A:** PVAT from either C57BL/6 or *db/db* mice does not affect smooth muscle tone of isolated resistance arteries (RAs) (white bar, tone in the absence of PVAT [$n = 6$]; black bar, tone in the presence of C57BL/6 PVAT [$n = 5$]; hatched bar, tone in the presence of *db/db* PVAT [$n = 7$]). DMax, maximal diameter. **B:** PVAT from either C57BL/6 or *db/db* mice does not affect acetylcholine (ACh) 10^{-7} -induced vasodilation, indicating that there is no general microvascular dysfunction (white bar, acetylcholine-dependent vasodilation without PVAT [$n = 5$]; black bar, acetylcholine-dependent vasodilation in the presence of C57BL/6 PVAT [$n = 4$]; hatched bar, acetylcholine-dependent vasodilation in the presence of *db/db* PVAT [$n = 6$]). **C:** Insulin-induced vasoreactivity in the absence and presence of PVAT from control mice. C57BL/6-PVAT unveils insulin-induced vasodilation, which is not present in the absence of PVAT (white squares, C57BL/6 RA, without PVAT [$n = 9$]; black squares, C57BL/6 RA in the presence of C57BL/6 PVAT [$n = 10$]). * $P < 0.05$. **D:** Insulin-induced vasoreactivity in the presence of PVAT from C57BL/6 or *db/db* mice. PVAT from *db/db* mice does not unveil the vasodilator properties of insulin as PVAT from control mice does (black squares, C57BL/6 RA in the presence of C57BL/6 PVAT [$n = 10$]; white circles, C57BL/6 RA in the presence of *db/db* PVAT [$n = 8$]). * $P < 0.05$. **E:** Western blot of Akt phosphorylation initiated by insulin in RAs in the presence of C57BL/6 PVAT or *db/db* PVAT. Insulin causes a more pronounced Akt phosphorylation in the presence of C57BL/6 PVAT than in the presence of *db/db* PVAT. * $P < 0.05$ compared with no insulin; # $P < 0.05$ compared with C57BL/6 PVAT.

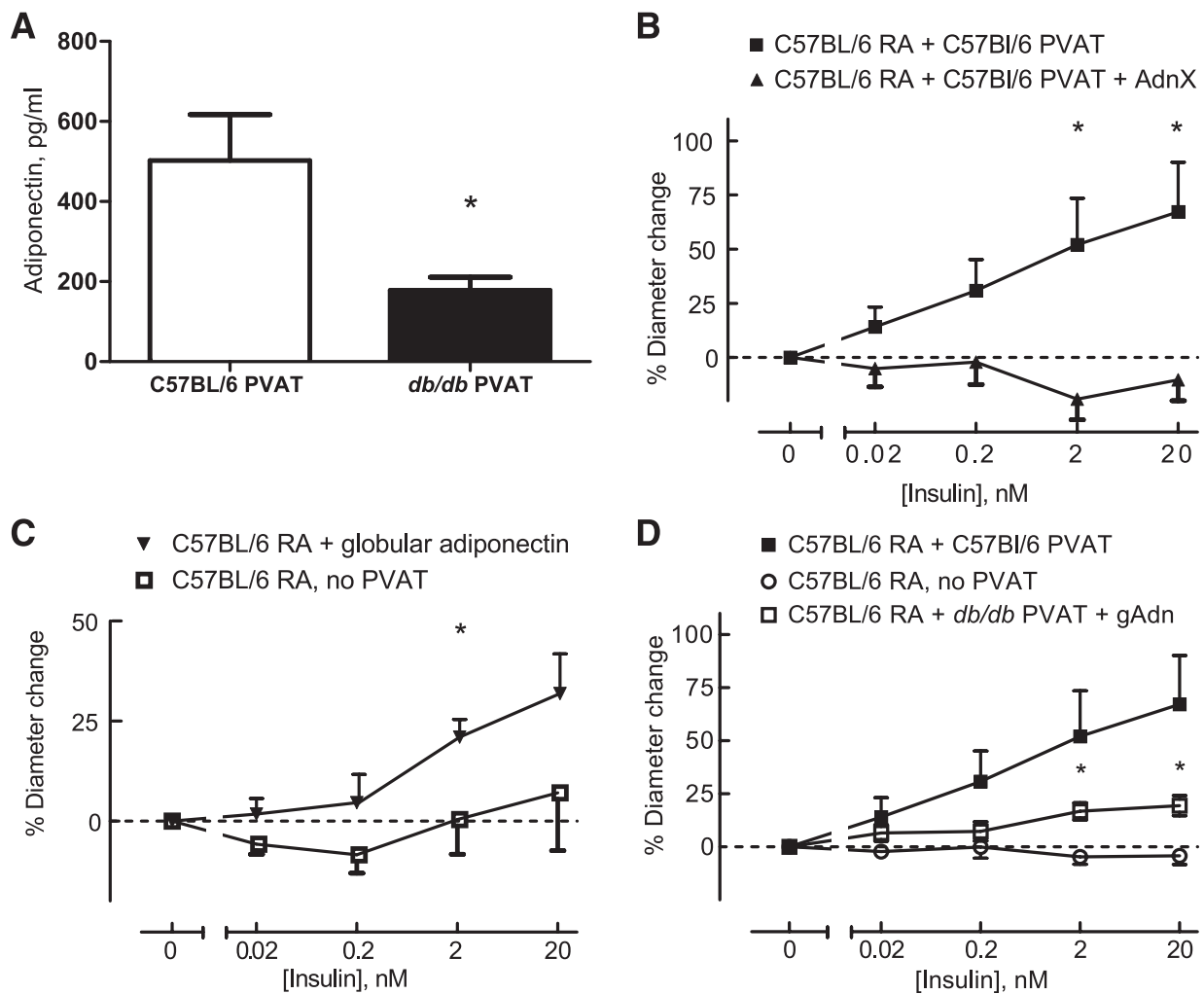


FIG. 4. Adiponectin plays a role in insulin-induced vasodilation, as seen with C57BL/6 PVAT. **A:** ELISA: adiponectin secretion by C57BL/6 PVAT ($n = 6$) (white bar) and *db/db* PVAT ($n = 6$) (black bar). $*P < 0.05$. **B:** Scavenging of the adiponectin secreted by control PVAT abolishes the vasodilator effect of insulin in the presence of control PVAT (black squares, C57BL/6 resistance artery [RA], with C57BL/6 PVAT [$n = 10$]; black triangle, C57BL/6 RA in the presence of C57BL/6 PVAT and a soluble adiponectin receptor fragment [$n = 6$]). $*P < 0.05$. **C:** Adiponectin, in the absence of PVAT, uncovers insulin-induced vasodilation (white square, C57BL/6 RA, without PVAT [$n = 9$]; black inverted triangle, C57BL/6 RA coincubated with globular adiponectin [$n = 5$]). $*P < 0.05$. **D:** Incubation of *db/db* PVAT with globular adiponectin unveils insulin-induced vasodilation. (Black squares, C57BL/6 RA with C57BL/6 PVAT [$n = 10$]; white circles, C57BL/6 RA with *db/db* PVAT [$n = 8$]; white squares, C57BL/6 RA with *db/db* PVAT and globular adiponectin 1 mg/mL [$n = 7$]). $*P < 0.05$ compared with C57BL/6 RA + *db/db* PVAT.

AMPK mediates control of insulin-induced vasodilation by PVAT in muscle. As AMPK is an important effector of adiponectin in vascular endothelium, we next examined the role of AMPK in the interaction of PVAT with insulin-induced vasoreactivity in isolated resistance arteries. Resistance arteries from C57BL/6 incubated with both PVAT from lean mice and compound C showed ($9 \pm 4\%$ at 2 nmol/L of insulin) vasodilation, a nonsignificant reduction ($P = 0.28$ vs. C57BL/6 resistance arteries with C57BL/6 PVAT) (Fig. 5A). However, there was no significant vasodilation compared with resistance arteries without PVAT.

AMPK phosphorylation was significantly increased in vessels exposed to both C57BL/6 PVAT ($P = 0.006$) and *db/db* PVAT ($P = 0.028$), compared with C57BL/6 resistance arteries without PVAT. There was, however, no difference in AMPK phosphorylation between vessels exposed to C57BL/6 PVAT and *db/db* PVAT (Fig. 5B).

Deletion of AMPK $\alpha 2$ in muscle resistance arteries disturbs insulin-induced vasoreactivity in the presence but not the absence of PVAT. To further examine the role of AMPK in insulin-induced vasodilation induced by PVAT,

resistance arteries from *AMPK $\alpha 2$ ^{-/-}* mice were exposed to PVAT from *AMPK $\alpha 2$ ^{+/+}* mice. Resistance arteries from *AMPK $\alpha 2$ ^{+/+}* littermates served as controls. In the latter group, unlike the parallel studies using arteries and PVAT from C57BL/6 mice, insulin did not induce a significant vasodilation (2 nmol/L; $-3 \pm 6\%$). There was no difference in insulin-induced vasoreactivity between *AMPK $\alpha 2$ ^{+/+}* and *AMPK $\alpha 2$ ^{-/-}* resistance arteries; both exhibited a neutral response (Fig. 5C). However, *AMPK $\alpha 2$ ^{-/-}* resistance arteries with *AMPK $\alpha 2$ ^{+/+}* PVAT exhibited vasoconstriction (2 nmol/L; $-26 \pm 6\%$; $P = 0.010$) (Fig. 5D), pointing to strain differences with C57BL/6 mice. This indicates the importance of AMPK in the interaction between PVAT and insulin-induced vasodilation. Tone development and acetylcholine-induced vasodilation were not different between the two groups (Fig. 5E and F).

JNK inhibition restores insulin-induced, adiponectin-dependent vasodilation in the presence of *db/db* PVAT. Because Akt phosphorylation in the presence of *db/db* PVAT was lower (Fig. 3E) and AMPK α phosphorylation is not different between C57BL/6 and *db/db* PVAT (Fig. 5B), adipokines other than adiponectin may also play

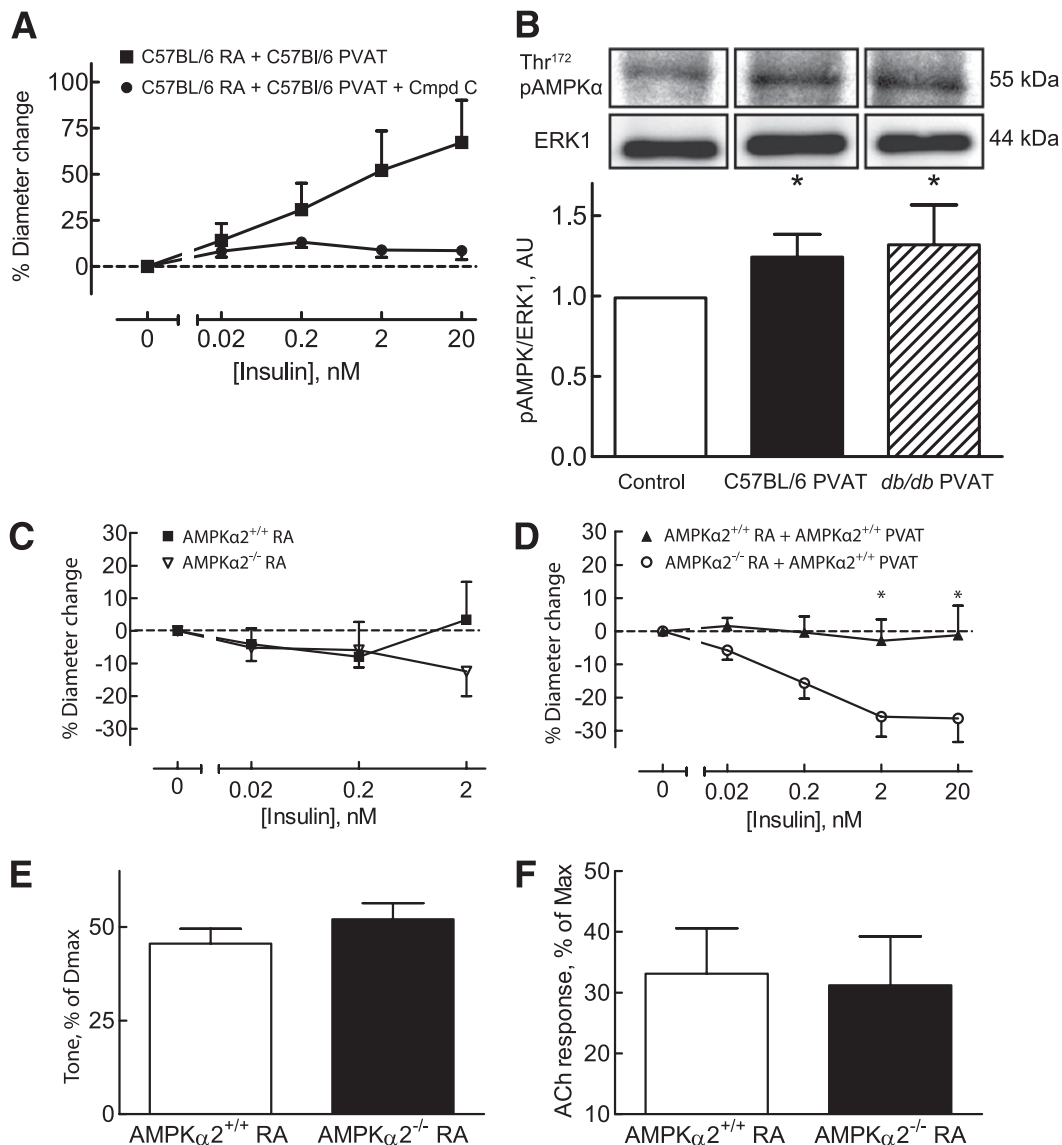


FIG. 5. The involvement of AMPK in insulin-induced vasodilation in the presence of PVAT through the secretion of adiponectin. **A:** The AMPK inhibitor Compound C (Cmpd C) inhibits the insulin-induced vasodilation nonsignificantly in the presence of C57BL/6 PVAT (black square, C57BL/6 resistance artery [RA], with C57BL/6 PVAT [$n = 10$]; black circle, C57BL/6 RA in the presence of C57BL/6 PVAT and compound C [$n = 7$]). **B:** PVAT from C57BL/6 mice induces significant threonine 172 phosphorylation of AMPK, as does PVAT from *db/db* mice. Threonine 172 phosphorylation of AMPK was, however, not significantly different between the two types of PVAT. $*P < 0.05$. **C:** *AMPK α 2^{+/+}* ($n = 7$) and *AMPK α 2^{-/-}* ($n = 7$) do not exhibit insulin-induced vasodilation or constriction. Black square, *AMPK α 2^{+/+}* RAs without PVAT; white inverted triangle, *AMPK α 2^{-/-}* RAs without PVAT. **D:** Knocking out *AMPK α 2* leads to insulin-induced vasoconstriction in the presence of PVAT from *AMPK α 2^{+/+}* wild-type mice (black triangles, *AMPK α 2^{+/+}* RA with *AMPK α 2^{+/+}* PVAT [$n = 6$]; white circles, *AMPK α 2^{-/-}* RA with *AMPK α 2^{+/+}* PVAT [$n = 8$]). $*P < 0.05$. **E:** Basal tone of *AMPK α 2^{+/+}* RAs is not different from *AMPK α 2^{-/-}* RAs. White bar, basal tone development of *AMPK α 2^{+/+}* RAs; black bar, basal tone development of *AMPK α 2^{-/-}* RAs. DMax, maximal diameter. **F:** Acetylcholine (ACh)-induced vasodilation is not different in *AMPK α 2^{+/+}* and *AMPK α 2^{-/-}* RAs in the presence of PVAT. White bar, acetylcholine-induced vasodilation of *AMPK α 2^{+/+}* RAs in the presence of PVAT; black bar, acetylcholine-induced vasodilation of *AMPK α 2^{-/-}* RAs.

a role in the change of insulin-induced vasodilation with *db/db* PVAT. To study whether PVAT from the muscle microcirculation produces additional vasoactive adipokines, incubation of a C57BL/6 resistance artery with C57BL/6 PVAT as well as *db/db* PVAT was performed (Fig. 6A). Dual incubation led to insulin-induced vasodilation ($26 \pm 9\%$; $P < 0.05$ vs. *db/db* PVAT). The vasodilation with dual PVAT incubation was smaller, however, than in the presence of C57BL/6 PVAT alone ($P = \text{NS}$) (Fig. 6A), suggesting interaction between the effects of C57BL/6 and *db/db* PVAT.

The vasodilator effect that was absent with *db/db* PVAT was restored by inhibiting inflammation using the

JNK-inhibitor L-JNKi (2 nmol/L insulin; $48 \pm 17\%$; $P < 0.001$ compared with *db/db* PVAT) and overlapping with the line of C57BL/6 PVAT, indicating that inflammation in either *db/db* PVAT or the acceptor resistance artery plays a role (Fig. 6B). Therefore, JNK opposes adiponectin-dependent vasodilator properties of PVAT in *db/db* mice. When adiponectin was blocked during incubation with *db/db* PVAT and L-JNKi, the insulin-induced vasodilation was completely inhibited (2 nmol/L; $1 \pm 6\%$; $P < 0.01$ vs. L-JNKi with *db/db* PVAT) (Fig. 6C). Treatment with L-JNKi did not restore insulin-stimulated Akt phosphorylation in the gracilis resistance arteries exposed to *db/db* PVAT ($P = \text{NS}$ vs. the non-pretreated resistance arteries) (Fig. 6D).

DISCUSSION

The principal findings of this study are that 1) PVAT uncovers insulin-induced vasodilation in muscle resistance arteries by secreting adiponectin and activating AMPK α 2 in the vessel wall, 2) in obese *db/db* mice, PVAT dramatically increases in muscle and loses its ability to induce insulin-mediated vasodilation, 3) this impaired interaction of PVAT with muscle resistance arteries is caused by a decrease in adiponectin release by PVAT and impairment of insulin-stimulated Akt activation, and 4) the blunted insulin-induced vasodilation with *db/db* PVAT can be restored by inhibition of JNK.

We show a direct regulatory effect of PVAT on insulin-induced vasoreactivity in muscle, shifting the balance of insulin's vasoactive effects toward insulin-induced vasodilation. Our finding that PVAT controls vasoreactivity is in line with previous observations in the aorta and adipose tissue microvessels that PVAT controls vascular tone by antagonizing vasoconstriction in response to norepinephrine, endothelin-1, and angiotensin II (11,28,29). We did not observe an effect of PVAT on basal smooth muscle tone, indicating that the interaction of PVAT with insulin-induced vasoregulation is specific. As direct effects of

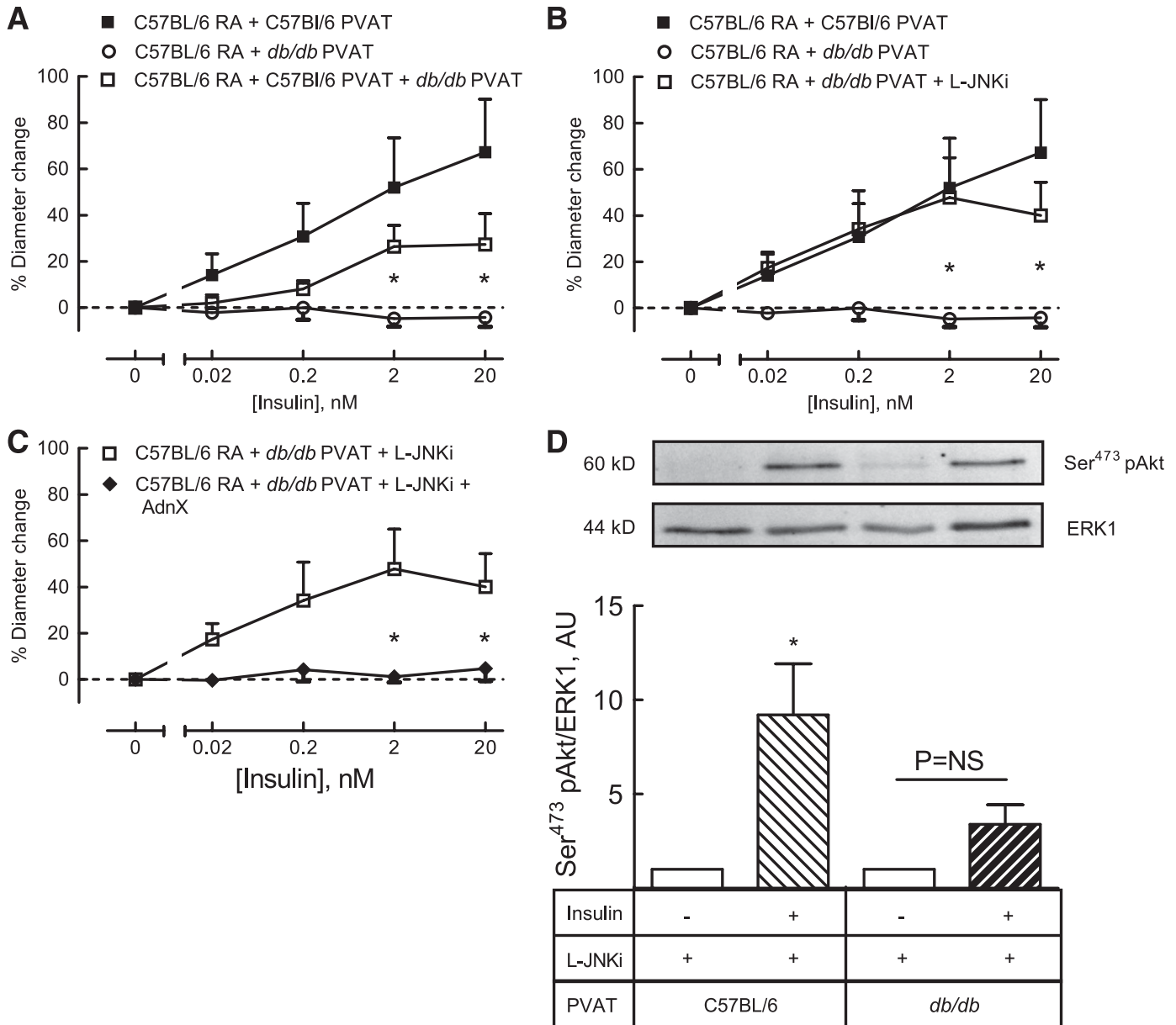


FIG. 6. A: Dual incubation of C57BL/6 PVAT and *db/db* PVAT partly restores insulin-induced vasodilation (black squares, C57BL/6 resistance artery [RA] with C57BL/6 PVAT [*n* = 10]; white circles, C57BL/6 RA with *db/db* PVAT [*n* = 8]; white squares, C57BL/6 RA with both C57BL/6 PVAT and *db/db* PVAT [*n* = 6]). **P* < 0.05 vs. C57BL/6 RA + *db/db* PVAT. B: Inhibition of JNK with L-JNKi in a setup with a C57BL/6 RA and *db/db* PVAT returns insulin-induced vasodilation to levels comparable with C57BL/6 PVAT (black squares, C57BL/6 RA with C57BL/6 PVAT [*n* = 10]; white circles, C57BL/6 RA with *db/db* PVAT [*n* = 8]; white squares, C57BL/6 RA with *db/db* PVAT and L-JNKi [*n* = 6]). **P* < 0.05 vs. C57BL/6 RA + *db/db* PVAT. C: The restoration of insulin-induced vasodilation by *db/db* PVAT with L-JNKi is mediated by adiponectin, because scavenging adiponectin completely abolishes this effect (black squares, C57BL/6 RA with C57BL/6 PVAT [*n* = 10]; white circles, C57BL/6 RA with *db/db* PVAT [*n* = 8]; white squares, C57BL/6 RA with *db/db* PVAT, L-JNKi, and the soluble adiponectin receptor fragment [*n* = 6]). **P* < 0.05 vs. C57BL/6 RA + *db/db* PVAT + L-JNKi. D: L-JNKi does not restore Akt phosphorylation at Ser⁴⁷³ by insulin during exposure to C57BL/6 PVAT, whereas insulin does increase Akt phosphorylation at Ser⁴⁷³ during exposure to C57BL/6 PVAT. AU, arbitrary units. **P* < 0.05.

PVAT on smooth muscle tone have been observed in adipose tissue and the aorta, the effects of PVAT on vaso-regulation vary among different vascular beds.

We have shown that release of adiponectin and activation of AMPK by PVAT are critical to its control of insulin-induced vasoregulation in muscle. Previous studies have indeed shown that adiponectin can activate AMPK in endothelial cells (30), and previous data from our group have shown that adiponectin uncovers insulin-induced vasodilation in muscle resistance arteries through AMPK (31). Our data are in good agreement with the earlier data of Greenstein et al. (11) who showed that PVAT effects on norepinephrine-induced vascular tone in subcutaneous adipose tissue are mediated by adiponectin. Although PVAT releases adiponectin, and a soluble fragment of the adiponectin receptor 1 (AdipoR1) inhibited PVAT control of insulin-induced vasoregulation, a role of other adipokines in PVAT-endothelial communication cannot be completely excluded. The effect of PVAT on insulin-induced vasodilation was partially mimicked by recombinant globular adiponectin, either in the absence or presence of *db/db* PVAT as well as by dual incubation of *db/db* and C57BL/6 PVAT. This suggests that other adipokines than adiponectin play a supporting role or stimulate the insulin-induced vasoconstrictor pathway. Indeed, obesity seems to promote secretion of adipokines by PVAT that inhibit insulin-induced vasodilation and others that enhance insulin-induced vasoconstriction, because the vasodilator effect of C57BL/6 PVAT was partially mimicked by adiponectin, and *db/db* PVAT decreases the vasodilator effect of C57BL/6 PVAT. Moreover, in *AMPK α 2^{+/+}*, no insulin-induced vasodilation was observed with PVAT; however, knocking out AMPK α 2 leads to insulin-induced vasoconstriction. This is further supported by equal amounts of AMPK α phosphorylation by C57BL/6 and *db/db* PVAT.

Although AMPK is an important effector of PVAT in vascular endothelium, we have observed AMPK-independent effects of PVAT. In AMPK α 2-deficient resistance arteries, PVAT uncovers insulin-induced vasoconstriction. Furthermore, PVAT from *db/db* mice enhances AMPK α phosphorylation in muscle resistance arteries but inhibits insulin-stimulated phosphorylation of Akt. These results suggest that AMPK α 2 activation is a necessary, but not sufficient, step to uncover insulin-induced vasodilation. Moreover, *db/db* PVAT must regulate insulin's vasoactive properties through a second mechanism, counteracting the insulin-induced vasodilation-stimulating effects of PVAT. The difference in net effects of PVAT between C57BL/6 and *AMPK α 2^{+/+}* mice strongly suggests that genetic factors determine adipokine secretion by PVAT.

Indeed, inhibition of inflammation with L-JNKi fully restored the vasodilator capacity of *db/db* PVAT (Fig. 6B), whereas blocking adiponectin with the soluble adiponectin receptor fragment completely abrogated this effect (Fig. 6C). Inflammation therefore plays an important role in the defective PVAT properties of *db/db* mice. Interestingly, L-JNKi did not restore insulin-induced Akt phosphorylation during exposure to *db/db* PVAT (Fig. 6D). This finding may be explained by increased adiponectin secretion by *db/db* PVAT during JNK inhibition, or inhibition of insulin's vasoconstrictor effects, which are independent from Akt (27). Indeed, inflammation of PVAT leads to a loss of its anticontractile properties (11,32). Nevertheless, adipokines other than adiponectin likely contribute to vasodilator effects of PVAT,

as supplementation of adiponectin to *db/db* PVAT could not completely restore insulin-induced vasodilation (Fig. 4D). Recently, a new class of adipokines, C1q/tumor necrosis factor (TNF)-related proteins, has been shown to activate AMPK in endothelial cells through the adiponectin receptor AdipoR1 (33). These C1q/TNF-related proteins could explain the AMPK phosphorylation and the vasodilator response to anti-inflammatory treatment with L-JNKi in the absence of increased Akt phosphorylation. The PVAT-derived adipokine that activates JNK in the muscle microcirculation remains to be identified in future studies. We have assessed secretion of the JNK agonists TNF- α and free fatty acids by muscle-derived PVAT but found only minor secretion of these adipokines (R.I.M., unpublished data). This finding does not exclude an autocrine role of TNF- α in regulating PVAT function.

Perspectives. Our results yield two novel insights into the pathogenesis of insulin resistance. First, we have shown that local depots of PVAT, which surround the resistance arteries in the muscle microcirculation, are capable of controlling insulin-dependent microvascular function. In humans, local accumulation of adipose tissue in the extremities is related to flow-mediated vasodilation and insulin sensitivity (21,34). These observations and the present dataset support our previous proposition (9) that adipokine-induced microvascular dysfunction in obesity is a paracrine, rather than an endocrine, process (9,35). In contrast to our initial hypothesis (9), we have found that altered adipokine secretion by intramuscular PVAT, rather than the formation of previously absent PVAT in muscle, impairs insulin-induced vasoregulation in obesity. Also, anti-inflammatory treatment restores insulin-induced vasodilation, at least partly, through adiponectin.

Second, our results provide a novel mechanism by which AMPK regulates insulin sensitivity. AMPK is well known as a "metabolic gauge" that controls both basal and insulin-stimulated glucose uptake (36). Paradoxically, AMPK α 2 controls muscle insulin sensitivity in vivo but not in isolated muscles (26). Our observation that *AMPK α 2^{-/-}* mice show impaired insulin-induced vasoregulation in the presence of PVAT, in combination with recent evidence that insulin-induced vasoregulation controls insulin sensitivity (2), suggests that AMPK α 2 controls muscle insulin sensitivity by interacting with insulin-induced microvascular recruitment. Further support for this notion comes from our recent observation that AMPK activation by AICA-riboside enhances muscle microvascular blood volume (15).

In conclusion, in this study, we show that PVAT unveils vasodilator responses to insulin through the secretion of adiponectin, which is antagonized in *db/db* PVAT by the inflammatory kinase JNK. Adiponectin activates AMPK α 2, which subsequently shifts the balance between vasodilation and vasoconstriction toward vasodilation. In obesity, the quantity of PVAT increases and adiponectin secretion diminishes. Due to the diminished adiponectin secretion and inflammatory burden, insulin no longer induces vasodilation. However, the insulin-stimulated vasodilation can be restored by inhibition of JNK.

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R.I.M. wrote the manuscript and researched and analyzed data. W.B. and C.-L.A.F.A. researched data and edited the manuscript. P.S. and E.A.R. supervised and edited the manuscript. J.S.Y., Y.M.S., V.W.M.v.H., and E.H.S. supervised, contributed to discussions, and edited the manuscript. B.V. created the *AMPK α 2^{+/+}* and *AMPK α 2^{-/-}* mice and edited the manuscript. E.C.E. researched data, supervised, contributed to discussions, and edited the manuscript. E.C.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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