Rictor in Perivascular Adipose Tissue Controls Vascular Function by Regulating Inflammatory Molecule Expression

Indranil Bhattacharya, Katja Drägert, Verena Albert, Emmanuel Contassot, Marlen Damjanovic, Asami Hagiwara, Lukas Zimmerli, Rok Humar, Michael N. Hall, Edouard J. Battegay, Elvira Haas

Objective—Perivascular adipose tissue (PVAT) wraps blood vessels and modulates vasoreactivity by secretion of vasoactive molecules. Mammalian target of rapamycin complex 2 (mTORC2) has been shown to control inflammation and is expressed in adipose tissue. In this study, we investigated whether adipose-specific deletion of rictor and thereby inactivation of mTORC2 in PVAT may modulate vascular function by increasing inflammation in PVAT.

Approach and Results—Rictor, an essential mTORC2 component, was deleted specifically in mouse adipose tissue (rictordel/−). Phosphorylation of mTORC2 downstream target Akt at Serine 473 was reduced in PVAT from rictordel/− mice but unaffected in aortic tissue. Ex vivo functional analysis of thoracic aortae revealed increased contractions and impaired dilation in rings with PVAT from rictordel/− mice. Adipose rictor knockout increased gene expression and protein release of interleukin-6, macrophage inflammatory protein-1α, and tumor necrosis factor-α in PVAT as shown by quantitative real-time polymerase chain reaction and Bioplex analysis for the cytokines in the conditioned media, respectively. Moreover, gene and protein expression of inducible nitric oxide synthase was upregulated without affecting macrophage infiltration in PVAT from rictordel/− mice. Inhibition of inducible nitric oxide synthase normalized vascular reactivity in aortic rings from rictordel/− mice with no effect in rictordel/0 mice. Interestingly, in perivascular and epididymal adipose depots, high-fat diet feeding induced downregulation of rictor gene expression.

Conclusions—Here, we identify mTORC2 as a critical regulator of PVAT-directed protection of normal vascular tone. Modulation of mTORC2 activity in adipose tissue may be a potential therapeutic approach for inflammation-related vascular damage. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: adipose tissue □ heart contractility □ inflammation □ nitric oxide synthase type II □ perivascular adipose tissue □ target of rapamycin complex 2

Adipose tissue not only stores excess of energy as triacylglycerols but also secretes numerous growth factors, cytokines, and hormones that are involved in overall energy homeostasis and metabolism.1 During obesity, adipose tissue mass increases and dysfunction occurs, favoring development of insulin resistance and cardiovascular disease.

A crucial intracellular regulator of fatty acid metabolism and cell growth is the serine/threonine kinase mammalian target of rapamycin (mTOR).2 mTOR exists in 2 multi-protein complexes, mTOR complex 1 (mTORC1) and mTORC2.3 The latter complex is characterized by its essential regulatory protein rictor and integrates signals from growth factors to regulate cell survival or cytoskeleton organization.4–7 mTORC2 phosphorylates AGC kinase family members, such as Akt at Serine 473.8

As full body knockout of rictor/mTORC2 is embryonically lethal,9 diverse tissue-specific knockout models of rictor have been generated to characterize the physiological functions of rictor in the adult mouse.10 Mice with specific ablation of rictor in adipose tissue have higher lean mass because of increased levels of insulin and insulin-like growth factor-1, display enhanced glucose metabolism, and are insulin resistant.10,11

Among the various and distinct adipose depots, perivascular adipose tissue (PVAT) not only serves as a structural support for most arteries but also secretes molecules to actively modulate vascular function.12,13 In 1991, Soltis and Cassis14 showed for the first time that PVAT has an anticontractile function. In 2002, Löhn et al15 reported PVAT to release a transferable vasoactive factor that acts on vascular smooth muscle cells via ATP-dependent K channels to mediate vasorelaxation. Since then, the list of dilatory or anticontractile molecules released from PVAT has expanded and now includes adipokines (eg, adiponectin),16 reactive oxygen species (eg, hydrogen peroxide),17 and others (eg, methyl palmitate,18 hydrogen sulfide,19 and angiotensin 1–720). In 2005, Yudkin et al21 proposed that obesity and its adverse effects cause PVAT to inflame and...
to secrete tumor necrosis factor-α (TNFα) and interleukin-6 (IL6), which resulted in loss of PVAT’s anticontractile function. In rat mesenteric arteries, these inflammatory cytokines impaired the anticontractile activity of healthy PVAT, and in mouse femoral arteries, endovascular injury upregulated inflammation in PVAT.

Thus, the progression of the inflammatory reaction in PVAT seems to be crucial in altering PVAT’s vasoactive properties; however, intracellular mechanisms that might control these reactions are not known. Recently, mTORC2-deficient mouse embryonic fibroblasts and rictor knockdown of dendritic cells exhibited a hyperinflammatory phenotype after lipopolysaccharide stimulation. In the present study, we have, therefore, tested the hypothesis that deletion of rictor in adipose tissue, and thereby inactivation of mTORC2 in PVAT, may modulate vascular function by increasing inflammation in PVAT.

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

Results
Adipose-Specific Deletion of Rictor Attenuates mTORC2 Signaling in Aortic PVAT
To analyze the role of mTORC2 in PVAT for vascular function, we used adipose cell-specific rictor knockout (rictorad−/−) and corresponding control (rictorfl/fl) mice, which were described previously. Adipose-specific rictor knockout was confirmed by significant reduction of rictor gene expression in epididymal fat (EFAT) from rictorad−/− mice (Figure I in the online-only Data Supplement). Next, rictor gene expression in aorta (tissue containing mainly smooth muscle and endothelial cells) and in PVAT surrounding the thoracic aorta (referred henceforth as PVAT) was examined. The expression of rictor was significantly reduced in the PVAT but remained unchanged in the aorta from rictorad−/− mice (Figure 1A). To evaluate mTORC2 activity, aortic rings with intact PVAT were stimulated with insulin. Subsequently, PVAT and aorta were separated and analyzed for Akt Ser473 phosphorylation in PVAT and aorta from rictor ad−/− mice (Figure 1A). To determine whether vascular relaxation in PVAT was impaired in rictorad−/− mice, PVAT from rictorfl/fl and rictorad−/− mice were stimulated with acetylcholine. Acetylcholine-induced relaxation in aortic rings with PVAT from rictorad−/− mice exhibited reduced Akt Ser473 phosphorylation after insulin stimulation indicating impaired insulin signaling (Figure 1B and 1C).

Increased Aortic Contraction and Impaired Endothelium-Dependent Relaxation in Rictorad−/− Mice in the Presence of PVAT
PVAT surrounding the arteries influences vascular function. To investigate whether adipose-specific rictor deletion modulate aortic contractions, aortic rings with or without PVAT were used. To test the contractile capacity of the smooth muscle cell layer by direct depolarization, all rings were initially treated with 100 mmol/L potassium chloride (KCl). In all rings, similar contractions independent of rictor and PVAT were observed (data not shown). Next, we investigated the contribution of rictor in PVAT to receptor-dependent vascular contraction. Aortic rings with or without PVAT were exposed to vasoconstrictor phenylephrine (PE) in a concentration-dependent manner. Aortic rings with PVAT from rictorad−/− mice exhibited 2.1-fold higher maximal contractions as compared with rings from rictorfl/fl mice after treatment with PE (rictorad−/−: 70.0±8.6% versus rictorfl/fl: 81.0±10.1% at 3x10−6 mol/L; Figure 2A). With the removal of PVAT, contractions to PE increased overall and maximal contractions were not significantly different between both groups of mice (rictorad−/−: 70.0±8.6% versus rictorfl/fl: 81.0±10.1% at 3x10−6 mol/L; Figure 2B). To determine whether vascular relaxation was also affected, we analyzed acetylcholine-induced endothelium-dependent relaxation. Acetylcholine-induced relaxation in aortic rings with PVAT was impaired in rictorad−/− mice (Figure 2C). This impairment was confirmed by the pD2 values, which suggests a delay in relaxation in rictorad−/− aortic rings with PVAT (rictorad−/−: 6.2±0.2 mol/L versus rictorfl/fl: 7.0±0.1 mol/L). On removal of PVAT, the differences were lost and the aortic rings relaxed normally (Figure 2D).
characterized contraction to KCl. Concentration-dependent (10–100 mmol/L) KCl-induced contractions in aortic rings with PVAT were similar between both groups of mice (Figure II in the online-only Data Supplement).

Hypothetically, increased aortic contraction with PVAT in rictord−/− mice might be because of reduced nitric oxide (NO) availability. To investigate this L-NAME, which preferentially inhibits endothelial NO synthase (NOS), was used.\textsuperscript{23} In the presence of L-NAME (300×10⁻⁶ mol/L), the contractile responses to PE increased in rings with PVAT from both mouse groups (Figure 2E). Nonetheless, PE-induced contractions were higher in aortic rings with PVAT from rictord−/− mice (1.5-fold; rictord−/−: 86.36±8.17% versus rictorfl/fl: 58.78±6.71% at 3×10⁻⁶ mol/L; Figure 2E).

To define the specificity of L-NAME for the different NO synthases, we used RAW264.7 cell line which exclusively expresses inducible NOS (iNOS/NOS2).\textsuperscript{28} NO measurements after LPS stimulation revealed that L-NAME at 300×10⁻⁶ mol/L also partially inhibited iNOS-induced NO production (≈40%; Figure III in the online-only Data Supplement). These results suggest that L-NAME is a nonselective NOS inhibitor. Expectedly, iNOS inhibitor 1400 W (10⁻⁶ mol/L) also partially inhibited iNOS-induced NO production to baseline levels (Figure III in the online-only Data Supplement).

In addition to NO, endothelium also releases other dilatory factors. Hence, experiments were performed with endothelium-denuded aortic rings with PVAT. Aortic contractions to PE or PGF\textsubscript{2α}, were significantly higher in rictord−/− mice compared with rictorfl/fl mice (Figure 2F and 2G). Taken together, these results suggest that the anticontractile effect mediated by rictor is for the most part endothelial-independent.

Loss of Rictor in Adipose Cells of PVAT Results in Upregulation of Inflammatory Mediators

To determine how loss of rictor in adipose cells of PVAT increases aortic contractions and impairs relaxation, we screened genes which are expressed in and secreted by PVAT. These included adipokines,\textsuperscript{29} reactive oxygen species,\textsuperscript{17,29} and inflammatory molecules.\textsuperscript{30} Among the adipokines, gene expression levels of adiponectin (adipoq) and lipocalin (lnc2) were not affected in the PVAT from rictord−/− mice (n=4–8). Interestingly, the expression of inflammatory genes, such as chemokine (C-C-motif) ligand 3 (Ccl3)/macrophage inflammatory protein protein 1α (Mip1α), and Tnfα, was increased in the PVAT from rictord−/− mice (Figure 3A).

![Image](http://www.ahajournals.org)

**Figure 2.** Rictor controls anticontractility in perivascular adipose tissue (PVAT). Aortic rings from rictord−/− and rictorfl/fl mice were used. Rings with PVAT (A) or without PVAT (B) were contracted with phenylephrine (PE; 10⁻⁶ to 3×10⁻⁴ mol/L) in a concentration-dependent manner (n=10–13). Rings with PVAT (C) or without PVAT (D) were precontracted with PE and treated with acetylcholine (AcH; 10⁻⁶ to 10⁻⁴ mol/L) in a concentration-dependent manner (n=7–13). E, Aortic rings with PVAT were pretreated with L-NAME (300×10⁻⁶ mol/L) and then contracted with PE in a concentration-dependent manner (n=5–13). F and G, Aortic rings with PVAT but without endothelium were treated in a concentration-dependent manner with PE or with 3×10⁻⁶ mol/L of PGF\textsubscript{2α} (n=6–9). All values are mean±SEM. *P<0.05 vs rictorfl/fl.

![Image](http://www.ahajournals.org)

**Figure 3.** Adipocyte-specific deletion of rictor increases expression of inflammatory molecules in perivascular adipose tissue (PVAT). A, Steady-state mRNA expression levels of adipokines, reactive oxygen species (ROS), adipocyte differentiation, and inflammatory molecules as determined by quantitative real-time polymerase chain reaction. Results are normalized to the levels in PVAT from rictorfl/fl mice (n=4–8). B, Protein concentrations of inflammatory cytokines released by PVAT were determined in conditioned media using Bioplex system (n=4). All values represent mean±SEM, *P<0.05 vs rictorfl/fl. IL indicates interleukin-6; and TNFα, tumor necrosis factor-α.
Protein analysis of conditioned media revealed increased release of IL6 (5-fold), Mip1α (5-fold), and Tnfα (4-fold) from PVAT of rictor<sup>−/−</sup> mice (Figure 3B).

**Increased iNOS Expression but Unchanged Leukocytes Levels in PVAT from Rictor<sup>−/−</sup> Mice**

To investigate whether the augmented expression of inflammatory molecules was associated with elevated macrophage infiltration in PVAT, we determined steady-state mRNA expression levels of macrophage-associated genes emr1 (F4/80), CD163, and Nos2/iNos. Expression of F4/80 and CD163 was similar between groups, whereas iNos expression increased significantly in PVAT from rictor<sup>−/−</sup> mice (Figure 4A). To validate this, we examined the expression of iNOS in cross sections of thoracic aorta with PVAT. Indeed, iNOS expression was higher in the PVAT from rictor<sup>−/−</sup> mice (Figure 4B). The uniform expression of iNOS across the PVAT suggests that adipocytes themselves and not macrophages are the predominant cells expressing iNOS. Moreover, iNOS staining in aortic medial layer was hardly visible.

Using flow cytometry, the infiltration of inflammatory cells was quantified. The percentages of total leukocytes and macrophages in the stromal vascular fractions were similar between groups in PVAT (Figure 4C). In EFAT, no differences in leukocyte and macrophage percentages in stromal vascular fractions were detected, but overall percentages of leukocytes and macrophages were increased ≈100% when compared with PVAT (Figure 4C).

**Inhibition of iNOS Restores Anticontractile Effect of PVAT**

We analyzed the effect of TNFα on PE-induced contractions, as this proinflammatory cytokine has been shown to counteract the anticontractile activity of PVAT. In aortic rings with PVAT from rictor<sup>−/−</sup> mice, contractions to PE (10<sup>−6</sup> mol/L) increased in the presence TNFα but not to a similar level as contractions in the rings from rictor<sup>−/−</sup> mice, which were significantly higher compared with untreated rings from rictor<sup>−/−</sup> mice (Figure IV in the online-only Data Supplement). TNFα stimulated iNos expression in 3T3-L1 adipocytes (Figure V in the online-only Data Supplement) as reported previously. To confirm our observation that iNOS is expressed mainly in PVAT (Figure 4B, only background staining in the medial layer of the aorta), we analyzed gene expression of iNos in aortic tissue and PVAT. Indeed, expression of iNos was only detected in PVAT but not in the aorta of rictor<sup>−/−</sup> mice using quantitative real-time polymerase chain reaction (CT value in PVAT: 29; aorta: no amplification). Thus, we investigated the possible role of iNOS for vascular function using iNos inhibitor 1400 W. In aortic rings with PVAT, contractions in rictor<sup>−/−</sup> mice were reduced in the presence of 1400 W (Figure 5A), reaching similar levels detected in rictor<sup>−/−</sup> mice (Figure 5B). In addition, treatment with 1400 W improved the endothelium-dependent relaxation in aortic rings with PVAT from rictor<sup>−/−</sup> mice (Figure 5C) to comparable levels observed in control rings (Figure 5D). Thus, the altered function in aortic rings with PVAT in rictor<sup>−/−</sup> mice was normalized after inhibition of iNOS. Taken together, our findings suggest that increased levels of TNFα in concert with iNOS-derived NO metabolites and possibly other inflammation-associated molecules counteract the intrinsic anticontractile function of PVAT from rictor<sup>−/−</sup> mice.

**Figure 4.** Elevated inducible nitric oxide synthase (iNOS) expression in perivascular adipose tissue (PVAT) from rictor<sup>−/−</sup> mice is independent of macrophage infiltration. A. Steady-state mRNA expression levels of emr1 (F4/80), CD163, and Nos2/iNos were determined by quantitative real-time polymerase chain reaction. Results are normalized to the levels in PVAT from rictor<sup>fl/fl</sup> mice (n=8–9). *P<0.05 vs rictor<sup>fl/fl</sup>. B. Immunofluorescence staining of iNOS in cross sections of thoracic aorta with PVAT from rictor<sup>fl/fl</sup> (top) and rictor<sup>−/−</sup> (bottom) mice. Arrowheads indicate aortic medial layer. Scale bar, 100 μm. Images are representative of n=3 mice. C. Percentages of macrophages (F4/80 and CD163 positive cells) and leukocytes (CD45.2 positive cells) in stromal vascular fraction (SVF) from PVAT and epididymal fat (EFAT) using flow cytometry (n=4–5). All values represent mean±SEM. *P<0.05.

**Figure 5.** Inhibition of inducible nitric oxide synthase (iNOS) in perivascular adipose tissue (PVAT) normalizes vascular reactivity in rictor<sup>−/−</sup> mice. A. and B. Aortic rings with PVAT from rictor<sup>−/−</sup> and rictor<sup>−/−</sup> mice were contracted with phenylephrine (PE) in the presence or absence of iNOS inhibitor 1400 W (10<sup>−6</sup> mol/L). rictor<sup>−/−</sup> pD2: 5.4±0.2 mol/L vs rictor<sup>−/−</sup>+1400 W pD2: 4.8±0.2 mol/L (n=11–13). *P<0.05 vs rictor<sup>−/−</sup> by pD2. C and D. Precontracted aortic rings with PVAT were relaxed using acetylcholine (ACh) in the presence or absence of 1400 W (n=7–13). All values are means±SEM. *P<0.05 vs rictor<sup>−/−</sup>.
Downregulation of Rictor Expression in Adipose Tissue After High-Fat Diet Feeding

Because depletion of rictor in adipose tissue is associated with increased expression of inflammatory genes and proteins in PVAT, we assessed the possible physiological relevance of rictor expression regulation after high-fat diet (HFD) feeding, a model of experimental obesity and chronic low-grade inflammatory disease.32

We analyzed rictor gene expression levels in PVAT and EFAT in control mice fed for 10 to 12 weeks with a HFD and with control diet. Steady-state mRNA expression levels of rictor were significantly downregulated in both types of adipose tissue after HFD (>30%; Figure 6A and 6B, left panels). Concomitantly, iNos expression was significantly increased in PVAT of these HFD fed mice, whereas in EFAT only an upward trend was observed (P=0.16). In June 2013, a search in published micro array data sets in NCBI Gene Expression Omnibus database33 revealed that rictor mRNA expression levels were also significantly lower in white adipose tissue from C57Bl6/J mice after 8 weeks of HFD feeding (accession number GSE30247; Figure VI in the online-only Data Supplement), whereas inflammatory genes were upregulated.34

Discussion

In the present study, we describe a hitherto unknown function of the serine and threonine kinase complex mTORC2 in controlling the anticontractile activity of PVAT primarily by regulating inflammation in the adipocytes. Downregulation of rictor, an essential mTORC2 component, in adipose tissue increased gene expression of Tnfa, Mip1α, and iNos in PVAT and, concurrently increased the secretion of IL6, MIP1α, and TNFα. Moreover, inhibition of iNOS restored the anticontractile activity of PVAT lacking rictor. Importantly, we demonstrate that in a model of low-grade chronic inflammation, such as HFD-induced obesity, gene expression of rictor in PVAT is downregulated, whereas that of iNOS is upregulated.

Studies addressing PVAT’s contribution to vascular functions have largely focused on identifying molecules released by PVAT in normal and in pathophysiological conditions, such as obesity or hypertension.13,30 Signaling enzymes in PVAT that control the expression and secretion of these secretory molecules are not yet known. To the best of our knowledge, this is the first study to show the contribution of an intracellular signaling molecule (rictor) in adipocytes of PVAT in regulating the anticontractile activity.

Jabs et al15 have shown that continuous treatment with the mTOR inhibitor rapamycin, also known as sirolimus, markedly reduced vasorelaxation to acetylcholine in rat aorta devoid of PVAT by inducing endothelial dysfunction associated with reduced NO availability. Possible underlying mechanism may be the direct inhibition of mTORC1. In endothelial cells, however, also mTORC2 assembly after long-term rapamycin treatment is compromised,28 impairing downstream signaling from Akt to endothelial NOS.27,38 In our study, depletion of rictor from adipose cells in PVAT increased aortic contractions in response to receptor-dependent vasoconstrictors and impaired vasorelaxation. On removing PVAT, the differences in contraction and dilation between the mice groups vanished, supporting the current model stating that PVAT-released molecules directly mediate vasoreactive effects.13

PVAT can exert its anticontractile effects via endothelium-dependent or endothelium-independent mechanisms.17,20 The endothelium-dependent effect involves production of NO by endothelial NOS, which is required to maintain vascular tone, regulate platelet aggregation and leukocyte adhesion.39 In our model, the anticontractile function of PVAT was still compromised after endothelium denudation in rictorad−/− mice compared with controls. Thus, endothelium-derived vasodilators do not play a major role in anticontractile effects mediated by mTORC2 in PVAT. Inherent contractile responsiveness of vascular smooth muscle cells in the aortic rings was also preserved, despite the loss of rictor in PVAT because KCI-induced contractions were identical. This suggests that anticontractile effects mediated via mTORC2 in PVAT are most likely caused by modulation of the secretory proteins affecting adjacent vascular cells without changing their inherent functionality.

Increased inflammation and oxidative stress in PVAT have been linked to promote vasoconstriction and endothelial dysfunction.40,41 In this regard, we screened the expression of reactive oxygen species, generating NADPH oxidase subunits and inflammatory cytokines. In PVAT lacking rictor, expression levels of iNos and inflammatory cytokines, such as IL6, MIP1α, and TNFα, were increased. Thus, in murine PVAT, decreased mTORC2 activity initiated a cascade of inflammatory pathways.

Vascular expression of iNOS occurs during pathological changes in the vascular wall such as atherosclerosis development, hypertension, and vascular injury.42 These pathologies are often associated with inflammatory processes which induce expression of iNOS in the vascular wall. Healthy blood vessels do not express iNOS.43 Consistent with this finding, we did not detect iNOS expression in thoracic arterial tissue, that is, endothelium plus vascular smooth muscle cells, without PVAT. Tian et al44 have demonstrated that inhibition of iNOS protects endothelium/NO-dependent vasoconstriction of thoracic aorta in aged rats. Vice versa, overexpression of

Figure 6. Differential effects of high-fat diet (HFD) feeding on expression of rictor and inducible nitric oxide synthase (iNos) in fat depots. Steady-state mRNA expression levels of rictor and iNos in PVAT (A) and epididymal fat (B) of mice fed with control diet (CD; n=5–8) or HFD (n=6–9). Results are normalized to the levels from mice fed CD. Values are means±SEM. *P<0.05 vs CD.
iNOS using gene transfer resulted in impaired NO-dependent vasorelaxation in carotid arteries. In our work, partial inhibition of iNOS by 40% using non-selective NOS inhibitor L-NAME was not sufficient to adjust PE-mediated contractions between groups, whereas specific and complete inhibition of iNOS using 1400 W completely restored vasodilation and anticontractile activity in rings with PVAT lacking adipose-specific rictor. We propose that PVAT from rictorde−/− mice has preserved vasodilator effects which are compromised by the action of upregulated procontractile mediators such as iNOS-derived NO metabolites and proinflammatory cytokines. As a consequence, the procontractile molecules counteract but cannot abolish the overall anticontractile activity exerted, which is ultimately lost only after removal of PVAT in rings from rictorde−/− mice.

The marked reduction in insulin-stimulated Akt phosphorylation in PVAT of rictorde−/− mice may not only be caused by reduced mTORC2 to Akt signaling but may also be attributed to insulin resistance in these mice, resulting in impaired insulin signaling already at the level of the insulin receptor substrates. Brown et al. demonstrated that expression of a constitutively active Akt variant normalizes the hyperinflammatory phenotype in rictor-deficient cells by restoring mTORC2 downstream signaling. Thus, these increased inflammatory expression profiles observed in our study may possibly be caused by defective Akt signaling in PVAT lacking rictor.

Infiltration of macrophages and T cells in PVAT has been reported in mice fed with HFD. In contrast, thoracic PVAT has also been reported to be fairly resistant to macrophage infiltration, to obesity-induced inflammation, and is nearly identical to interscapular brown adipose tissue. In line with these findings, we found in our study about half as many leukocytes and macrophages in vascular stromal fractions of PVAT as compared with those of EFAT. These observations argue against an intrinsically proinflammatory depot in thoracic PVAT. Moreover, there was no difference in macrophage presence in PVAT between rictorde−/− and rictorfl/fl mice. Thus, we suggest that ablation of rictor in adipocytes initiates expressional changes to upregulate release of inflammatory molecules in the adipocyte itself which counteract PVAT’s anticontractile function.

The hyperinflammatory phenotype in PVAT caused by rictor deletion in adipocytes resembles obesity-induced changes. Increased expression of inflammatory cytokines, such as TNFα and IL6 in PVAT of obese patients, has been implicated to reduce its anticontractile activity. In line, a previous study by Meijer et al. showed that inhibition of inflammation restored anticontractile function of microvascular PVAT in a genetic model of obesity.

Intriguingly in our study, experimental obesity in mice induced via HFD feeding per se downregulated expression of rictor in thoracic PVAT, whereas iNos expression was upregulated. Considering the complexity and the interplay of different molecules and cells involved in obesity-induced adipose tissue inflammation, it was beyond the scope of the present study to elucidate mechanisms underlying the downregulation of rictor expression in this context. We speculate that several inflammatory molecules, including TNFα, induce a series of signaling cascades in perivascular adipocytes resulting in downregulation of rictor expression. Furthermore, reduced mTORC2 assembly and signaling, in turn, might further advance the ongoing inflammatory response. Thus, mTORC2 signaling is highly likely to participate in the complex regulation of obesity-associated adipose tissue inflammation.

In conclusion, the present findings assign a decisive function to rictor in PVAT in protecting arteries from inflammatory stress and in controlling normal vascular function. Stabilizing expression of rictor in adipose tissue during obesity and other metabolic rearrangements might, therefore, embody a novel treatment approach to combat inflammation-associated vascular damage.

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Disclosures

None.

References


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Online Supplementary Data

Supplemental Figure I: Steady state mRNA expression levels of rictor in epididymal fat were determined using quantitative real-time PCR in rictor^fl/fl and rictor^ad-/mice. Data represent means±SEM, n=5; *P<0.01.
Supplemental Figure II: KCl-induced contractions. Aortic rings with PVAT from rictor\(^{fl/fl}\) or rictor\(^{ad-/}\) mice were contracted with KCl in a concentration-dependent manner (10-100 mmol/L). Data represent means±SEM, n=4-5.
Supplemental Figure III: Inhibition of LPS-induced NO production by 1400W and L-NAME in macrophages. Murine macrophage RAW 264.7 cells were treated with 1400W (10⁻⁶ mol/L) or L-NAME (300x10⁻⁶ mol/L). After 45 minutes, cells were stimulated with LPS (1 µg/ml) as indicated. Conditioned media was collected and nitrate levels were determined. Data represent means±SEM, n=3; *P<0.05 vs. LPS only.
Supplemental Figure IV: TNFα increases PE-mediated contraction. Aortic rings with PVAT from rictor$^{fl/fl}$ mice were pre-treated with or without TNFα (10 ng/ml) and then contracted with PE (10^{-6} mol/L). As reference, aortic rings with PVAT from rictor$^{ad-/}$ mice were used. Values are means±SEM, n=4; *P<0.05 vs. rictor$^{fl/fl}$ without TNF.
Supplemental Figure V: TNFα stimulation increases gene expression of iNOS. Steady state mRNA expression levels of iNos were determined by quantitative real-time PCR in 3T3-L1 adipocytes stimulated with TNF (10 ng/ml). Values represent means±SEM, n=5. *P<0.05
Supplemental Figure VI: High-fat diet feeding decreases rictor gene expression in adipose tissue. GEO data set analysis (accession number GSE30247) for rictor gene expression in white adipose tissue from C57BL6J mice fed for 8 weeks with high-fat (HFD) or control diet (CD). Values are means±SEM of the rictor gene with the array ID 1441753_at, n=4; *P<0.05.
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Online Supplementary Materials and Methods

Mice
The mice used in the present study were described before in detail.¹ Briefly, mice with adipose-specific deletion of rictor (rictor<sup>ad−/−</sup>) were generated by crossing rictor<sup>fl/fl</sup> mice with C57BL/6J mice expressing Cre recombinase under the control of the adipocyte-specific fabp4aP2 gene promoter (purchased from JAX Laboratories, Bar Harbor, Maine, USA). Male mice (5 months) were used for all experiments except for flow cytometry analysis for which female mice (5-6 months) were used. All mice were backcrossed at least 5 times with the C57BL/6J strain. Littermates with the lox/lox genotype (rictor<sup>fl/fl</sup>) were used as control group in all experiments. Mice were housed at the institutional animal facilities (University Hospital Zurich and Biozentrum Basel, Switzerland, respectively) with a 12 hour light/dark cycle. Animals had access to standard chow (4.5% calories from fat; Kliba Nafag, Kaiseraugust, Switzerland) and to water ad libitum. Another group of male mice 8 weeks of age were fed high-fat diet (60% calories from fat; Harlan Research Diets) for 10-12 weeks. All mice used were genotyped with specific primers using PCR and standard protocols. Before sacrifice, mice were weighed and anesthetized by i.p. injection (xylazine: 100; ketamine: 23; and acepromazine: 3.0; in mg/kg body weight) and exsanguinated via cardiac puncture. Mice used for vascular function and insulin stimulation experiments were starved overnight. All mouse experiments described here were approved by the Kantonales Veterinaeramt of Zuerich and Basel-Stadt and, Switzerland, respectively.

Cell Culture
3T3-L1 cells were cultured in DMEM medium (Biochrome AG, Berlin, Germany) supplemented with 10% FCS and differentiated to adipocytes as described.² Briefly, post-confluent 3T3-L1 cells were treated with 3-isobutyl-1-methylxanthine (500 µmol/L), dexamethasone (0.25 µmol/L), insulin (10 µg/ml) and rosiglitazone (2 µmol/L) for 2 days. Thereafter, cells were treated with insulin (1 µg/ml) for next 2 days. The cells were then maintained in DMEM medium with 10% FCS for 6 additional days and the media was
changed every 2nd day. Differentiated adipocytes were kept overnight in DMEM medium with 0.5% FCS and then stimulated without or with TNFα (10 ng/ml) for 24 hours. RAW264.7 cells were cultured in DMEM medium with 10% FCS.

Nitric Oxide Measurement
Nitric oxide is converted to nitrate and nitrite in the cell and is released in the conditioned media. RAW264.7 cells were treated with lipopolysaccharide from Escherichia coli 0111:B4 (1 µg/ml; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 24 hours in the absence or in the presence of L-NAME (300x10⁻⁶ mol/L) or 1400W (1x10⁻⁶ mol/L). In the conditioned media, nitrite concentrations were determined using Griess reagent (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and optical density was measured at 540 nm. The amount of nitrite in the culture media was quantified using sodium nitrite as a reference standard.

RNA Isolation and Quantitative PCR
RNA isolation, cDNA preparation and real-time quantitative PCR were performed from epididymal fat (EFAT), thoracic PVAT and aortae using standard protocols. Briefly, RNA from fat tissues was extracted using RNeasy lipid tissue mini kit and from aortae using RNeasy fibrous tissue kit according to the protocol of the manufacturer (Qiagen, Hombrechtikon, Switzerland). Total RNA was transcribed into cDNA using WT (Whole Transcript)-Ovation™ Pico RNA Amplification System from NuGEN (Bemmel, Netherlands). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using specifically designed mouse primer pairs for the genes analyzed in the present study (Table S1). Gene expression in EFAT and PVAT was normalized to acidic ribosomal phosphoprotein (Arbp) and in aortic tissue to β-actin using the comparative C(T) method.³

Vascular Function Analysis
Experiments were performed as described before.⁴,⁵ After sacrifice, thoracic aortae was dissected, placed in cold (4°C) Krebs-Ringer bicarbonate solution (in mmol/l: NaCl 118.6; KCl 4.7; CaCl₂ 2.5;MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25.1; EDTA₉Na₂Ca 0.026; glucose 10.1). To check the effect of PVAT on vascular function, aortae was either cleaned of adhering PVAT or was left intact. Aorta with or without fat was cut into equal-sized rings (2.5 mm of length) under a dissection microscope (Olympus SZX9, Volketswil, Switzerland), and care was taken not to damage the endothelium during preparation. Aortic rings were mounted on tungsten wires (100 µm in diameter) and placed vertically in water-jacketed organ chambers containing 10 ml of Krebs buffer maintained at 37°C and pH 7.4 with constant gassing (95% O₂ and 5% CO₂). One end of each wire was fixed to a stainless steel rod, and the other end was connected to force transducers (Hugo Sachs Electronik, March-Hugstetten, Germany).
Rings were stretched in a stepwise manner until a resting tension of 3.5 grams was achieved. Stretched rings were allowed to equilibrate for 30 minutes before exposing with KCl (100 mmol/L) until a stable contractile response was reached. The rings were exposed to phenylephrine (PE; 10⁻⁹- 3x10⁻⁶ mol/L), prostaglandin₂αₙ (PGF₂α; 3x10⁻⁶ mol/L) or acetylcholine (ACh; 10⁻⁹-10⁻⁴ mol/L) in a concentration-dependent manner. To block nitric oxide synthase (NOS) selected aortic rings were treated with either N⁶-nitro-L-arginine methyl ester (L-NAME; 300x10⁻⁶ mol/l) or with iNOS inhibitor (1400 W, 10⁻⁶ mol/L). To check the vascular smooth muscle cells-dependent contractile responses, aortic rings with PVAT were exposed to increasing concentration of KCl (10 to 100 mmol/L).

For endothelial denudation experiments, selected rings were exposed for 10 seconds to 1% Triton X-100 before mounting into the organ chamber. Endothelial denudation was tested by treating PE-precontracted rings with ACh followed by treatment with sodium nitroprusside (10⁻⁵ mol/L), a nitric oxide donor.

**Insulin stimulation and Immunoblotting**

Aortic rings with PVAT were mounted to organ chambers and stretched as described above. After 30 minutes of equilibration, tissues were stimulated with insulin (1µmol/L). After 10 minutes, tissues were transferred into ice cold Krebs Ringer buffer and PVAT was separated from the aortic tissue. Both tissues (PVAT or aorta without PVAT) were homogenized using Qiagen tissue lyser in lysis buffer containing Tris-HCl (50 mmol/L, pH 7.4), NaCl (150 mmol/L), EDTA (1 mmol/L), NP-40 (1%), protease inhibitor, and phosphatase inhibitor cocktail (Sigma, Buchs, Switzerland). Equal amounts of protein were loaded for SDS-PAGE, transferred to nitrocellulose and analyzed with the indicated antibodies. Immunoblotting was performed using the antibodies against phospho Akt (pSer473), Akt and phospho Erk1/2 (Cell Signaling, Danver, MA, USA) and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and corresponding HRP-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK). Labeled proteins were visualized on X-ray films using a chemiluminescence reaction. Quantification of blots was performed by using the software ImageJ 1.40g (NIH, USA).

**Bioplex Assay**

Thoracic aortic PVAT was excised and incubated in a 96-well plate containing 200µl Phenol Red-free and serum-free DMEM/Ham’s F-12 medium (BioConcept Amimed, Allschwill, Switzerland) for 48h. Conditioned media was collected, shock-frozen in liquid nitrogen and stored at -80°C. Concentrations of MIP-1α, TNFα and IL-6 levels in the conditioned media were determined using BioPlex Pro Mouse cytokine Group 1 (customized 3-plex assay, BioRad, Hercules CA, USA) according to the recommendations of the manufacturer. Data
were analyzed using the BioPlex Manager 4.1.1 software. Cytokine concentration in PVAT was quantified based on Logistic-5PL regression standard curve. Values were normalized to protein content in the conditioned media.

**Immunofluorescence analysis of cryosections**
Thoracic aortae with PVAT were embedded in Tissue Tec (Sakura, Germany) and stored at –80°C. Cross sections (10 μm) were cut using a cryostat and mounted on superfrost plus slides (Thermo Fisher, Germany). Slides were used directly for immunofluorescence analysis or stored at -80°C until needed. Before staining, slides were dried at room temperature (RT) for 20 minutes and fixed with 4 % PFA in PBS (pH 7.4) for 10 minutes. Slides were washed twice for 5 minutes in PBS (pH 7.4). Sections were incubated in blocking buffer (0.1% Tween 20, 5% goat serum in PBS) for 60 minutes at RT to block non-specific binding of immunoglobulin. Sections were incubated with iNOS primary antibody overnight at 4°C (ab15323; 1:350 in blocking buffer; Abcam, Cambridge, United Kingdom) in a humidified chamber. Next day, slides were washed three times for 5 minutes in wash buffer (0.1% Tween20 in PBS). Sections were incubated with Alexa 568 secondary antibody (A-11036; Invitrogen, Lucerne, Switzerland) diluted 1:500 in blocking buffer for 30 minutes at RT in the dark and humidified chamber. Slides were rinsed in wash buffer three times for 5 minutes. Coverslips were mounted with Dako Fluorescence mounting medium (Dako, Baar, Switzerland) onto slides. Slides were dried at least 1 day before imaging and kept at 4°C. Images were acquired using CLSM Leica SP5 microscope and LAS-AF 2.6.3.8173 software (Leica, Heerbrugg, Switzerland).

**Isolation of the stromal vascular fraction from adipose tissue and flow cytometry**
PVAT from thoracic aortae and EFAT were harvested, and digested enzymatically by incubation in a solution containing collagenase type D from clostridium histolyticum (0.5 mg/ml, Roche Diagnostics, Indianapolis, USA) dissolved in PBS supplemented with CaCl₂ 2.5 mmol/L for 1 hour at 37°C with constant agitation (900 rpm). After 30 minutes, tissues were passed 10 times through an 18g needle and, at the end of the incubation period, through a 30 μm sterile cell strainer (Miltenyi Biotec, Bergisch Gladbach, Germany) to yield a single cell suspension corresponding to the vascular stromal fraction. Cells were washed and suspended in flow cytometry buffer (0.5% BSA, 1mM EDTA in PBS) containing of the following antibodies: fluorescein isothiocyanate anti-CD45.2 (1:200; Biolegend, Fell, Germany) and APC-AlexaFluor750 anti-F4/80 (1:100, eBiosciences, San Diego, USA). After immunostaining (incubation with labeled antibodies for 40 minutes at 4°C in the dark), cells were washed 2 times in flow cytometry buffer and cell populations were analyzed by BD FACS Canto run with DIVA software (Becton Dickinson, Allschwil, Switzerland). Data were
analyzed with FlowJo software 8.5.2, (Tree Star). Percentages of macrophages (CD45+ F4/80+) in the stromal vascular fraction from the different adipose tissues was determined.

**Statistical Analysis**

Data are expressed as means ± standard error of the mean (SEM). Vascular function experiments were analyzed with ANOVA for repeated measurements followed by a Bonferroni post-hoc test. Contractions are given as a percentage of contraction to 100 mmol/L KCl, and dilations as a percentage of the maximal pre-contraction. pD2 (-log EC50) values were calculated with non-linear regression analysis. Comparisons between groups for remaining experiments were analyzed by two-tailed Student’s t-test. Differences were considered statistically significant at values of P <0.05. All statistical analysis was performed using GraphPad Prism 5.04 program for Windows (GraphPad software, San Diego, CA, USA).

**References**

4. Bhattacharya I, Mundy AL, Widmer CC, Kretz M, Barton M. Regional heterogeneity of functional changes in conduit arteries after high-fat diet. *Obesity (Silver Spring)*. 2008;16:743-748
**Supplementary Table 1**

**Primer list for quantitative real-time PCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
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<tr>
<td><em>Arbp</em></td>
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</tr>
<tr>
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