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Vascular TNF-α/Rho-kinase Signaling Mediates Vasoconstriction and Blood Pressure Elevation in Overweight

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Table of Content

1. Used abbreviations

%	Percent
°C	Degree Celsius
DNA	Desoxy-ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IL	Interleukin
MJ	Megajoule
MCP-1	Monocyte chemotactic protein-1
mg	Miligram
ml	Milliliter
mM	Millimolar
mmHg	Milimeters of mercury
mN	Millinewton
min	Minutes
mM	Millimolar
M	Molar
mRNA	Ribo-Nucleic-Acid
MYPT1	Myosin phosphatase target subunit 1
nm	Nanometer
µg	Microgram
µl	Microlitre
µm	Micrometer
pMYPT1	Phosphorylated myosin phosphatase target subunit 1
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROCK2	Rho-associated, coiled-coil containing protein kinase 2
SMC	Smooth muscle cells
TNF-α	Tumor necrosis factor-α

Let food be your medicine and medicine be your food.

-Hippocrates (400 B.C.)

2. Thesis overview

Globally, more than 1 billion people have excess weight[1]. Similar to obesity, the overweight status is associated with increased cardiovascular risk[2-3]. Large epidemiological studies demonstrate that overweight is associated with hypertension, an established cardiovascular risk factor[3-4] with prevalence reaching 60%[5]. However, more than in obesity, the molecular mechanisms underlying blood pressure elevation in overweight remain unclear.

The association among overt obesity, cardiovascular risk and proinflammatory cytokines is well known[6-10]. In particular, tumor necrosis factor-α (TNF-α) has been widely linked to obesity[11-14] and also overweight-related pathologies[11-12, 15]. Apart from the adipose tissue being a known source of inflammation[8, 16-17], TNF-α was recently reported to be increased in the vasculature of obese individuals[18], suggesting that vascular proinflammatory signaling might importantly contribute to the vascular features associated of obesity status. Interestingly, TNF-α is also linked to the pathology of hypertension[19] and not only obese but also overweight individuals are known to exhibit features of vascular dysfunction that are typically associated as well with hypertension, such as increased vasoconstriction[20-21] and endothelial dysfunction[21-22].

Given the link between hypertension and microvascular inflammation[21, 23-24] via its effects on vasoconstriction[21, 25], we hypothesized that the proinflammatory cytokine TNF-α plays a role in the development of cardiovascular risk in overweight via an effect on vasoconstriction and blood pressure elevation. Targeting microvascular dysfunction in overweight could reduce blood pressure and therefore cardiovascular risk, beyond common antihypertensive treatment. However, the underlying molecular mechanism remains uncertain.

One suggestion for a key molecular mechanism for this microvascular dysfunction is vascular proinflammatory signaling, since inflammatory processes can modulate vascular function [21, 25-26]. The present thesis analyses the development of cardiovascular risk factors in a mouse model of overweight. We hypothesize that in overweight vascular proinflammatory signaling increases vasoconstriction resulting in blood pressure elevation.

This work describes results indicating that vascular TNF-α/Rho-kinase signaling plays a role in vasoconstriction and blood pressure elevation in overweight. TNF-α/Rho-kinase signaling may thus represent a new therapeutic target that functions independently of the overweight state.

3. Introduction

The association of excess weight and blood pressure elevation and the search for a link

Modern times have brought a huge supply of food rich in saturated fat, among others leading to excessive weight gain, affecting both adults and children. The most common measure used to define excess body weight is still the body mass index (BMI), due to its simplicity. The index is defined as the weight in kilograms divided by the height in meters squared (kg/m^2). The National Institutes of Health and the World Health Organization have defined overweight as a BMI between 25.0 and 29.9 kg/m^2; and obesity as having a BMI greater than 30.0 kg/m^2[27]. When overweight/obesity is established, the association with increased cardiovascular risk is well known[2, 6, 10, 28-29]. In this context, arterial hypertension is extensively recognized as an important cardiovascular risk factor, increasing the risk for a variety of cardiovascular diseases including coronary artery disease, stroke, heart failure and peripheral vascular disease[30-35].

The association of excess weight and hypertension is known since long time[36-37]. There is a clear positive relationship between blood pressure levels and body weight[38-40]. In large cohorts such as in the INTERSALT study for example, a 10 kg difference in body weight was associated on average with a 3.0 mmHg difference in systolic pressure[39]. In a meta-analysis from Staessen et al., a decrease in body weight by 1 kg resulted in a reduction of systolic pressure by 1.2 mmHg[40]. Recently, not only the excess weight defined as increased BMI but also the accumulation of visceral fat, independent of body weight, has its importance recognized and the waist circumference has been included to estimate cardiovascular risk[41-42]. The visceral fat is now accepted as an active tissue, source of hormones and cytokines with crucial influence on lipid and glucose metabolism, being associated to a chronic low-grade inflammatory state[16, 43-47].

Along with metabolic disturbances, the association of fat tissue[48] and more specifically of the visceral fat tissue with arterial hypertension has been pointed out in several studies[49-51]. The mechanisms involved in the physiopathology of hypertension associated to excess weight conditions are still an area of investigation. Some of the identified mechanisms include activation of the sympathetic nervous system[52-53] and of the renin-angiotensin–aldosterone system[54-55], neuroendocrine mechanisms related to peptides

such as leptin[56] and more recently, the influence of insulin resistance on microvascular function[57-58].

However, although microvascular dysfunction and inflammation are important features shared by excess weight states and hypertension, this link has been poorly explored. The increasing development in the field of vascular biology has shown that the vasculature is a highly responsive organ. The two main cells types of the vasculature – the smooth muscle and endothelial cells - are sensitive to a great variety of stimuli including shear stress, pressure, neurohumoral factors, cytokines and many other molecules that generate vascular responses[59]. Therefore, the role played by the vessel itself in the physiopathology of overweight/obesity-related vascular complications such as hypertension, cannot be neglected.

Microcirculation and blood pressure control

The microcirculation consists of the smallest arteries, the arterioles, capillaries, and venules[60], therefore including the so-called resistance arteries, which consist mainly of smaller arteries less than 400 μm and arterioles less than 100 μm in lumen diameter[61]. The resistance arteries regulate the systemic vascular resistance[62]. In this thesis, small mesenteric arteries from mice were used to assess the function of the resistance vasculature[62-64]. Resistance vessels are the key site where the blood pressure control occurs, between large arteries (the conductance arteries) and capillaries. This control is achieved through the contractile phenotype of the vascular smooth muscle cells (VSMCs)[65-66].

Regulation of VSMCs contractility – The Rho/Rho-kinase pathway

The regulation of VSMCs contractility depends on the balance between vasodilators and vasoconstrictors, being controlled by several hormonal autocrine and paracrine actions. In any type of stimulus, all smooth muscle cells use cross-bridge cycling (between actin and myosin) to develop force[67-68]. In VSMCs, contractile activity is initiated by a calcium ions (Ca^{+2})-calmodulin interaction to stimulate phosphorylation of the light chain of myosin II (MLC_{20}). This phosphorylation is determined by the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activities. MLCK is activated by Ca^{+2}-calmodulin, thus dependent on intracellular Ca^{+2} concentration. On the other hand, MLCP works independently of Ca^{+2}-calmodulin, being mainly modulated by the RhoA/Rho-kinase pathway[69].

The Ras homolog gene family member A (RhoA) is a member of the Rho family of GTPases (enzymes that hydrolyze guanosine triphosphate (GTP) to guanosine

diphosphate (GDP)), a family of small signaling G proteins. The guanine nucleotide-binding proteins or G proteins, integrate signals between receptors and effector proteins and work as molecular switches: when they bind GTP, they are 'on', and when they bind GDP, they are 'off'. They respond to cell surface receptors for cytokines, growth factors, adhesion molecules and G-protein-coupled receptors[70-71]. The activation of RhoA leads to activation of Rho-kinase (ROCK1 and ROCK2), the most studied downstream effector protein of RhoA.

The RhoA/Rho-kinase pathway is a key regulator of several cellular processes such as cytoskeletal regulation, migration, adhesion, cell cycle and cytokine production[72]. In VSMCs, the Rho-kinases regulate contractility by direct phosphorylation of MLC_{20} and phosphorylation and inactivation of the myosin phosphatase target subunit 1 (MYPT1) of MLCP[73-74]. Inhibition of MLCP leads to an increase in phosphorylation of MLC_{20} thereby keeping the contractile state, without any changes in intracellular Ca^{+2} concentrations (Figure 1). This enhancement of the contractile response to Ca^{+2} is normally called “calcium sensitization” [75-76].

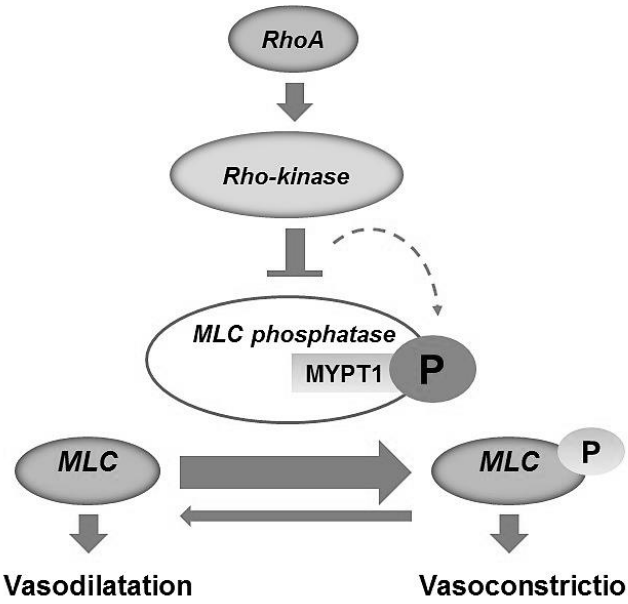


Figure 1. *Activation of Rho-kinase by RhoA leads to phosphorilation of the MLC phosphatase (MYPT1 site), causing its inactivation and therefore avoiding the dephosphorylation of MLC, favoring vasoconstriction.*

The Rho/Rho-kinase pathway in disease

Since the discovery of the essential role of RhoA and its downstream target Rho-kinase in the regulation of vascular tone, alterations in this pathway have been reported to be associated with cardiovascular diseases[77-79], including arterial hypertension[80-81].
Hilgers et al. described an enhanced RhoA/Rho-kinase signaling in resistance arteries from angiotensin-induced hypertensive rats[82] and Failli et al. reported increase in Rho-kinase expression and activity in aortas from diabetic rats[83]. The pyridine derivative Y-27632 selectively inhibits Rho-kinase and it was reported to correct hypertension in hypertensive rat models[84-85].

The first report suggesting that Rho-kinase could be upregulated in human hypertension was in 2001, when Masumoto et al. found that forearm vasodilator response to fasudil was significantly greater in hypertensive patients than in normal subjects[86]. Much evidence has accumulated implicating activation of Rho family proteins in the pathogenesis of essential arterial hypertension in humans[87-88]. Moreover, an increased Rho-kinase activity was reported to be associated with components of metabolic syndrome such as BMI, waist circumference, fasting glucose, C-reactive protein and triglyceride levels[89].

Recently, a role for the RhoA/Rho-kinase pathway in the adipose tissue was suggested by Nakayama et al. and Hara et al. Their studies linked inflammation and activation of the pathway showing that this interaction induced inflammatory cytokine expression in adipocytes[90-91], providing an important link between inflammation and Rho-kinase in obesity context.

It is know that the proinflammatory cytokine TNF-α can activate the Rho/Rho-kinase pathway in different cell types such as microvascular endothelial cells[92-93], bladder and airway smooth muscle cells[94]. Incubation of bronchial smooth muscle cells with TNF-α increased their contractility, as the result of an increase in Ca^{2+} sensitivity through MLC_{20} phosphorylation[95]. Later on, this TNF-α action in airways was reported to be mediated by TNFR1[96].

Following this line of reasoning, we hypothesized that a similar mechanism involving TNF-α and the Rho-kinase pathway could take place in the vasculature of mice fed a high-fat diet, thereby providing an explanation for vascular changes observed in a diet-induced overweight model.

The vasculature is target and source of TNF-α

TNF-α is a proinflammatory cytokine mainly synthesized by monocytes and macrophages. It plays a key role regulating immune responses and energy[97], lipid and glucose metabolism[98-99]. The combination of TNF-α with its two cell membrane receptors p55 and p75 (known as TNFR1 and TNFR2) induces a variety of signals that depend on TNF-α concentration and on cell type[100-102]. At low concentrations in tissues TNF-α has favorable effects, such as the increase of host defense mechanisms. At high concentrations TNF-α can lead to excess inflammation, like for example in septic shock, when large amounts of TNF-α are released.[97]. TNF-α mediates inflammatory, immune, proliferative and cytotoxic effects in various cell types[103]. The vasculature, including endothelial cells and VSMCs is a major site for the action of TNF-α.

TNF-α and endothelium

Characteristics of endothelial cells when they are target for TNF-α action have been well described. On endothelial cells, TNF-α is known to induce inflammatory responses by enhancing adhesion molecule expression and cytokine secretion, therefore participating actively in leukocyte adhesion and in the pathogenesis of vascular diseases[104-107]. More recently, microarray data showed that TNF-α induces distinct gene expression of chemokines, cytokines, and cell surface molecules in microvascular and macrovascular human endothelial cells[108]. The presence of quiescent VSMCs seems to be crucial in the modulation of the activation of endothelium by TNF-α[109].

The endothelium modulates vascular tone through several factors, including nitric oxide (NO), prostacyclin and endothelium-dependent hyperpolarizing factor (EDHF).

TNF-α impairs endothelium-dependent and NO-mediated vasodilatation in different vascular beds[110-111], thus influencing contractility in VSMCs. Nitric oxide is a free radical generated by NO synthase (NOS) and is the major mediator of endothelium-dependent relaxation[112-113]. TNF-α regulates NOS expression and activity, although the overall regulation is still discussed and it is probably in a time-dependent way and dependent on the cell type[114]. However, it is mostly accepted that TNF-α decreases the bioavailability of NO to induce relaxation of smooth muscle in the vasculature[115].

EDHF also plays an important role in regulating vascular tone and vascular reactivity, particularly in resistance vessels[116]. As well, the role of TNF-α in EDHF-mediated vascular dysfunction is controversial[117-118], although there is strong evidence that TNF-α diminishes EDHF, via the inhibition of cytochrome P450 enzyme activity[119]. Finally,

incubation of endothelial cells with TNF-α was reported to cause release of endothelin-1, an important vasoconstrictor[120].

Endothelial cells can also be a source of TNF-α, in conditions such as in atherosclerotic plaques[121], in human umbilical vein endothelial cells[122-123] and brain microvascular endothelial cells[124]. With all these effects, TNF-α is an important modulator of vascular function, mainly through its actions on endothelium.

TNF-α and VSMCs

TNF-α has been implicated in contractility disorders – mainly increased contractility - associated to smooth muscle cells. The structure and function of smooth muscle cells are essentially the same in different organs such as stomach, intestines, bladder, airways, male and female reproductive tracts, lymphatic and blood vessels [68].

Experimental administration of TNF-α can induce preterm labour[125] and increase expression of contraction-associated proteins in the labour cascade, increasing uterine contractile activity[126]. TNF-α also increased contractility in *in vitro* studies with cultured human uterine smooth muscle cells [127], in a dose-dependent manner[128]. In cultured human detrusor smooth muscle cells, TNF-α treatment caused upregulation of two kinins receptor subtypes B_1 and B_2. Kinins are potent mediators of inflammation that, among others, cause smooth muscle contraction[129]. In this context, the airways consist on the organ where smooth muscle cell reactivity is mostly studied, due to its importance to the understanding of bronchial asthma pathogenesis.

Since the concept of asthma as an inflammatory disease was established, there is growing association between TNF-α and bronchial hyperreactivity, a characteristic feature of asthma[130-133]. Some studies depicted a role for TNF-α affecting directly the smooth muscle cell function. Amrani et al. performed studies in cultured human airway smooth muscle cells showing that TNF-α significantly enhanced the Ca^{+2} responsiveness to bronchoconstrictor agents[134-135] and that this alteration in Ca^{+2} homeostasis occurred through activation of TNFR1[136]. Amrani et al. also suggested that the mechanisms may be due to a direct modulatory effect on G protein-mediated signal transduction[137]. Later on, the underlying mechanism to explain the TNF-α signaling leading to bronchial hyperreactivity included that TNF-induced Ca^{2+} sensitization was, at least partly, the result of an increase in myosin light $chain_{20}$ (MLC20) phosphorylation and a subsequent increase in Ca^{2+} sensitivity of the myofilaments [95]. Hunter et al. in 2003 reported that

TNF-induced Ca^{2+} sensitization of MLC20 phosphorylation in airway smooth muscle cells from both guinea pig and human is regulated by activation of RhoA and inhibition of the smooth muscle myosin light chain phosphatase activity, probably via Rho-kinase activation. This Ca^{2+} sensitization caused by TNF-α was predominantly via the TNFR1 and occurred in a time course compatible with the TNF-induced airway hyperreactivity[96]. Besides other molecules, the Rho-kinase pathway is definitely implicated in the physiopathology of asthma[138].

Concerning the vasculature, TNF-α has been consistently shown to be a chemoattractant in VSMCs[139-141]. Important roles for TNF-α have been described in VSMCs concerning migration[142], proliferation[101, 143-145] and apoptosis[143, 146-147]. In normal vessels, TNF-α is not expressed[148]. The idea that local TNF-α production by VSMCs might initiate vascular inflammation was first reported by Warner S. et al. in 1989[141]. They reported that cultured VSMC can express TNF-α gene and secrete biologically active TNF-α. Therefore VSMCs could be a local source of TNF-α in the vessel wall and thereby intensify, maintain or propagate locally inflammatory responses that originate systemically[141]. Later on, TNF-α was localized in human arterial wall with atherosclerosis, where TNF-α concentrations in the arteries were about 200 times higher than in the corresponding serum samples[149], strengthening the importance of vascular inflammation in the atherosclerosis process. For example, formation of the neointima is characterized by an inflammatory process with expression of numerous inflammatory cytokines including TNF-α by the expanding population of VSMCs found within atherosclerotic plaques[121, 148]. TNF-α serves as a marker when there is a change from the physiological contractile phenotype of VSMCs to the secretory/migrating phenotype, after acute vascular injury. The expression of TNF-α by VSMCs is reported in balloon-injured arteries[150-151] and in graft coronary disease[152-153]. In a mouse model of myocardial ischemia-reperfusion showed that myocardial ischemia initiated TNF-α expression in vascular smooth muscle cells, inducing vascular oxidative stress, independent of neutrophil activation[110].

TNF-α has its importance well recognized in modulating vascular inflammation within pathological VSMC populations, such as in atherosclerosis. However, the direct effects of TNF-α on one of the main functions of VSMC, vasoconstriction, has not been well explored.

TNF-α as a vasoconstrictor cytokine

Depending on the studied vascular bed and on the inflammatory condition, TNF-α can cause vasodilatation and hyporesponsiveness to vasoconstrictors[154-155], induce vasoconstriction or increase the response to vasoconstrictor agents and impair endothelium-dependent vasodilatation[25, 156-158].

Studies have showed that TNF-α elicits vasoconstriction in different vascular beds. In murine models, TNF-α mediated the vasoconstrictive effect of hemolyzed blood in hemolysis-induced cerebral vasospasm, and neutralization of TNF-α by administration of infliximab prevented and resolved the vasospasm[159]. Moreover, by means of endothelin-1, a focal intrastriatal injection of TNF-α caused acute reduction in cerebral blood volume through vasoconstriction[158].

In rat lungs, TNF-α was implicated in the development of pulmonary hypertension during sepsis, potentiating the vasoconstriction induced by platelet-activating factor, by a mechanism that is neutrophil independent but thromboxane dependent[156]. In skeletal muscle arterioles in aged rats, inhibition of TNF-α abolished augmented vasoconstriction response induced by angiotensin II, as this response is related to a proinflammatory state[160]. Furthermore, reports of infusion of TNF-α in rats caused within 15 min an elevation in the levels of ET-1 associated with coronary vasoconstriction[161] and induced renal vasoconstriction and hypofiltration by means of reduction in NO bioavailability in mice[162]. In a report from Wagner et al. TNF-α was infused directly into the bronchial airway of sheep, causing bronchial vasoconstriction by the secondary release of endothelin-1[163].

In pregnant rats, TNF-α enhances vascular contraction, therefore is a possible mediator of increased vascular resistance associated with pregnancy-induced hypertension[157, 164]. In line, TNF-α is elevated in the plasma of preeclampsia women and placental ischemia promotes the release of cytokines such as TNF-α[165].

In humans, TNF-α was reported to constrict as well arterial segments such as the temporal artery, in an endothelium-dependent way[166-167]. Scherer et al. proposed a connection between hearing loss and microvascular ischemia, involving activation of vascular sphingosine-1-phosphate signaling by TNF-α. In the cochlear microvasculature of gerbils, TNF-α reduced cochlear blood flow. In this same report a small group of patients with acute hearing loss was treated with the anti-TNF-α antibody etanercept and

most of them recovered[25]. Bolz et al. and Scherer et al. suggested that TNF-α can directly result in enhanced vascular tone and sensitivity by augmenting the myogenic tone of resistance arteries[25, 168].

Thus, TNF-α is able to generate vascular dysfunction – a known cardiovascular risk factor[169-171].

Vascular dysfunction and inflammation: Hallmarks shared by excess weight and arterial hypertension

Hypertension and vascular dysfunction

Hypertension is associated with increased peripheral vascular resistance[172], caused by conditions that produce decreased lumen size. These conditions may be structural, mechanical, and functional[169]. Concerning function, the association between vascular dysfunction and arterial hypertension is well known[60, 173-176].

In hypertension, vascular dysfunction is mostly related to increased peripheral resistance, due to enhanced vasoconstriction[177]. The imbalance towards vasoconstriction occurs through several ways. For example, the existence of endothelial damage in hypertension is largely reported[178-181], which primarily reduces the availability of vasodilators and thus favours vasoconstriction. Nevertheless, the importance of an enhanced state of vasoconstriction, independent of endothelial function, is also recognized in experimental and human hypertension. Since long time, the association of human hypertension with increased responsiveness to vasoconstrictors is known[182-183].

In experimental studies the onset of hypertension in spontaneously hypertensive rats (SHR) agreed with increased vasoconstriction elicited by noradrenaline in mesenteric arteries[184-185]. Augmented vasoconstriction has also been reported in response to angiotensin II[186]. In cultured VSMCs from SHR, calcium concentration responses to angiotensin II are enhanced[187].

In a new line of findings, it was recently reported that mineralocorticoid receptors present in VSMCs can directly contribute to vascular contraction and hypertension, being one of the unique studies to attribute a key direct role for VSMCs in hypertension[188]. But the exact mechanisms underlying the increased vascular reactivity and hypertension remain unresolved.

Hypertension as an inflammatory disease

Along with the study of the role of inflammation in the atherosclerosis process, in the last decades the search for a link between inflammation and hypertension is subject of intense investigation[24, 189-190]. The evidence comes from clinical and experimental studies. Hypertensive patients show increase of proinflammatory markers and cytokines in blood such as C-reactive protein, TNF-α and IL-6, as well as adhesion molecules such as monocyte chemotactic protein-1 (MCP-1)[191-192]. A chronic low-grade inflammatory status is linked to hypertension and also to the pre-hypertension condition, suggesting that inflammation could be involved in the development of hypertension[23, 193-195]. This association remains significant after adjustment for other cardiovascular risk factors such as dyslipidemia, excess weight and insulin resistance. However, it is still a challenge to define the exact role of inflammation in the physiopathology of hypertension.

In hypertension, inflammatory cells have been reported to accumulate in tissues such as vasculature[24, 196], kidney[197] and brain[198]. Concerning the vasculature, macrophages are involved in perivascular inflammation and seem to be a source of vascular oxidative stress and to influence vascular remodeling[199-201]. The involvement of the innate immune system in vascular inflammation is also well recognized, such as the role of T cells, including their activation by the central nervous system[196, 202-204]. The mechanisms through inflammatory process may be involved in the development of hypertension also include endothelial dysfunction[181, 205] and arterial stiffness[206-207], with oxidative stress and consequent reduced availability of nitric oxide being a key feature[208].

The endothelium is a fundamental regulator of vascular tone. In hypertensive patients endothelium dysfunction is a hallmark, with reduced vasodilatation and increased vascular tone associated with a proinflammatory and prothrombotic state. Low-grade inflammation localized in vascular tissue is thus accepted as a significant contributor to the pathophysiology of hypertension[180-181]. The evidence that drugs commonly used to treat hypertension, such as statins, angiotensin converting enzyme inhibitors and angiotensin II receptor blockers have anti-inflammatory properties and can improve outcomes, reinforce a key role for inflammation[209-210].

Overweight and obesity are as well considered inflammatory conditions, being highly associated with cytokines. Therefore, it is plausible that the inflammatory milieu strongly influences blood pressure levels in these contexts.

Role of TNF-α in hypertension

Several studies report a positive independent association between TNF-α with hypertension[211-213].

The association between higher levels of TNF-α was seen even in prehypertensive states, as reported in the ATTICA study[193] and in young adults with mild hypertension[214], suggesting that inflammatory pathways are already activated in early stages of hypertension. Interestingly, in a sample of 196 healthy subjects randomly chosen, there was an independent association of TNF-α levels with higher blood pressure levels, after adjustment for body weight, sex, age and others, suggesting that TNF-α could be an independent risk factor for hypertension[215].

Some reports show that increased levels of TNF-α are associated with impaired endothelial function, which explains in some cases the presence of increased vasoconstriction, as for example in cerebral vasospasm, where TNF-α plays a key role, contributing to the recruitment of other inflammatory mediators[159, 216-217].

Essential systems in blood pressure regulation such as the sympathetic nervous system and the renin-angiotensin system interact with proinflammatory cytokines, such as IL-6 and TNF-α. The sympathetic nervous system stimulates the release of cytokines and sympathetic nerves may also serve as a source of cytokines[218]. TNF-α plays as well an important role in angiotensin II-dependent and other forms of hypertension[196, 219-221]. The interactions between TNF-α and angiotensin II is known to promote vascular inflammation[222]. Administration of angiotensin II for 2 weeks causes increases in salt and water intake, increase in blood pressure and cardiac hypertrophy in mice. TNF-$\alpha^{-/-}$ mice were reported to be protected against these responses, suggesting that TNF-α plays a mechanistic role in mediating the effects induced by angiotensin II[222-223]. In angiotensin high-salt hypertension, treatment with the anti-TNF-α etanercept regulated the increase in blood pressure[224]. Angiotensin II-induced hypertension has been reported to increase the production of TNF-α by T lymphocyte. Moreover, treatment with etanercept during angiotensin II infusion prevented both hypertension and increase in vascular superoxide[196].

Nevertheless, a direct molecular mechanism linking the presence of vascular TNF-α and increase in vasoconstriction has not yet been described.

Excess weight and vascular dysfunction

The concept of adipose tissue as a merely storage of fatty acids has been replaced over the last years. In the hypertrophied adipocyte, there is decreased expression of adipokines with anti-inflammatory properties like adiponectin, and secretion of free fatty acids and inflammatory factors that may contribute to the increased cardiovascular risk linked to obesity[225-226] (Figure 2).

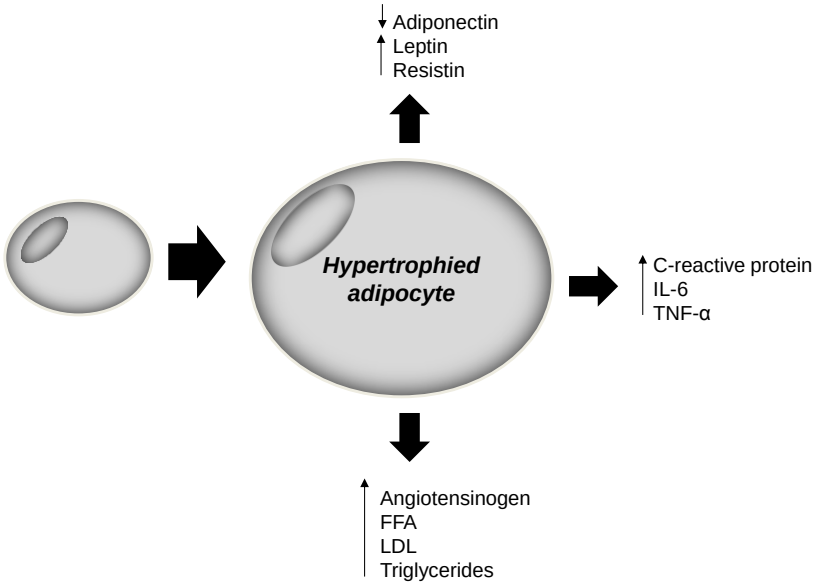


Figure 2. *The hypertrophied adipocyte: a source of cytokines, hormones and lipids.*

Similarly to hypertension, the association of vascular dysfunction and obesity is well known[20, 58, 227]. Obesity is associated to infiltration of the expanded adipose tissue by macrophages[228] and increased levels in proinflammatory cytokines[229-230]. This highly active adipose tissue concentrates in the upper body and visceral cavity. This dysfunctional adipose tissue influences vascular function in a complex network[20] (Figure 3), therefore it is difficult to distinguish the specific roles each play through excess weight progression and when and how far the adipose tissue or a vascular signaling itself is contributing to bad vascular outcomes.

The first indication for increased cytokine release in obesity was provided by the identification of increased expression of TNF-α in the adipose tissue of obese mice in the early 1990s[11]. TNF-α is expressed in and secreted by adipose tissue and its levels correlates with the degree of adiposity and the associated insulin resistance[8, 15]. Therefore, TNF-α can be assumed as inflammatory contributor of the elevated cardiovascular risk in obesity, since it is related to the low-grade inflammatory state in obesity[12-13, 98, 231].

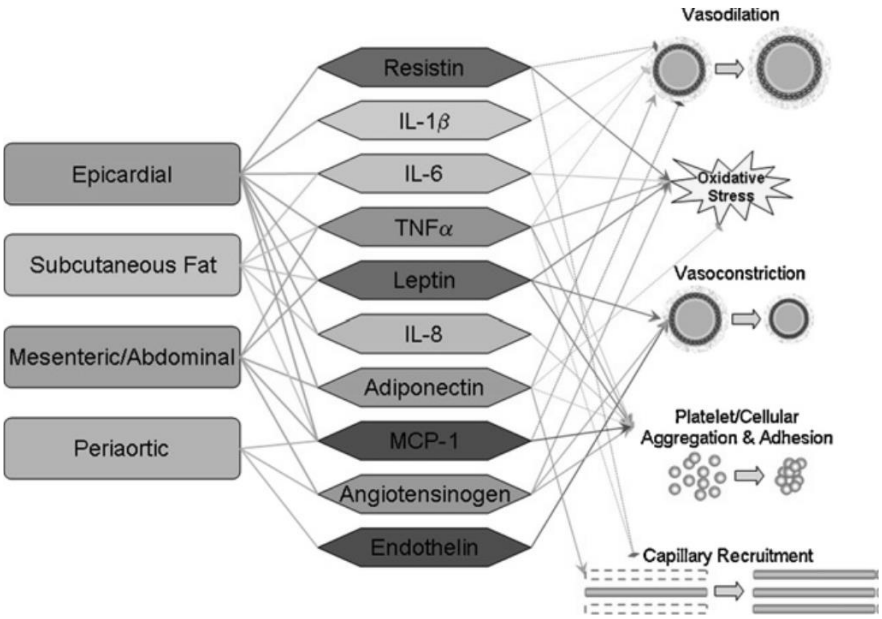


Figure 3. *Figure extracted from P.A. Stapleton et al.[20]: a schematic representation of adipose tissue depositions, the adipokines, inflammatory markers and other factors that are released from these tissues, and their identified impact on elements of vascular function. Pointed arrows indicate potentiating effects on vascular outcome, while flat headed arrows indicate inhibitory effects on vascular outcome.*

Apart from the specific effects on endothelium and VSMCs already here described, concerning the obesity context TNF-α has been as well associated with impaired capillary recruitment, elevated adhesion of polymorphonuclear leukocytes to microvessels and induction of oxidative stress[20].

Recently, the proinflammatory state promoted by the adipose tissue around the vessels, the so-called perivascular adipose tissue (PVAT), has been proposed to importantly influence the vasculature due to the close relationship, virtually without any barrier (Figure 4). The paracrine and vasocrine signaling originated from a dysfunctional PVAT might connect the obesity-associated pathologies to vascular diseases[232-233].

In obesity, PVAT becomes inflamed and secretes vasoconstrictor factors and proinflammatory adipokines such as TNF-α, inducing vascular dysfunction[234-236].

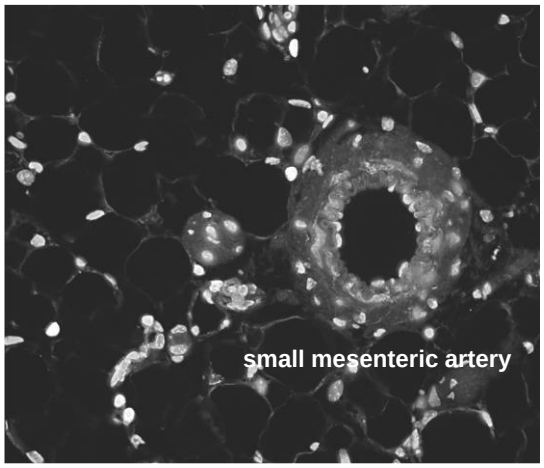


Figure 4. *A small mesenteric artery and surrounding PVAT. The close anatomical relationship suggests that mediators secreted by PVAT can easily access the blood vessel wall.*

In this background, Greenstein et al. demonstrated that PVAT from small arteries from obese patients lost the anti-contractile mechanisms present in the healthy PVAT, which results in an enhanced vasoconstriction state, probably involving vascular inflammation elicit by TNF-α[233]. Its apparently elevated production by PVAT shows that circulating TNF-α measured in serum may give unrepresentatively low levels, in comparison to local active concentrations.

The sympathetic nervous system is also involved in the physiopathology of excess weight-related vascular outcomes. Sympathetic neural hyperactivity has been shown to reduce vasodilatation, increase vascular resistance and decrease blood flow[237-238].
In summary, the physiopathological mechanism behind the relationship between excess weight and increased cardiovascular risk is certainly multifactorial. However, a relevant role for vascular dysfunction itself, independent of the adipose tissue, has not been yet described in the context of overweight.

The proposal of vascular dysfunction as a key interface between blood pressure elevation and excess weight is novel and challenging. A better understanding of this complex process may bring new ideas to understand the ongoing concept that the overweight status is as dangerous as obesity[239-240]. Therefore, the focus of this thesis is to study vascular features associated to increased cardiovascular risk in a mouse model of overweight induced by high-fat diet where some of the important factors present in obesity status that influence vascular function are not yet present, such as an inflamed adipose tissue, insulin resistance and systemic inflammation.

4. Aims of the study

The following questions and respective aims will be addressed to gain insight in the physiopathology of cardiovascular risk factors associated to overweight:

Is there a good mouse model to mimic the overweight condition with cardiovascular risk factors?

1. To characterize a mouse model for overweight
2. To describe the vascular features in overweight
3. To identify possible determinants of the vascular features

What is the role played by the adipose tissue on overweight concerning the vascular features?

4. To characterize the fat tissue in overweight, when obesity has not yet developed and its role on the vascular features.

What is the mechanism behind the vascular features associated to overweight?

5. To describe a mechanism linking the vascular features and the overweight status.

5. Materials and Methods

Animals and diets

Male wild-type C57BL/6J (WT) mice, tumor necrosis factor receptor 1 knockout (TNFR1$^{-/-}$) and toll-like receptor 4 knockout (TLR4$^{-/-}$) with C57BL/6J background mice were used for the studies. Mice were housed in temperature-controlled cages (20°C to 22°C) and maintained on a 12/12-hour light/dark cycle. The TNFR1$^{-/-}$ (B6.129-*Tnfrsf1a*^{tm1Mak}) and the TLR4$^{-/-}$mice were originally purchased from the Jackson Laboratory (Bar Harbor, Me., USA) and bred in the Central Animal Facility (University Hospital Innenstadt, Ludwig-Maximilians-University Munich). WT mice were obtained from Charles River Laboratories (Sulzfeld, Germany).

At the age of 12 weeks mice were placed either on a normal diet containing 12.8 MJ/kg metabolizable energy with 9% of its energy from fat (V1534-000 Ssniff, Soest, Germany) or 2 weeks of high-fat diet containing 20.1 MJ/kg metabolizable energy with 51% of its energy from fat (E15126-34 Ssniff, Soest, Germany). The ingredients of both diets are given in Table 1. The standard diet derived its fat from soy oil, whereas the high-fat diet derived its fat from beef tallow. Animals had *ad libitum* access to water and diets. The principles of the NIH Guide for the Care and Use of Laboratory Animals as well as the German Law on the Protection of Animals were followed.

Ingredients of normal diet and high-fat diet		
Ingredient	**Normal diet**	**High-fat diet**
Crude nutrients	proportion (%)	
Crude proteins	19.0	20.7
Crude fat	3.3	30.2
Crude fiber	4.9	5.0
Dry substance	87.7	96.7
Sodium	0.24	0.19
Metabolizable energy	MJ/kg	
	12.8	20.1
	% of energy	
Fat	9.0	51.0
Carbohydrate	58.0	26.0
Protein	33.0	23.0
Both diets contain vitamins, trace elements and minerals.		

Tabel 1. *Composition of diets used in this thesis.*

Materials

Chemicals, reagents and kits:

Acetic acid	Sigma-Aldrich, Missouri, USA
Acetone	Sigma-Aldrich, Missouri, USA
Acetylcholine	Sigma-Aldrich, Missouri, USA
BCA Protein Assay Reagent Kit Pierce	Thermo Fisher Scientific, Waltham, USA
β – Mercaptoethanol	Carl Roth, Karlsruhe, Germany Bovine Serum Albumin
Calcium chloride	Sigma-Aldrich, Missouri, USA
CelLytic MT, Tissue Lysis/Extraction Reagent	Sigma-Aldrich, Missouri, USA
CelLytic MT, Cell Lysis/Extraction Reagent	Sigma-Aldrich, Missouri, USA
Complete Ultra, Protease inhibitor cocktail	Roche, Basel, Switzerland
Distilled water (sterile)	B. Braun, Melsungen, Germany
DTT (Dithiothreitol)	Sigma-Aldrich, Missouri, USA
Dulbecco's Modified Eagle Medium High Glucose	PAA, Linz, Austria
Dulbecco's PBS	PAA, Linz, Austria
EDTA (Ethylenediaminetetraacetic acid)	Sigma-Aldrich, Missouri, USA
Elastase	Sigma-Aldrich, Missouri, USA
Eosin	Carl Roth, Karlsruhe, Germany
Etanercept (Enbrel®)	Pfizer, New York, USA
Ethanol (70%, 96%, 100%)	Apotheke Klinikum r.d. Isar, Munich, Germany
Fasudil	LC laboratories
Fetal Bovine Serum	Sigma-Aldrich, Missouri, USA
Glucose	Sigma-Aldrich, Missouri, USA
HEPES (hydroxyethyl piperazineethanesulfonic acid)	Sigma-Aldrich, Missouri, USA
HBSS (Hanks' balanced salt solution*)*	Sigma-Aldrich, Missouri, USA
Insulin ELISA	Shibayagi Co. Ltd.
IL-6 ELISA	Peprotech, Hamburg, Germany
Isofluran (Forane®)	Baxter, Illinois, USA
iQ SYBR Green Supermix	BioRad, Hercules, USA
iScript cDNA Synthesis Kit	BioRad, Hercules, USA
Luminata Forte Western Substrate	Millipore, Billerica, USA
Magnesium sulfate 7-hydrate ($MgSO_4$ $7H_20$)	Carl Roth, Karlsruhe, Germany
Methanol	Klinikum r.d. Isar‘s pharmacy, Munich, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
MOPS (3-(N-Morpholino)-Propansulfonsäure)	Sigma-Aldrich, Missouri, USA

Noradrenaline	Sigma-Aldrich, Missouri, USA
Paraformaldehyde	Sigma-Aldrich, Missouri, USA
Paraffin	Carl Roth, Karlsruhe, Germany
Pentobarbital	Sigma-Aldrich, Missouri, USA
Phosphatase inhibitor cocktail (Phosstop)	Roche, Basel, Switzerland
Ponceau S	Sigma-Aldrich, Missouri, USA
Potassium chloride (KCl)	Sigma-Aldrich, Missouri, USA
Pravastatin (Pravachol®)	Bristol-Myers Squibb, New York, USA
Primers	Sigma-Aldrich, Missouri, USA
Protein marker, Precision Plus	BioRad, Hercules, USA
Sodium chloride (NaCl)	Sigma-Aldrich, Missouri, USA
Sodium chloride solution 0.9%	B. Braun, Melsungen, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth, Karlsruhe, Germany
Sodium pyruvate	Sigma-Aldrich, Missouri, USA
Sodium palmitate	Sigma-Aldrich, Missouri, USA
Soybean trypsin inhibitor	Life Technologies, Carlsbad, USA
Recombinant mouse TNF-α	Millipore, Billerica, USA
Recombinant mouse adiponectin	Millipore, Billerica, USA
Rho-associated kinase activity assay	Cell Biolabs, San Diego, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
TaqMan Protein Assay	Applied Biosystems, Foster City, USA
TNF-α ELISA	Peprotech, Hamburg, Germany
Tween 20	Carl Roth, Karlsruhe, Germany
Xylol	Klinikum r.d. Isar‘s pharmacy, Munich, Germany
Y-27632 compound	Sigma-Aldrich, Missouri, USA

Antibodies:

Anti-mouse TNF-α	Santa Cruz, Santa Cruz, USA
Anti-mouse CD115	eBioscience, San Diego, USA
Anti-mouse CD45	eBioscience, San Diego, USA
Anti-mouse GR-1	eBioscience, San Diego, USA
Anti-mouse GAPDH	Cell signaling, Beverly, USA
Anti-mouse MYPT1	Millipore, Billerica, USA
Anti-mouse phosphorylated MYPT1	Millipore, Billerica, USA
Anti-goat horseradish peroxidase conjugated-donkey	Santa Cruz, Santa Cruz, USA
Goat anti-mouse adiponectin polyclonal antibody	R&D Systems, Minneapolis, USA

Secondary antibody anti-rabbit	Santa Cruz, Santa Cruz, USA

Buffers used in myograph experiments (concentration in mM):

MOPS buffer: NaCl 145, KCl, 4.7, $CaCl_2$ 3.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 0.02

Calcium-free MOPS buffer: NaCl 147, KCl, 4.7, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 1.00

K+ 125 mM MOPS buffer, Ca^{2+} 0 mM: NaCl 26.5, KCl 125, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 1.00

K+ 125 mM MOPS buffer, Ca^{2+} 0.5 mM: NaCl 27.2, KCl 125, $CaCl_2$ 0.5, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 0.02

K+ 125 mM MOPS buffer, Ca^{2+} 1.0 mM: NaCl 26.7, KCl 125, $CaCl_2$ 1.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 0.02

K+ 125 mM MOPS buffer, Ca^{2+} 2.0 mM: NaCl 25.7, KCl 125, $CaCl_2$ 2.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 0.02

K+ 125 mM MOPS buffer, Ca^{2+} 3.0 mM: NaCl 24.7, KCl 125, $CaCl_2$ 3.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, Pyruvate 2.0, Glucose 5.0, MOPS 3.0, EDTA 0.02

Concentrations given in the text refer to the final organ bath concentrations.

Machines and Instruments:

Autoclave, H+P/Varioklav	Thermo Fisher Scientific, Waltham, USA
Incubator, Heracell	Heraeus, Osterode, Germany
ELISA Reader, Multiskan FC	Thermo Fisher Scientific, Waltham, USA
FLUOstar Omega Multidetection Microplate Reader	BMG Labtech, Ortenberg, Germany
Freezer	Thermo Fisher Scientific, Waltham, USA
iBlot 7-Minute Blotting System	Invitrogen, Carlsbad, USA
Immobilon-P Transfer Membrane	Millipore, Billerica, USA
Kryostat, Leica CM 3050	Leica, Wetzlar, Germany
Microscope, Leica MZ 16	Leica, Wetzlar, Germany
Microtome, Leica RM 2145	Leica, Wetzlar, Germany

NanoDrop, ND-1000	Thermo Fisher Scientific, Waltham, USA
pH-Meter	Sartorius, Göttingen, Germany
Photometer, Multiscan FC	Thermo Fisher Scientific, Waltham, USA
Real Time PCR System, MyiQ	BioRad, Hercules, USA
Water purification system	Millipore, Billerica, USA
Tissue lyser	Qiagen, Hilden, Germany
Thriller thermoshaker incubator	Peqlab, Erlangen, Germany
Thermo EC 3000P Programmable Controller	Thermo Fisher Scientific, Waltham,U SA
Precise weighting machine	Sartorius, Göttingen, Germany
Water bath with thermostat, SW 21	Julabo, Seelbach, Germany
Wire Myograph, Model 620M	Danish Myograph Technology, Aarhus, Denmark
Centrifuge, Biofuge/Multifuge 3 L-R	Thermo Fisher Scientific, Waltham, USA
1.5T MRI System Achieva	Philips Medical Systems, Best, The Netherlands
Blood pressure monitor BP1	World Prec. Instruments, Sarasota, USA
Clamp forceps	Aesculap, Tuttlingen, Germany
Microsurgery forceps	Fine Science Instruments, North Vancouver, Canada
Microsurgery scissors	Fine Science Instruments, North Vancouver, Canada

Accessories and supplies:

Cell strainer	BD, Heidelberg, Germany
Centrifuge tubes	Eppendorf, Hamburg, Germany
Serum separator tubes	Microvette, USA
Falcons	BD, Heidelberg, Germany
Cuvettes	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Polyvinylidene difluoride (PVDF) membranes	Millipore, Billerica, USA
Syringes	B. Braun, Melsungen, Germany
Tissue culture flasks	TPP Techno Plastic Products, Switzerland
Tungsten wire (40 µm)	Danish Myograph Technology, Aarhus, Denmark

Methods

Therapeutic interventions

- WT mice were treated with the anti-TNF-α antibody etanercept 10 mg/kg twice per week as intraperitoneal injection. Corresponding control groups received etanercept as intraperitoneal injection.
- One group of WT mice were treated with HFD plus concomitant treatment with the Rho-kinase inhibitor fasudil 30 mg/kg/day in drinking water.
- WT mice were treated with HFD plus concomitant treatment with pravastatin, 50 mg/kg/day in drinking water. Corresponding control groups received pravastatin in drinking water.
- In another set of WT mice on HFD, the HFD was withdrawn and normal diet was returned for 2 weeks. Their control groups either received normal diet or were kept on HFD for 4 weeks.

Magnetic resonance imaging

Whole body magnetic resonance imaging was performed on mice anesthetized with intraperitoneal pentobarbital and placed in prone position onto a 47-mm microscopy surface coil inside the clinical 1.5 T MRI. An axial multi-slice turbo spin echo (TSE) sequence (resolution 0.25 x 0.25 x 0.35 mm^3, 140 slices, echo time (TE) = 100 ms, repetition time (TR) = 1000 ms) was applied to allow signal suppression from tissue other than fat. The whole body images were reconstructed using OsiriX DICOM viewer.

Organ harvesting and blood pressure measurement

During isoflurane anesthesia mice were fixed on a heat-controlled plate and the left carotid artery was exposed. An intra-arterial pressure transducer was inserted in the left carotid artery under sterile conditions and continuous intra-arterial blood pressure was measured for 15 minutes. Thereafter animals were sacrificed and the entire intestine with vascular arcades was immediately excised and kept in cold MOPS buffer for myograph experiments. All animals were analyzed concerning changes in whole body weight and epididymal fat pad mass, as a parameter to assess visceral fat status. Serum was obtained in serum separator tubes and stored at -80°C for subsequent biochemical measurements.

Biochemical measurements

Serum fasting total cholesterol, triglycerides and glucose levels were measured by enzymatic methods (Roche Diagnostics). Fasting insulin was measured by ELISA. Calculation of the insulin resistance index [homeostasis model assessment (HOMA-IR)] was made using fasting insulin and glucose values: [insulin (picomoles per liter) x glucose (millimolar)] /22.5.[241-242] Serum TNF-α and IL-6 were measured by ELISA.

Isolation of vascular smooth muscle cells and culture experiments

Primary vascular smooth muscle cells (VSMCs) were isolated by enzymatic digestion in a solution containing collagenase type II 1mg/ml, soybean trypsin inhibitor 1mg/ml (Life Technologies), elastase 0,744u/ml in Hanks' Balanced Salt Solution (HBSS). Isolated aortas from 2 mice were cleaned from PVAT and predigested for 10min. Subsequently adventitia was removed, aortas were cut lengthwise and intima was removed by gentle scraping. Aortas were enzymatically digested for 1h at 37˚C[243]. After digestion aortas were passed through 100um cell strainer and left undisturbed on 3 wells of a 48 well plate for 1 week. Until passage number 3, cells were cultivated in Dulbecco`s Modified Eagle Medium **(**DMEM) medium with 20% fetal bovine serum. After the 3rd passage VSMCs were cultivated in DMEM medium containing 4,5mg/l Glucose, 2mM L-glu, supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 10% fetal bovine serum. In one set of experiment VSMCs were treated with TNF-α 40 and 100ng/ml for 6 hours. In another set, TNF-α was used in concentrations of 100ng/ml for 6 and 12 hours. Palmitate was used in the concentration of 200µM for 12 hours.

Histology

Epididymal fat pads were fixed in paraformaldehyde, embedded in paraffin and subsequently stained with hematoxylin-eosin. For each mouse the area of 50 randomly chosen adipocytes was measured in 5 representative sections using Image J software at 10x magnification. Mean values given in pixels were compared. Analysis of infiltrating macrophages and lymphocytes in the fat pads was performed by immunohistochemical staining for CD45. Total CD45-positive cells were counted in 10 randomly chosen fields per mouse and averaging the number. Immunostaining for TNF-α was performed in mesenteric vascular beds and surrounding adipose tissue.

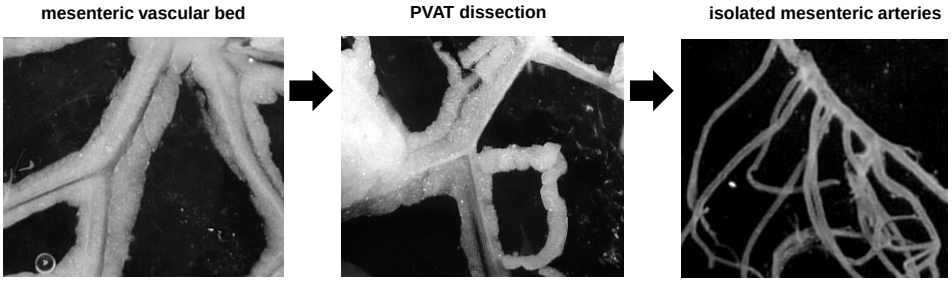
Two-photon microscopy

Small mesenteric arteries and surrounding adipocytes were visualized using a Leica SP5 II MP two-photon laser scanning microscope coupled to a water dipping 20X;NA 1.00

objective and a pre-chirped Ti:Sa laser (Spectra Physics) tuned to 840nm[244]. Four Hybrid detectors (HyD) were spectrally tuned for optimal detection efficiency and low bleed through of signal: Second Harmonic Generation of collagen (HyD1: 400-425nm), autofluorescence of adipocytes and GR1/eFluor450 (HyD2: 445-500nm), autofluorescence of adipocytes/elastin, and CD115/Alexa488 (HyD3: 515-555nm) CD45/nanocrystal-605nm (HyD4: 590-625nm). Additional image processing was performed using Leica LAF AF 3.0 and ImagePro number of inflammatory cells was determined in 3D datasets in the adipose tissue directly surrounding the small mesenteric arteries (up to a distance of three times the average adipocyte diameter from the small artery). Quantification of inflammatory cells was performed by detection of the number of CD45 /CD115 and CD45/Gr-1 positive cells in each arterial segment recorded in 3D datasets (n = 3 arterial segments per mouse; n = 4 mice per group). The total number of positive cells is presented as inflammatory cells per adipocyte. Adipocyte volume was determined by measuring their maximal diameter in the corresponding arterial segment assuming spherical cell shape.

RNA isolation and real-time PCR

Dissected mesenteric PVAT and epididymal fat tissue were snap-frozen in liquid nitrogen and stored at -80ºC. The amount of 80 mg was used for RNA isolation using RNeasy Lipid Tissue mini kit according to the manufacturer's protocol. The mesenteric arterial tree was rapidly cleaned of surrounding PVAT under a dissection microscope:



The arterial tree was snap-frozen in liquid nitrogen, stored at -80ºC and used later on for RNA isolation, performed with RNeasy Mini kit according to the manufacturer's protocol. Quality of RNA was assessed in agarose gel stained with ethidium bromide. Complementary DNA was synthesized using iScript cDNA Synthesis Kit, according to manufacturer's protocol. Quantitative reverse transcriptase PCR was performed using SYBR Green I. Sense and anti-sense cDNA primers were as follows:

Gene	***Forward***	***Reverse***
GAPDH	TCGGTGTGAACGGATTTGGC	TTTGGCTCCACCCTTCAAGTG
TNF-α	CCAAAGGGATGAGAAGTTCC	GGCAGAGAGGAGGTTGACTTT
IL-6	CTGGGAAATCGTGGAAATGAG	ACTCTGGCTTTGTCTTTCTTG
TLR4	ATTCCCTCAGCACTCTTGATT	AGTTGCCGTTTCTTGTTCTTC
MCP-1	GCTGTAGTTTTTGTCACCAAG	GATTTACGGGTCAACTTCACA
RhoA	CTCTCTTATCCAGACACCGAT	CAAAAACCTCTCTCACTCCATC
ROCK1	AAGGCGGTGATGGCTATTATG	TCCTCTACACCATTTCTGCCC
ROCK2	ATGTGATTGGTGGTCTGTAGGT	AGCTGCCGTCTCTCTTATGTTA

The ddCt algorithm method was applied for quantification, including the normalization either to beta-actin or GAPDH of each sample. Values are reported as fold of controls [245].

Protein lysates preparation

The whole mesenteric vascular tree was rapidly isolated and cleaned from PVAT to obtain only the arteries, (as similarly prepared for the RNA isolation). Per sample, mesenteric arteries or PVAT from one mouse were immediately lysed in lysis buffer (CellLytic MT Mammalian Tissue lysis/extraction reagent) containing phosphatases and proteases inhibitors tablets. The lysate was cleared by centrifugation at 15.000g for 15 min at 4°C. The supernatant was collected. Protein concentration was measured by a bicinchoninic acid protein assay reagent kit and immediately used for Rho-associated kinase activity and TaqMan protein assays.

Cultured VSMCs were harvested with lysis buffer (CellLytic Cell lysis/extraction reagent) containing phosphatases and proteases inhibitors tablets. The lysate was cleared by centrifugation at 15.000g for 15 min at 4°C. The supernatant was collected. Protein concentration was measured by a bicinchoninic acid protein assay reagent kit and used for Rho-associated kinase activity and pMYPT1/MYPT1 Western blotting.

Western Blot

Total adiponectin and adiponectin multimers were determined by western blotting in serum. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was

performed. In brief, serum proteins were separated by 10% SDS-PAGE under non-reducing and non-heating conditions, and transferred to nitrocellulose membranes. Membranes were blocked with Tris Buffered Saline (TBS)-Tween 20 containing 5% skim milk and incubated with a goat anti-mouse adiponectin polyclonal antibody (1:500). After being washed, membranes were incubated with horseradish peroxidase conjugated-donkey antigoat antibody (1:4000). Bands were visualized by using lumi-light western blotting substrate, and the image was acquired with a Kodak IS440CF Imaging Station. Densitometry analysis was performed with Adobe Photoshop software. Relative distributions of adiponectin multimers were calculated by dividing band density by total density.

Total MYPT1 and pMYPT1 were determined by western blotting in primary aortic smooth muscle cells. Primary VSMCs were starved overnight with DMEM medium containing 2% fetal bovine serum and subsequently treated with TNF-α 40 and 100ng/ml for 6 hours. Sixty ug of protein per sample was loaded per lane and resolved by SDS-PAGE under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membranes using iBlot transfer device. Membranes were blocked by treatment with 5% bovine serum albumin in TBS and subsequently probed with antibodies against MYPT1 (1:800), phosphorylated MYPT1 (p-MYPT1, 1:80), GAPDH (1:15000) and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000). Immunoreactivity was visualized by adding Luminata Forte Western Substrate and detected by Intas imaging system.

Rho-associated kinase activity

Rho-associated kinase activity was measured by an enzyme immunoassay in mesenteric arteries, mesenteric PVAT and epididymal fat. Experiments were performed according to the manufacturer's protocol, using 10 µg of protein lysate. Values are reported as percentage of activity related to controls.

TNF-α protein expression

TNF-α protein expression was measured using TaqMan Protein assay in mesenteric arteries using a mouse TNF-α biotinylated antibody, according to the manufacturer's instructions. Protein lysates from mesenteric arteries were incubated with two pools of TNF-α antibody labeled with two types of oligonucleotides. Subsequently, oligonucleotides ligation was performed and the prepared templates were amplified and

analyzed by quantitative PCR using TaqMan probes. Pure TNF-α was used as positive control. Values are reported as fold of controls.

Wire myograph and contractility studies

The mesenteric circulation from rodents influences regulation of peripheral vascular resistance and is widely accepted as a model to access microcirculation function[63, 246], with good correlation to the human omental microcirculation[247].

Basic protocol

First to second order branches from superior mesenteric artery (270-330 µm) were cut into 2 mm long rings and mounted in a 4-channel wire myograph. Each vessel segment was mounted on two tungsten wires (40 µm diameter) in the organ chamber filled with MOPS buffer (Figure 5).

Vessels were pre-stretched to a tension representing a blood pressure of 13.3 kilopascal and equilibrated at this tension for 30 minutes at 37°C[185]. Subsequently, the organ bath solution was changed for a fresh pre-heated MOPS buffer and vascular functions were analyzed. During the experiments, the diameter of vessels is kept constant, so the vessels are examined under isometric conditions.

The myograph gives output readings as absolute tension generated elicited by vasoconstriction/vasodilatation in millinewton (mN).

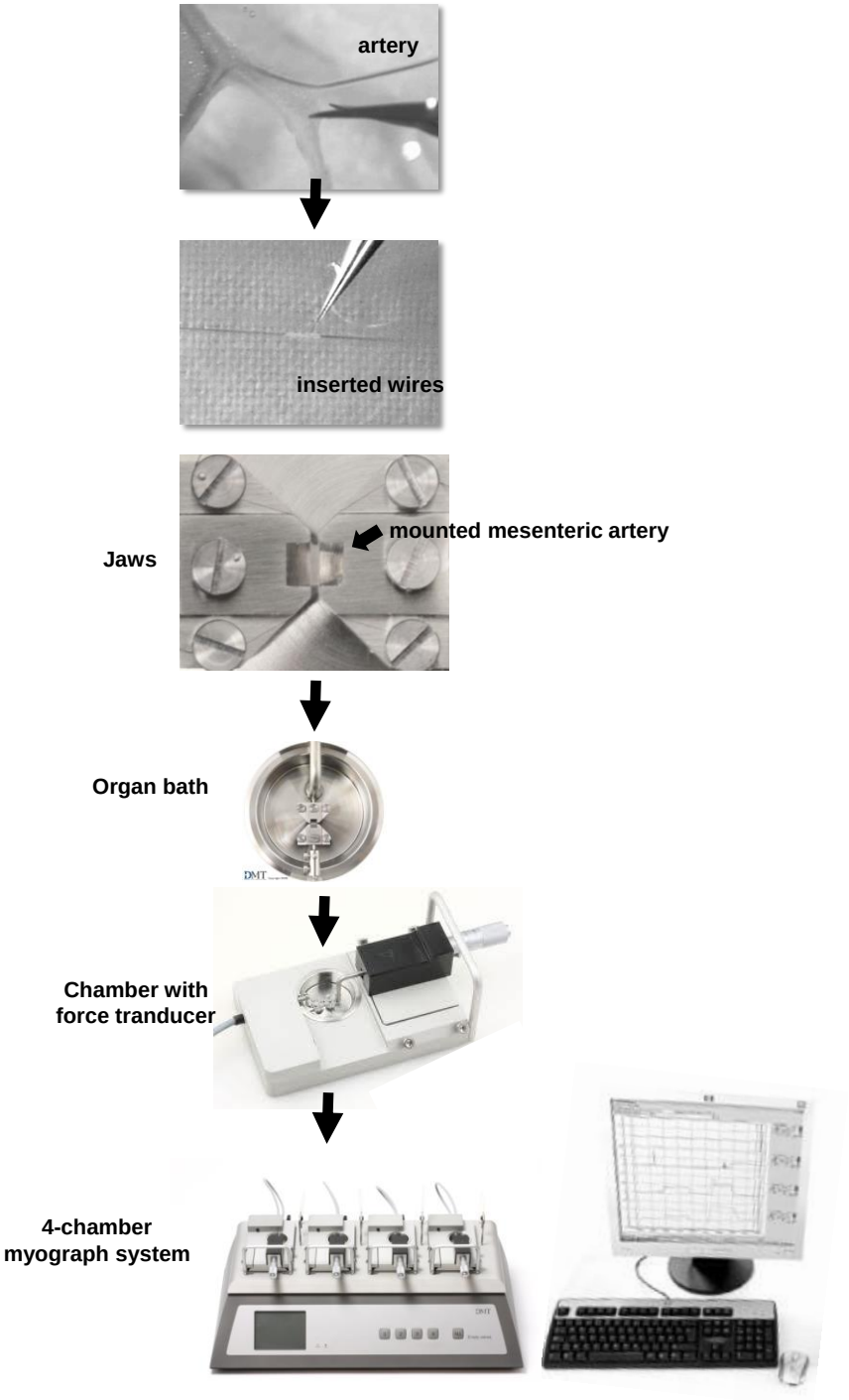


Figure 5. *The wire myograph (620M) system. After cleaned from PVAT, small mesenteric arteries arteries are mounted in the myograph chamber with two tungsten wires. Each organ bath is connected to a force transducer. The device allows the study of 4 vessels in parallel.*

For testing viability, vessels were subjected to noradrenaline-induced constriction followed by acetylcholine (Figure 6). Noradrenaline is a widely used constricting agent that exerts a well-sustained contractile response by activating α1-adrenoceptors. The main mechanism for α1- agonist-mediated vasoconstriction involves the release of Ca^{+2} from the sarcoplasmic reticulum and subsequent depolarization by activation of chloride channels. This results in augmented extracellular Ca^{+2} influx through voltage gated L-type Ca^{+2} channels in the plasma membrane of the VSMC leading to an increase in intracellular Ca^{+2} concentration and vasoconstriction[248].

Acetylcholine is the classical compound exerting endothelium-dependent vasodilatation due to stimulation of muscarinic receptors in the presence of an intact endothelium. Acetylcholine leads to liberation of nitric oxide from endothelium and also opening of the potassium channels in the VSMC thereby leading to hyperpolarization[249]. Vessels with endothelium-dependent relaxation to acetylcholine above 70% of the maximal noradrenaline vasoconstriction were considered with intact endothelium.

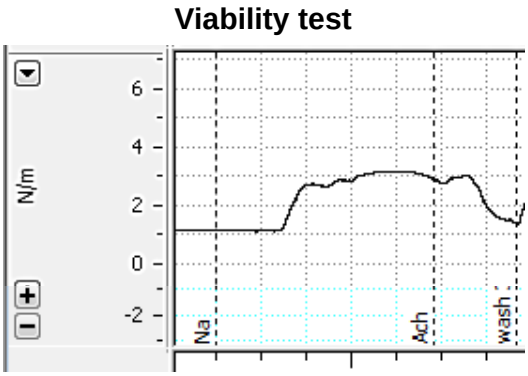


Figure 6. *In the viability test the small mesenteric artery is challenged with 10^{-5} mol/L of noradrenaline (Na) followed by 10^{-5} mol/L of acetylcholine (Ach). The generated force is displayed on the left axis in millinewtons.*

After washing out with MOPS buffer and resting for 15 minutes, noradrenaline and acetylcholine dose-response curves were performed (Figure 7).

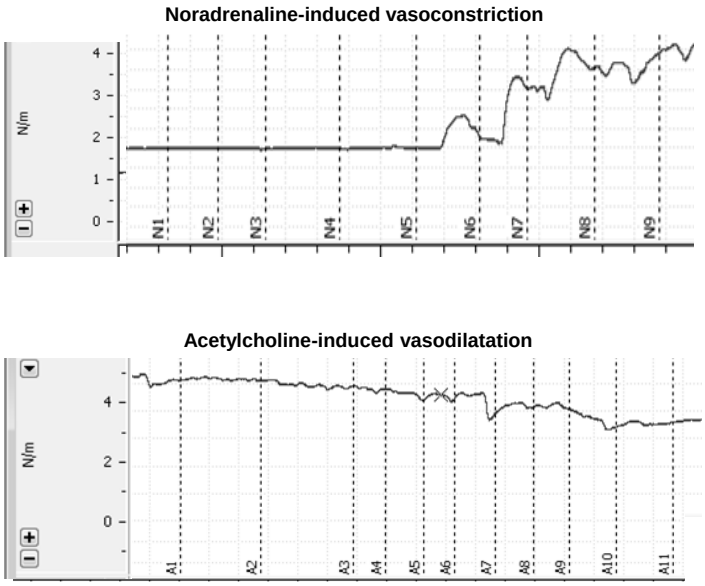


Figure 7. *Noradrenaline-induced vasoconstriction and acetylcholine-induced vasodilatation in the wire myograph.*

Noradrenaline was used in stepwise increases in concentration, from 10^{-9} to 10^{-5} mol/L (N1 to N9 in Figure 7), followed by acetylcholine dose-response curve from 10^{-10} to 10^{-5} mol/L (A1 to A11 in Figure 7). Following the dose-response curves, vessels were washed with MOPS buffer and left resting for 20 minutes.

Next, calcium-induced vasoconstriction was performed, directly followed by sodium nitroprusside-induced vasodilatation (Figure 8). Vessels were first depleted of calcium with a calcium-free MOPS buffer plus high ethylenediaminetetraacetic acid (EDTA) concentration (0/4.7 mmol/L potassium buffer). Then a high-potassium calcium-free depolarizing solution plus high EDTA concentration was used (0/125 mmol/L potassium buffer), followed by stepwise increases in calcium concentration in the chamber, always under depolarizing conditions (0.25-3.0 mmol/L calcium + 125 mmol/L potassium buffers).

To study endothelium-independent vasodilatation, sodium nitroprusside (NO donor) was used in concentrations from 10^{-10} to 10^{-5} mol/L (S1 to S11 in the myograph curves in Figure 8).

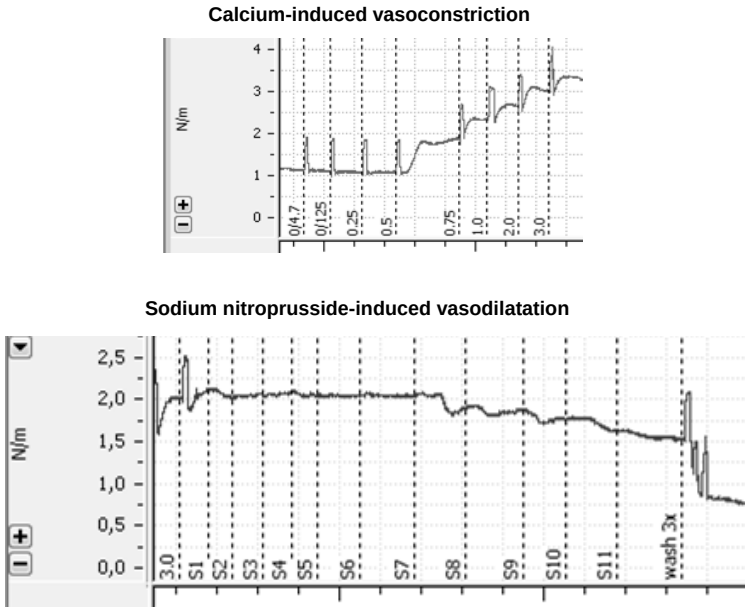


Figure 8. *Calcium-induced vasoconstriction and sodium nitroprusside-induced vasodilatation dose-response curves.*

Incubation with TNF-α

Small mesenteric arteries from WT mice (on normal diet) were incubated with TNF-α in the concentration of 20ng/ml for 30 minutes. Noradrenaline-induced vasoconstriction was investigated before and after TNF-α exposure. Control experiments were also performed to investigate the influence of endothelium and of the incubation time on vascular responses.

Incubation with Y-27632 (Rho-kinase inhibitor)

Small mesenteric arteries from control and overweight WT mice were incubated in the chamber with the Rho-kinase inhibitor Y-27632 (1μM for 10 minutes). Y-27632 was dissolved in distilled water. Control experiments were also performed to investigate the influence of the incubation time on vascular responses. Calcium-induced vasoconstriction was performed before and after incubation with Y-27632.

Removal of endothelium

In some experiments, endothelium was removed by gently rubbing (one time) the intimal surface of the small mesenteric artery with a human hair. The effectiveness of the procedure was confirmed by the subsequent absence of relaxation to acetylcholine 10^{-5} mol/L (Figure 9).

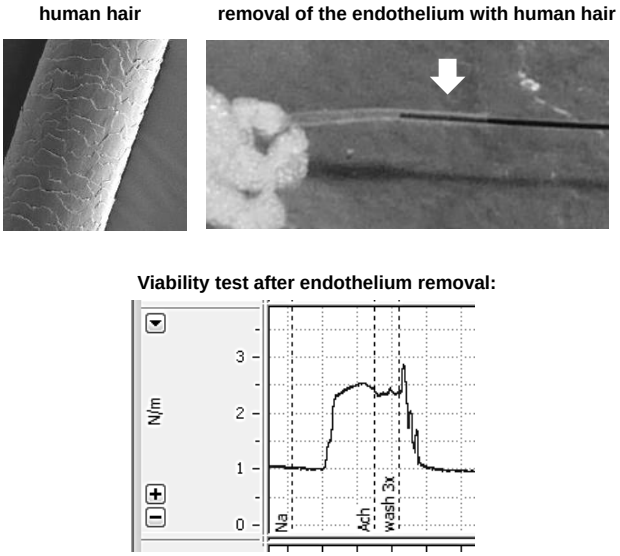


Figure 9. *Flattened scales seen in human hair. Gentle rubbing of the intimal surface of the artery with a human hair removes the endothelial layer, seen in the absence of relaxation to acetylcholine in the viability test.*

Statistical analysis

All data are presented as mean ± SEM. In cumulative dose-response curves of myograph experiments, logEC50 of each mouse (2-4 arteries) was calculated. Differences between logEC50 were calculated using a non-linear regression analysis. For comparisons of absolute tensions among groups, two-way ANOVA test with Bonferroni post hoc test was used. Student's t test was used for comparisons between two groups. In other experiments, for comparisons between groups one-way ANOVA with Tukey post hoc test was used.

All tests were performed with GraphPad Prism ® 5.0. A probability value <0.05 was considered statistically significant.

6. Results

The obesity model

With the aim to address cardiovascular risk factors present in overweight condition, we investigated initially the vascular features of a well-studied diet-induced obesity model.
As expected, WT mice fed a HFD for 8 weeks developed features of obesity. There was markedly difference in percentage of body weight gain: control WT mice presented 17% ± 4.0 and obese WT mice presented 24.2% ± 2.0, $P<0.05$.

Intra-abdominal adipose tissue was importantly augmented. Particularly the visceral fat tissue represented here by the mesenteric PVAT and the epididymal fat pads were significantly increased compared to controls. There was important adipocyte hypertrophy with mean adipocyte area 64% bigger in obese WT mice. The control group was age- and body weight-matched (Figure 10).

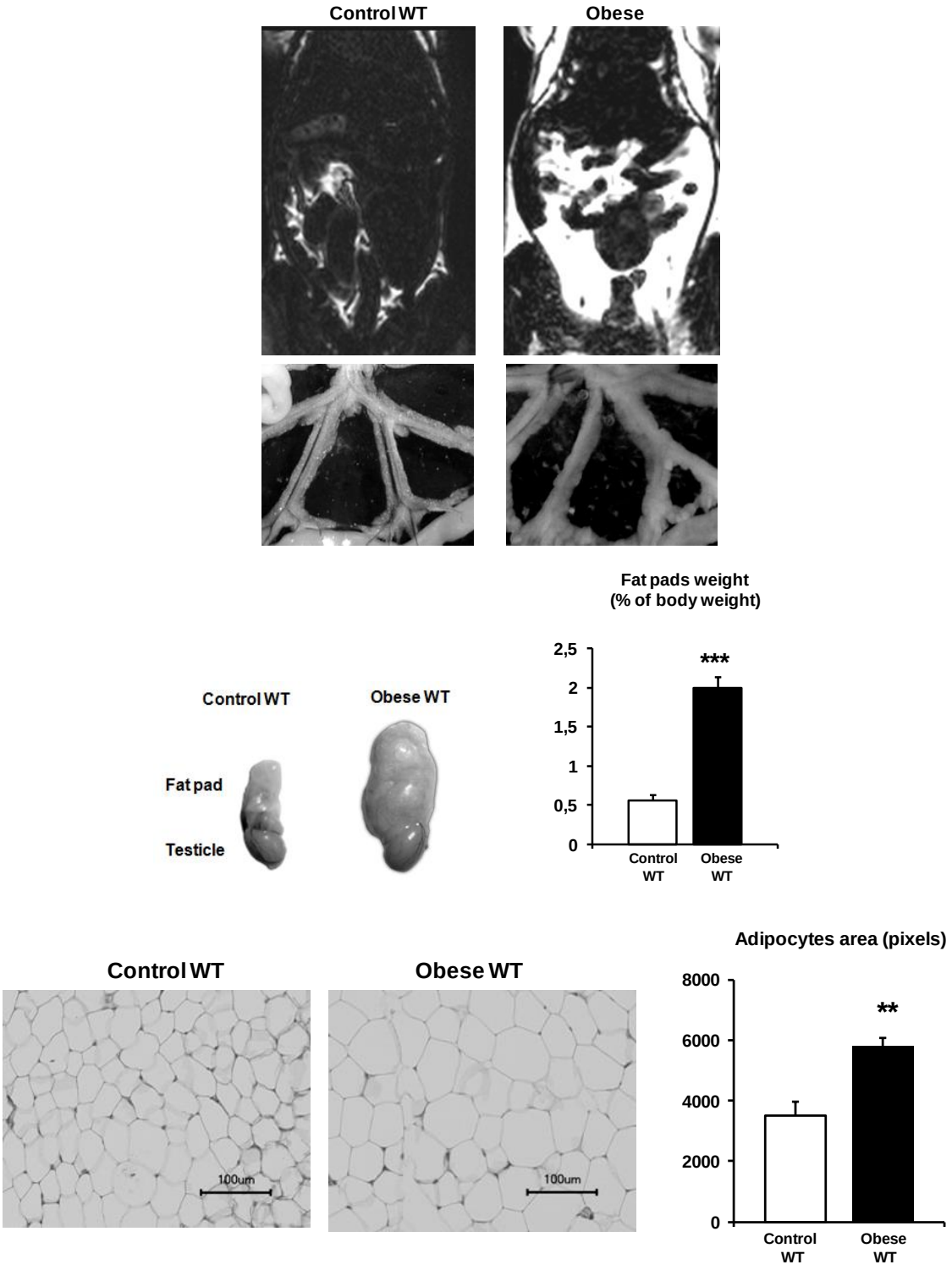


Figure 10. *High-fat diet for 8 weeks in WT mice resulted in marked increase of visceral fat, apart from increase in total body weight. White areas represent fat depots in magnetic resonance imaging (n = 3/group). Mesenteric PVAT and epididymal fat pads were also markedly increased with significant increase in adipocyte size. n = 10 mice/group, **P<0.01, ***P < 0.001.*

The obese mice presented with important hypercholesterolemia, insulin resistance and systemic inflammation mediated by TNF-α (Figure 11). Serum triglycerides and IL-6 levels were not different between groups.

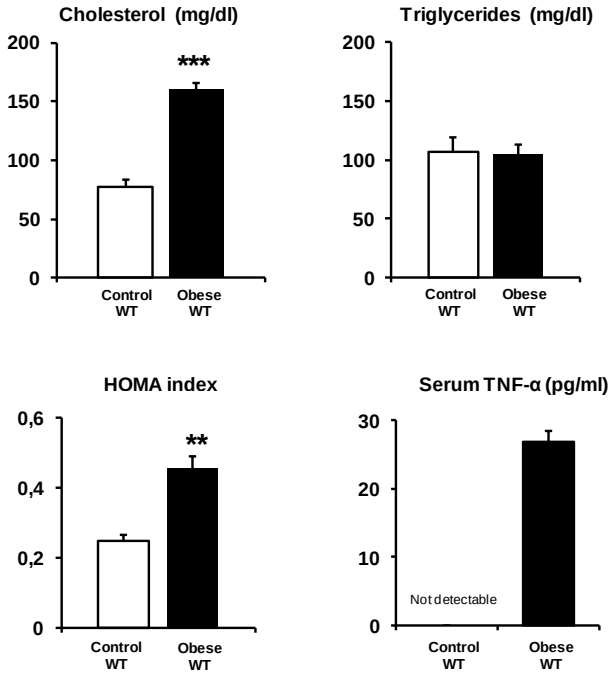


Figure 11. *Metabolic results after 8 weeks of HFD. n = 10 mice/group, **P<0.01, ***P < 0.001.*

Concerning the vascular features, small mesenteric arteries from obese WT mice had increased vasoconstriction responses to noradrenaline, shown by both measurement of sensitivity (control WT: logEC50 -6.170 vs. obese WT: logEC50 -6.664, ****P*<0.001) and of absolute tension, ***P*<0.01. There was significant increase in mean blood pressure values in the obese group: control WT: 70.5 ± 2.1 mmHg vs. obese WT: 82.7 ± 1.6 mmHg, ***P*<0.01.

In endothelium-denuded arteries, the increase in vasoconstriction was still seen, suggesting that these responses are at least partly independent of endothelium. The bigger shift to the left seen in endothelium-denuded arteries suggests that endothelium function partly influences vasoconstriction responses in obesity (Figure 12).

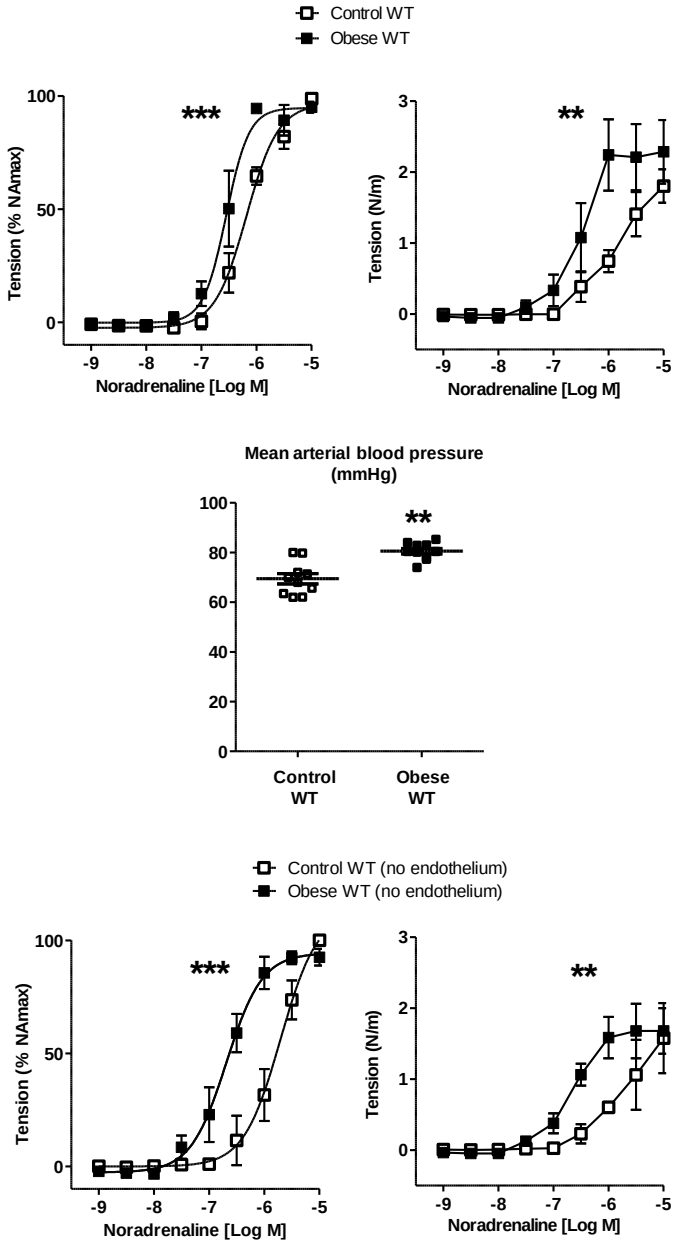


Figure 12. *Presence of cardiovascular risk factors such as increased noradrenaline-induced vasoconstriction (sensitivity and absolute tension, n=2-3 arteries/mouse; 10 mice/group) and blood pressure elevation. Results in endothelium-denuded arteries (sensitivity and absolute tension, n=2-3 arteries/mouse; 4-6 mice/group). **P < 0.01, ***P < 0.001.*

Concerning the vasodilatation features, relaxation induced by acetylcholine was diminished in relation to controls, suggesting endothelial dysfunction. Relaxation responses to an external NO donor - sodium nitroprusside - were increased (Figure 13).

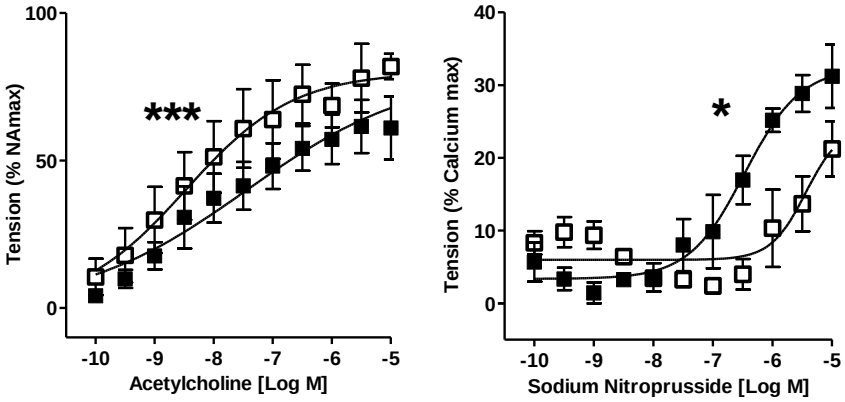


Figure 13. *Endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) relaxation responses (n=2-3 arteries/mouse, 10 mice/grou), *P < 0.05, ***P < 0.001.*

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In obesity, the adipose tissue works as an active secretory tissue, serving as an important source of inflammation. In mouse models, the epididymal fat pads are one of the parameters used to assess visceral fat. The expression of proinflammatory genes was not increased after 8 weeks of HFD in epididymal fat pads (Figure 14).

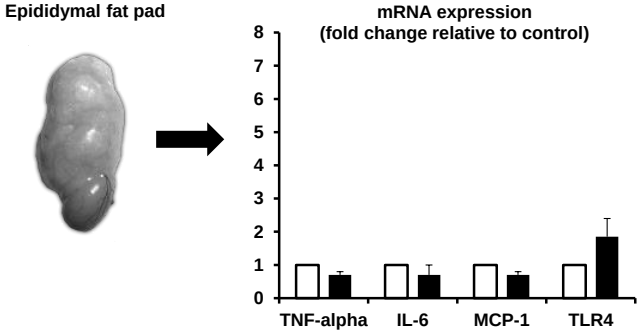


Figure 14. *Expression of inflammatory genes in epididymal fat tissue. N = 4-6 mice/group, white columns control WT and black columns obese WT mice. Data represent the mean ± s.e.m. from 2-3 independent experiments.*

We checked as well the expression of inflammatory genes in the mesenteric PVAT. Differing from the epididymal fat tissue, this fat depot showed signs of activation of inflammation, with significant increase in gene expression of MCP-1 and TLR4 and a trend toward increasing in TNF-α expression (Figure 15).

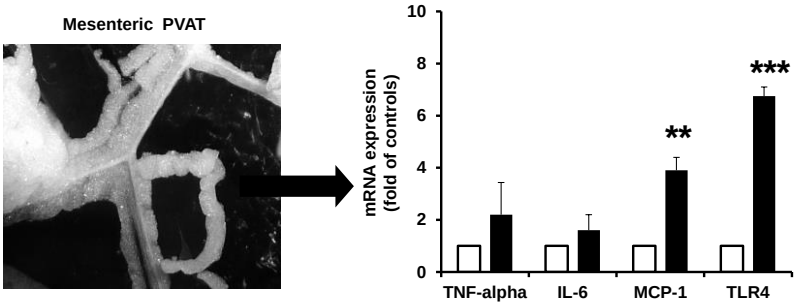


Figure 15. *Increased gene expression of inflammatory genes in mesenteric PVAT (n= 4-6 mice/group, white columns control WT and black columns obese WT mice). Data represent the mean ± s.e.m. from 2-3 independent experiments; **P <0.01; ***P <0.001.*

Thus, mice fed a HFD for 8 week presented cardiovascular risk factors such as increased vasoconstriction (partly independent of endothelium integrity) and blood pressure elevation. These findings were associated with hypercholesterolemia, systemic inflammation, insulin resistance and adipose tissue inflammation, being difficult to separate the roles each play, since all these factors influence vascular function.

The overweight model

An ideal mouse model to study the clinical condition of overweight should reflect mild body weight gain and increased cardiovascular risk. To distinguish from obesity, large weight gain, insulin resistance and systemic inflammation should be not apparent. The 2 weeks period of HFD fulfilled these characteristics for the overweight condition.

After a short period of HFD, a modest weight gain was achieved: the control group presented body weight gain of 9.0 % ± 0.9 and the overweight group of 13.8 % ± 0.7, ****P*<0.001. There was increase in abdominal fat, seen in magnetic resonance. Mesenteric PVAT was already markedly increased and epididymal fat pads were twice heavier. There was significant hypertrophy of adipocytes, with a mean area increase of 50% (Figure 16).

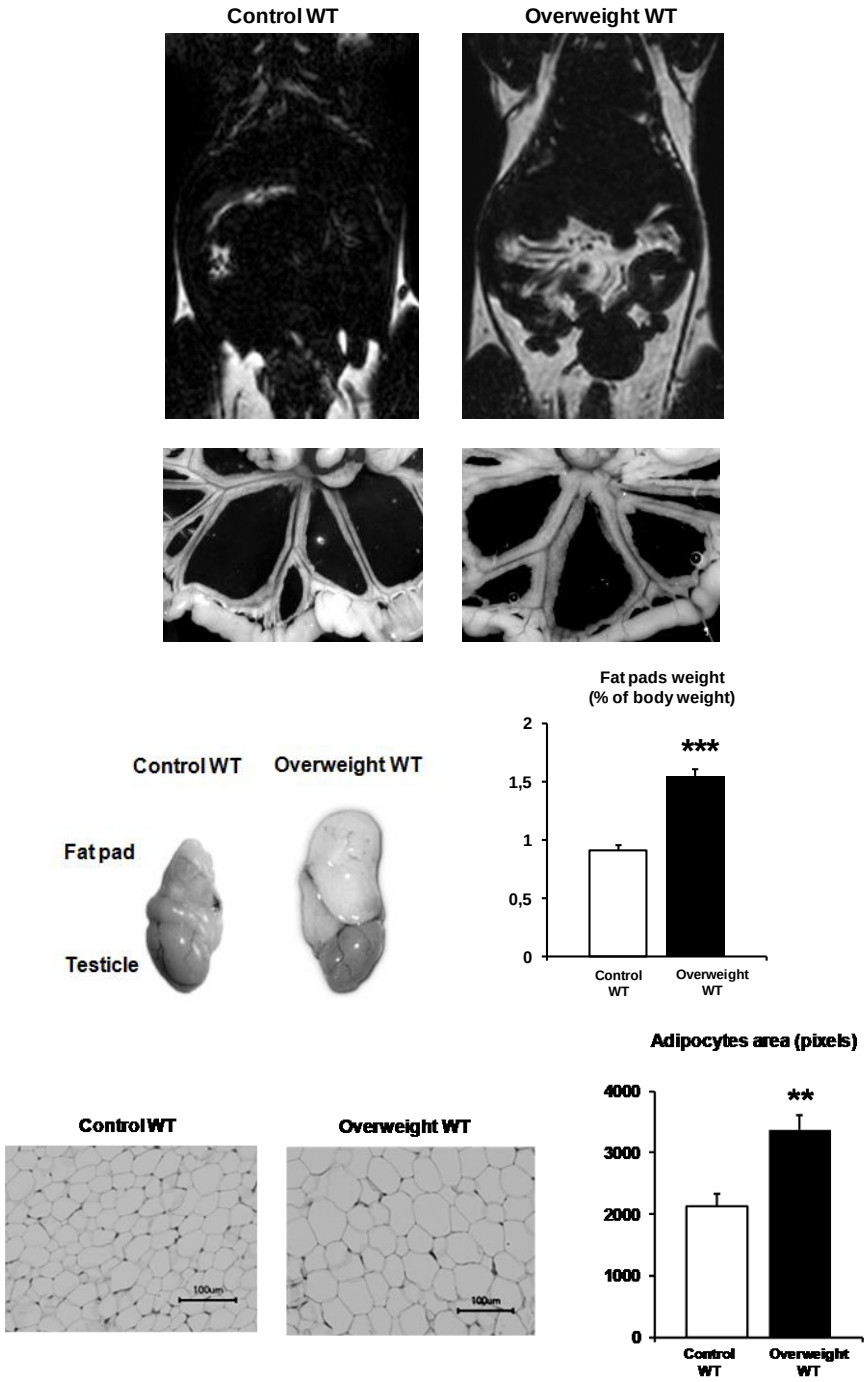


Figure 16. *High-fat diet for 2 weeks in WT mice resulted in overweight. White areas represent fat depots in magnetic resonance imaging (n = 3/group). There was increase in mesenteric PVAT, epididymal fat pads and adipocytes size. N=15-20 mice/group, **P <0.01, ***P <0.001.*

Contrasting with the obese WT mice, the overweight WT mice had no insulin resistance and no systemic inflammation. TNF-α and IL-6 were not detectable in serum. Hypercholesterolemia was as well important, as in obesity (Figure 17).

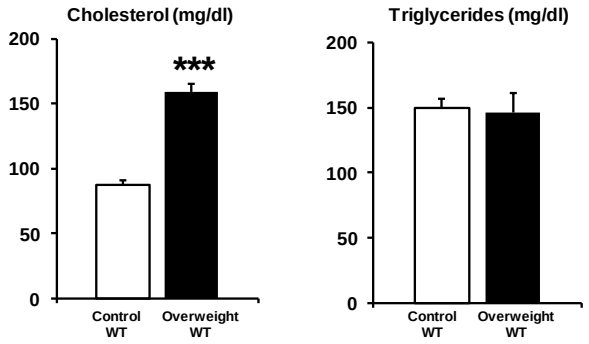


Figure 17. *Overweight mice presented total hypercholesterolemia and no difference in triglyceride levels. N=15-20 mice/group, ***P < 0.001.*

The vascular features in this model were similar to the obese group, fed a HFD for 8 weeks. Vascular sensitivity to noradrenaline was significantly increased: control WT logEC50: -6.166 vs. overweight WT: -6.487, *P*<0.001) as well as absolute tension responses, *P*<0.001. Blood pressure levels were significant increased in the overweight group (Figure 18).

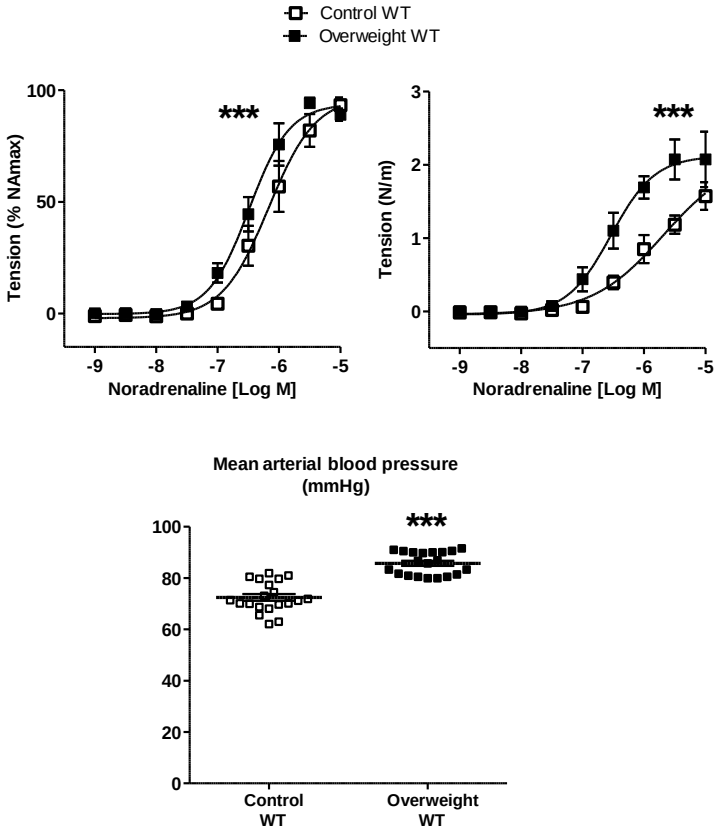


Figure 18. *Presence of cardiovascular risk factors such as increased noradrenaline-induced vasoconstriction (sensitivity and absolute tension, n=2-3 arteries/mouse; 20 mice/group) and blood pressure elevation (20 mice/group) after 2 weeks of HFD. ***P < 0.001.*

A very similar result was obtained in endothelium-denuded arteries, suggesting an independence of endothelium in vasoconstriction responses, especially because endothelium-dependent and independent relaxation responses did not differ between groups (Figure 19).

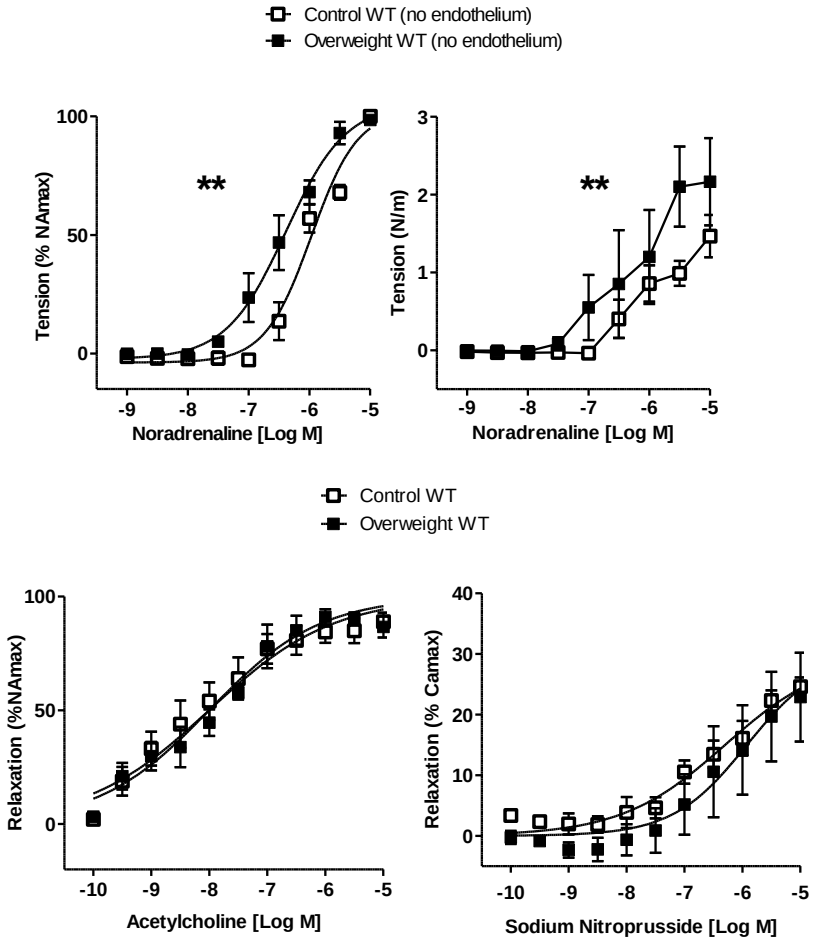


Figure 19. *In endothelium-denuded arteries, vasoconstriction values (sensitivity and absolute tension) were still significantly higher in the overweight group (n=6 mice/group), **P<0.01. No difference between groups in relaxation responses induced by acetylcholine and sodium nitroprusside (n=20 mice/group).*

Recently, PVAT under normal conditions has been described to have protective vascular properties and when inflamed like in obesity, might influence vascular reactivity[250]. For that reason we investigated in our overweight model whether the PVAT would have any effect in vasoconstriction responses. Vascular responses were examined in arteries cleaned from PVAT or not. The results did not show any difference between control and

overweight, suggesting that PVAT has not lost its vasodilator properties, therefore, not influencing the vascular responses observed in our model (Figure 20).

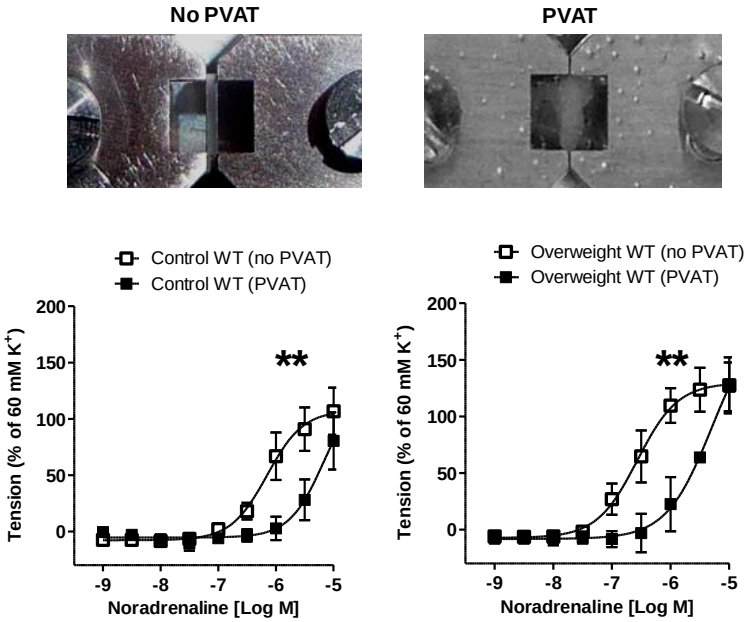
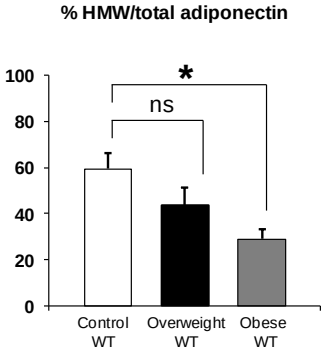


Figure 20. *In the myograph chamber, the PVAT left around the vessel showed to influence vasoconstriction response in a similar manner after 2 weeks of HFD. (n=05 mice/group). **P < 0.01.*

Furthermore, the protective effect of the normal PVAT has been credited to a vasodilator effect of adiponectin[233]. In overweight WT, adiponectin and in particular high-molecular weight (HMW) adiponectin, was not changed as compared to controls. However, in obese mice HMW adiponectin was significantly decreased (*P<0.05, ns=not significant):



In summary, the HFD-induced overweight mouse model showed already the same cardiovascular risk factors present in obese mice, without confounding obesity-related factors and without influence of PVAT, which point to a direct role for the vasculature.

Localization of the inflammation

Following our hypothesis of a vascular proinflammatory signaling occurring in overweight, the next step was to characterize and localize the inflammation, considering that the overweight state did not elicit systemic inflammation. As in obesity setting TNF-α is a key inflammatory mediator, we focused in this cytokine. The presence of TNF-α was analyzed in isolated mesenteric arteries, mesenteric PVAT and epididymal fat pads.

Mesenteric arteries from overweight WT were cleaned from PVAT, veins and lymphatic vessels. TNF-α gene expression represented by mRNA expression had two-fold increase in comparison to control WT. In line with this, a TaqMan protein assay revealed a two-fold increase of TNF-α protein level in mesenteric of overweight WT (Figure 21).

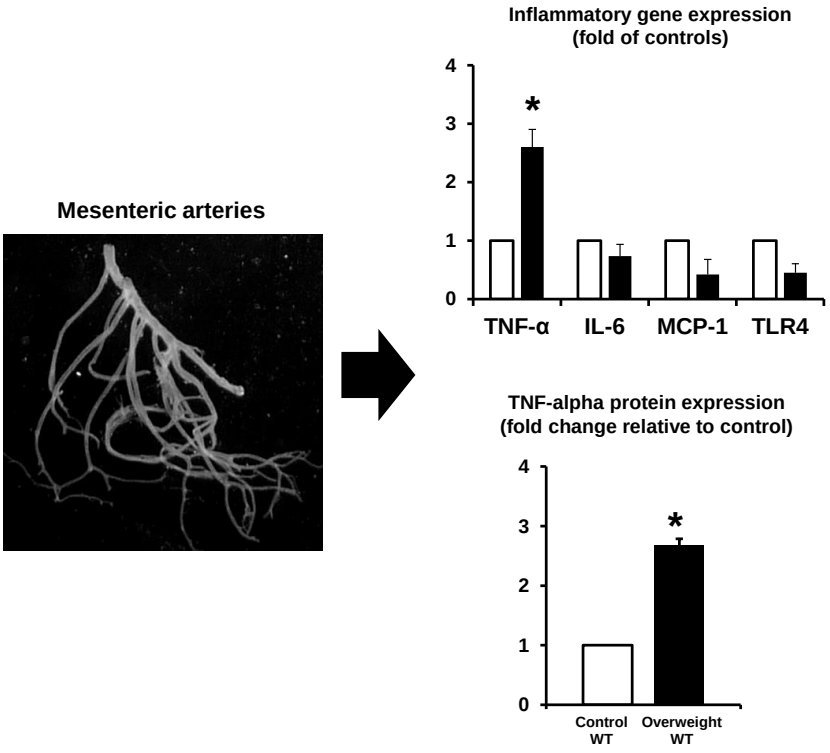
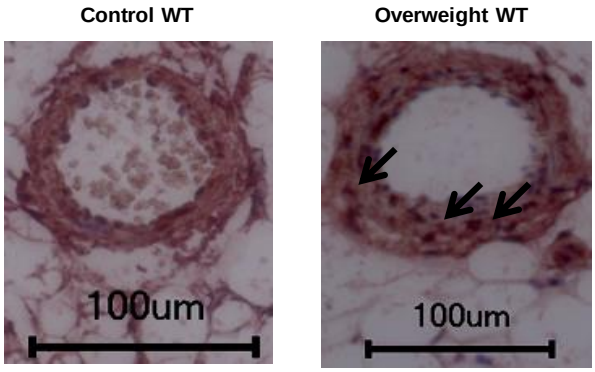


Figure 21. *Overweight WT (black columns) had increased expression of TNF-α mRNA and protein in mesenteric arteries compared to controls (white columns) *P < 0.05.*

This finding was further corroborated by the positive immunostaining of mesenteric arteries for TNF-α (black arrows):



Since we hypothesized that the vasculature would be the main responsible for increasing vasoconstriction and blood pressure, we checked the inflammatory status of the adipose tissue after 2 weeks of HFD, to exclude it as a main source of inflammation. Visceral fat depots represented by epididymal fat pads and mesenteric PVAT did not show any significant elevation of inflammatory genes. In mesenteric PVAT there was a trend to increased levels of MCP-1 and TLR4 (Figure 22).

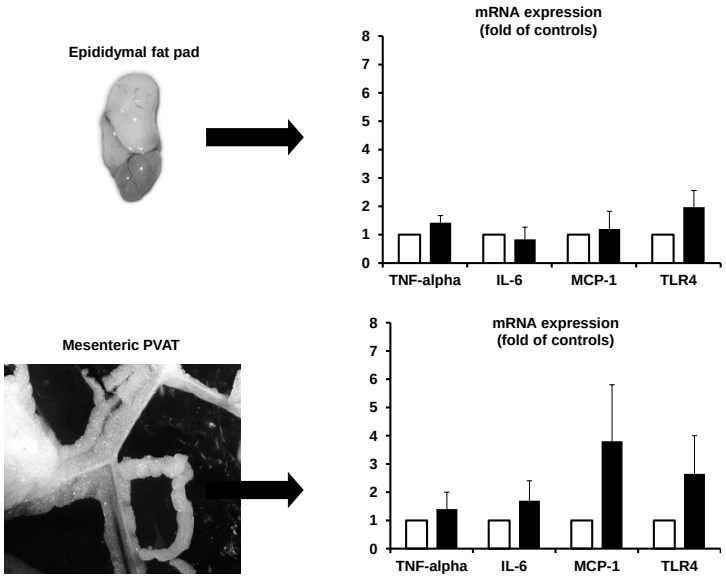


Figure 22. *Overweight WT mice (black columns) had no elevation of TNF-α in visceral adipose tissue (epididymal and PVAT). Data represent the mean ± s.e.m. from 3 independent experiments.*

In addition, investigation of inflammation was performed in mesenteric PVAT by histology. Infiltration of positive cells for CD45 was greater in obese compared to overweight mice (Figure 23).

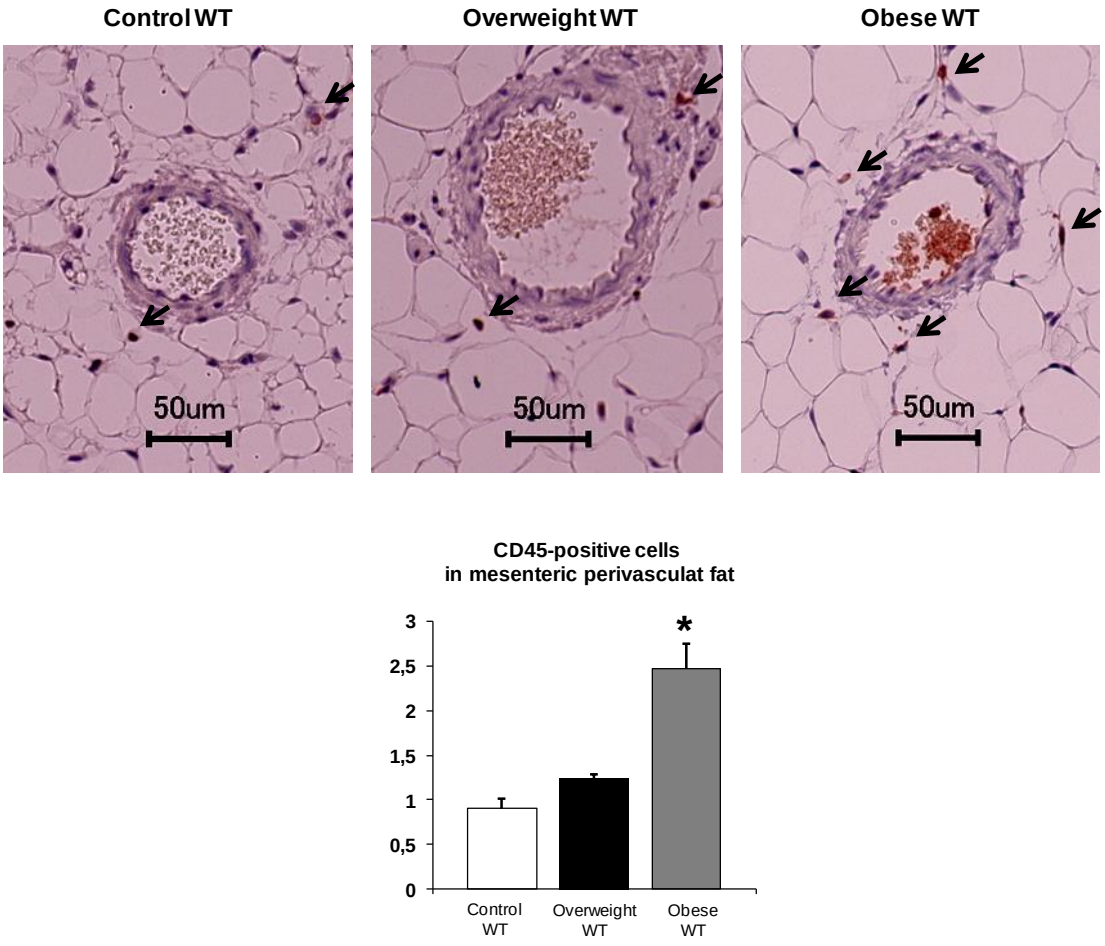


Figure 23. *Infiltration of CD45-positive cells in mesenteric PVAT. Obese mice had significantly larger number of positive cells.*

In two-photon laser scanning microscopy, small mesenteric arteries with PVAT were visualized and quantification of inflammatory cells was performed. The number of positive cells for CD45 /CD115 (CD115 is a marker for monocytes and macrophages) and CD45/Gr-1 (Gr-1 is a marker for neutrophil) in each arterial segment had a trend toward higher in obese mice (Figure 24).

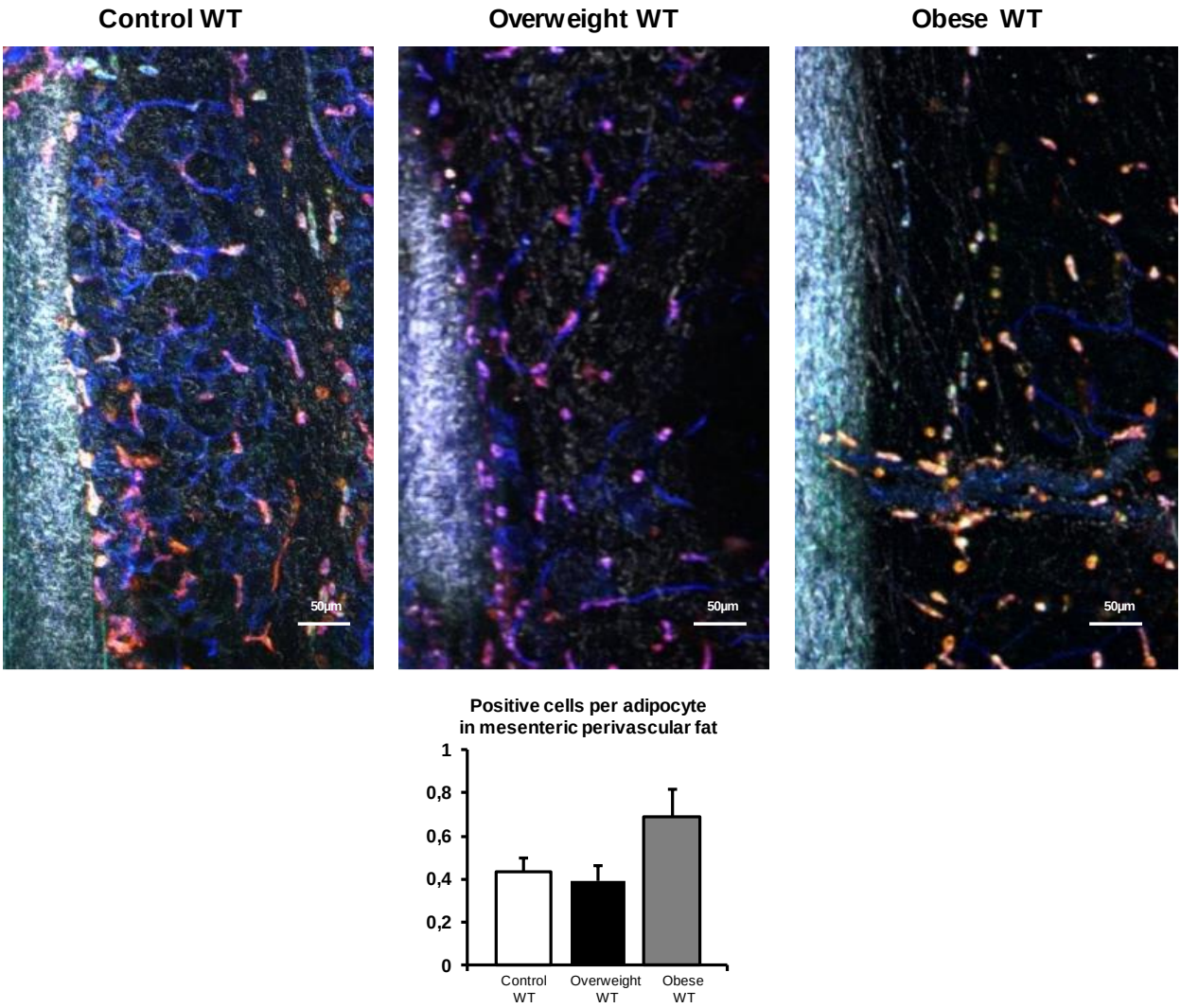


Figure 24. *Representative images of two-photon microscopy a small mesenteric artery and correspondent PVAT. The number of positive cells for CD45/CD115 and CD45/Gr-1 staining are shown. Overweight mice did not present with increased number of positive cells. In the obese mice group there was a trend toward quantitatively more positive cells (P=0.06). The column graph represents the number of positive cells per arterial segment examined (3 segments/mouse, n=4 mice/group, error bars, s.e.m.).*

We concluded that inflammation derived from the vasculature represented by TNF-α, plays a main role in overweight and is directly involved in the vascular findings, although the increase is visceral fat is already prominent.

Role of TNF-α on vasoconstriction

TNF-α ex vivo

In order to test the hypothesis that TNF-α plays a role in vascular dysfunction, we initially used mesenteric arteries from control WT in the myograph. The arteries were incubated with TNF-α 20ng/ml for 30 min. The incubation increased noradrenaline-induced vasoconstriction, suggesting that TNF- α has a direct effect on vasoconstriction (Figure 25).

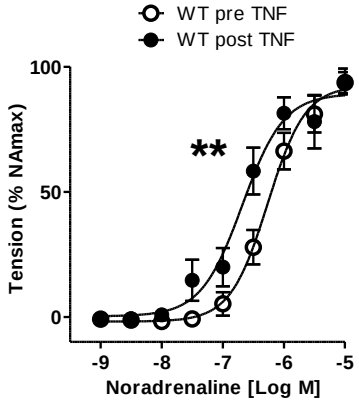


Figure 25. *Noradrenaline dose-response curve in mesenteric arteries from WT mice on normal diet. logEC50 pre TNF-α: -6.265; post TNF-α: -6.680, **P < 0.01.*

TNF-α loss-of-function models

To answer the question whether *in vivo* interventions with TNF-α signaling would result in prevention of vascular changes induced by high-fat diet, we used two different strategies to block TNF-α: anti-TNF-α antibody (etanercept) and global TNFR1$^{-/-}$ mice.

Overweight WT mice plus etanercept treatment

In this experiment, WT mice received HFD concomitantly with treatment with the TNF-α antibody etanercept for 2 weeks, 10 mg/kg twice per week intraperitoneal injection.

Upon exposure to HFD, pharmacological blockade of TNF-α did not interfere with the development of overweight, leading to a similar metabolic phenotype to untreated overweight WT. After 2 weeks, the fat pads weight and the amount of mesenteric PVAT were increased. The control group in this setting was made with WT under normal diet plus etanercept (Figure 26).

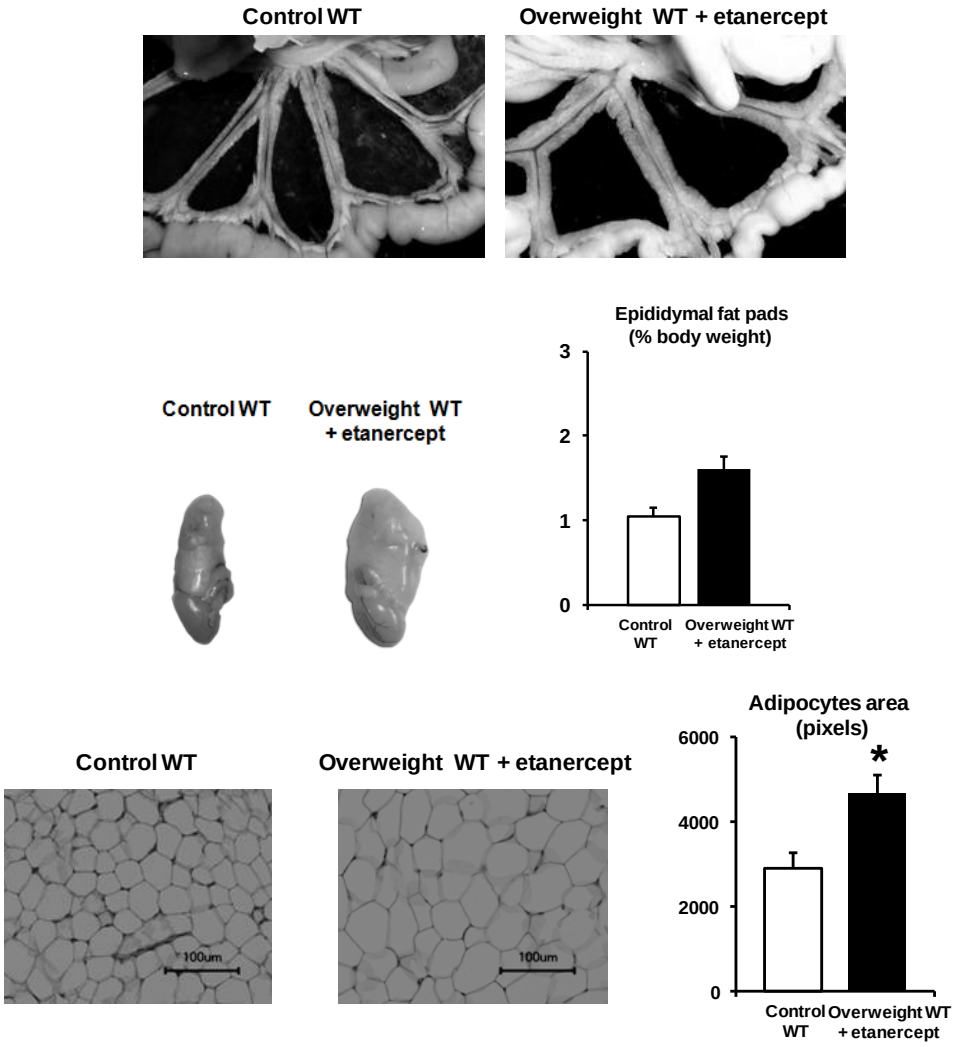


Figure 26. *High-fat diet for 2 weeks plus etanercept treatment in WT mice did not prevent the increase in visceral fat. (n=5 mice/group, *P<0.05, **P<0.01).*

However, the vascular effects of high-fat diet were prevented by etanercept treatment. Noradrenaline-induced vasoconstriction was not changed and there was no blood pressure elevation (Figure 27).

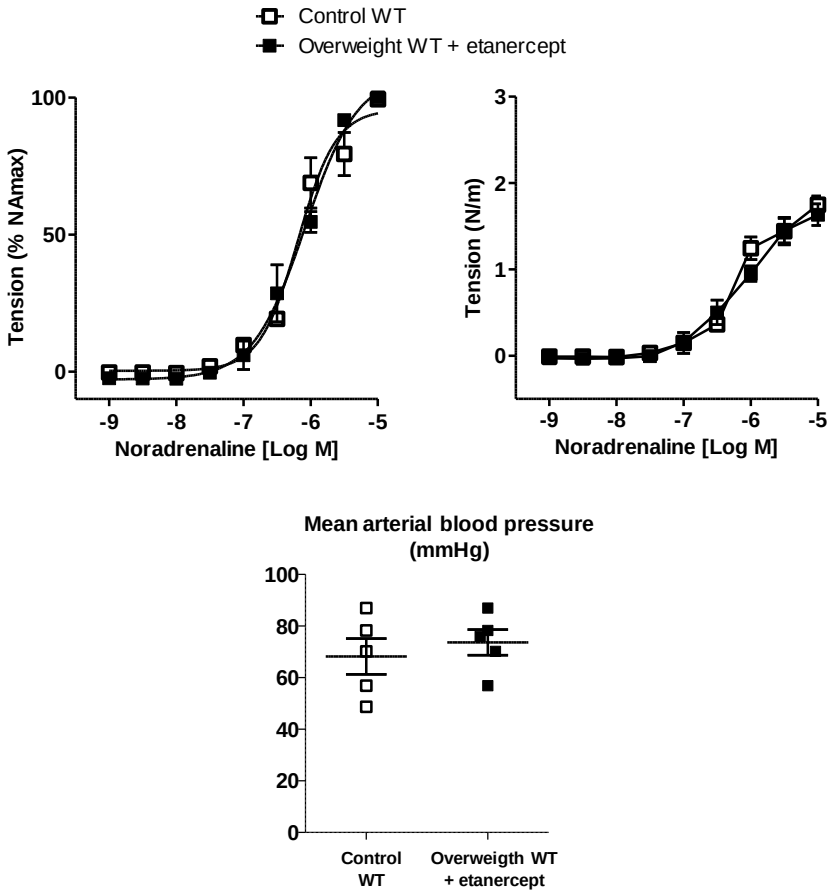


Figure 27. *High-fat diet for 2 weeks plus etanercept treatment in WT mice resulted prevention of the onset of increased vasoconstriction and blood pressure elevation. Noradrenaline-induced vasoconstriction in mesenteric arteries (sensitivity and absolute tension) (n=5 mice/group) and mean arterial blood pressure were not different between groups (n=5 mice/group).*

Overweight TNFR1$^{-/-}$ mice

To confirm the findings with etanercept treatment, TNFR1$^{-/-}$ mice were used as an alternative strategy to block the action of TNF-α. They received HFD for 2 weeks and again the increase in adiposity was not prevented (Figure 28).

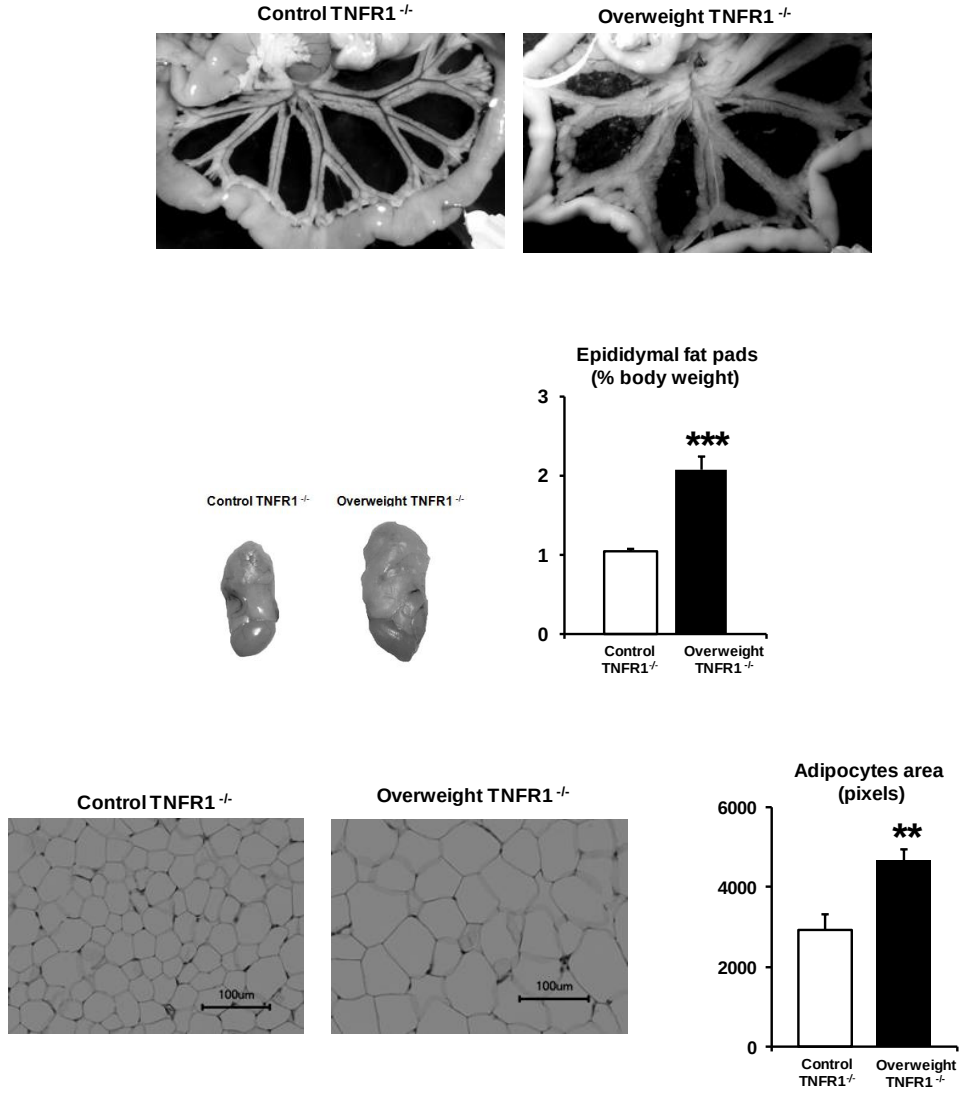


Figure 28. *High-fat diet for 2 weeks in TNFR1 $^{-/-}$ mice did prevent increase in visceral fat. N=6 mice/group, **P<0.01, ***P<0.001.*

Similarly to the result obtained with etanercept treatment, the absence of TNFR1 protected against the increase in vasoconstriction and blood pressure (Figure 29).

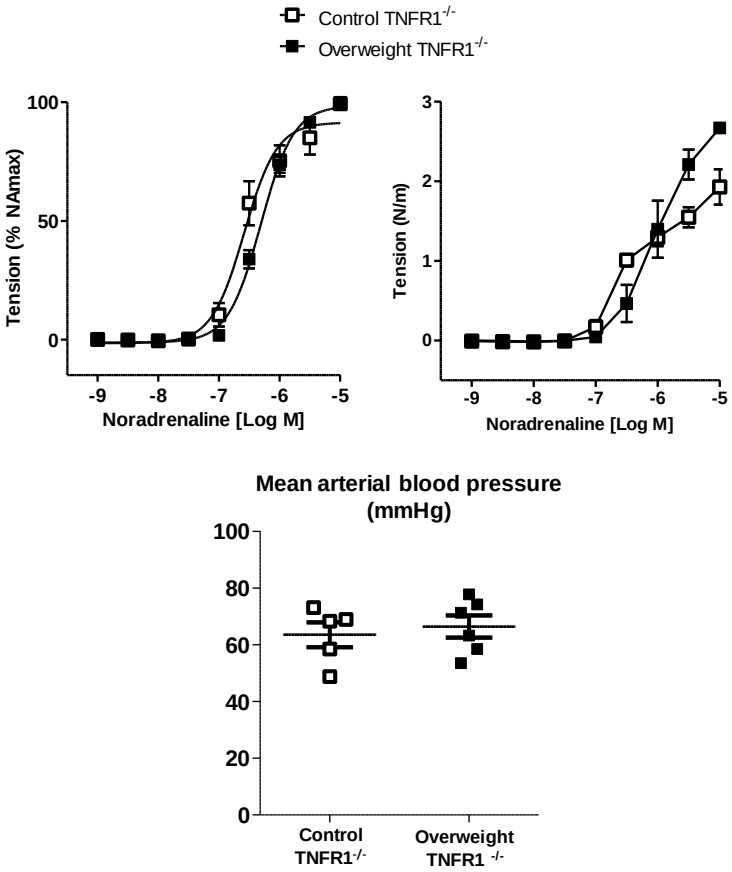


Figure 29. *High-fat diet for 2 weeks in TNFR1$^{-/-}$ mice resulted prevention of the onset of cardiovascular risk factors. Noradrenaline-induced vasoconstriction in mesenteric arteries (sensitivity and absolute tension, n=6 mice/group) and mean arterial blood pressure were not different between groups (n=6 mice/group).*

In summary, these data indicate that TNF-α signaling importantly participates in the vascular features observed in overweight.

A possible mechanism for vascular TNF-α signaling in overweight vascular changes: Rho-kinase activation

We next focused on identifying vascular TNF-α downstream signaling events mediating vasoconstriction and blood pressure elevation, considering that one of the main regulators of contractility of the VSMCs, the RhoA/Rho-kinase pathway, can be activated by TNF-α[96].

Whole mesenteric arterial beds from WT mice on normal diet was incubated with TNF-α (20 and 100ng/ml, 30 minutes) and Rho-kinase activity was measured. TNF-α caused more than two-fold increase in activation of Rho-kinase (Figure 30).

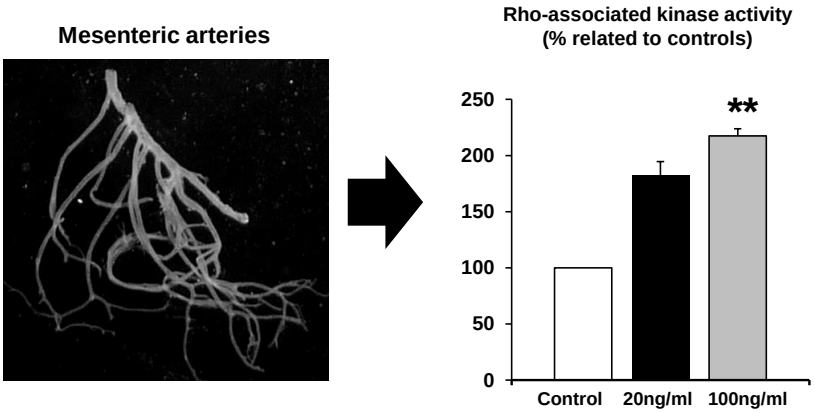


Figure 30. *TNF-α increased Rho-kinase activity in the vasculature. Rho-kinase activity in isolated mesenteric arteries from control WT after incubation in culture medium with TNF-α (20 and 100ng/ml, 30 minutes). Data represent the mean ± s.e.m. from 3 independent experiments.*

Apart from the important vascular signaling, activation of the RhoA/Rho-kinase pathway is also described in hypertrophied adipocytes[91]. Therefore we studied the visceral fat tissues. In overweight mice, expression of RhoA protein, ROCK1 and ROCK2 was not increased in adipose tissues, only a trend to increase in the PVAT. Rho-kinase activity was increased only in mesenteric arteries, suggesting that in the overweight condition the vasculature might be the main source of Rho-kinase and not the adipose tissue (Figure 31).

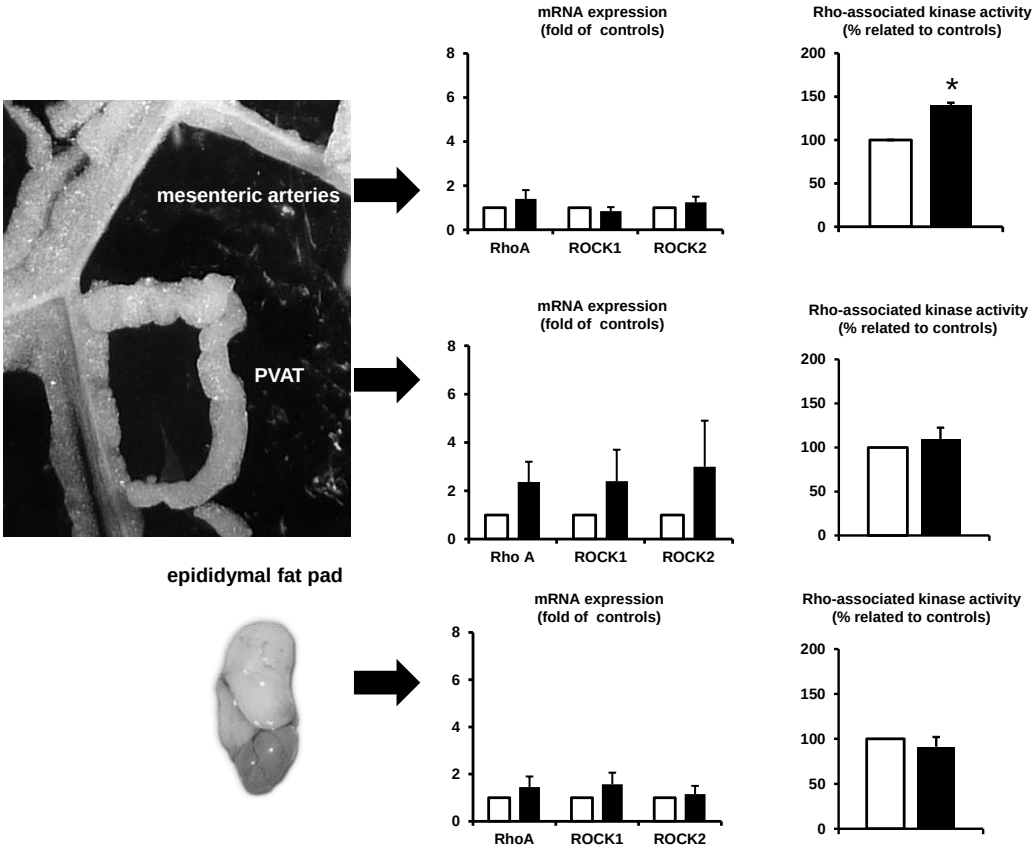


Figure 31. *RhoA/Rho-kinase pathway in mesenteric arteries and visceral fat tissues of overweight WT mice. White columns represent control WT and black columns overweight WT mice. There was a significantly higher Rho-kinase activity in mesenteric arteries, but not in PVAT or epididymal fat tissues. Data represent the mean ± s.e.m. from 2-4 independent experiments, *P<0.05.*

On the other hand, obese WT mice had a markedly different profile. After 8 weeks of HFD the RhoA/Rho-kinase pathway continued to have its activity increased in the mesenteric arteries, which supports the vascular findings at this time point. In addition, the visceral fat tissues PVAT and epididymal fat showed signs that the pathway was activated (Figure 32).

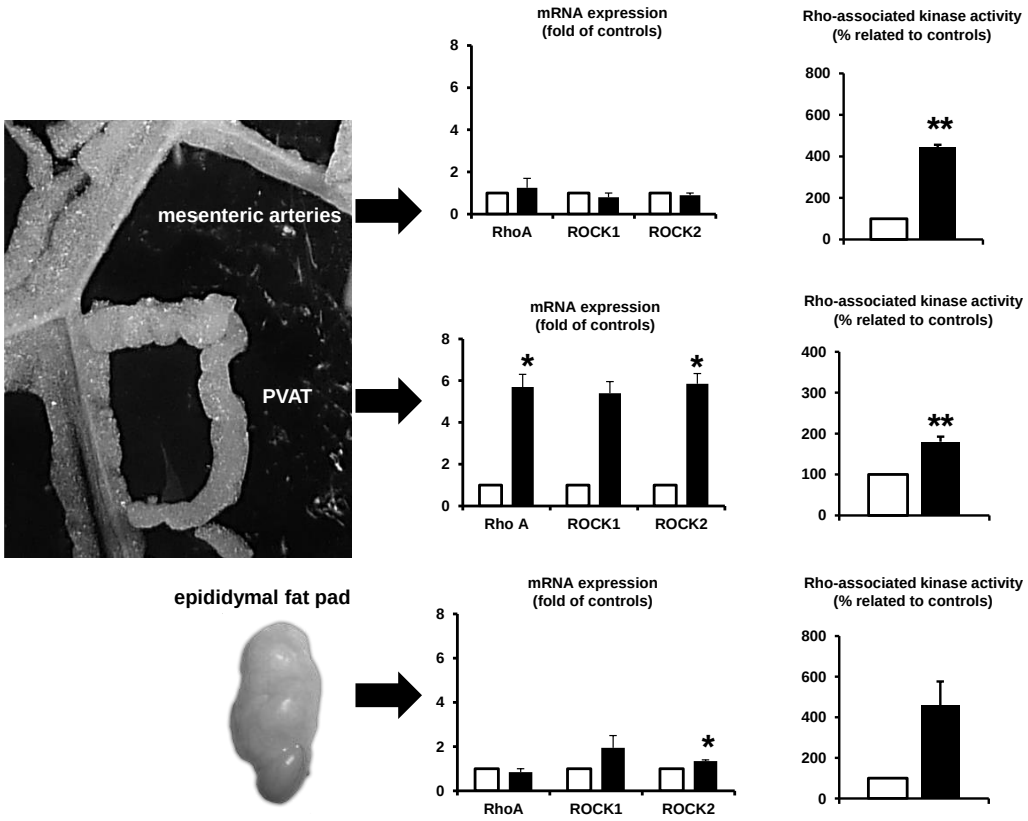
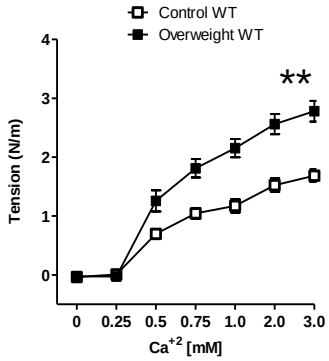


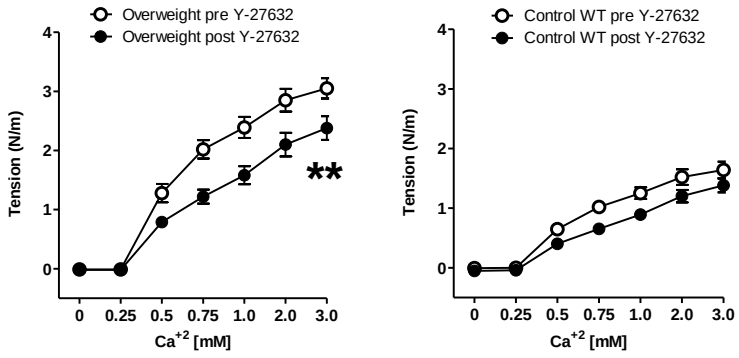
Figure 32. *RhoA/Rho-kinase pathway in mesenteric arteries and visceral fat tissues of obese mice. White columns represent control WT and black columns obese WT mice. There was a significantly higher Rho-kinase activity in mesenteric arteries and in PVAT and a trend in epididymal fat. Data represent the mean ± s.e.m. from 2 independent experiments, *P<0.05, **P<0.01.*

Calcium-induced vasoconstriction

Calcium is the most important intracellular messenger that triggers smooth muscle contraction and undergo critical regulation of the Rho-kinase pathway[69]. In line with the vascular TNF-α/Rho-kinase activation, calcium-induced vasoconstriction was augmented in overweight WT mice as compared to controls:



Using the Rho-kinase inhibitor Y-27632 in mesenteric arteries of overweight mice, calcium-mediated vasoconstriction returned to a level similar to the one in normal diet. By contrast, in arteries from WT on normal diet the Y-27632 compound caused only a mild reduction of vasoconstriction:



In parallel to the ameliorated noradrenaline sensitivity and blood pressure levels by concomitant treatment with TNF-α antibody and HFD, in this group calcium-mediated vasoconstriction was also reduced. Similarly, TNFR1$^{-/-}$ mice upon HFD did not show augmented calcium-induced vasoconstriction. Furthermore, vascular Rho-kinase activity was not increased neither in overweight WT mice treated with etanercept nor in overweight TNFR1$^{-/-}$ mice (Figure 33).

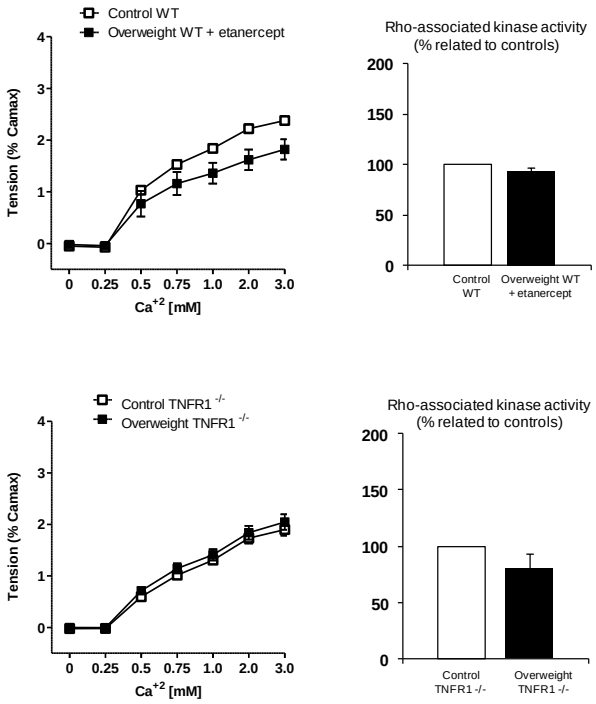


Figure 33. *Calcium-induced vasoconstriction and Rho-kinase activity in overweight WT mice treated with etanercept and in overweight TNFR1$^{-/-}$ mice (n= 6 mice/group).*

Rho-kinase activity in VSMCs treated with TNF-α

Experiments in cultured VSMCs treated with TNF-α supported the hypothesis of activation of Rho-kinase in this site (Figure 34).

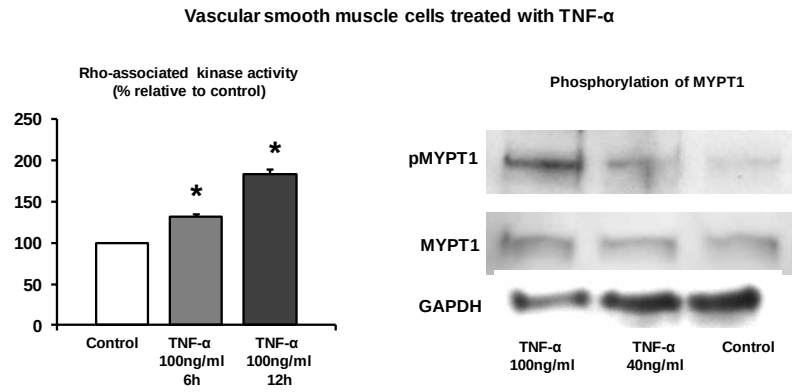


Figure 34. *Rho-associated kinase activity in vascular smooth muscle cells treated with vehicle (control) or TNF-α 100ng/ml for 6 and 12 hours. Western blot analysis showed increased phosphorylation of MYPT1 in cells treated with TNF-α 100ng/ml. Cells were treated with vehicle (control) or TNF-α 100 and 40ng/ml for 6 hours. Error bars, s.e.m., *P <0.05.*

In vivo treatment with fasudil

Finally, concomitant treatment of overweight mice with the specific Rho-kinase inhibitor fasudil for 2 weeks resulted in significantly less increase in noradrenaline-vasoconstriction, with a very low mean blood pressure, suggesting once more a key role for Rho-kinase in the vascular responses observed in overweight (Figure 35).

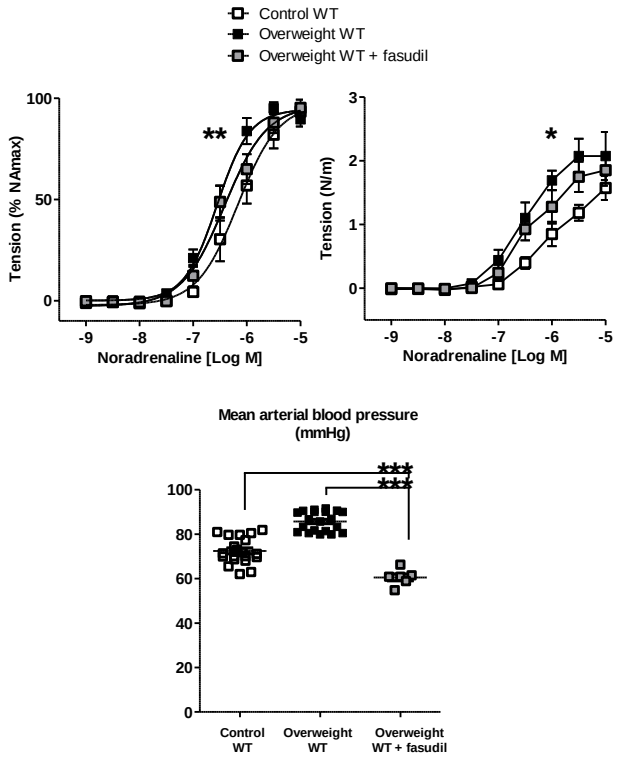


Figure 35. *Effect of fasudil treatment in overweight WT mice. Fasudil prevented increased noradrenaline-induced vasoconstriction and blood pressure elevation. N= 6 mice/group, *P<0.05, **P<0.01, ***P<0.001.*

It was important to exclude the participation of an important adrenergic signaling and of increased activation of sphingosine-kinase in enhancing calcium sensitivity.

Calcium sensitivity was investigated in the myograph setting by a pretreatment with phentolamine to block the adrenoceptors, followed by induction of intracellular calcium release with vasopressin. Mesenteric arteries of overweight WT mice showed an earlier and stronger response to intracellular calcium release independent of adrenergic signaling suggesting higher calcium sensitivity (Figure 36).

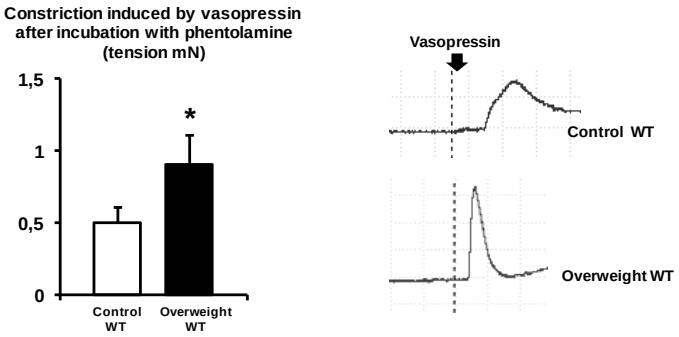
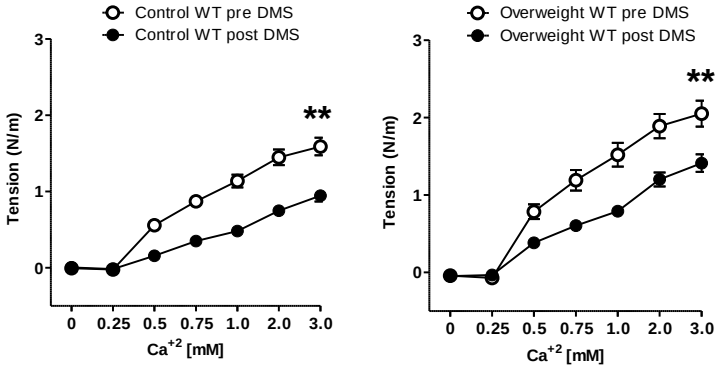


Figure 36. *Overweight WT mice showed increase in calcium sensitivity, even after blockade of adrenoceptors. N=2-3 arteries/mouse, 5 mice/group, *P<0.05.*

TNF-α can induce vascular smooth muscle Rho-kinase activation directly or by activation of sphingosine kinase (Sphk) and subsequent sphingosin-1-phosphate production[25]. Therefore SphK was blocked with DMS in mesenteric arteries of control and overweight WT mice. Calcium-mediated vasoconstriction was reduced in both diets to the same extent, suggesting no differential participation of SphK in vascular Rho-kinase activation in overweight:



In summary, a downstream signaling involving vascular activation of Rho-kinase in overweight is strongly suggested by these results.

Interventional approaches to antagonize the vascular effects of HFD

As we demonstrated the role of vascular inflammation for increased vasoconstriction and blood pressure elevation in overweight, we next aimed to investigate the effect of interventions with anti-inflammatory properties – statin treatment and withdrawal of the HFD - on the vascular findings in overweight.

Pravastatin treatment

Statin is an HMG-CoA reductase inhibitor that we used to stimulate pleiotropic anti-inflammatory effects on overweight WT mice. Pravastatin was added to drinking water in the concentration of 50mg/kg/day. The control group received normal diet plus pravastatin. At the end of 2 weeks, there was had no effect in preventing visceral fat gain and adipocyte hypertrophy (Figure 37).

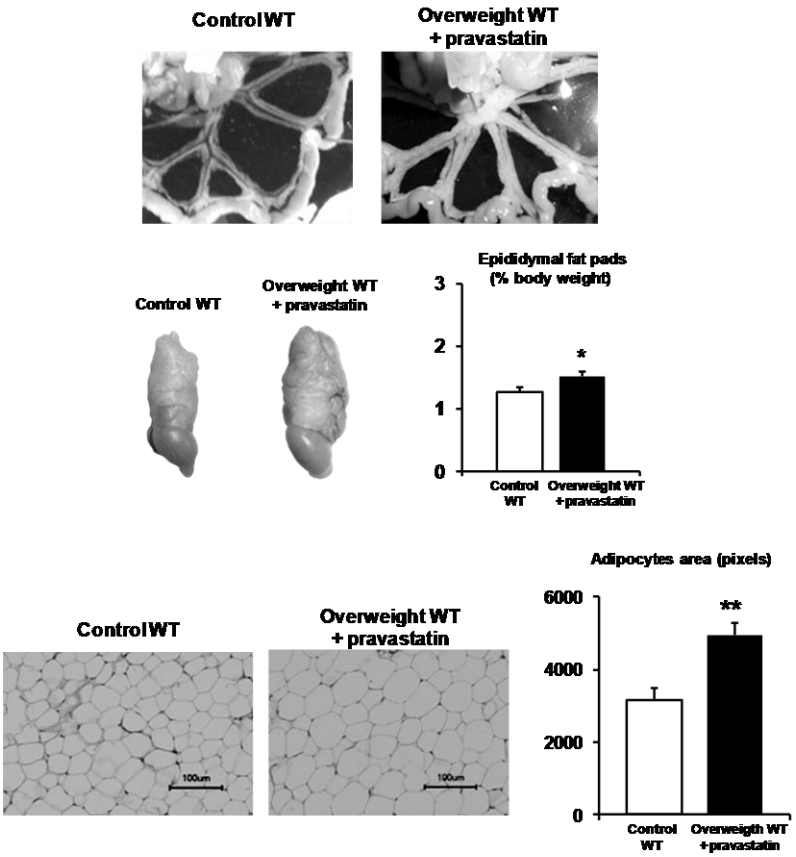
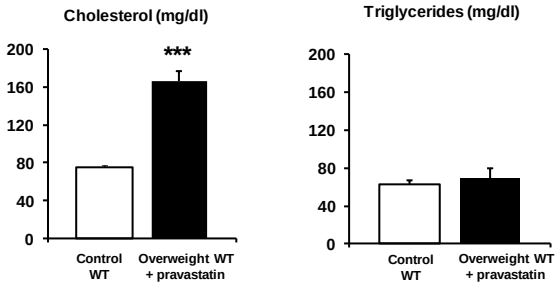
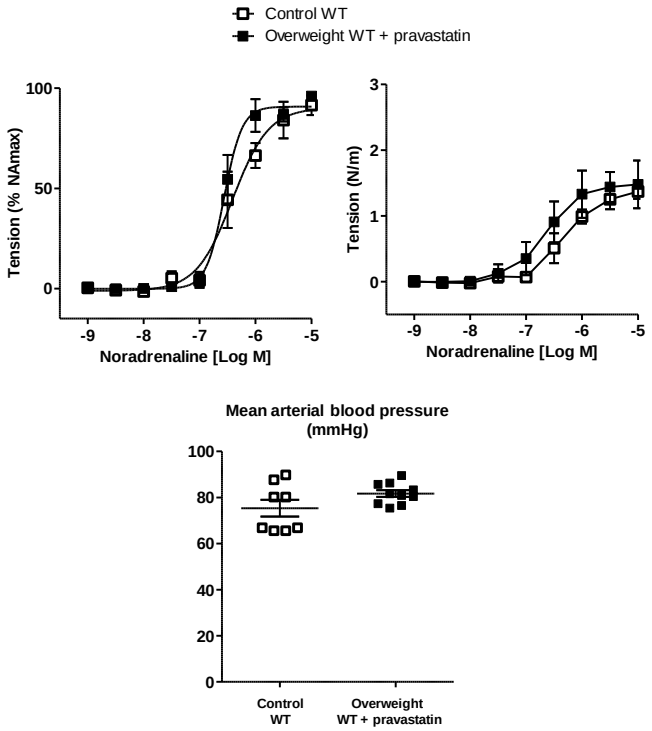


Figure 37. *Pravastatin treatment did not prevent increase in adiposity in overweight WT mice. N=10 mice/group, *P < 0.05, **P<0.01.*

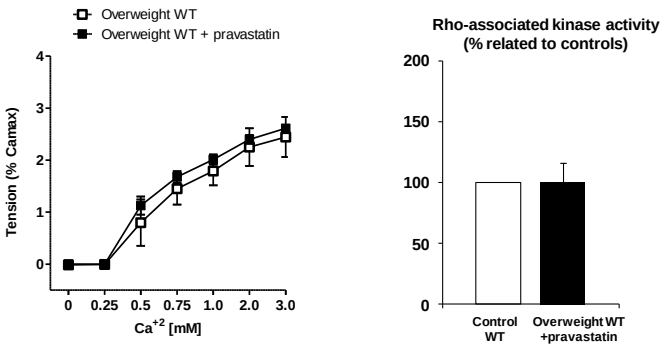
Importantly, pravastatin treatment did not affect the lipid profile *(n = 10 mice/group, *** P < 0.001)*:



However, it protected from increase in vasoconstriction and blood pressure *(n = 8-10 mice/group):*



In line, calcium-induced vasoconstriction was not different between groups and no increase in Rho-kinase activity was detected in small mesenteric arteries:



Return to normal diet

As a diet rich in saturated fat is itself a potential source of inflammation, we checked whether the return from HFD to normal diet would lead to reversal and recovery of vascular function and blood pressure. After 2 weeks, WT mice had the diet switched from HFD to normal diet, as well for 2 weeks. In these mice, visceral fat gain was not significantly reduced as compared to the group kept continuously on HFD (Figure 38).

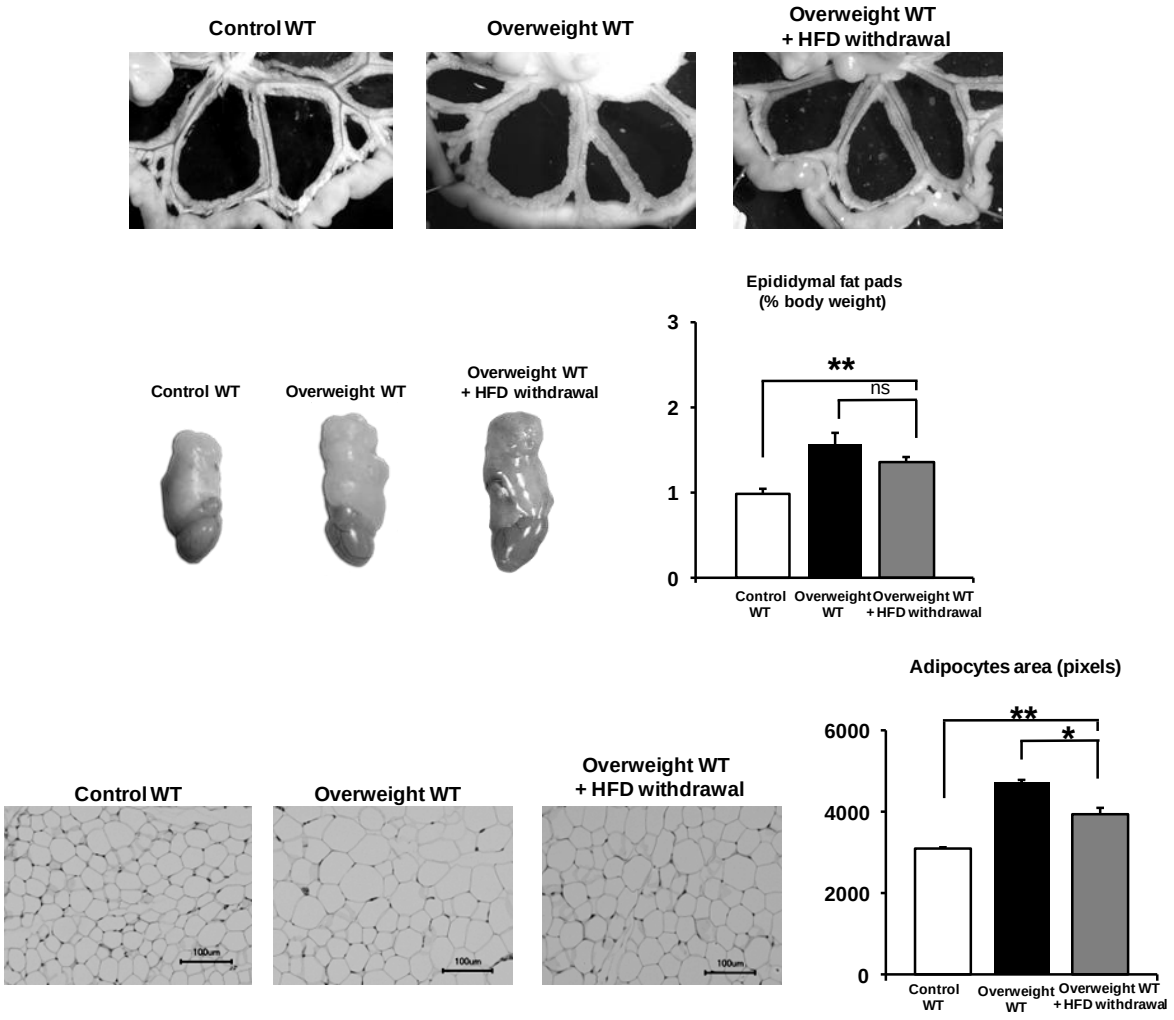
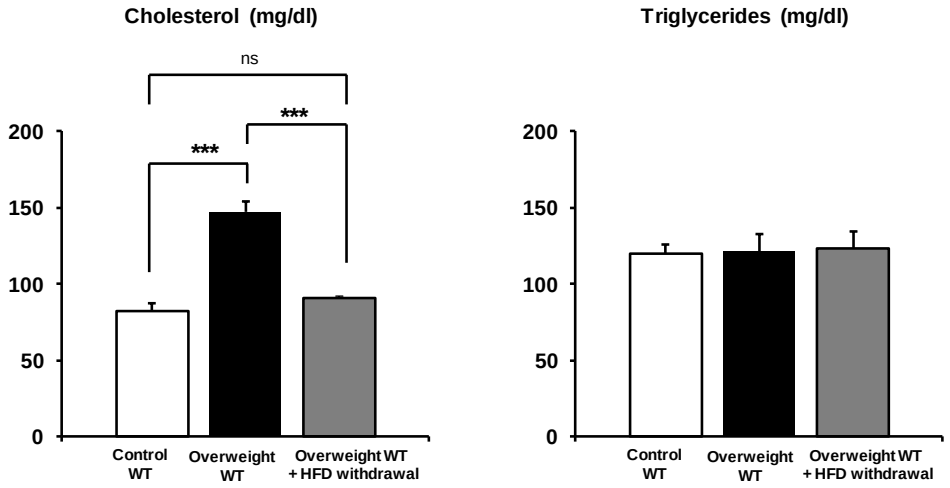


Figure 38. *After 2 weeks of HFD, overweight WT mice were returned to normal diet for 2 weeks. As control mice served aged-matched WT mice on normal diet and on HFD for 4 weeks. N= 10/group, ns=not significant, *P<0.05, **P<0.01.*

Levels of cholesterol were significantly reduced after HFD withdrawal (N=10 mice/group, ns=not significant, ****P* <0.001)*:*



Concerning the vascular features, noradrenaline-induced vasoconstriction was significantly ameliorated (logEC50 overweight -6.360 vs. HFD withdrawal -6.088, **P*<0.05). The mean blood pressure was significantly lower after diet switch as compared to continuous HFD, although still significantly elevated as compared to controls on normal diet (Figure 39).

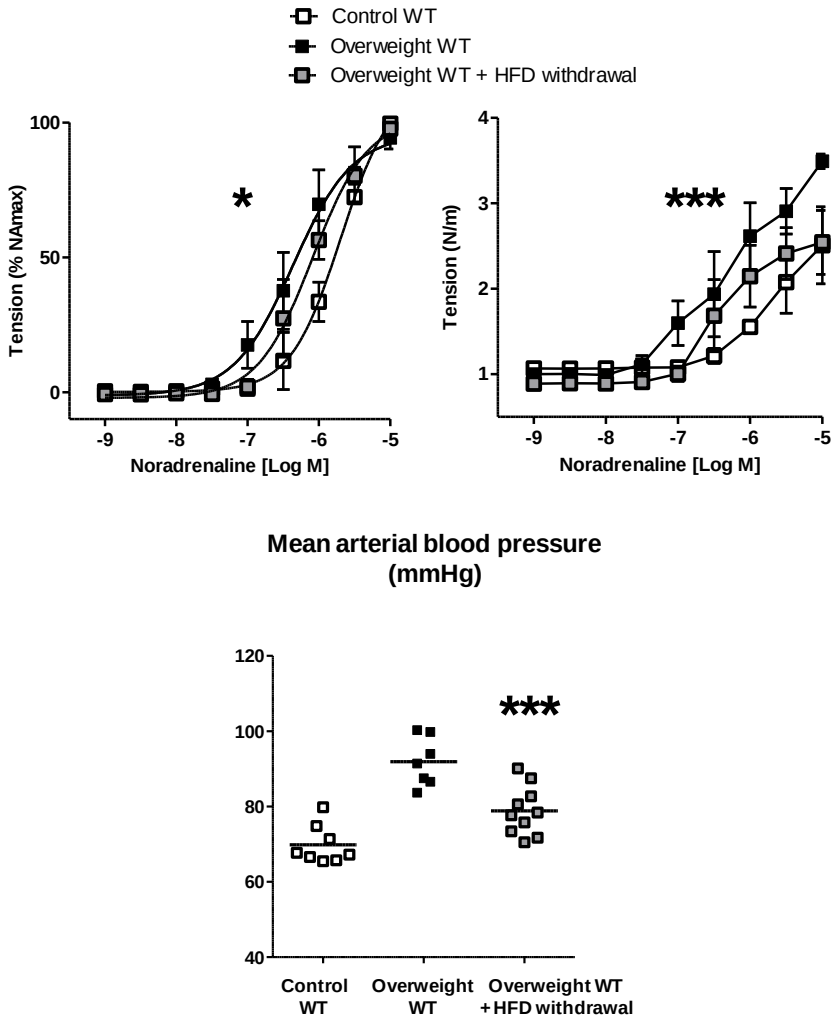


Figure 39. *Increase in vasoconstriction was significantly ameliorated by HFD withdrawal (n=10 mice group, *P<0.05 and ***P<0.001refer to HFD withdrawal vs. the other groups). In line, blood pressure elevation was significantly ameliorated by HFD withdrawal (n= 7-10/group, ***P <0.001).*

HFD withdrawal partially ameliorated as well the elevated calcium-induced vasoconstriction and caused decrease in Rho-kinase activity (Figure 40).

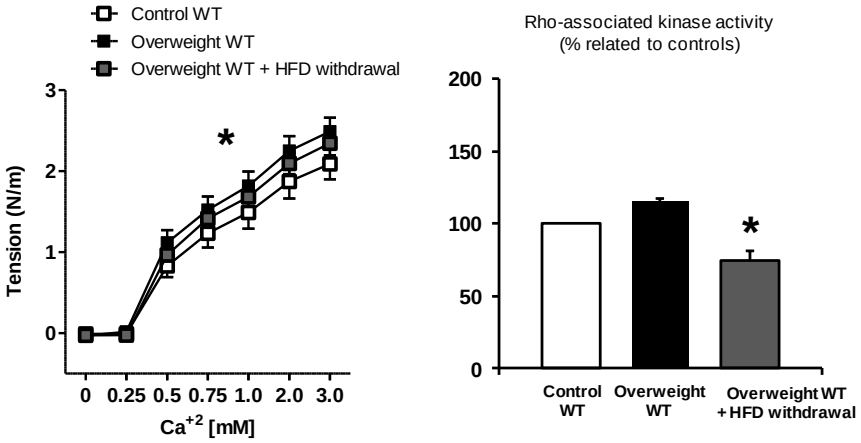


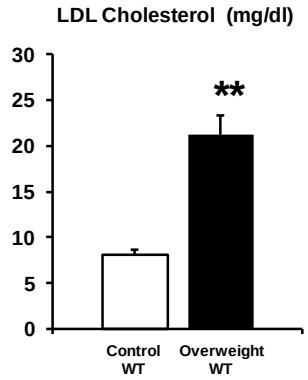
Figure 40. *Calcium-induced vasoconstriction and Rho-kinase activity in mesenteric arteries. The intervention of withdrawing the HFD for 2 weeks partially corrected the increased calcium-induced vasoconstriction. *P<0.05 refers to HFD withdrawal vs. the other groups. Activation of Rho-kinase was significantly reduced compared to the other groups (*P<0.05).*

Possible sensing nutrients from the HFD for the vascular signaling

A diet rich in saturated fat is an important source of lipids that work as ligands to activate proinflammatory signalings[251-252]. After having identified a vascular signaling in overweight that could be successfully blocked, we investigated two possible causes for the vascular inflammatory signaling.

Cholesterol and free fatty acids

We found elevated levels of total cholesterol in all groups fed a HFD. Although most of the cholesterol found in the C57/BL6 strain is composed by HDL-cholesterol, the amount of LDL-cholesterol is also significantly higher in overweight WT:



LDL-cholesterol is known to be a ligand for the toll-like receptor 4 (TLR4), in its oxidized form[253]. Therefore we treated VSMCs from WT mice with oxidized LDL-cholesterol. We saw an increase in gene expression of ROCK1, which did not occur in cells from TLR4$^{-/-}$ mice (Figure 41).

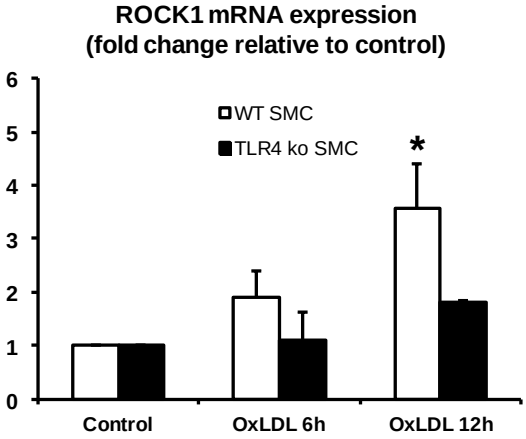
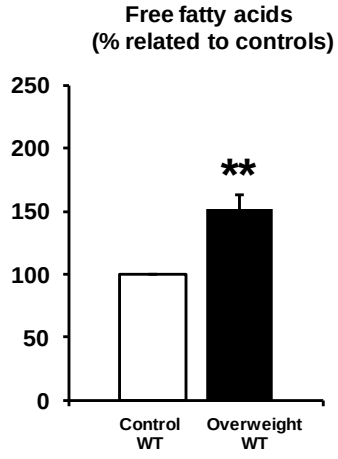


Figure 41. *ROCK1 gene expression in smooth muscle cells treated with oxidized LDL. Results represent results of two independent experiments. *P<0.05*

Apart from hypercholesterolemia, overweight mice had as well an important increase in the serum levels of free fatty acids (FFAs):



FFAs are also known ligands for TLR4. As palmitate is one of the most abundant saturated FFA found in serum, we tested the effect of palmitate in cultured VSMCs and in mesenteric arteries *ex vivo* and we saw an increase in Rho-kinase activity (Figure 42).

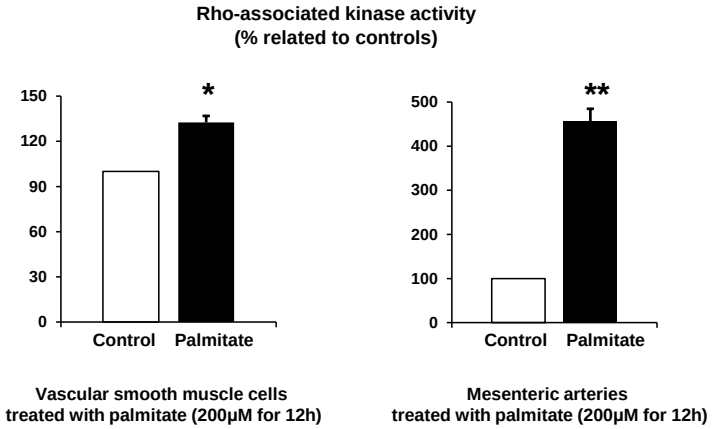
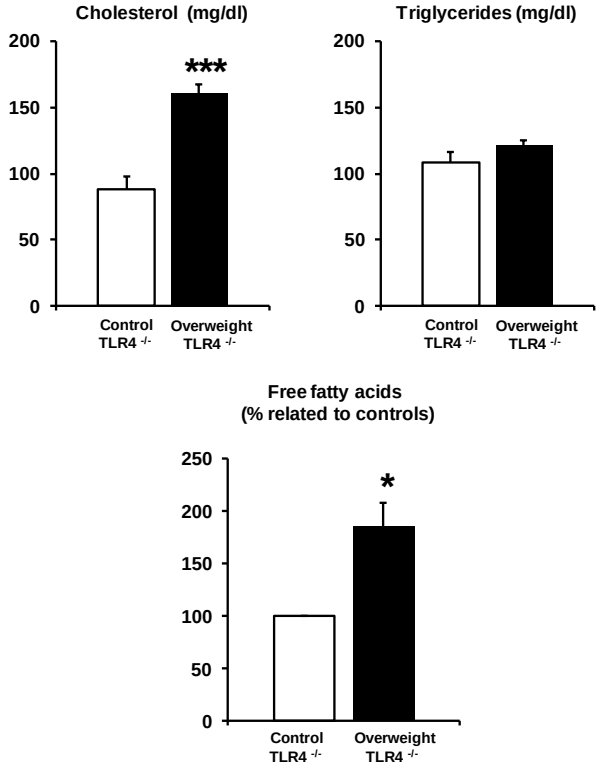


Figure 42. *VSMCs and mesenteric arteries (3 mice/group) treated with sodium palmitate 12 hours. Data represent the mean ± s.e.m. from 2 independent experiments, *P<0.05, **P<0.01*

Next, we examined the effect of HFD in TLR4$^{-/-}$ mice. TLR4$^{-/-}$ mice developed overweight, with increase in visceral adipose tissue and body weight in similar proportions to WT mice. After 2 weeks of HFD, similarly to WT mice, they presented significant hypercholesterolemia and elevated FFA levels (6 mice/group, *P<0.05, ***P<0.001):



However, they showed to be protected against the vascular effects of HFD (Figure 43).

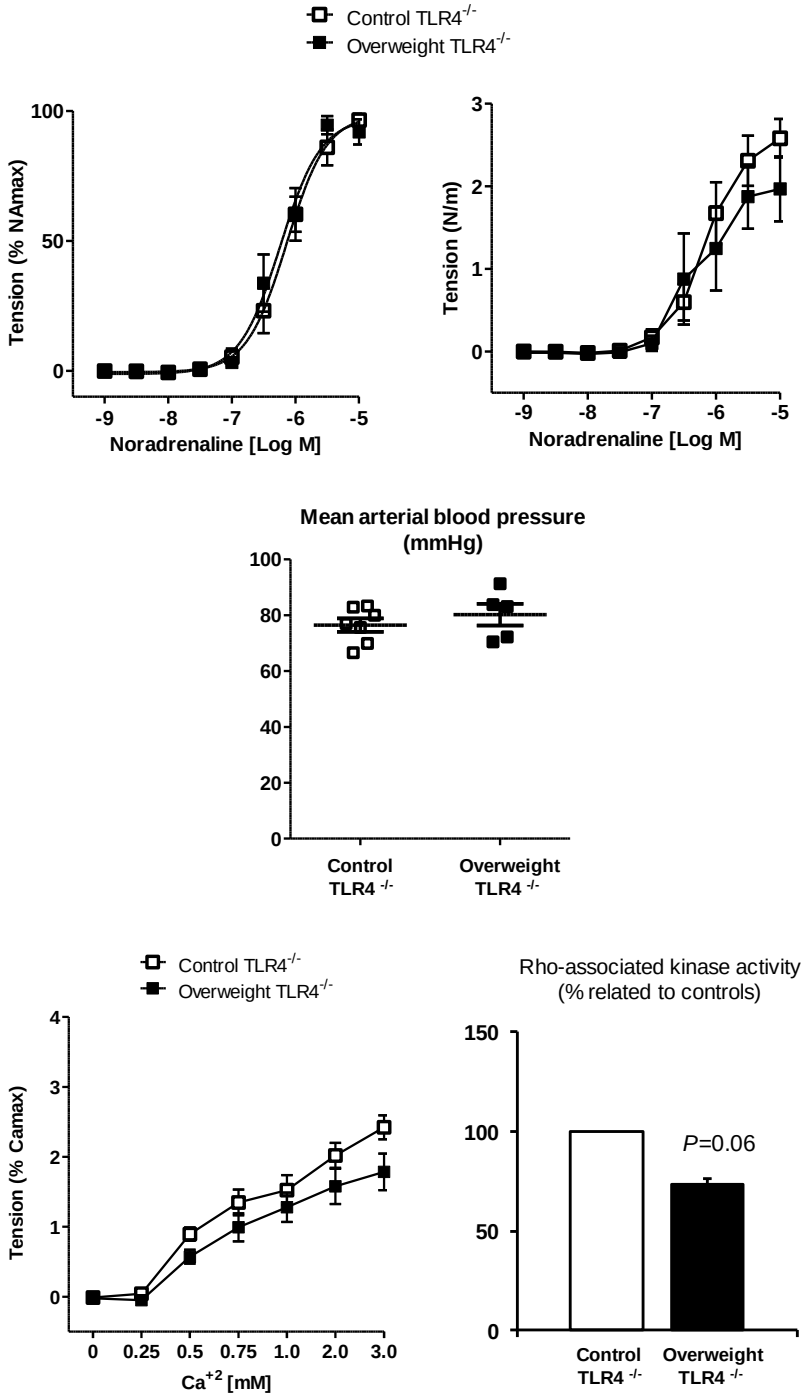


Figure 43. *Noradrenaline-induced vasoconstriction and blood pressure were not different between controls and overweight TLR4 -/- mice. Calcium-induced vasoconstriction and Rho-kinase activity were not increased in overweight TLR4 -/- mice.*

7. Discussion

This work describes in a mouse model a mechanism by which high fat intake for a short period gives rise to vascular features related to increased cardiovascular risk. A mouse model to create a parallel with the clinical condition of overweight was induced with high-fat diet, presenting with increased vasoconstriction and blood pressure elevation. In overweight mice, although increased adiposity was present, vascular but not adipose tissue inflammation contributed to these vascular findings. A vascular signaling involving TNF-α and activation of Rho-kinase mediated this mechanism. Different strategies either prevented or partially reversed the vascular effects of HFD, without affecting weight gain.

Overweight mice present cardiovascular risk factors

Despite numerous studies on animal models of obesity, to this point the overweight condition is not well defined or characterized in a mouse model with concomitant vascular alterations. The C57BL/6J mouse develops obesity in response to high caloric intake typically from saturated fat and is a relevant experimental model to study human diet-induced obesity[254-255]. In line with this literature, after 8 weeks of HFD the WT mice – the obese group - developed features such as hypercholesterolemia, disturbance of glucose metabolism and systemic inflammation. The PVAT presented signs of activation of inflammation. It is known that hypercholesterolemia[256], insulin resistance[257], systemic inflammation[258] and PVAT inflammation[233, 250] interfere on vascular function and generate features associated to increased cardiovascular risk. In addition, as well great attention has been paid to endothelium dysfunction, an early marker of atherosclerotic[170] and other cardiovascular diseases[171] and obesity is also associated with endothelial dysfunction[259-260]. However, the observed increase in vasoconstriction responses was present in experiments with and without intact endothelium, suggested that this response was strongly dependent on the function of VSMCs. Along with this microvascular dysfunction, there was a 14% increase in mean arterial blood pressure value. A certain degree of endothelial dysfunction was seen in these experiments, accompanied by enhanced response to sodium nitroprusside, a NO donor. This was in line with the literature. Obesity is associated with impaired NO bioavailability with a compensatory increase in soluble guanylyl cyclase (sGC)[261], the major cellular receptor for NO. This justifies the increased response to sodium nitroprusside.

As we wanted to investigate the vascular findings in a metabolic environment without the described confounders, we chose the time point of 2 weeks to perform further mechanistic investigations. After 2 weeks of HFD the WT mice - the overweight group - presented with weight gain, including important increase in visceral fat mass, along with increased vasoconstriction and elevation of blood pressure levels. Thus, we found this time point suitable to be used as a model to mimic the human clinical condition classified as overweight.

Interestingly, we observed that the endothelium function was still preserved and did not influence the observed increased vasoconstriction. In line with this finding, the NO donor sodium nitroprusside did not elicit different relaxation responses. This suggests that no compensatory mechanism involving increase in sCG activity was present yet, as NO availability was still preserved.

Concerning the role played by the PVAT, we did not observe any influence on vasoconstriction responses after this short period of HFD. Adiponectin, a hormone secreted by adipocytes, has been addressed as counter regulatory part of TNF-α. Lower levels of adiponectin, in particular the high-molecular weight (HMW) adiponectin, have been linked to the metabolic syndrome[262], and Greenstein et al. reported a vascular effect dependent of adiponectin in obese subjects[233]. In PVAT from small arteries of obese patients, the vasodilator effect of adiponectin was lost. In our study, after short-term HFD, a role for adiponectin on vascular function was not observed in myograph experiments. In particular, we did not observe a shift in adiponectin fractions in serum, which might be also an indicator that adiponectin action is not warranted at this stage in our model.

Vascular TNF-α and the role on vascular responses

The proinflammatory cytokine TNF-α was present in overweight mice arteries. The association between cytokines, cardiovascular risk and obesity[8-9, 29] is well known. In our overweight mice the mesenteric arteries had elevated TNF-α, that has also been reported to be an adipocyte-derived cytokine[11, 13, 15]. However, TNF-α expression was not increased neither in PVAT nor in epididymal fat pads in overweight mice. This finding corresponds to the observation of Virdis et al., who demonstrated the existence of a vascular generation of TNF-α in small human visceral arteries, in individuals with severe abdominal obesity. However, in this report, the increased production of TNF-α was shown to reduce the NO availability, therefore the small arteries showed reduced

endothelium-dependent vasodilatation. Along with these findings the obese group presented increased serum levels of TNF-α[18].

An important role for TNF-α in influencing vascular function in overweight is confirmed by the results demonstrated in this work. To understand the molecular mechanisms involving vascular TNF-α in overweight, we showed in *ex vivo* myograph experiments that TNF-α elicits vasoconstriction in mesenteric arteries of WT mice on normal diet. A pharmacological approach to antagonize TNF-α in the course of HFD prevented the increase in vasoconstriction and blood pressure without affecting the overweight status.
As overweight TNFR1 deficient mice were similarly protected, the role of vascular TNF-α in overweight-induced vasoconstriction and blood pressure elevation was further strengthened. These results are in line with a report of a mouse model of vasculitis, where treatment with a TNF-α antibody lowered blood pressure[263].

The involvement of the RhoA/Rho-kinase pathway

We investigated the role of a specific mechanism involving the RhoA/Rho-kinase pathway in vasoconstriction responses and consequently blood pressure levels. The RhoA/Rho-kinase pathway is known to be a key regulator of vasoconstriction[264] and was found to be upregulated in hypertensive states and other cardiovascular diseases[77]. Furthermore, TNF-α (through TNFR1) has been reported to activate RhoA in airway smooth muscle cells[96]. However, there is currently no evidence of similar signaling occurring in vascular smooth muscle cells.

We demonstrated *ex vivo* that TNF-α activates Rho-kinase in mesenteric arteries from WT mice on normal diet. In this line, mesenteric arteries of overweight WT presented with increased TNF-α expression along with increased Rho-kinase activity. Recently, a vicious circle was described in hypertrophied adipocytes involving adipocyte stretch, Rho-kinase signaling and inflammation of adipose tissue. They reported that lipid accumulation in adipocytes activated the RhoA/Rho-kinase signaling, at least in part through mechanical stretch. RhoA/Rho-kinase signaling would be implicated in inflammatory changes in adipose tissue in obesity, contributing and aggravating features of obesity such as weight gain, adipose tissue inflammation and insulin resistance[91]. In parallel with these published data, our obese mice indeed showed signs of RhoA/Rho-kinase activation, in arteries and in visceral fat tissue, suggesting that this might be contributing to the vascular signaling, later on in obesity development. Interestingly,

although already hypertrophied, visceral adipose tissues in overweight mice did not show evidence of Rho-kinase activation. This suggests that the Rho-kinase vascular signaling was more important, or at least, not influenced by Rho-kinase activation in the surrounding hypertrophied adipocytes.

The RhoA/Rho-kinase pathway regulates especially calcium-mediated vasoconstriction. In line, vasoconstriction induced by increase in extracellular calcium concentration was increased in overweight WT mice and blocked in a more effective way by a Rho-kinase inhibitor, strengthening the hypothesis of over activation of Rho-kinase, leading to an increased sensitivity of the calcium-dependent contractile apparatus. Furthermore, the functional link between TNF-α and Rho-kinase activity was evidenced as overweight WT mice treated with etanercept and overweight TNFR1$^{-/-}$ mice did not present increase in vascular Rho-kinase activity, vasoconstriction and blood pressure. *In vivo* treatment of overweight WT mice with fasudil prevented increase in blood pressure and attenuated the increase in vasoconstriction.

Blood pressure elevation related to obesity has among its suggested mechanisms activation of the sympathetic nervous system, which regulates total and regional peripheral vascular resistance[265-266]. The increase in calcium-induced vasoconstriction we observed in overweight was still present after blockade of the adrenoceptors with phentolamine and release of intracellular calcium by vasopressin, suggesting independency of the adrenoceptors.

To cover another aspect from vasoconstriction regulation, we investigated the role of sphingosine-kinase. Rho-kinase activity induced by TNF-α has been described to happen either directly or by activation of SphK[168]. Blockade of SphK in arteries in the myograph caused a similar decrease in arteries from both control WT and overweight WT, suggesting absence of upregulation of SphK activity in overweight. Therefore, a SphK-dependent mechanism remained unlikely, favoring a direct TNF-α effect on Rho-kinase activity. In summary, these findings strongly suggest that the calcium contractile apparatus was sensitized in overweight, due to activation of Rho-kinase by TNF-α. Supporting our idea of the link between TNF-α/Rho-kinase and bad microvascular outcomes, a recent study in early diabetic retinopathy described that Rho-kinase is a key mediator of TNF-α signaling in retinal microvessels[92]. Together, our data offer the vascular TNF-α/Rho-kinase signaling as an explanatory link between overweight and bad cardiovascular outcomes.

Interventional strategies

Blockade of TNF-α action through etanercept and lack of TNFR1 avoided increased vasoconstriction and blood pressure elevation in overweight WT mice. Thus, we investigated whether we could break the link between the vascular inflammatory signaling and blood pressure increase with strategies that could be reasonable to apply in the clinical setting. We questioned in how far unspecific anti-inflammatory treatment with a HMG-CoA reductase inhibitor[267] or with the withdrawal of high-fat content in diet for a short period of time would have a considerable impact on the vascular features.

The use of statin

Statins are largely used in patients with hypercholesteremia and overweight, both in primary and secondary prevention[268]. They have been shown to have pleiotropic anti-inflammatory effects that include decrease in levels of TNF-α[267], C-reactive protein[269] and improve of vascular inflammation through lipid independent mechanisms[268, 270], such as the reduction of IL-6 release in VSMCs[271-272]. Besides anti-inflammatory effects, statins modulate the RhoA/Rho-kinase signaling[78]. Kansui et al. reported that treatment with statin reduced the contractile response associated with Rho/Rho-kinase in arteries of hypertensive rats[273]. Administration of 80mg of atorvastatin per day to patients with stable atherosclerosis caused a rapid and significant reduction in leukocyte Rho-kinase activity, within 2 weeks of treatment, independent of cholesterol reduction[274]. Thus, one of the beneficial effects of statin therapy might be due to inhibitory effects on Rho-kinase activity.

Statins are reported to reduce blood pressure levels, although the exact mechanisms remain unclear. It is known that they can increase nitric oxide bioavailability, affecting the expression and activity of endothelial nitric oxide synthase[275-276] and consequently modulating Rho-kinase-mediated vascular contraction[277]. Statins also decrease serum levels of endothelin-1[278], downregulate the expression of angiotensin II type-1 receptor and reduce vascular endothelial production of reactive oxygen species[279]. However, apart from all the endothelial benefits, the effect in reducing Rho-kinase activity in the VSMCs should be considered as a potential explanation for the hypotensive effect, especially because it has been demonstrated that Rho-kinase can be modulate by statins independently of the nitric oxide synthase[277]. In the clinical setting recent meta-analysis studies still show conflicting data. In a meta-analysis of 18 trials and 5628 subjects randomized to receive either statins or placebo, statin therapy did not lead to

significant reductions in blood pressure, in normotensive or hypertensive patients[280]. On the other hand, larger meta-analysis reports have shown a modest but significant effect in reducing blood pressure, independent of the lipid-lowering effect. In a meta-analysis including 20 trials and 828 patients systolic blood pressure was significantly lower in patients on statin than in those on placebo or control. Interestingly, the higher the baseline blood pressure, the greater was the effect of statins[281]. More recently, a large meta-analysis of 40 studies examining 22511 controls and 22602 patients taking statins showed that the mean systolic blood pressure in the statin group decreased by 2.62 mmHg and diastolic blood pressure by 0.94 mmHg. In hypertensive patients, the decrease in blood pressure was bigger[282]. Therefore, statins can be assumed to have a relatively small but significant effect in reducing blood pressure and might provide additional cardiovascular protection.

In our model pravastatin reduced blood pressure and noradrenaline-induced vasoconstriction in a similar manner as the TNF-α signaling blockade, without affecting the extent of fat gain and without lowering cholesterol levels. It is known that statins have different lipid-lowering effects depending on the mouse strain and in C57BL/6J mice it was not expected to lower cholesterol[283], which turned into an advantage to study the pleiotropic effects. We observed that Rho-kinase activity was not increased in mesenteric arteries from the overweight group treated with statin, as well the vasoconstriction responses to calcium were not different from the control group. These results suggest that a vascular modulatory effect resulting in lower blood pressure might be expected from the use of statin in overweight, independent of other benefits.

Withdrawal of high-fat diet

Studies on the acute effects of HFD intake provide some background to support the idea of this experiment. Independent of the degree of overweight/obesity and even in healthy subjects, the acute ingestion of saturated fat has been described as a trigger of inflammation. A single meal rich in fat is enough to increase plasma levels of pro-inflammatory cytokines such as interleukin-6 (IL-6)[284], IL-17[285], TNF-α[285] and soluble intercellular adhesion molecule-1[286]. Recently, TNF-α was reported to reach maximum concentrations in healthy subjects 8h after a high-fat meal consumption[287]. In subjects with metabolic syndrome TNF-alpha levels increased more than in healthy subjects, after a single high-fat meal[288].

Over the last few years, it has been proposed that high-fat diet, especially the ones with high content of saturated fat, can cause postprandial inflammation by means of low-grade endotoxemia[289]. Endotoxin derived from gut leads to subsequent endotoxemia. A high-fat meal in healthy men increased in 50% the endotoxin concentrations and induced cellular activation via TNF-α, increasing the endothelial cell expression of E-selectin[252]. Thus, dietary fat intake even as a single meal causes acute inflammation and is able to modulate endotoxemia[290]. On a repeated basis, this is certainly part of a cascade for the establishment of diseases that have inflammation involved in their physiopathology, such as diabetes and cardiovascular diseases.

Here it is appropriate to refer once more to the parallel we made with published studies on bronchial smooth muscle cells. Inflammation of the airways mediated by TNF-α activates the Rho-kinase pathway and triggers constriction, causing bronchospasm[96]. Related to this mechanism, it is now accepted that dietary saturated fat can trigger asthma and a reduction in intake reduces airway inflammation[291-292]. Moreover, adherence to the Mediterranean diet tended to be associated with lower occurrence of asthma[293].

According to our hypothesis, if the dietary habit is strongly involved in the development of a localized vascular signaling in overweight state, the return to a normal diet should result in a protective effect, independent of the adipose tissue. In this case, targeting the effect of high-fat diet on vascular inflammation could represent not only a preventive strategy, but as well a therapeutic alternative. We aimed to describe in how far the withdrawal of the HFD for a short time would influence the vascular features, even when the overweight state would still be present. Therefore we exchanged HFD to standard diet after two weeks and allowed the mice to adapt for two weeks on normal diet. Indeed, the return to normal diet in overweight WT mice partially treated the increase in vasoconstriction and reduced blood pressure without significantly altering the overweight status. Of note, total cholesterol levels decreased significantly. This finding supports a potential link between hypercholesterolemia and blood pressure elevation in overweight, in line with epidemiological observations showing a high prevalence of elevated cholesterol levels in patients with hypertension[294-296]. Hypercholesterolemia can cause vascular dysfunction. It has been shown that hypercholesterolemia is associated with endothelial cell dysfunction, decrease of nitric oxide bioavailability and elevated oxidative stress, creating an important proinflammatory condition[297-298]. Nevertheless, in the other

strategies we used such as etanercept treatment or statin treatment, the protective effect on vascular outcomes was independent of hypercholesterolemia.
We suggest that the downstream vascular signaling that involves TNF-α and/or Rho-kinase can be targeted, independent of lowering cholesterol levels. Feasible strategies such as statin treatment and low-fat diet might prevent or at least ameliorate increase in vasoconstriction and blood pressure elevation associated to overweight.

Possible triggers of the vascular TNF-α/Rho-kinase signaling

Although not part of the scope of this work, we discuss here some possible causes for the vascular findings. Food rich in saturated fat is an important source of nutrients that could work as triggers for the vascular TNF-α/Rho-kinase signaling described, such as cholesterol and free fatty acids.

Cholesterol and free fatty acids – ligands for toll-like receptor 4

In our model we can consider cholesterol and free fatty acids as ligands for TLR4 and therefore triggers for the vascular signaling involving TNF-α.
TLR4 is an evolutionarily ancient pattern recognition receptor that plays a key role in the innate immune response, as TLR4 is the receptor for lipopolysaccharide. Ligation of these receptors initiates the activation of nuclear factor-kappa B in a variety of cell types, resulting in the expression of a wide array of proinflammatory genes, such as TNF-α [299]. Interestingly, lipids can activate members of the Toll-like receptor family.

There is now strong evidence linking TLR4-mediated inflammation and metabolic signals in conditions such as obesity, insulin-resistance and diabetes[300]. In obesity, it is well described a vicious cycle in the hypertrophied adipocytes involving release of saturated fatty acids, activation of Toll-like receptors and inflammatory changes in adipose tissue[98, 301-302]. Moreover, HFD can alter the gut microbiota composition and increase intestinal permeability through the induction of TLR4, thereby accelerating obesity[303].

Concerning the vascular field, TLR4 is constitutively expressed in VSMCs[304] and overexpressed in VSMCs of atherosclerotic arteries, even in regions with few inflammatory cells[305]. Studies point to an important role of TLR4 in intimal hyperplasia[306], atherosclerotic lesion progression[307] and arterial hypertension[308-309]. Supporting a role for TLR4 in the connection between metabolic and vascular features, type 2 diabetic mice with mutated TLR4 do not develop hyperglycemia and hypertension, although they had

increased body weight[310]. Therefore the link between TLR4-mediated nutrient sensing and cardiovascular disease is a novel and promising field of research.
Apart from free fatty acids, OxLDL cholesterol also serves as ligand for TLR4. This supports the involvement of TNF-α. It has been described that hypercholesterolemia and inflammation alter constriction responses directly by acting in the VSMC, increasing calcium sensitivity through upregulation of the RhoA/Rho-kinase pathway[256, 311-313]. Furthermore, the stimulation of RhoA by OxLDL was reported to contribute to vasospasm in atherosclerotic arteries[314].

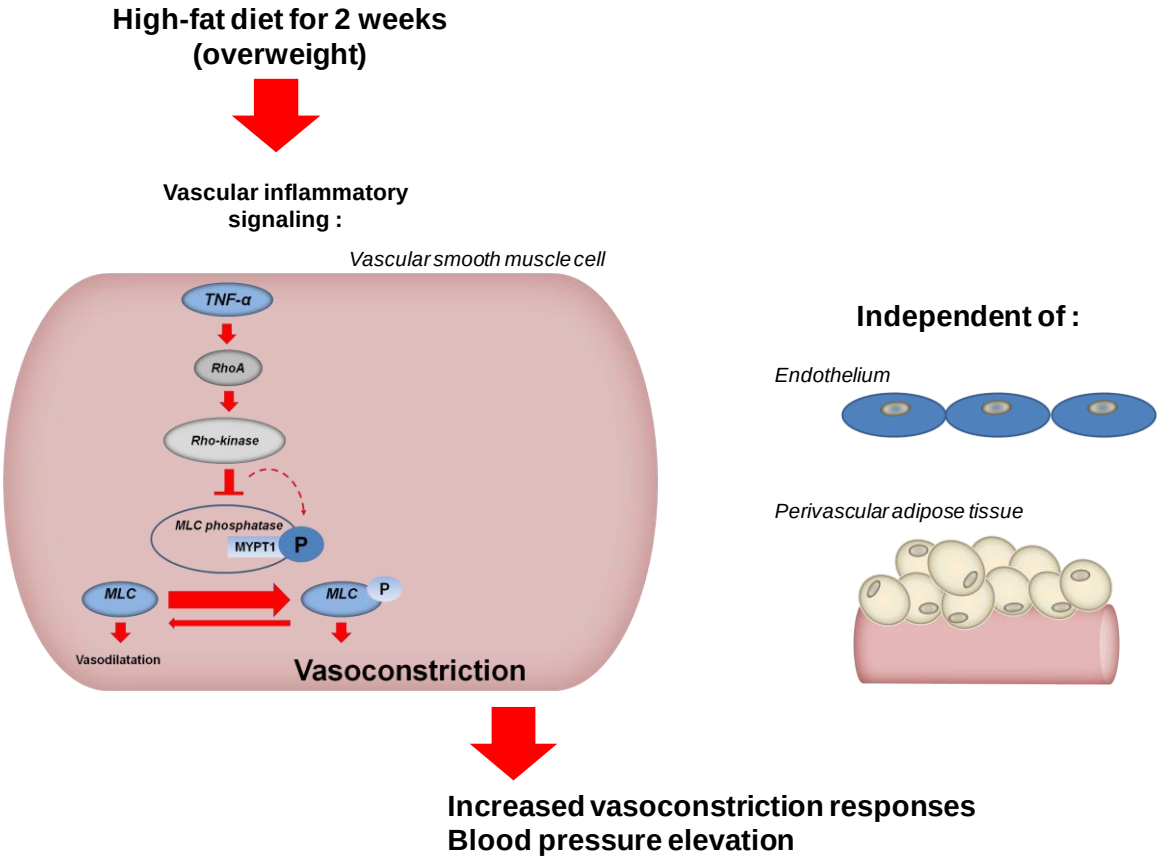
In our study the vascular features in overweight could be prevented independent of elevated cholesterol levels in the cases of blocking TNF-α or inhibition of Rho-kinase activity with statin. However these findings can only mean that the downstream signaling was blocked. In addition, the protection seen with the HFD withdrawal was accompanied by lower cholesterol levels. Therefore, hypercholesterolemia as a trigger for the vascular signaling cannot be excluded in this model, although the C57BL6/J fed a HFD is not an ideal model to study dyslipidemia, as this strain develops hypercholesterolemia mainly due to increase in the HDL fraction[315].

Our results showing the protection of overweight TLR4$^{-/-}$ mice against the vascular effects of HFD suggest a key role for TLR4 in cardiovascular risk factors found in overweight. Thus, it is conceivable that in our study the HFD is working as a source of TLR4 ligands, such as cholesterol and free fatty acids.

8. Summary

These results demonstrate that mice fed a high-fat diet for a short period such as 2 weeks results in overweight with increased vasoconstriction and blood pressure elevation through a mechanism involving a vascular TNF-α/Rho-kinase signaling. The vasculature and the adipose tissue can behave as independent sources of inflammation. Our data strongly suggest that the vasculature can be regarded as an independent factor contributing to cardiovascular risk in overweight. These findings pave the way for improved therapeutic strategies beyond antihypertensive drugs and weight loss, to specifically target vascular proinflammatory signaling as a means of preventing early cardiovascular consequences of overweight.

Schematic representation of the mechanism proposed in this thesis:



9. Clinical significance

Consumption of food rich in saturated fat is a dominant habit of millions of people in modern societies. Overweight is a typical consequence of this western diet and it is associated with blood pressure increase. The exact mechanisms linking high-fat diet, overweight and elevated cardiovascular risk are yet not fully understood and involve important networks making the interaction between immune responses and sensing derived from nutrients. Our mouse model of overweight with cardiovascular risk factors that can be ameliorated through anti-inflammatory strategies brings new insights and might represent a potential target for interventions in human overweight aiming to decrease cardiovascular risk. The need to significantly reduce adiposity to improve cardiovascular risk is not confirmed by our experiments. To reduce vascular proinflammatory signaling seems a realistic therapeutic strategy that can be accomplished by HMG-CoA reductase inhibitors or by a reduced fat content in the diet.

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PUBLICATIONS

- ***Increased visceral adiposity is associated with coronary artery calcification in male patients with chronic kidney disease.***

  Aoqui C, Cuppari L, Kamimura MA, Canziani ME.
  Eur J Clin Nutr. 2013 Mar 27. doi: 10.1038/ejcn.2013.66. [Epub ahead of print

- ***Hemoglobin levels in hemodialysis patients treated with epoetin: a Brazilian experience.***

  Ammirati AL, Watanabe R, **Aoqui C**, Draibe SA, Carvalho AB, Abensur H, Drumond SS, Moreira J, Bevilacqua JL, Silva AC, Tatsch F, Canziani ME.
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- ***Deceased organ donation in Brazil: how can we improve?***

  Medina-Pestana JO, Sampaio EM, Santos TH, **Aoqui CM**, Ammirati AL, Caron D, Galvao-Pereira M.
  Transplant Proc. 2007 Mar; 39(2):401-2.

- ***Role of Toll-like receptor 4 in the modulation of blood pressure and vascular contractility***

  Eißler R., Sollinger D., Chmielewski S., **Aoqui C**., Schmaderer C., Liskova S., Zicha J., Bluijssen H., Witzke O., Benz M., Scherer E., Lutz J., Heemann U., Baumann M.
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ABSTRACTS IN CONGRESSES RELATED TO THIS WORK:

- ***Sphingosine-1-phosphate in Calcium Sensitization of Small Mesenteric Arteries in Spontaneously Hypertensive Rats*** *(POSTER)*

  Aoqui C, Eißler R, Chmielewski S , Schmaderer C, Sollinger D, Heemann U, Baumann M
  21st annual scientific meeting of the European Society of Hypertension (ESH), Milan, Italy (June, 2011)

- ***Short time of high fat intake increases vascular constriction – possible role of local inflammation*** *(ORAL PRESENTATION)*

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PAPER RELATED TO THIS WORK (*Attached)*
Status in December 2013: *Submitted*

- ***Vascular TNF-α/Rho-kinase Signaling Mediates Vasoconstriction and Blood Pressure Elevation in Overweight***

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Abstract

Objective-Large epidemiological trials have demonstrated that overweight is associated with increased cardiovascular risk and has a high prevalence of hypertension. However, the exact molecular underpinnings linking overweight to blood pressure elevation remain unclear. We hypothesized that vascular proinflammatory signaling independent of adipose tissue inflammation, contributes to the onset of blood pressure elevation in overweight.

Approach and Results-To study the human condition of overweight, we defined overweight in a mouse model in which male C57/Bl6 mice were fed a high-fat diet for 2 weeks. We showed that increased vasoconstriction and blood pressure elevation following brief exposure of mice to a high-fat diet is dependent on vascular TNF-α signaling, leading to increased Rho-kinase activity. Inhibition of TNF-α signaling normalized vascular Rho-kinase activity, vasoconstriction and blood pressure elevation without affecting overweight. Interestingly, statin treatment or return to normal diet were similarly protective in ameliorating the effects of vascular signaling in overweight.

Conclusions-These results indicate that vascular TNF-α/Rho-kinase signaling plays a role in vasoconstriction and blood pressure elevation in overweight. Our findings pave the way for improved therapeutic strategies specifically targeting vascular TNF-α/Rho-kinase signaling to prevent early cardiovascular consequences associated to overweight.

Non-standard Abbreviations and Acronyms	
PVAT	perivascular adipose tissue
HFD	high-fat diet
$TNFR1^{-/-}$	tumor necrosis factor receptor 1 knockout
MOPS	3-(N-morpholino)propanesulfonic acid
HOMA	homeostasis model assessment

Introduction

Globally, more than 1 billion people have excess weight[1]. Similar to obesity, overweight is associated with increased cardiovascular risk[2-3]. Large epidemiological studies demonstrate that overweight is associated with hypertension, an established cardiovascular risk factor[3-4] with prevalence

reaching 60%[5]. The molecular mechanisms underlying blood pressure elevation in overweight, however, remain unclear.

Several epidemiological studies have demonstrated an association between proinflammatory cytokines, cardiovascular risk and obesity[6-7]. In particular, tumor necrosis factor-α (TNF-α) has been widely linked to obesity[8-11] and also overweight-related pathologies[8-9, 12]. Interestingly, TNF-α is also linked to the pathology of hypertension[13] and its levels are clearly increased in the vasculature of obese individuals[14]. Overweight individuals are known to exhibit features of vascular dysfunction typically associated with hypertension, such as increased vasoconstriction[15-16] and endothelial dysfunction[16-17]. Given the link between hypertension and vascular inflammation[16, 18-19] via its effects on vasoconstriction[16, 20], we hypothesized that the proinflammatory cytokine TNF-α plays a role in the development of cardiovascular risk in overweight via an effect on vasoconstriction and blood pressure elevation. However the underlying molecular mechanism remains unclear. Targeting vascular dysfunction in overweight could reduce blood pressure and therefore cardiovascular risk beyond common antihypertensive treatment. One suggestion for the underlying molecular mechanism for vascular dysfunction is vascular proinflammatory signaling, as inflammatory processes can modulate vascular function [16, 20-21].

Here we analyse the development of cardiovascular risk factors in a mouse model of overweight. We hypothesized that in overweight vascular proinflammatory signaling increases vasoconstriction resulting in blood pressure elevation.

Materials and Methods

Animal Procedures

Male wild-type C57BL/6J (WT) and tumor necrosis factor receptor 1 knockout ($TNFR1^{-/-}$) mice were housed in temperature-controlled cages (20°C to 22°C) and maintained on a 12/12-hour light/dark cycle. At the age of 12 weeks mice were placed (10-12/group) *ad libitum* either on a normal diet (4% crude fat) or 2 weeks of high-fat diet (HFD, 30% crude fat, Ssniff, Germany). Additionally, WT mice were treated with HFD plus concomitant treatment either with pravastatin (Bristol-Myers Squibb, USA, 50mg/kg/day in drinking water) or

etanercept (Abbott, USA, 10 mg/kg twice per week intraperitoneal, ip, injection). Corresponding control groups received normal diet plus either pravastatin in drinking water or vehicle/etanercept as ip injection.

In one set of experiments with WT mice, the HFD was withdrawn and normal diet was returned for 2 weeks. Their control groups either received normal diet or were kept on HFD for 4 weeks. The TNFR1$^{-/-}$mice (B6.129-*Tnfrsf1a*^{tm1Mak}) were purchased from the Jackson Laboratory (Bar Harbour, Me., USA) and bred in the Central Animal Facility (University Hospital Innenstadt, Ludwig-Maximilians-University Munich). WT mice were obtained from Charles River Laboratories (Sulzfeld, Germany).

During isoflurane anesthesia mice were fixed on a heat-controlled plate, and the left carotid artery was exposed. An intra-arterial pressure transducer was inserted in the left carotid artery under sterile conditions and continuous intra-arterial blood pressure was measured for 15 minutes (Blood Pressure Monitor BP1, World Prec. Instruments, USA). Thereafter, animals were sacrificed and the entire intestine, including vascular arcades, was immediately excised and stored in cold 3-(N-morpholino)propanesulfonic acid (MOPS) buffer for wire-myography.

Changes in whole body weight and epididymal fat pad weight were assessed as parameters of visceral fat status. Serum was obtained in serum separator tubes (Microvette, USA) and stored at -80°C for subsequent biochemical measurements and western blot.

The principles of the NIH Guide for the Care and Use of Laboratory Animals as well as the German Law on the Protection of Animals were followed.

Magnetic resonance imanging

Whole body magnetic resonance imaging (MRI) was performed on mice anesthetized with ip pentobarbital and placed in prone position onto a 47-mm microscopy surface coil inside the clinical 1.5 T MRI System (Achieva 1.5T, Philips Medical Systems, Best, The Netherlands). An axial, multi-slice, turbo spin echo (TSE) sequence (resolution 0.25 x 0.25 x 0.35 mm^3, 140 slices, echo time (TE) = 100 ms, repetition time (TR) = 1000 ms, FOW) was applied

to suppress signal from tissue other than fat. The whole body images were reconstructed using OsiriX DICOM viewer.

Biochemical measurements

Serum fasting total cholesterol, triglycerides and glucose levels were measured by enzymatic methods (Roche Diagnostics). Fasting insulin was measured by ELISA kit (Shibayagi Co. Ltd.). Calculation of the insulin resistance index [homeostasis model assessment (HOMA-IR)] was done using fasting insulin and glucose values: [insulin (picomoles per liter) x glucose (millimolar)] /22.5.[22-23] Serum TNF-α was measured with murine ELISA kit (Peprotech).

Histology

Epididymal fat pads and mesenteric vascular beds with perivascular adipose tissue (PVAT) were fixed in paraformaldehyde, embedded in paraffin and subsequently stained with hematoxylin-eosin. For each mouse the area of 50 randomly chosen adipocytes was measured in 5 representative sections using Image J software (National Institutes of Health, USA) at 10x magnification. Mean values given in pixels were compared. Analysis of infiltrating leukocytes in epididymal fat pads and in mesenteric vascular beds was performed by immunohistochemical staining for CD45 (Beckton & Dickenson, USA). Total CD45-positive cells were counted in 10 randomly chosen arteries per whole mesenteric vascular bed per mouse and averaging the number.

Two-photon microscopy

Small mesenteric arteries and correspondent PVAT were visualized using a Leica SP5 II MP two-photon laser scanning microscope coupled to a water dipping 20X ;NA 1.00 objective and a pre-chirped Ti:Sa laser (Spectra Physics) tuned to 840nm[24]. Four Hybrid detectors (HyD) were spectrally tuned for optimal detection efficiency and low bleed through of signal: Second Harmonic Generation of collagen (HyD1: 400-425nm), autofluorescence of adipocytes and GR1/eFluor450 (HyD2: 445-500nm), autofluorescence of adipocytes/elastin, and CD115/Alexa488 (HyD3: 515-555nm) CD45/nanocrystal-605nm (HyD4: 590-625nm). Additional image processing was performed using Leica LAF AF 3.0 and ImagePro number of

inflammatory cells was determined in 3D datasets in the PVAT of the small mesenteric arteries (up to a distance of three times the average adipocyte diameter from the small artery).

Quantification of inflammatory cells was performed by detection of the number of CD45 /CD115 and CD45/Gr-1 positive cells in each arterial segment recorded in 3D datasets (n = 3 arterial segments per mouse, n = 4 mice per group). The total number of positive cells is presented as inflammatory cells per adipocyte (adipocyte volume was determined by measuring their maximal diameter in the corresponding arterial segment assuming spherical cell shape.

RNA isolation and real-time PCR

Dissected mesenteric PVAT and epididymal fat tissue were snap-frozen in liquid nitrogen and stored at - 80ºC. Tissue (80 mg) was used for RNA isolation using RNeasy Lipid Tissue mini kit (Qiagen) according to the manufacturer's protocol. The mesenteric arterial tree was rapidly cleared of PVAT under a dissection microscope, snap-frozen in liquid nitrogen, stored at - 80ºC and used later on for RNA isolation, performed with RNeasy micro kit (Qiagen). Quality of RNA was assessed in agarose gels stained with ethidium bromide. Complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's protocol. Quantitative reverse transcriptase PCR was performed using SYBR Green I (MyiQ ICycler, Bio-Rad). Sense and anti-sense cDNA primers are depicted in Table I in Data Supplement. The ddCt algorithm method was applied for quantification, including the normalization to GAPDH of each sample[25]. The results are expressed as fold of controls from two to four independent assays.

Tissue lysates

Protein extracts were isolated from mesenteric tissues and epididymal fat tissue. The whole mesenteric vascular tree was rapidly isolated and cleaned to obtain separated arteries and PVAT. Per sample, snap-frozen mesenteric arteries, epididymal fat tissue and PVAT from one mouse were lysed in buffer (CellLytic MT Mammalian Tissue lysis/extraction reagent, Sigma) containing proteases inhibitors (Roche).The lysate was cleared by centrifugation at

16.000g for 15 min at 4°C. The supernatant was collected. Protein concentration was measured by a bicinchoninic acid protein assay reagent kit (Pierce) and used for Rho-associated kinase activity and TaqMan protein assays.

Rho-associated kinase activity

Rho-associated kinase activity was measured by an enzyme immunoassay (Cell Biolabs) in mesenteric arteries from all mice groups and after incubation with TNF-α in *in vitro* experiments. In overweight WT mice Rho-kinase activity was measured as well in epididymal fat pads and PVAT. Experiments were performed according to the manufacturer's protocol, using 10 µg of protein lysate. Values are reported as percentage relative to controls.

TNF-α protein expression

TNF-α protein expression was measured using TaqMan Protein Assay (Applied Biosystems) in mesenteric arteries using a mouse TNF-α biotinylated antibody (R&D Systems), according to the manufacturer's instructions. Protein lysates from mesenteric arteries were incubated with two pools of TNF-α antibody labeled with two types of oligonucleotides. Subsequently, oligonucleotide ligation was performed and the prepared templates were amplified and analyzed by quantitative PCR using TaqMan probes. Pure antigen (TNF-α, Invitrogen) was used as positive control. The results are expressed as fold of control from two independent assays.

Wire myograph and vasoconstriction studies

First to second order branches from superior mesenteric artery (270-330 µm) were cut into 2 mm long rings and mounted in a 4-channel wire myograph (Model 620M, Danish Myo. Technology). Each vessel segment was mounted on two tungsten wires (40 µm diameter) in the organ chamber filled with MOPS buffer. MOPS buffer consisted of (in mM) NaCl 145, KCl 4.7, $CaCl_2$ 3.0, $MgSO_4$ 1.17, NaH_2PO_4 1.2, pyruvate 2.0, EDTA 0.02, MOPS 3.0, and glucose 5.0. Vessels were pre-stretched to a tension representing a blood pressure of 13.3 kilopascal and equilibrated at this tension for 30 minutes at 37°C.[26] Subsequently the organ bath solution was changed for a fresh pre-heated MOPS buffer and vascular functions were analyzed.

Vessels were subjected to noradrenaline-induced vasoconstriction followed by acetylcholine for viability testing. During the experiments, vessel diameter was kept constant for examination under isometric conditions[26]. After washing out with MOPS buffer and resting for 15 minutes, noradrenaline and acetylcholine dose response curves were performed. After washing out with MOPS buffer and resting again for 15 minutes, calcium sensitivity was assessed by stepwise increases in calcium concentration (0 - 3 mmol/L) in the chamber, under depolarizing conditions (125 mmol/L potassium). Vessels were first depleted of calcium with a calcium-free MOPS buffer plus EDTA that consisted of (in mM) NaCl 147, KCl 4.7, $MgSO_4$ 1.17, NaH_2PO_4 1.2, pyruvate 2.0, glucose 5.0, MOPS 3.0, EDTA 1.0. This solution was then substituted for a high-potassium, calcium-free depolarizing solution plus EDTA (NaCl 26.5, KCl 125, $MgSO_4$ 1.17, NaH_2PO_4 1.2, pyruvate 2.0, glucose 5.0, MOPS 3.0, EDTA 1.0).

In separate sets of experiments, vessels from WT mice on normal diet and HFD were incubated in the chamber with the Rho-kinase inhibitor Y-27632 (1µM, 10 minutes). Arteries from the same animals were used for time controls. The protocol to study vasoconstriction was performed before and after incubation.

In another set of experiments, mesenteric arteries of WT mice receiving normal diet were exposed to TNF-α (Millipore, USA) 20ng/ml for 30 minutes and noradrenaline-induced vasoconstriction was investigated before and after TNF-α exposure. Arteries from the same WT mice but without TNF-α treatment were used for time control. Further control experiments were performed to investigate the influence of endothelium and incubation time on vascular responses. In some experiments, endothelium was removed by gently rubbing the intimal surface with a wire, to obtain acetylcholine-induced vasodilation of less than 20%.

The myograph gives output readings as absolute tension generated elicited by vasodilation or vasoconstriction in millinewton (mN). Data are presented as absolute tension and in percentaqe related to the maximal tension developed. Noradrenaline was obtained from Fluka, Switzerland; acetylcholine, Y-27632 and all the others chemical components of myograph

buffer solutions were from Sigma-Aldrich, USA. Y-27632 was dissolved in distilled water. Concentrations given in the text refer to final bath concentrations.

To examine the vasoconstriction dependent of intracellular calcium stores, we first made a blockade of α1-receptors with phentolamine10 μM. This excludes the contribution from nerve-released noradrenaline[27]. After 10 minutes vasoconstriction was induced with vasopressin 1 μM[28].

Histology

Immunostaining for TNF-α was performed in mesenteric vascular beds with adipose tissue. Immediately after dissection, the mesenteric vessels together with perivascular adipose tissue (PVAT) were placed in 4% paraformaldehyde in phosphate buffered saline for 24 hours and subsequently processed to paraffin wax blocks. Consecutive 5μm sections were de-waxed, rehydrated and immunostained for pro-inflammatory cytokine TNF-α (Abcam, Cambridge, UK; dilution 1: 100). Antigen retrieval was performed followed by blocking of endogenous peroxidase and nonspecific protein binding with Dako blocking solutions. Tissue sections were incubated with primary antibodies for 18 hours followed by anti-rabbit/anti-mouse EnVision-HRP (Dako) and finally by Vector SG chromogen kit (Vector Laboratories, Burlingame, CA), used to disclose the presence of TNF-α.

Isolation of smooth muscle cells

Primary smooth muscle cells (SMC) were isolated by enzymatic digestion in a solution containing collagenase type II 1mg/ml, soybean trypsin inhibitor 1mg/ml (Life Technologies), elastase 0,744u/ml (Sigma) in HBSS (Life Technologies). Isolated aortas from 2 mice were cleaned from PVAT and predigested for 10min. Subsequently adventitia was removed, aortas were cut lengthwise and intima was removed by gentle scraping. Aortas were enzymatically digested for 1h at 37˚C[29]. After digestion aortas were passed through 100um cell strainer (BD Falcon) and left undisturbed on 3 wells of a 48 well plate for 1 week. Until passage number 3 cells were cultivated in DMEM (PAA) medium with 20% FBS. After 3rd passage SMC were cultivated in DMEM (PAA) medium containing 4,5mg/l Glucose, 2mM L-glu,

supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 10% FBS (PAA).

Western Blot

Total adiponectin and adiponectin multimers were determined by western blotting in serum. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS**-**PAGE**)** was performed. In brief, serum proteins were separated by 10% SDS-PAGE under non-reducing and non-heating conditions, and transferred to nitrocellulose membranes. Membranes were blocked with TBS-Tween 20 containing 5% skim milk and incubated with a goat anti-mouse adiponectin polyclonal antibody (1:500, R&D Systems). After being washed, membranes were incubated with horseradish peroxidase conjugated-donkey antigoat antibody (1:4000, Santa Cruz Biotechnology). Bands were visualized by use of lumi-light Western blotting substrate (Roche Diagnostics), and the image was acquired with a Kodak IS440CF Imaging Station. Densitometry analysis was performed with Adobe Photoshop software. Relative distributions of adiponectin multimers were calculated by dividing band density by total density.

Rho-kinase phosphorilates myosin phosphatase target subunit 1 (MYPT1), inactivating myosin phosphatase activity, favoring the phosphorilated status of myosin light chain, thereby promoting vasoconstriction[30]. Total MYPT1 and phosphorylated MYPT1 were determined by western blotting in primary aortic smooth muscle cells. Primary SMC were starved overnight with DMEM medium containing 2%FBS and subsequently treated with TNF-α 40 and 100ng/ml for 6 hours. Cells were homogenized in a lysis buffer (CeLytic, Sigma-Aldrich) containing phosphatases and proteases inhibitors (Roche). Protein concentration was determined using a bicinchoninic acid protein assay kit (Perce). 60ug of protein per sample was loaded per lane and resolved by SDS-poly-acrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were transferred onto PVDV membrane using iBlot transfer device (Life Technologies). Membranes were blocked by treatment with 5% BSA (PAA) in TBS and subsequently probed with antibodies against MYPT1 (1:800, EMD Millipore), phosphorylated MYPT1 (p-MYPT1, 1:80, EMD Millipore), GAPDH (1:15000, EMD Millipore) and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000, Santa

Cruz). Immunoreactivity was detected by adding Luminata Forte Western Substrate (EMD Millipore) and detected by Intas imaging system

Data Analysis and Statistical Procedures

Data are presented as mean ± SEM. In myograph experiments differences between logEC50 in cumulative dose-response curves were calculated using a non-linear regression analysis. For comparisons between absolute tensions two-way ANOVA with Bonferroni post hoc test was used. For comparisons between more than two groups one-way ANOVA with Tukey post-hoc test was used. In all other experiments comparing two groups, Student's t-test was used. A probability value <0.05 was considered statistically significant (GraphPad Prism ® 5.0).

Results

A mouse model for overweight

To address the putative role of vascular TNF-α signalling in overweight-induced hypertension, we developed a mouse model of overweight. As expected, wild-type (WT) mice fed HFD for 8 weeks were obese (**Fig. 1A)**. serum TNF-α was elevated and insulin resistance was present (**Fig. 1B**). We observed increased vasoconstriction (**Fig. 1C**) and elevated blood pressure (**Fig. 1D**). Overweight mice fed HFD for 2 weeks had significantly heavier epididymal fat pads along with accumulated mesenteric PVAT accompanied by hypertrophy of adipocytes (**Fig.1A)**. Interestingly, while overweight WT mice also had increased total cholesterol levels, common confounders associated with obesity, such as insulin resistance and systemic inflammation, were absent (**Fig.1B**). The small mesenteric arteries of overweight WT mice had an increased response to noradrenaline as shown by measurement of absolute tension and EC50 (control WT: logEC50 -5.921 vs. overweight WT: logEC50 -6.556, *P*<0.001) (**Fig.1C**). A similar result was obtained in endothelium-denuded arteries, demonstrating that the vasoconstriction response was independent of endothelium (**Figure IA in the Data Supplement**). We examined as well the role of PVAT in this model. The data strongly suggest that after 2 weeks of high-fat diet the PVAT keeps its anticontractile properties, very similarly to controls. Thus, PVAT did not

seem to influence vascular function in our overweight model (**Figure IB in the Data Supplement**). Overweight WT mice also had significantly increased blood pressure values (control WT: 72 ± 1.3 mmHg vs. overweight WT: 85 ± 1.0 mmHg, *P*<0.001) (**Fig.1D**). Unlike the overweight, the mesenteric PVAT from the obese group showed signs of inflammation (**Fig. 2A-E**). Thus, our 2-week HFD-induced mouse model exhibits an overweight phenotype without confounding obesity-related factors but already with increased vasoconstriction and blood pressure elevation.

Vascular TNF-α contributes to increased noradrenaline-induced vasoconstriction and blood pressure in overweight mice.

In order to test the hypothesis that TNF-α plays a role in vascular dysfunction, we first analyzed the expression of the cytokine in our overweight mouse model. Mesenteric arteries from overweight WT mice had a two-fold increase in expression of both TNF-α mRNA and TNF-α protein levels (**Fig. 3A**). This finding was corroborated by positive immunostaining of mesenteric arteries for TNF-α (**Figure IIA in the Data Supplement**). Overweight WT mice had no elevation of TNF-α in visceral adipose tissue, as shown by analysis of mesenteric PVAT and epididymal fat pads (**Fig. 3A**).

Functional analysis of the effect of TNF-α on small mesenteric arteries from control WT mice was carried out using wire myography. Incubation of the arteries with TNF-α for 30 min increased noradrenaline-induced vasoconstriction (logEC50 pre TNF-alpha: -6.265; post TNF-alpha: -6.680, *P*<0.01), suggesting a direct effect of TNF-α on the vasculature (**Fig. 3B**). In contrast, no such effect was observed in TNF-α loss-of-function models either using an anti-TNF-α antibody (etanercept) or $TNFR1^{-/-}$ mice. Pharmacological or genetic loss of TNF-α signaling did not interfere with the development of overweight and hypercholesterolemia, leading to a metabolic phenotype identical to untreated overweight WT mice. However, increased vasoconstriction and blood pressure elevation were not observed (**Fig. 3C and 3D**). Arteries with similar endothelial function were used for all vasoconstriction studies.

Adiponectin is suggested as an endogenous opponent to TNF-α in the control of vascular biology[31]. In our overweight WT mice, however, levels of adiponectin and, in particular HMW adiponectin, were unchanged compared to controls (**Figure IIB in the Data Supplement**). In contrast, obese mice

(after 8 weeks of HFD) had significantly reduced HMW adiponectin (**Figure IIB in the Data Supplement**). Thus, although HMW adiponectin may influence vascular biology in obese mice, the effect of TNF-α signaling in overweight mice is independent of adiponectin action.

Vascular TNF-α downstream signaling in overweight mice

One candidate to mediate the signaling events downstream of TNF-α in overweight-induced vasoconstriction and blood pressure elevation is the RhoA/Rho-kinase pathway. MYPT1 is phosphorylated by Rho-kinase and thereby inactivates myosin phosphatase activity, favoring the phosphorylated status of the myosin light chain, leading to vasoconstriction[32]. To assess the role of this pathway, we measured Rho-kinase activity in overweight WT mice and found that activity was increased in mesenteric arteries but not epididymal or mesenteric PVAT (**Fig. 4A**). Incubation of mesenteric arteries from control WT mice with TNF-α led to a significant increase in Rho-kinase activity in mesenteric arteries, with even low doses (20ng/ml) causing an almost two-fold increase (**Fig. 4B**). In contrast, vascular Rho-kinase activity was not increased in overweight WT on TNF-α antibody treatment or in overweight TNFR1$^{-/-}$ mice (**Fig. 4C**). In addition, vascular smooth muscle cells treated with TNF-α presented increased Rho-kinase activity and increased phosphorilation of myosin phosphatase target subunit 1 (MYPT1) (**Figure IIC in the Data Supplement**). Taken together, these data suggest that the Rho-kinase pathway mediates TNF-α-induced vasoconstriction and elevated blood pressure in a mouse model of overweight-induced hypertension.

Consistent with a vascular TNF-α-induced activation of Rho-kinase, calcium-induced vasoconstriction was also augmented in overweight WT mice compared to control WT animals (**Fig. 4D**). In order to test the relevance of Rho-kinase activation in these responses, calcium sensitivity was investigated in the wire myograph. We first blocked adrenoreceptors with phentolamine, and then vasopressin was added to elicit vasoconstriction that is dependent on release of intracellular calcium. Mesenteric arteries from overweight WT mice had an earlier and stronger response to intracellular calcium release that was independent of adrenergic signaling, suggesting higher calcium sensitivity in these arteries (**Figure III in the Data**

Supplement). Treatment with TNF-α antibody prevented increased calcium-mediated vasoconstriction in overweight mice (**Fig. 4E**). Similarly, overweight TNFR1$^{-/-}$ mice did not show augmented calcium-induced vasoconstriction (**Fig. 4E**). Upon blockade of Rho-kinase activity with the specific inhibitor Y-27632 in mesenteric arteries of overweight WT mice, calcium-induced vasoconstriction returned to a level similar to that found in control WT mice. As expected, treatment with Y-27632 caused only a mild reduction of vasoconstriction in arteries from control WT mice (**Fig. 4F**). Taken together, these data further support a role for vascular Rho-kinase activity as a link between TNF-α and vascular changes in overweight mice.

Pravastatin and return to normal diet prevented/reversed increased vasoconstriction and blood pressure levels in overweight mice

Given the relation between TNF-α signaling and overweight-induced vascular pathology, we investigated whether we could break the link between vascular inflammation and blood pressure increase in our mouse model. We first investigated the effects of pravastatin, an HMG-CoA reductase inhibitor commonly used in patients with high cardiovascular risk[33] and that is known to have pleiotropic effects[33-35], including modulation of the RhoA/Rho-kinase signaling pathway[36-37]. Similarly to etanercept and lack of TNFR1, pravastatin had no effect on weight gain or cholesterol level (**Fig. 5A and 5B**), however it prevented the increase of vascular Rho-kinase activity and protected against vasoconstriction and blood pressure elevation in WT overweight mice (**Fig. 5C**). Interestingly, returning overweight mice to a normal diet for two weeks resulted in prevention of Rho-kinase activity increase and a significant reduction in noradrenaline sensitivity (overweight mice, logEC50 -6.360; HFD withdrawal, logEC50 -6.088; $P<0.05$). Blood pressure was also significantly lower than in mice kept on HFD; however, vasoconstriction and blood pressure still remained significantly elevated compared to control mice on normal diet (**Fig. 6A**). This effect was observed despite overweight features not being significantly different from mice that had continued the HFD (**Fig. 6B and 6C**). Notably, with the return to normal diet, cholesterol decreased significantly (**Fig. 6C**) Thus, return from HFD to normal diet partially recovered vascular function and decreased blood pressure, without reversing overweight. Such observations are consistent with reports that diets rich in

saturated fat may themselves be a source for inflammation, including for TNF-α, either directly or via metabolic endotoxemia[38-40].

Discussion

We showed that a short-term HFD intake generates a mouse model of overweight with increased vasoconstriction and elevated blood pressure. In overweight mice, vascular but not adipose tissue inflammation contributed to increased vasoconstriction and blood pressure elevation. We demonstrated that vascular TNF-α/Rho-kinase signaling mediates this mechanism. Different strategies either prevented or partially reversed the increase in vasoconstriction and blood pressure without affecting weight gain.

Despite numerous studies in animal models of obesity, the overweight condition has not been well characterized in a mouse model with concomitant vascular alterations. The C57/Bl6 mouse on prolonged HFD is an established model to study diet-induced obesity[41]. A short-term HFD in C57/Bl6 mice presents already after two weeks with weight gain, including visceral fat gain. Therefore, we propose it as a model mimicking the human clinical condition classified as overweight. Importantly, this mouse model of overweight shows an increased vasoconstriction and blood pressure without the presence of confounders such as disturbance of glucose metabolism and systemic inflammation.

The proinflammatory cytokine TNF-α was present in arteries of overweight mice. The association between cytokines, cardiovascular risk and obesity[6-7, 42] is well known. In our overweight mice the mesenteric arteries had elevated TNF-α, that has also been reported to be an adipocyte-derived cytokine[8, 10, 12]. However, TNF-α expression was not increased in mesenteric PVAT and epididymal fat in overweight mice. This finding corresponds to the observation of Virdis et al., who demonstrated a prominent TNF-α staining in visceral arteries of obese individuals[14]. To understand the molecular mechanisms involving vascular TNF-α in overweight, we showed in *ex vivo* myograph experiments that TNF-α elicits vasoconstriction in mesenteric arteries of WT mice on normal diet. A pharmacological approach to antagonize TNF-α in the course of HFD prevented the increase in vasoconstriction and blood pressure without affecting overweight. This is in

line with a report of a mouse model of vasculitis, where treatment with a TNF-α antibody lowered blood pressure[43]. As overweight TNFR1 deficient mice were similarly protected, the role of vascular TNF-α in overweight-induced vasoconstriction and blood pressure elevation was further strengthened. In order to understand the mechanism involved in the vascular effects elicited by TNF-α we investigated adiponectin, discussed as regulatory counterpart of TNF-α. Lower levels of adiponectin, in particular the high-molecular weight (HMW) adiponectin, have been linked to metabolic syndrome[44], and Greenstein et al. reported an adiponectin-dependent vascular effect in obese subjects[31]. However, our data do not indicate a role for adiponectin after short-term HFD in mice. In particular, as the protective HMW adiponectin fraction was not significantly reduced in overweight mice.

The RhoA/Rho-kinase pathway is known to be a key regulator of calcium signaling during vasoconstriction[45], and can be upregulated in hypertensive states and other cardiovascular diseases[46]. Furthermore, TNF-α (through TNFR1) has been reported to activate RhoA in airway smooth muscle cells[47]. However, there is currently no evidence of similar signaling occurring in vascular smooth muscle cells. We demonstrated that TNF-α activates Rho-kinase in mesenteric arteries *ex vivo* and *in vitro* in cultured vascular smooth muscle cells from WT mice on normal diet. In this line, mesenteric arteries of overweight WT presented not only with increased TNF-α expression but also with increased Rho-kinase activity. Similarly, adipose tissue of overweight WT without TNF-α expression had also no increase in Rho-kinase activity. The functional link between vascular TNF-α expression and Rho-kinase activity was evidenced as overweight WT treated with TNF-α antibody and overweight mice with genetic deletion of TNFR1 had lower vascular Rho-kinase activity, reduced vasoconstriction and lower blood pressure. Taken together these data offer vascular Rho-kinase activity as a link between TNF-α and blood pressure increase in overweight mice.

Next we investigated whether we could break the link between vascular inflammation and blood pressure increase. Statins have pleiotropic anti-inflammatory effects[33-35] that include the modulation of Rho-kinase signaling[36-37]. In overweight mice pravastatin prevented blood pressure increase similarly to TNF-α blockade without affecting the extent of fat gain.

Finally, we targeted HFD as a potential source for inflammation[48-50]. Indeed, the return to normal diet in overweight WT mice reduced blood pressure without significantly altering the overweight state, although the cholesterol levels decreased significantly. This finding suggests a potential link between hypercholesterolemia and blood pressure elevation in overweight, in line with epidemiological observations showing a high prevalence of elevated cholesterol levels in patients with hypertension[51-53]. These data support the idea that blood pressure increase in overweight can be prevented or at least ameliorated by targeting a downstream vascular signaling through different strategies not necessarily dependent on significant weight loss.

In summary, our results demonstrate that short-term HFD in mice results in overweight with increased vasoconstriction and blood pressure elevation through a mechanism involving vascular TNF-α/Rho-kinase signaling. These findings pave the way for improved therapeutic strategies beyond antihypertensive drugs and weight loss to specifically target vascular proinflammatory signaling as a means of preventing the early cardiovascular consequences of overweight.

Significance

Overweight is a common and increasing problem in western societies and is associated with blood pressure increase. A better understanding of the mechanisms involved in the increase of blood pressure will provide opportunities for therapeutic interventions to decrease cardiovascular risk, beyond weight loss. Our mouse model for the clinical condition of overweight showed that vascular inflammation is a major player for blood pressure increase and might represent a potential target for interventions in human overweight aiming to decrease cardiovascular risk.

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Disclosures

The authors declare that they have no conflict of interest.

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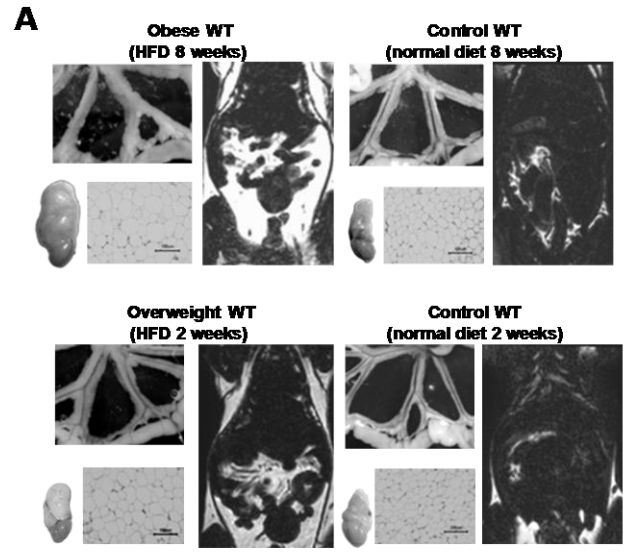
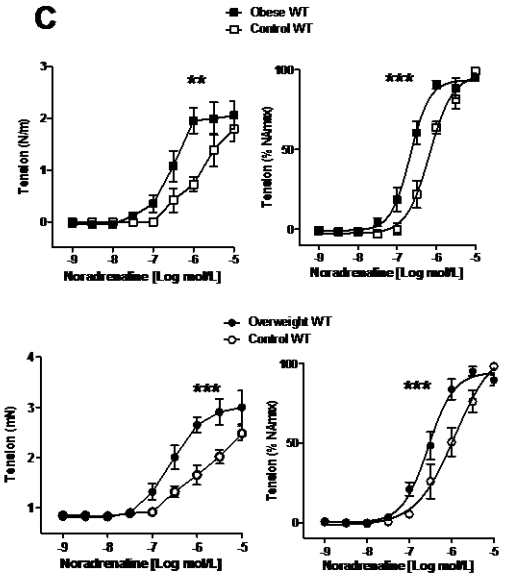
Figures and Figure Legends

Figure 1





B

	Obese WT (HFD 8 wks)	Control WT (normal diet 8 wks)	Overweight WT (HFD 2 wks)	Control WT (normal diet 2 wks)
Body weight gain (%)	27.8 ± 2.0***	17 ± 4.0	13.8 ± 0.7***	9.0 ± 0.9
Fat pad weight (% of body weight)	2.0 ± 0.1***	0.6 ± 0.1	1.5 ± 0.06***	0.9 ± 0.04
Increase of adipocytes area (% related to control)	64 ± 7***	na	51 ± 2***	na
Cholesterol (mmol/L)	3.9 ± 0.1***	2.1 ± 0.1	4.2 ± 0.2***	2.5 ± 0.1
Triglycerides (mmol/L)	1.17 ± 0.1	1.19 ± 0.1	1.13 ± 0.2	1.20 ± 0.1
HOMA Index	0.45 ± 0.03	0.24 ± 0.02**	0.25 ± 0.1	0.20 ± 0.1
Serum TNF-alpha (pg/ml)	27 ± 1.5	not detectable	not detectable	not detectable

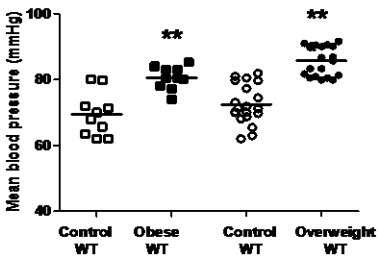


Figure 1. Effect of HFD for 8 (obese) and 2 weeks (overweight) on WT mice. Similar to obese WT, overweight resulted in onset of cardiovascular risk factors such as increased vasoconstriction and blood pressure elevation. **A,** Mesenteric PVAT, epididymal fat pads and adipocyte hypertrophy. White areas represent fat depots in magnetic resonance imaging (n=3-6/group). **B,** Comparative table of weight and metabolic parameters (n=10-15/group). **C-D,** Vasoconstriction (absolute tension and sensitivity) and mean arterial blood pressure pressure were significantly higher in obese and in overweight WT (n=10/obese WT group and 20/overweight WT group). Error bars, s.e.m., **P* <0.05, ***P* <0.01, ****P* < 0.001 compared to the corresponding controls, na=not applicable.

Figure 2

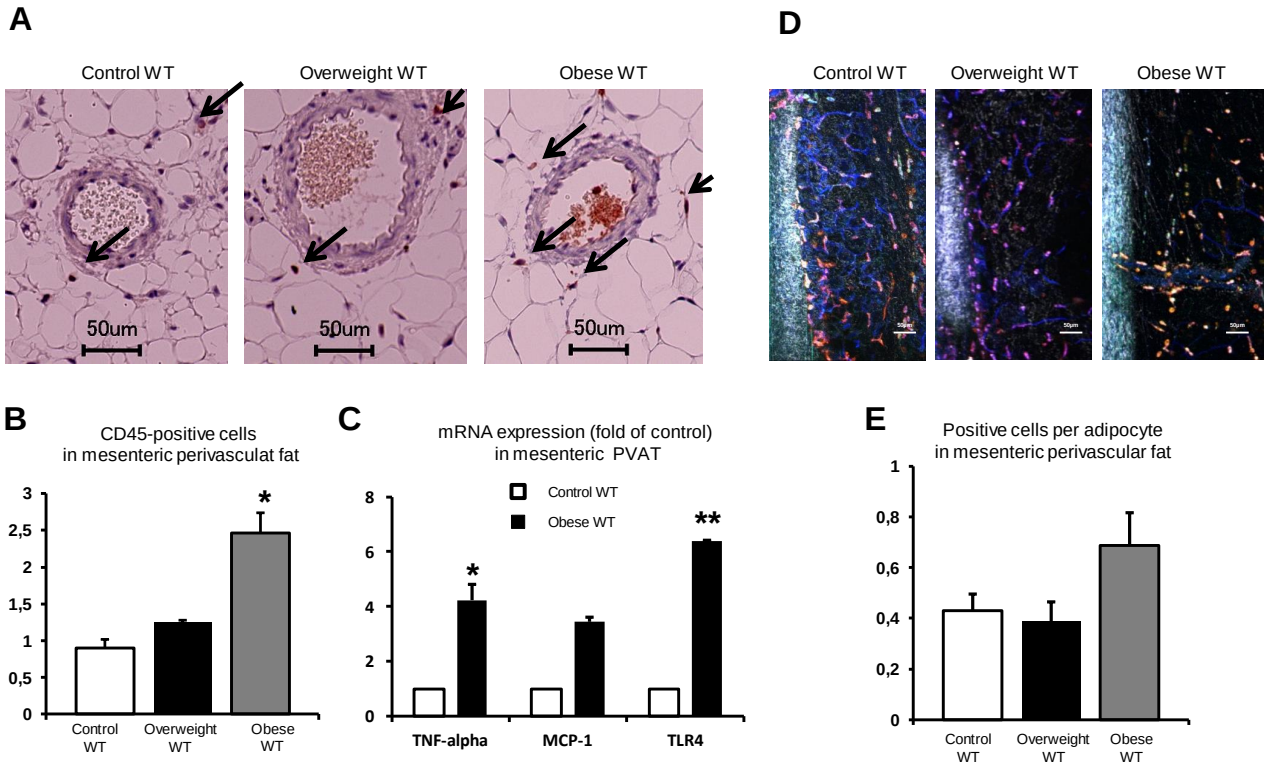


Figure 2. Inflammation in mesenteric perivascular adipose tissue (PVAT). **A,** Representative images from CD45 immunohistochemical staining of mesenteric PVAT. **B,** The number of CD45-positive cells was not different between overweight mice and controls, but it was increased in obese mice. **C,** Increased expression of inflammatory genes in mesenteric PVAT (n=6/group, error bars, s.e.m., **P*<0.05, ***P*<0.01). **D-E,** Representative images of two-photon microscopy a small mesenteric artery and correspondent PVAT. Positive cells for CD45/CD115 and CD45/Gr-1 staining are shown. Overweight mice did not present with increased number of positive cells. In the obese mice group there was a trend toward quantitatively more positive cells (*P*=0.06). The column graph represents the number of positive cells per arterial segment examined (3 segments/mouse, n=4 mice/group, error bars, s.e.m.).

Figure 3

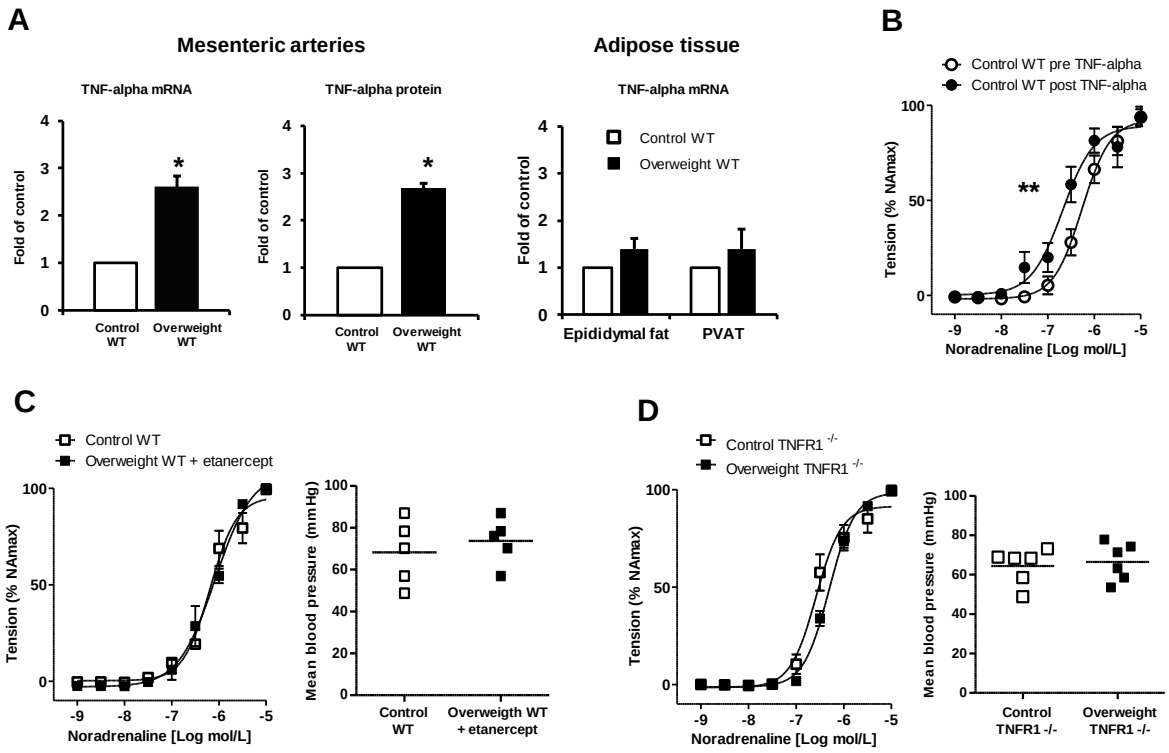


Figure 3. Role of TNF-α in the development of cardiovascular risk factors associated with overweight. **A,** Expression of TNF-α mRNA and protein in mesenteric arteries and adipose tissue (epididymal fat pads and mesenteric PVAT), n=6/group. **B,** Mesenteric arteries from control WT were incubated with TNF-α (20ng/ml, 30 minutes) in the wire myograph and presented increased noradrenaline-induced vasoconstriction (n=6/group). **C-D,** Increase in noradrenaline-induced vasoconstriction and mean arterial blood pressure was prevented in overweight WT mice plus treatment with etanercept (10 mg/kg twice per week intraperitoneally, n=5/group) and in overweight TNFR1$^{-/-}$ mice, n=6/group. Error bars, s.e.m., **P* <0.05 ,***P* <0.01.

Figure 4

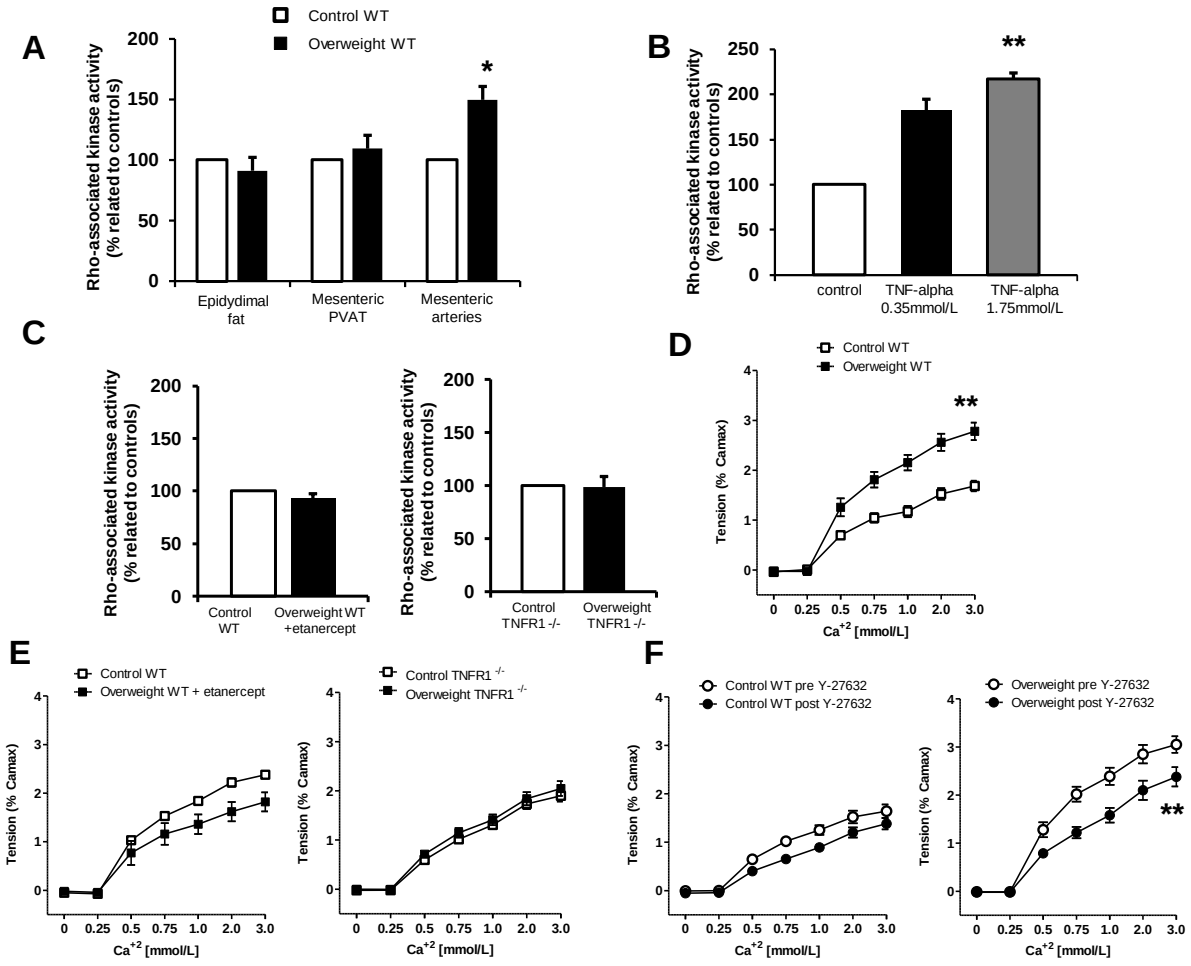
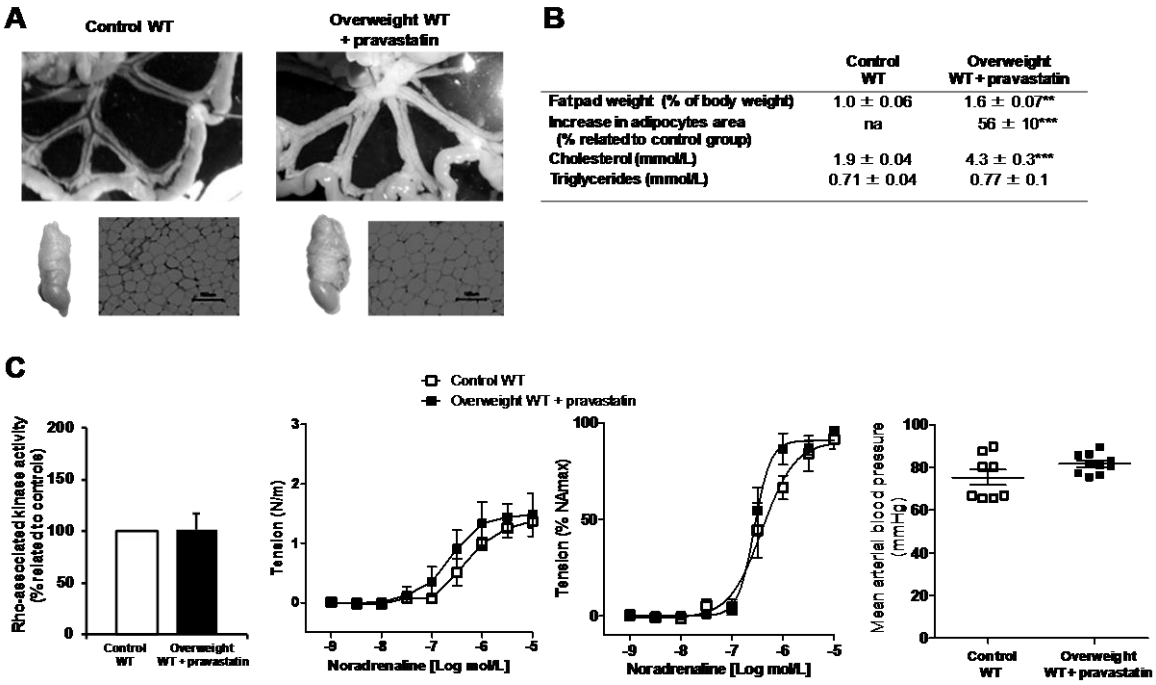


Figure 4. TNF-α increases Rho-kinase activity in the vasculature. **A,** Increased Rho-kinase activity in mesenteric arteries but not in epididymal or in mesenteric PVAT of overweight WT mice (n=8/group). **B,** Increased Rho-kinase activity in isolated mesenteric arteries from control WT after incubation in culture medium with TNF-α (0.35 and 1.75mmol/L, 30 minutes). **C,** Rho-kinase activity was not increased in overweight WT treated with etanercept or in overweight TNFR1$^{-/-}$ (n=6/group). **D,** Increased calcium-induced vasoconstriction in small mesenteric arteries of overweight WT (n=20/group). **E,** Calcium-induced vasoconstriction was not increased in overweight WT treated with etanercept or in overweight TNFR1$^{-/-}$ (n=6/group). **F,** Blockade of Rho-kinase activity (Y-27632, 1μmol/L for 10 minutes) in mesenteric arteries mounted in the wire myograph had a more pronounced effect in decreasing calcium-induced vasoconstriction in the overweight WT group (n=6/group). Error bars, s.e.m., **P* <0.05 ,***P* <0.01.

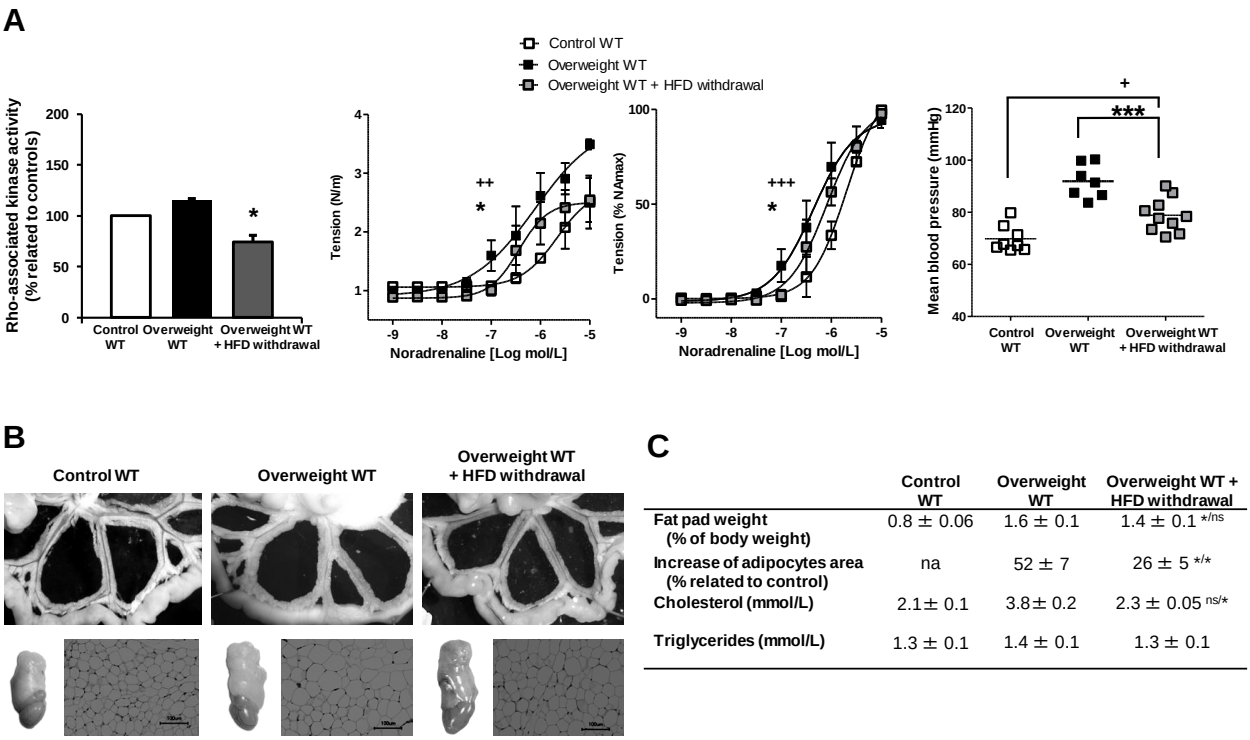
Figure 5



	Control WT	Overweight WT + pravastatin
Fatpad weight (% of body weight)	1.0 ± 0.06	1.6 ± 0.07**
Increase in adipocytes area (% related to control group)	na	56 ± 10***
Cholesterol (mmol/L)	1.9 ± 0.04	4.3 ± 0.3***
Triglycerides (mmol/L)	0.71 ± 0.04	0.77 ± 0.1

Figure 5. Effect of pravastatin treatment on overweight WT mice. **A,** Mesenteric PVAT, epididymal fat pads and adipocyte hypertrophy. Treatment with pravastatin (50mg/kg/day in drinking water) did not prevent fat gain (n=6/group). **B,** Comparative table of weight and metabolic parameters (n=6/group, ***P* <0.01; ****P* <0.001; na=not applicable). **C,** Increases in Rho-kinase activity, vasoconstriction and blood pressure (8-10/group) were prevented by treatment with pravastatin in overweight WT mice.

Figure 6



	Control WT	Overweight WT	Overweight WT + HFD withdrawal
Fat pad weight (% of body weight)	0.8 ± 0.06	1.6 ± 0.1	1.4 ± 0.1 $^{*/ns}$
Increase of adipocytes area (% related to control)	na	52 ± 7	26 ± 5 $^{*/*}$
Cholesterol (mmol/L)	2.1± 0.1	3.8± 0.2	2.3 ± 0.05 $^{ns/*}$
Triglycerides (mmol/L)	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1

Figure 6. Effect of return to normal diet on overweight WT mice. After 2 weeks of HFD, overweight WT mice were returned to normal diet for 2 weeks (n=10/group). Aged-matched WT mice on normal diet and on HFD for 4 weeks were used as controls. **A,** Rho-kinase activity was not increased after HFD withdrawal. Increase in vasoconstriction and blood pressure elevation were partially prevented (^{+}P<0.05, ^{++}P<0.01, ^{+++}P<0.001 = HFD withdrawal vs. Control WT and *****P<0.05**, *****P<0.001 = HFD withdrawal vs. Overweight WT, n=7-10/group) Error bars, s.e.m.. **B,** Mesenteric PVAT, epididymal fat pads and adipocyte hypertrophy (magnification 200x). **C,** Comparative table of weight and metabolic parameters. HFD withdrawal partially prevented visceral fat gain at the end of 4 weeks, compared to the overweight group on continuous HFD (*****P<0.05, *** P<0.001 and ns=not significant; before slash refer to HFD withdrawal vs. Control WT and following the slash refer to HFD withdrawal vs. Overweight WT, n=7-10/group).

Data Supplement

Supplemental Table I – Primer sequences

Gene	Forward	Reverse
GAPDH	TCGGTGTGAACGGATTTGGC	TTTGGCTCCACCCTTCAAGTG
TNF-α	CCAAAGGGATGAGAAGTTCC	GGCAGAGAGGAGGTTGACTTT
TLR4	ATTCCCTCAGCACTCTTGATT	AGTTGCCGTTTCTTGTTCTTC
MCP-1	GCTGTAGTTTTTGTCACCAAG	GATTTACGGGTCAACTTCACA

Figure I

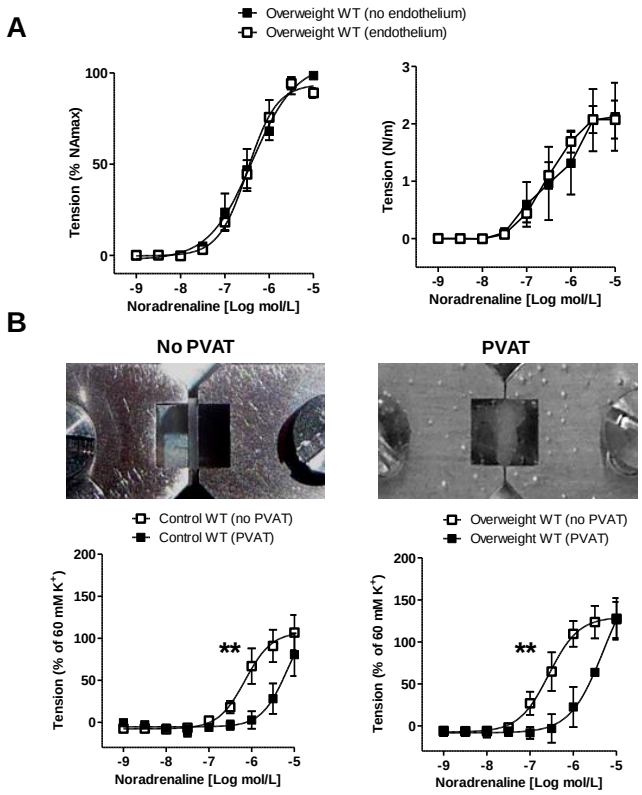


Figure I. Effect of endothelium and perivascular adipose tissue (PVAT) on vasoconstriction of small mesenteric arteries from overweight WT mice. **A,** Endothelium-denuded arteries presented similar vasoconstriction level, suggesting the after 2 weeks of high-fat diet the endothelium function did not influence noradrenaline-induced vasoconstriction. **B,** Arteries examined in the wire myograph without and with PVAT. The anticontractile properties of the PVAT were still present in the overweight WT group, with similar effect to the one observed in the control WT mice. **P<0.01, n=2-3 arteries/mouse, 4 mice/group.

Figure II

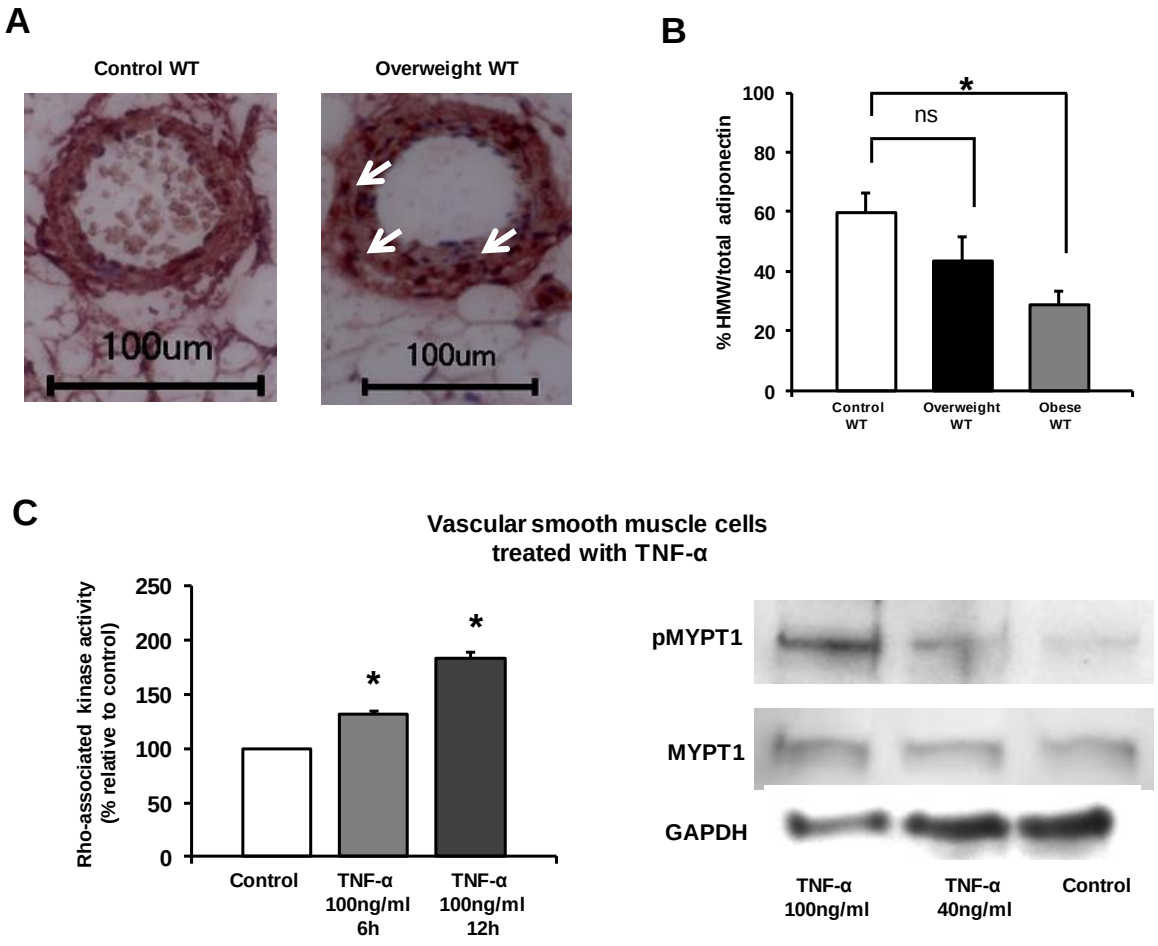


Figure II. TNF-α staining, adiponectin measurement and activation of Rho kinase/phosphorilation of MYPT1. **A,** Representative images of positive immunostaining for TNF-α in small mesenteric arteries. **B,** High-molecular weight (HMW) adiponectin level detected by Western blot in serum was significantly reduced in obese but not overweight WT mice (n=6/group, error bars, s.e.m., ns=not significant, **P* <0.05). **C,** Rho-associated kinase activity in vascular smooth muscle cells treated with vehicle (control) or TNF-α 100ng/ml for 6 and 12 hours. Western blot analysis showed increased phosphorilation of MYPT1 in cells treated with TNF- α 100ng/ml. Cells were treated with vehicle (control) or TNF-α 40 and 100ng/ml for 6 hours. Error bars, s.e.m., *P* <0.05**.**

Figure III

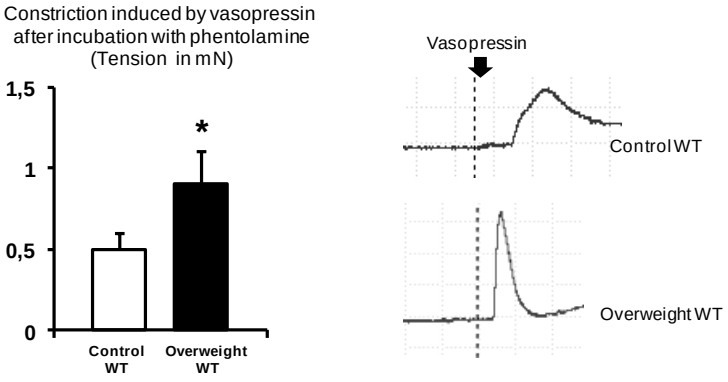


Figure III. Role of intracellular calcium in vasoconstriction response in overweight. After blocking adrenoreceptors with phentolamine, intracellular-calcium-dependent vasoconstriction (elicited by vasopressin) was higher in overweight WT mice. Error bars, s.e.m., *P<0.05.