

## **chapter 1 - introduction**

*adapted from*

*Microvascular dysfunction: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension.*

*Microcirculation 19: 5-18, 2012*

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## **Opening remarks**

This thesis describes the studies performed at the VU medical center during my PhD project. It is the latest in a line of projects testing our group's main hypothesis: the microvasculature as a pivot at the heart of clustering cardiometabolic risk factors. Although initially meant to focus on a specific aspect of this hypothesis, e.g. the effects of endothelin-1 in blood pressure control and whole body glucose uptake, the project gradually evolved to address several other aspects of the microvasculature. As such, I was given the unique opportunity to study the microcirculation in the broadest sense, from intracellular signaling to epidemiological cohorts, using in-vitro, ex-vivo and in-vivo settings, in mice and men. These different angles of investigation build upon research-lines initiated at the departments of physiology and internal medicine, combining pre-clinical and clinical *methods of questioning* to expose the central paradigm.

Throughout this introductory text you will find boxes highlighting several unanswered questions concerning the central paradigm. We will entertain these questions in the remaining chapters.

## **Introduction**

The obesity pandemic is paralleled by a catastrophic increase in the prevalence of cardiometabolic disease. Obesity has been implicated in the rising prevalence of the metabolic syndrome, a cluster of risk factors including hypertension and insulin resistance, which confers an increased risk for type 2 diabetes and cardiovascular disease (1). Although this is well recognized, the underlying mechanisms have only started to unravel. Obesity-associated microvascular dysfunction is hypothesized to explain part of the clustering of cardiovascular risk factors, predisposing obese subjects to cardiovascular disease (2).

### **The microcirculation - physiology**

The microcirculation is generally taken to include vessels less than ~150  $\mu\text{m}$  in diameter. This definition includes the smallest arteries, arterioles, capillaries, and venules. A primary function of the microcirculation is to optimize supply of nutrients and oxygen within tissues in response to prevailing local demand. Adequate perfusion via the microcirculatory network is thus essential for the integrity and normal function of tissues and organs. According to Poiseuille, it is at the level of the microcirculation that the largest drop in hydrostatic pressure occurs. As a result, the microcirculation determines most of overall peripheral vascular resistance, and is thus an intricate part of blood pressure control.

Microvascular dysfunction, e.g. decreased microvascular vasoreactivity in response to vasoactive stimuli at the level of both resistance vessels and the nutritive capillary beds, has been established in obesity, hypertension and insulin resistance. Importantly, these microvascular abnormalities appear to represent a generalized condition that affects multiple tissues and organs. Not only peripheral microvascular function in skin and muscle, but also coronary, retinal and renal microvascular function are affected (3,4,5).

### **Hypertension as a result of microvascular dysfunction**

In most forms of experimental and clinical hypertension peripheral vascular resistance is increased in proportion to the increase in blood pressure (3). This increase in peripheral vascular resistance reflects changes in the microcirculation, specifically arterioles. In several tissues both microvascular endothelium-dependent vasodilatation and capillary density has been found to correlate inversely with blood pressure in hypertensive and normotensive subjects (2,6,7,8). Whereas it has been known for many years that increased wall-to-lumen ratio and microvascular rarefaction can be secondary to sustained elevation of blood pressure (3), there is also evidence that abnormalities in the microcirculation precede and are thus a causal component of high blood pressure. Microvascular rarefaction, similar in magnitude to the rarefaction observed in patients with established hypertension, can already be demonstrated in subjects with mild intermittent hypertension and in normotensive

subjects with a genetic predisposition to high blood pressure (9,10). Moreover, in hypertensive subjects, capillary rarefaction in muscle has been shown to predict the increase in mean arterial pressure over two decades (11). More recently, a smaller retinal arteriolar diameter has been shown to predict the occurrence and development of hypertension in a prospective, population-based study of normotensive middle-aged persons (12,13). Other, indirect, evidence comes from studies demonstrating that inhibitors of angiogenesis and especially inhibitors of VEGF/VEGFR-2 signaling cause arterial hypertension, which is paralleled by microvascular rarefaction (14), see box.

Are the changes in capillary density and blood pressure during VEGF receptor tyrosine kinase inhibition directly related? If so, are these changes reversible?

In addition, calculations by mathematical modeling of in vivo microvascular networks predict an exponential relationship between capillary and arteriolar number and vascular resistance (15). Total vessel rarefaction up to 42% (within the range observed in hypertensive humans) can increase tissue vascular resistance by 21% (16). In a microvascular network maturation model, rarefaction of vessels below a critical diameter was shown to be important in determining the mature network structure and its response to hypertension (17). It was shown that there was a network density threshold below which resistance to flow dramatically increased. In addition, simulating hypertension in a mature and already compromised network leads to further rarefaction (18).

Our understanding of the role of obesity-associated microvascular abnormalities in the development of hypertension has been enhanced by studies in the obese Zucker rat, in which a defective leptin receptor gene causes excessive food intake and leads to obesity, hypertension, and type 2 diabetes. The obese Zucker rat shows microvascular remodeling and rarefaction in skeletal muscle before any elevation of blood pressure has occurred, and rarefaction still occurs if the increase in blood pressure is prevented by treatment with hydralazine, a direct-acting smooth muscle relaxant (19). Rarefaction in this situation, therefore, is not a consequence of hypertension. Thus, it seems likely that microvascular abnormalities in obesity can both result from and contribute to hypertension, and a “vicious cycle” may exist in which the microcirculation maintains or even amplifies an initial increase in blood pressure (20). However, according to the Borst-Guyton concept, chronic hypertension can occur only if renal function is abnormal with a shift in the renal pressure-natriuresis relationship (21,22). In the absence of the latter, increased peripheral resistance only temporarily raises blood pressure, to be followed by an increase in renal sodium excretion restoring blood pressure towards normal. Importantly, therefore, subtle renal microvascular disease (23) as well as a reduced number of nephrons (24) may reconcile the Borst-Guyton concept with the putative role of vessel rarefaction in the etiology of high blood pressure (25).

Can renal microvascular injury - as manifested by salt sensitivity of blood pressure - explain the relationship between impaired early development and increased cardiovascular risk later in life?

This may also explain the observed salt sensitivity of blood pressure in insulin resistant subjects (26). In agreement with a central role for generalized microvascular dysfunction as a link between salt sensitivity, insulin resistance and hypertension recent data suggest an association between salt sensitivity and microvascular dysfunction independent of hypertensive status. More importantly, microvascular function, at least statistically, largely explained associations of salt sensitivity with both insulin resistance and elevated blood pressure (25).

In summary, microvascular dysfunction, by affecting peripheral vascular resistance and renal function, may initiate the pathogenic sequence and subsequently maintain or amplify the initial increase in blood pressure. It may also explain salt-sensitivity of blood pressure, associated with insulin resistance.

### **Insulin resistance as a result of microvascular dysfunction**

Recent evidence indicates that insulin delivery to the skeletal muscle interstitium is the rate limiting step in insulin-stimulated glucose uptake by skeletal muscle, and is much slower in obese insulin-resistant subjects than in normal subjects (27). Interestingly, insulin acts on the vasculature at different levels, potentially regulating its own delivery to muscle interstitium (27,28,29): (A) relaxation of resistance arteries/arterioles to increase total blood flow; (B) relaxation of pre-capillary arterioles (through changes in vasomotion) to increase the microvascular exchange surface perfused within skeletal muscle (microvascular/capillary recruitment) and (C) trans-endothelial transport (TET) of insulin.

#### *Insulin's effect on total blood flow*

Insulin increases total blood flow and blood volume in skeletal muscle (28,30). Mainly because the ability of insulin to dilate skeletal muscle vasculature is impaired in a wide range of insulin-resistant states (e.g. obesity, hypertension, type 2 diabetes), Baron et al. (30) introduced the novel concept that insulin's vasodilatory and metabolic actions (i.e. glucose disposal) are functionally coupled. However, despite the compelling nature of these findings, the concept that insulin might control its own access and that of other substances, particularly glucose, has been challenged (31). In experiments with lower doses of insulin and shorter time courses of insulin infusion, it was shown that insulin-mediated changes in total blood flow appear to have time kinetics and a dose dependence on insulin different from those for the effect on glucose uptake. In addition, studies in which glucose uptake has been measured during hyperinsulinemia and manipulation of total limb blood flow with different vasodilators have shown that total limb blood flow could be increased in either normal or insulin-resistant individuals, yet there was no increase in insulin-mediated glucose uptake (27,28,29). Induction of endothelial dysfunction with subsequent impairment of insulin-induced increases in total limb blood flow also does not decrease insulin-mediated glucose uptake (32). These discrepant findings have been ascribed to the fact that various vasoactive agents may change total flow but have distinct effects on the distribution of perfusion within the microcirculation (i.e. nutritive versus non-nutritive, see below). In addition, it should be appreciated that increasing total blood flow will have little or no impact on total glucose uptake by the tissue in the absence of an appreciable arterial–venous concentration gradient, as is the case in insulin resistance states (27). However, expansion of the endothelial surface area available for exchange of insulin, glucose or other nutrients through the recruitment of additional microvasculature within muscle can enhance nutrient delivery to the tissue, even under circumstances where the extraction ratio is small, provided there is a demonstrable intravascular–interstitial gradient (27,33).

#### *Insulin-induced microvascular/capillary recruitment*

Clark et al. (28) have introduced the concept that distribution of blood flow in nutritive ('feeding') compared to non-nutritive ('shunting') vessels, independent of total muscle flow, may affect insulin-mediated glucose uptake. By elegant studies in rats, applying different techniques to measure capillary recruitment (1-methylxanthine metabolism) and microvascular perfusion (contrast enhanced ultrasonography (CEU)) (figure 1) and laser Doppler flowmetry, they could demonstrate that insulin induces changes in muscle microvascular perfusion consistent with capillary recruitment (28). This capillary recruitment is associated with changes in skeletal muscle glucose uptake independent of changes in total blood flow, requires lower insulin concentrations than necessary for changing total blood flow, and precedes muscle glucose disposal (28,33). Moreover, insulin-mediated capillary recruitment is impaired in obese Zucker rats (34). Other indirect evidence also supports the concept that the in vivo effect of

insulin is determined, at least in part, by insulin's own effect to reach metabolically active tissues by changing local blood flow distribution patterns. Recently, the effects of systemic insulin infusion on transport and distribution kinetics of the extracellular marker [14C]inulin were studied in an animal model that allowed access to hindlimb lymph, a surrogate for interstitial fluid (35). Insulin, at physiological concentrations, augments the access of the labeled inulin to insulin-sensitive tissues. In addition, access of macromolecules to insulin-sensitive tissues is impaired during diet-induced insulin resistance (36). These data suggest that insulin redirects blood flow from non-nutritive vessels to nutritive capillary beds, resulting in an increased and more homogeneous overall capillary perfusion termed "functional capillary recruitment". The latter would enhance the access of insulin and glucose to a greater mass of muscle for metabolism. Consistent with such a mechanism in humans, insulin increases microvascular blood volume as measured with CEU or positron emission tomography, and concomitantly enhances the distribution volume of glucose in human muscle (27,28,37). Subsequently, capillary recruitment was reported in the forearm of healthy humans following a mixed meal and was found to follow closely the time-dependent rise in plasma insulin (38). In addition, insulin-mediated microvascular recruitment in the forearm was shown to be impaired in obese women when they were exposed to a physiological insulin clamp (39). By directly visualizing capillaries in human skin, it has been demonstrated that systemic hyperinsulinemia is capable of increasing the number of perfused capillaries (2,6). Comparable to insulin-mediated microvascular recruitment in the forearm (39), the action of insulin on capillary recruitment is impaired in obese subjects (6,40).

Can the microvascular bed in skin serve as a proxy for the microvascular bed in skeletal muscle?

Further insight into the complex relationships between vasodilatation, blood flow, and capillary recruitment was gained through measurement of the capillary permeability-surface (PS) for glucose and insulin. PS for a substance describes its capacity to reach the interstitial fluid. This depends on the permeability and the capillary surface area, of which the latter in turn depends on the amount of perfused capillaries. A recent investigation employing direct measurements of muscle capillary permeability showed that PS for glucose increased after an oral glucose load, and a further increase was demonstrated during an insulin infusion (41). Importantly, the increase of PS was observed without any concomitant change in total blood flow. It was concluded that the insulin-mediated increase in PS seen after oral glucose is important for the glucose uptake rate in normal muscle (41). Interestingly, the transcapillary delivery of insulin to the muscle interstitium and the onset of insulin action to stimulate glucose uptake were equally delayed among obese, insulin-resistant individuals (42). In a recent study, using the same technique, the metabolic and vascular effects of the nitric oxide vasodilator metacholine were investigated in a group of obese, insulin resistant and insulin sensitive individuals during glucose-stimulated physiological hyperinsulinemia (43). The results demonstrated that, in obesity, even in the absence of measurable increments in total forearm blood flow, capillary recruitment (i.e. PS<sub>glucose</sub>) and forearm glucose disposal increased in response to a glucose challenge, which effect was blunted in the insulin resistant individuals. Subsequently, it was demonstrated that in the obese, insulin-resistant subjects, an intrabrachial metacholine infusion attenuated the impairment of muscle microvascular recruitment and the kinetic defects in insulin action. To date, there is one study where the hypothesis that insulin increases delivery to muscle has been challenged (44). During hyperinsulinemic euglycemic clamps, transport parameters and distribution volumes of [14C]inulin (a polymer of D-fructose of similar molecular size to insulin) were determined in healthy, non-obese subjects. The results suggest that, in contrast to earlier findings of the same group performed in a canine model (35,36), physiological hyperinsulinemia does not augment access of macromolecules to insulin-sensitive tissues in healthy humans. The study is somewhat hampered by the fact that microvascular perfusion was not assessed at the

same time, in contrast to earlier mentioned studies (41,42,43). Insulin's effect on capillary recruitment are considered to be caused by insulin-mediated effects on precapillary arteriolar tone and/or on arteriolar vasomotion (27,28,29).

#### *Insulin's effect on vasomotion*

Vasomotion is a spontaneous rhythmic change of arteriolar diameter that almost certainly plays an important role in ensuring that tissue such as muscle is perfused sufficiently to sustain the prevailing metabolic demand by periodically redistributing blood from one region of the muscle to another (45). It is an important determinant of the spatial and temporal heterogeneity of microvascular perfusion and, therefore, most likely of the number of perfused capillaries (45,46).

Are vasomotion and capillary recruitment related?

It has been suggested that vasomotion is regulated by both local vasoactive substances and influences of the central nervous system. The contribution of different regulatory mechanisms can be investigated by analyzing the contribution of different frequency intervals to the variability of the laser Doppler signal. Stefanovska et al. have analyzed the reflected laser Doppler signal from skin to provide indirect assessment of vasomotion (47,48). In humans they have interpreted the spectrum as follows: (1) 0.01–0.02 Hz, which is thought to contain local endothelial activity; (2) 0.02–0.06 Hz, which is thought to contain neurogenic activity; (3) 0.06–0.15 Hz, which is associated with the myogenic response of the smooth muscle cells in the vessel wall; (4) 0.15–0.4 Hz, which is the frequency interval of respiratory function; and (5) 0.4–1.6 Hz, which contains the heart beat frequency. Local hyperinsulinemia during cathodal iontophoresis of insulin affects microvascular vasomotion by increasing myogenic activity (49). Similarly, rat muscle studies showed the main increase due to insulin to be myogenic (50). On the other hand, systemic hyperinsulinemia has been shown to affect microvascular vasomotion by increasing endothelial and neurogenic activity in skin and muscle (2,46), and that particularly the contribution of endothelial and neurogenic activity to microvascular vasomotion is impaired in insulin-resistant obese individuals (51).

Can the association between adiposity and vasomotion be explained by adipokines?

#### *Insulin's effect on TET*

Finally, TET is a third potential site for regulating insulin delivery (27). Recent in vivo and in vitro findings suggest that insulin crosses the vascular endothelium via a trans-cellular, receptor-mediated pathway, and emerging data indicate that insulin acts on the endothelium to facilitate its own TET (52). It is still unclear whether capillary recruitment and TET of insulin are related or function independently.

#### *Impairment of insulin-mediated microvascular recruitment – vascular insulin resistance*

All together, these data illustrate the importance of the microcirculation in regulating nutrient and hormone access to muscle, and raise the possibility that any impairment in capillary recruitment may cause an impairment in glucose uptake by muscle.

Insulin resistance in skeletal muscle is characterized by the diminished ability of insulin to initiate intracellular PI3k-dependent signaling. However, insulin receptors and insulin signaling are not exclusively restricted to skeletal myocytes, but are also found in vascular cells. Insulin directly targets the endothelial cell where it stimulates NO release from the vascular endothelium in a PI3K-dependent manner that involves the Akt-mediated phosphorylation of eNOS, which leads to vasodilatation (53). Alternatively, insulin also activates the mitogen-activated protein kinase pathway in endothelial cells, which enhances the generation of the vasoconstrictor ET-1 via ERK1/2 signaling (53,54). In healthy subjects the vasodilatory signal predominates, but if signaling from the insulin receptor to eNOS is inhibited pharmacologically or downregulated by insulin resistance-associated factors such as TNF- $\alpha$  and fatty acids, (which appears to affect the PI3K-dependent pathway more than the ERK1/2 pathway, see below), this can lead to impaired insulin-mediated vasodilatation or even insulin-stimulated vasoconstriction. In this manner, vascular insulin resistance may contribute to the development of hypertension and impaired overall insulin-stimulated glucose uptake (29,55,56).

In obese rats, the insulin-signaling pathways are selectively impaired: insulin-mediated activation of PI3-kinase, Akt and eNOS is impaired, but insulin-mediated activation of ERK1/2 is intact/relatively preserved (57,58). Recently, it has been demonstrated that impaired insulin signaling in endothelial cells, due to reduced IRS2 expression and insulin-induced eNOS phosphorylation, caused attenuation of insulin-induced capillary recruitment and insulin delivery, which in turn reduced glucose uptake by skeletal muscle (56). Moreover, restoration of insulin-induced eNOS phosphorylation in endothelial cells completely reversed the reduction in capillary recruitment and insulin delivery in tissue-specific knockout mice lacking IRS2 in endothelial cells and mice fed a high-fat diet. As a result, glucose uptake by skeletal muscle was restored in these mice. These results show that insulin signaling in endothelial cells plays a pivotal role in the regulation of glucose uptake by skeletal muscle. Notably, during obesity induced by high fat feeding, inflammation and insulin resistance developed in the vasculature well before these responses were detected in muscle, liver, or adipose tissue (59). This observation suggests that the vasculature is more susceptible than other tissues to the deleterious effects of nutrient overload and may play a pathophysiological role in inducing insulin resistance. The contribution of insulin signaling to the regulation of blood pressure in different states of insulin resistance is less unequivocal (60).

In healthy humans, insulin has also been shown to stimulate both ET-1 and NO activity at the level of the resistance vessels of forearm (61). Moreover, obese, hypertensive humans show an insulin-induced vasoconstriction (62) as well as increased ET-1-dependent vasoconstrictor tone and decreased NO-dependent vasodilator tone at the level of the resistance arteries (63). Increased circulating levels of endothelin have been described in obesity and increased endogenous endothelin activity contributes to the impaired endothelium-dependent vasodilatation that characterizes this state (64,65). Furthermore, increased endogenous endothelin action contributes to insulin resistance in skeletal muscle of obese humans, likely through both vascular and tissue effects (65,66). However, endothelin-antagonism alone seems not sufficient to normalize vascular insulin sensitivity in obese subjects, suggesting that endothelin alone does not account for vascular insulin resistance in humans (67). On the other hand, metacholine, a NO vasodilator, seems to improve muscle capillary recruitment and forearm glucose uptake to physiological hyperinsulinemia in obese, insulin resistant individuals (43).

Taken together, shared insulin-signaling pathways in metabolic and vascular target tissues with complementary functions seem to provide a mechanism to couple the regulation of glucose with hemodynamic homeostasis.

### **Obesity and vascular insulin resistance**

The predominant feature of obesity is the seemingly uncontrolled expansion of adipose tissue. The view of adipose tissue as mere passive energy stores has changed dramatically. Adipose tissue is now generally regarded as the body's biggest endocrine organ (68), as adipocytes (and infiltrated inflammatory cells) are found to produce a heterogeneous group of (vaso)active substances, collectively known as adipose tissue derived

cytokines or adipokines. These include, among many others, cytokines (i.e. tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (69, 70,71)) and hormones (i.e. adiponectin (72)). Dysregulated production and secretion of these adipokines by adipose tissue may play an important role in shifting the balance from insulin mediated vasodilation towards vasoconstriction as described above.

Obesity-related microvascular dysfunction and insulin resistance may well be caused by altered signaling from adipose tissue to blood vessels, which impairs the balance of NO- and ET-1 production in the microvascular endothelium. (Vascular) insulin resistance in obesity is manifested through complex, heterogeneous mechanisms that can involve increased fatty acid flux, microhypoxia in adipose tissue, ER stress, secretion of adipocyte-derived cytokines and chronic tissue inflammation (73,74,75). A discussion of all of these factors in detail is beyond the constraints of this review, and below we focus largely on the interactive role of fatty acids, angiotensin II, inflammation (particularly TNF- $\alpha$ ) and the adipokine adiponectin on the pathogenesis of (vascular) insulin resistance.

#### *Vascular insulin resistance and free fatty acids (FFA).*

By use of magnetic resonance spectroscopy, FFA-induced insulin resistance in humans has been shown to result from a significant reduction in the intramyocellular glucose concentration, suggestive of glucose transport as the affected rate-limiting step (76). The current hypothesis, supported by data from protein kinase theta (PKC- $\theta$ ) knock-out mice, proposes that fatty acids, upon entering the muscle cell, activate PKC- $\theta$ . PKC- $\theta$  activates a serine kinase cascade leading to the phosphorylation and inactivation of IRS-1 (77). Since the technique of magnetic resonance spectroscopy only identifies a gradient from extracellular to intracellular glucose in muscle cells, it remains to be proven that the gradient did not occur between the plasma and interstitial glucose and thus reflects a rate-limiting step of glucose delivery induced by fatty acids. Interestingly, studies suggest that glucose delivery contributes to sustaining the transmembrane glucose gradient and, therefore, is a determinant of glucose transport (78). This would be consistent with the finding in rats that FFA elevation concomitantly impairs insulin-mediated muscle capillary recruitment and glucose uptake (79). In lean humans, FFA elevation has been shown to reduce whole body glucose uptake and to impair insulin-mediated capillary recruitment in skin (40) and skeletal muscle (80), while in obese individuals FFA lowering has opposite effects (40). Moreover, changes in capillary recruitment statistically explained ~29% of the association between changes in FFA levels and insulin-mediated glucose uptake (40).

A defect involving fatty-acid-induced impaired insulin signaling through the same PKC- $\theta$  mechanism in endothelial cells, which in turn may negatively influence the balance between insulin-mediated vasodilation and vasoconstriction, may be responsible for the impaired capillary recruitment. In support of such a mechanism, PKC- $\theta$  has been shown to be present in the endothelium of muscle resistance arteries of both mice and humans and to be activated by physiological levels of insulin and pathophysiological levels of palmitic acid (69). By genetic and pharmacological inhibition of PKC- $\theta$  activity in mice, it was demonstrated that activated PKC- $\theta$  induces insulin-mediated vasoconstriction by the inhibition of insulin-mediated Akt activation, which results in a reduction of vasodilatation, and by the stimulation of insulin-mediated ERK1/2 activation, resulting in enhanced ET-1-dependent vasoconstriction (figure 2)(69). These data are consistent with a role for FFA-induced microvascular dysfunction in the development of obesity-associated disorders (40).

#### *Vascular insulin resistance and angiotensin II (AngII).*

Another potential mechanism between adipose tissue and the microvasculature is the renin-angiotensin system (RAS). Obese individuals are characterized by increased activity of the RAS (81). Adipocytes are rich sources of angiotensinogen, the precursor protein of angiotensin II (AngII), and possess all the enzymes necessary to produce AngII (82). These findings suggest the existence of a local renin-angiotensin system in adipose tissue.

Moreover, the amount of angiotensinogen mRNA in adipose tissue is 68% of that in the liver, supporting an important role for adipose angiotensinogen in AngII production (83).

Angiotensin II causes vasoconstriction via the type 1 receptor (AT1R) and vasodilatation through the type 2 receptor (AT2R). Both are expressed in muscle microvasculature (84) and in vitro studies have repeatedly shown that angiotensin II (AngII) impairs vascular insulin signaling and reduces insulin-stimulated NO production via the AT1R (85,86,87). AngII also increases the expression of interleukin 6 and TNF- $\alpha$ , as well as oxidative stress via the nuclear factor B pathway, which may also impair insulin signaling. Therefore, insulin resistance and RAS activation could cooperatively facilitate microvascular vasoconstriction. This provides a plausible explanation for repeated clinical trial findings that AT1R blockade decreases blood pressure and improves insulin sensitivity in patients with insulin resistance (88,89,90). Surprisingly, acutely raising AngII systemically also improves muscle glucose disposal thought to be secondary to the hemodynamic effects of AngII (91,92). Neither study, however, examined the microvascular changes. It has been hypothesized that these seemingly discordant findings may reflect the differential effects of AngII via AT1Rs and AT2Rs. Chai et al. demonstrated, that AngII, acting on both AT1R and AT2R, regulates basal skeletal muscle perfusion, glucose metabolism, and oxygenation in rats (84). Basal AT1R tone restricts muscle microvascular blood volume, a measure of microvascular surface area and perfusion and glucose extraction, whereas basal AT2R activity increases muscle microvascular blood volume and glucose uptake via a NO-dependent mechanism. Interestingly, administration of the AT1R blocker losartan increased muscle microvascular blood volume in rats by >3-fold and hindleg glucose extraction simultaneously increased by 2- to 3-fold. Human data examining the effects of AngII and AT1R blockers on microvascular function are scarce. Using the microdialysis technique, AngII has been shown to decrease local blood flow in a dose-dependent manner in skeletal muscle tissue (93). Recently, it has been demonstrated that acute infusion of AngII impairs insulin-induced capillary recruitment, as assessed with capillary microscopy, but enhances insulin stimulated whole-body glucose disposal (94). Moreover, acute AT1R blockade with irbesartan, but not acute calcium channel blockade with felodipine, increased functional capillary density during hyperinsulinemia in mildly hypertensive individuals despite similar blood pressure reductions (95). This beneficial effect of irbesartan on microvascular perfusion was however not associated with increased insulin mediated glucose uptake. In contrast, 26-weeks treatment with the AT1R blocker valsartan improved whole body glucose uptake, but had no effect on capillary density in fasting conditions (i.e. fasting insulin levels) (96). The latter study did not assess insulin-induced capillary recruitment. The human data, therefore are not unequivocal. It should be realized that there is cross talk between the RAS and insulin signaling at multiple levels and it remains possible that AngII may have simultaneous direct vascular and metabolic effects that may not necessarily be coupled.

#### *Vascular insulin resistance and inflammation.*

In parallel with the perturbations in fatty acid metabolism, adipocyte microhypoxia and ER stress precipitate a series of events that result in the recruitment of a specific population of pro-inflammatory, M1-like macrophages into adipose tissue (75). Activation of these macrophages leads to the release of a variety of chemokines (which recruit additional macrophages) and pro-inflammatory cytokines by the adipocytes. In turn, these cytokines change the milieu of secreted circulating adipokines, which then have endocrine or paracrine effects on the vasculature (73). In the past years, several adipokines have been shown to alter vascular tone and vessel wall inflammation. Adipokines that act directly on vascular endothelium include TNF- $\alpha$ , IL-6, leptin and adiponectin (73). Of the adipokines, TNF- $\alpha$  has been best characterized for its action in inducing metabolic insulin resistance through inflammatory pathways, with consequent effects on IRS-1 and Akt phosphorylation (97). TNF- $\alpha$  can certainly produce local and downstream endothelial activation and inhibition of nitric oxide production in small vessels. In rats, TNF- $\alpha$  elevation concomitantly impairs insulin-mediated muscle capillary recruitment and glucose uptake (99). Moreover, in isolated skeletal muscle resistance arteries, TNF- $\alpha$  impairs the vasodilator effects but

not the vasoconstrictor effects of insulin through activation of intracellular enzyme c-Jun N-terminal kinase (JNK) and impairment of insulin-mediated activation of Akt (figure 2)(100). This selective inhibition of the vasodilator effects of insulin results in insulin-mediated vasoconstriction in the presence of TNF- $\alpha$ . JNK has been shown to regulate whole-body insulin sensitivity as well as insulin-mediated cell signaling (101). In cultured bovine aortic endothelial cells, TNF- $\alpha$  induces insulin resistance in the phosphatidylinositol 3-kinase/Akt/eNOS pathway and enhances ERK1/2 phosphorylation (102). In humans, the TNF- $\alpha$  gene locus contributes to the determination of obesity and obesity-associated hypertension (103). Recent interesting evidence is that insulin sensitivity is improved by treatment through neutralizing TNF- $\alpha$  with the monoclonal antibody, infliximab, in patients with ankylosing spondylitis (104), indicating that TNF- $\alpha$  is indeed an important adipokine that may be at least partially responsible for an insulin resistant state. Notably, compared to healthy controls, patients with ankylosing spondylitis had impaired microvascular endothelium-dependent vasodilatation and capillary recruitment, which was normalized following anti-TNF- $\alpha$  treatment (105). Morphological studies reveal substantial differences in inflammation between subcutaneous and intra-abdominal (visceral) fat depots. Abdominal adipose tissue contains more monocytes and macrophages and expresses more TNF- $\alpha$  than subcutaneous adipose tissue in obesity (71,106). In accordance, increased visceral adipose tissue and trunk/extremity skinfold ratio were shown to be associated with an increased inflammation score, which combined information on concentrations of C-reactive protein, IL-6, and TNF- $\alpha$ . However, circulating TNF- $\alpha$  is associated with capillary recruitment in some (107), but not all studies (108). This may be explained by the fact that TNF- $\alpha$  may not be a good candidate as a systemic fat-derived signal, due to its low circulating concentration (70). A new source of TNF- $\alpha$  which has recently been identified is perivascular adipose tissue around coronary arteries (109,110). This implies that TNF- $\alpha$  is produced in the vicinity of the vascular endothelium and may mean that circulating levels of TNF- $\alpha$  underestimate the biologically relevant concentrations of this cytokine. In this context, we have suggested a regulatory role for local production of adipokines in deposits of fat around arterioles, so called muscle perivascular adipose tissue (figure 2 and figure 3) (73,111).

### **Perivascular adipose tissue (PVAT)**

Our group has demonstrated that there is a cuff of adipose tissue around the origin of nutritive arterioles, isolated from cremaster muscles from obese Zucker rats (73,111). Using a variety of insulin signaling pathway inhibitors, we have shown that in these animals, the PI3-kinase insulin signaling pathway is impaired, and nitric oxide production is suppressed (73). This has led to proposal that in states of obesity, the adipokines secreted from perivascular adipose tissue (PVAT) may signal to the vessel wall, both locally ('paracrine') and downstream ('vasocrine'), through outside-to-inside signaling (111). PVAT around nutritive arterioles may inhibit the effects of systemic insulin on local vasodilation, consequently inhibiting nutritive blood flow and insulin action.

Recently, we were able to start to confirm this hypothesis through work done in lean and obese mice (112). We found that, ex-vivo, in a pressure myograph, PVAT controls insulin-induced vasoreactivity in the muscle microcirculation through secretion of adiponectin and subsequent AMPK signaling. PVAT from obese mice inhibits insulin-induced vasodilation, which can be restored by inhibition of JNK, a mediator of TNF- $\alpha$ -induced inflammation.

Are adiponectin's insulin-sensitizing effects in vivo exerted through AMPK?

In conclusion, the obesity-associated adipokine profile which includes elevated TNF- $\alpha$  concentrations and decreased adiponectin concentrations among others, is a likely suspect linking (perivascular) adipose tissue with

defects in microvascular function, at least in part, by influencing insulin signaling and thereby insulin's vascular effects.

### **Outline of the thesis**

The aim of the presented studies was to obtain further insight in microvascular vasoregulation with specific emphasis on the roles of endothelin-1, VEGF and adiponectin. In this thesis, I will address different aspects of blood pressure control and insulin mediated glucose uptake, our two main microvascular interests, from several angles in various study designs (see also boxes throughout the introduction section). We start with two methodological considerations. In *chapter 2* we investigate whether we can confirm the implied relationship between vasomotion and capillary recruitment. In *chapter 3a*, we compare insulin mediated microvascular reactivity in skin and skeletal muscle. Can skin microvasculature be used as a proxy for the study of systemic effects? *Chapter 3b* investigates the possibilities for a new application of an established technique to assess skeletal muscle perfusion in blood pressure research.

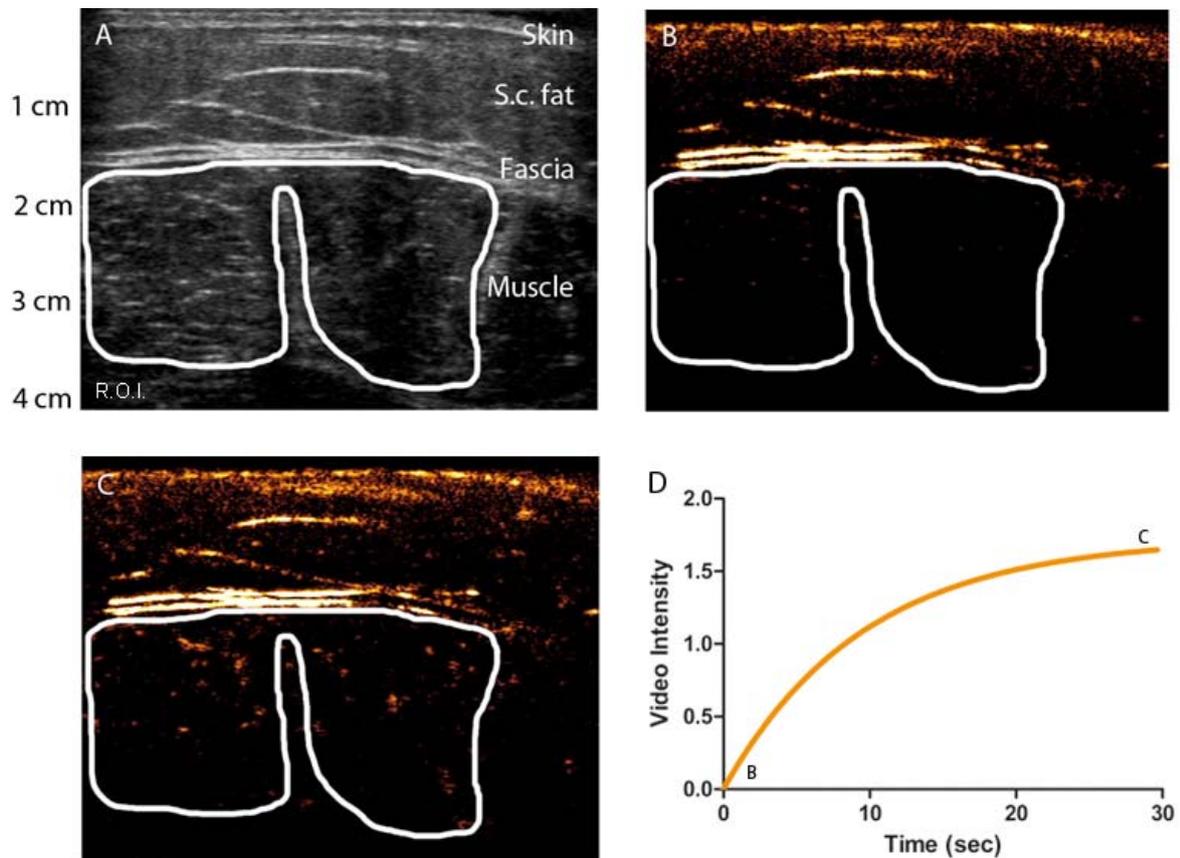
The next three chapters focus on the role of the microvasculature in blood pressure control. *Chapter 4a* describes the acute effects of a VEGF-R inhibitor, sunitinib, on capillary density and blood pressure in patients with metastatic renal cell cancer. *Chapter 4b* addresses whether these effects are reversible after cessation of the drug. In *chapter 5*, we investigate the relationship between birth weight and salt-sensitivity of blood-pressure, a measure of renal microvascular injury, during adulthood. Such a relationship would corroborate the suggestion that microvascular dysfunction precedes cardiometabolic risk factors, perhaps even starting during early development.

In *chapter 6*, we attempt to disentangle the main phenotypic characteristics of the metabolic syndrome with regards to vascular and metabolic insulin sensitivity.

The third and final part of this thesis focuses on microvascular function in a metabolic context, and especially on the role of adiponectin and its substrate AMPK. In *chapter 7* we use spectral analysis of skin laser Doppler measurements to investigate relationships between BMI, adiponectin and vasomotion in a healthy population based cohort. In *chapter 8*, we try to elucidate via in-vitro, ex-vivo and in-vivo experiments how adiponectin exerts its insulin-sensitizing effects via AMPK $\alpha$ 2. Finally, *chapter 9* summarizes and discusses the findings presented in this thesis.

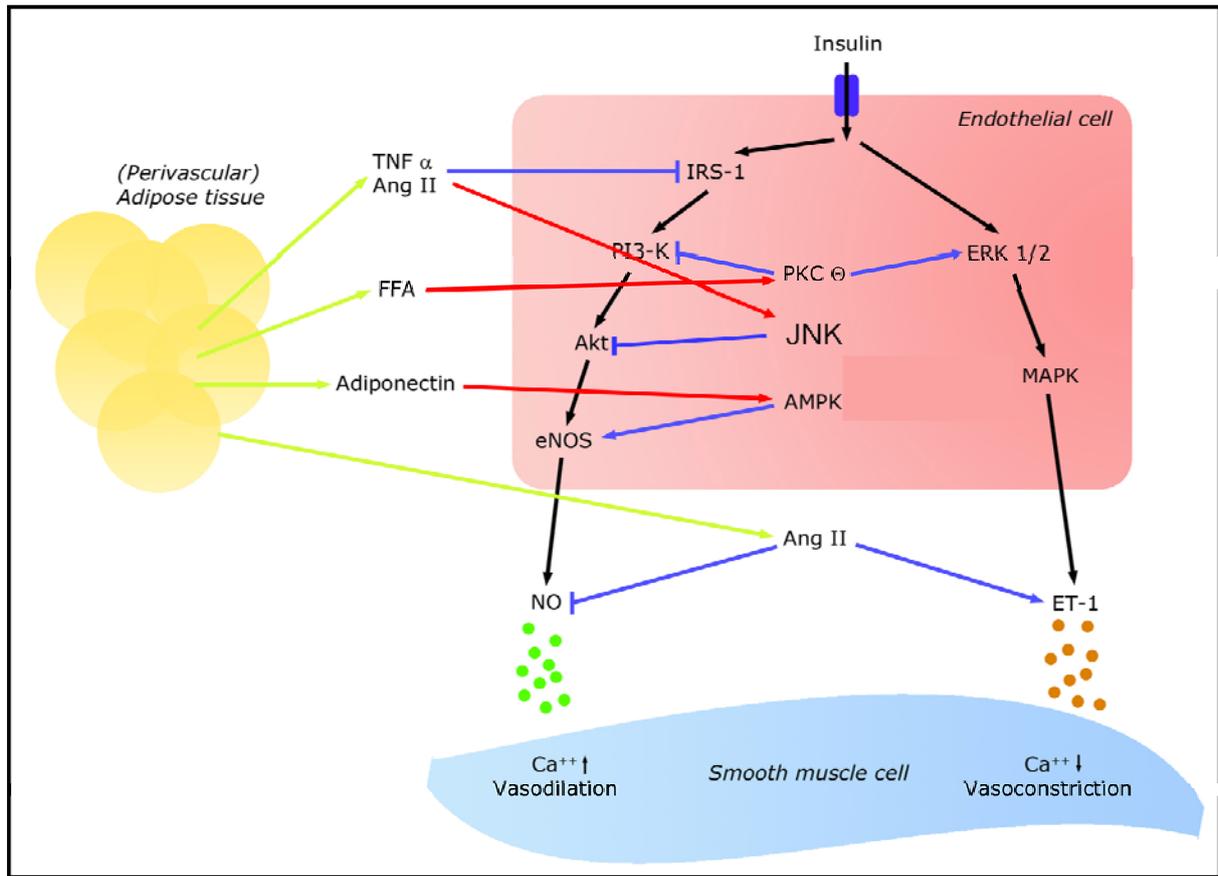
## Figures

figure 1



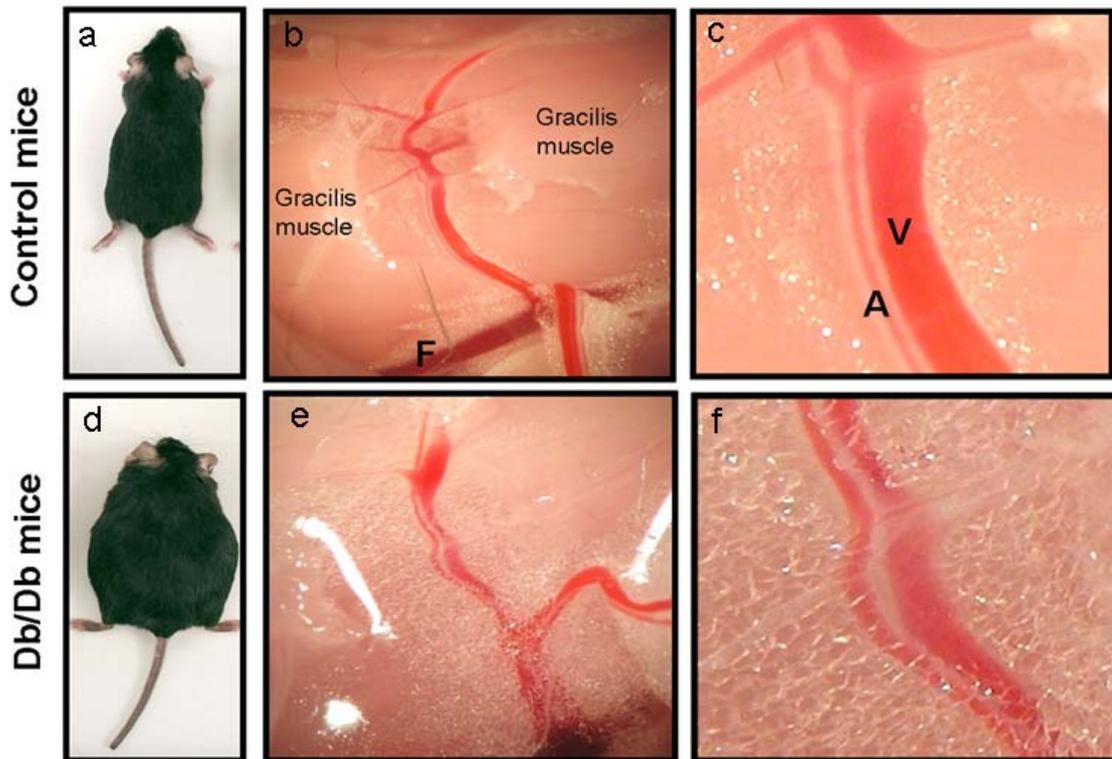
Measurement of microvascular blood volume using contrast enhanced ultrasound (CEU). A. Cross section of the flexor muscles of the human forearm. B. After destruction of – steady state – contrast-microbubbles in the region of interest with a high intensity ultrasound burst. C. After full replenishment of skeletal muscle microvasculature with microbubbles. D. Inflow-curve showing video-intensity reach plateau = microvascular blood volume. S.c.fat, subcutaneous fat; ROI, region of interest.

figure 2



Effect on insulin signaling by TNF- $\alpha$  or free fatty acids (FFA). Normal insulin signaling is mediated by either insulin receptor substrate (IRS), Akt, eNOS, and NO production leading to vasodilation or by ERK1/2 and ET-1 production leading to vasoconstriction. TNF- $\alpha$  and FFA affect the insulin signaling pathway by the activation of JNK or PKC $\theta$ , leading to impaired Akt activation induced by TNF $\alpha$  and FFA, and increase in ERK1/2 activation by FFA, both of which lead to insulin-mediated vasoconstriction in muscle resistance arteries (Cell Tissue Res. 335: 165, 2009)

figure 3



Increased local perivascular adipose tissue in skeletal muscle arterioles of Db/Db mice. After dissection of the gracilis muscle, the vasculature of the corresponding muscle in the control and Db/Db mice (B, E) becomes visible (A artery, V vein, F femoral artery). At higher magnification, the artery and vein can be distinguished (C, F). The Db/Db mice (D-F) possess more and larger fat cells surrounding the gracilis artery compared with control mice (A-C). Bars 1 mm (B, E), 0.25 mm (C, F). (Cell Tissue Res. 335: 165, 2009)

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