Investigation of effect of hyperinsulinaemia on adipose tissue microvasculature

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Declaration

No part of this thesis has been submitted in support of an application for any other degree or qualification at the University College London or any other university or institute. All the work presented is my own and all collaborations have been acknowledged.

Signature:

Date:

Abstract

Background: Obesity in Qatar is amongst the highest globally and constitutes a serious health risk. Obese individuals are at greater risk of vascular disease compared to the lean, especially in the insulin resistant state. The impact of obesity on endothelial vasomotor function is also adipose tissue depot-dependent, with the visceral environment being more pathogenic. It is, however, unclear how severe the impact of vascular dysfunction is in a relatively young and obese population. Further, it is becoming apparent that obesity-associated vascular dysfunction is heterogeneous. Recent research has focussed on different groups of obese subjects in order to elucidate the mediators of the differential cardiometabolic risk. In pathological obese (PO) subjects, sub-cutaneous adipocyte dysfunction and inflammation have been reported along with adipocyte hypertrophy.

This led to the following hypotheses:

1. The enlarged adipocytes are more susceptible to hypoxia due to decreased capillary density of the depot and changes in vascular tone. Hypoxia leads to cell necrosis and the formation of immunological foci.
2. The more hypoxic and inflamed fat depot secretes less adiponectin, which then may determine the increased systemic insulin resistance and dyslipidaemia seen in the PO.

Specifically differences between metabolically healthy but obese (MHO) and pathologically obese (PO) in relation to capillary density and vascular function was determined.

To facilitate the overall objectives, in this study:

1. A cohort of MHO and PO subjects were identified.
2. Vascular differences in the adipose tissue of PO versus MHO by functional studies (myography) and histological assessment of vascular density were carried out, and,
3. Mechanisms that underlie these differences were investigated.

Methods: Patients were recruited from the local hospital and blood and adipose tissue (Omental, OM; Subcutaneous, SC) samples collected. Fasting plasma glucose and insulin were assayed to determine insulin resistance status. Vascular function

was assessed by wire myography. Cumulative concentration-response curves were generated for various vasoconstrictors (e.g. noradrenaline, potassium chloride), and vasodilators (e.g. acetylcholine, Sodium nitroprusside (SNP), and prostaglandin E_2). Relaxation to acetylcholine was recorded in the absence or presence of N_ω-Nitro-L-arginine methyl ester (L-NAME), indomethacin, diclofenac, $BaCl_2$, apamin+charybdotoxin, and arginine.

mRNA expression of hypertension associated genes in stromal vascular fractions (SVFs) of both depots was assessed by real time RT-PCR. Paraffin-embedded tissues were used for histological studies.

Results: OM arterioles were less sensitive to noradrenaline-mediated vasoconstriction compared with SC (log EC50 -5.9±0.2 vs. -6.5±0.1, $p<0.05$).

Vasorelaxation to acetylcholine was attenuated in OM vessels compared with SC vessels ($p<0.01$). In contrast, relaxation to SNP was greater in OM compared with SC vessels ($p<0.01$). Acetylcholine curves for insulin-sensitive patients were less attenuated compared with insulin-resistant patients. L-NAME, apamin, charybdotoxin, and $BaCl_2$ caused right-ward shifts of the acetylcholine curves, while indomethacin, diclofenac and arginine produced the reverse.

In the whole group, COX2 mRNA, but not eNOS and COX1, were up regulated in OM compared with SC SVFs. However, when analyzed separately, in the OM compared to SC SVFs, of the MHO several genes were unregulated (AGT, ARG2, CLIC5, EPHX2, ITPR1 and PRKG1), while in the PO only two were significantly different between the depots (CLIC5 and PDE3B).

Conclusions: Hyperinsulinaemia in adipose tissue microvessels was associated with i) vasocontractile insensitivity to noradrenaline and ii) to changes in NO-mediated vasodilation, at least partially mediated through components of the COX2 pathway.

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Contents

Figures:

Abbreviation:

ADRF …… Adipocyte-derived relaxing factor

Ang II …… Angiotensin II

AT …… Adipose tissue

BAT …….. Brown adipose tissue

Ca …… Calcium

cGMP …… Cyclic guanosine monophosphate

Cl …… Chlorid

COX …… Cyclooxygenase

CRP ……..... C-Reactive protein

CVD…… Cardio-vascular disease

DO…….. Diabetic obese

ECM ……. Extracellular matrix

EDHF …… Endothelium derived hyperpolarizing factor

ET-1……... Endothelin-1

eNOS …… endothelial nitric oxide synthase

EGF ……. Epidermal Growth Factor

ERK ……. Extracellular signal-regulated kinase

EVG……. ELASTIC-VAN GIESON

FFA…… Fatty acid flux

FGF …… Fibroblast Growth Factor

HIF-1 ……. Hypoxia-inducible factor-1

ICAM-1 ……. Intercellular adhesion molecule-1

IL-6 …… Interleukin-6

IGF ……. Insulin-like Growth Factor

IRS ……. Insulin receptor substrate

K ……. Potassium

Kv ……... Voltage-gated potassium channel, (A type of potassium channel)

LPL…….. Lipoprotein lipase

MAPK…….. Mitogen-active protein kinase

MCP-1 …….. Monocyte chemoatractant protein-1

MIF…… Migration inhibitory factor

MHO ……. Metabolically healthy but obese

mRNA …….. Messenger RNA

Na …… Sodium

NA……. Noradrenaline

NO……. Nitric oxide

PAI-1…… Plasminogen activator inhibitor-1

PGl_2 …….. Prostacyclin

PDK …….. Phosphoinositide-dependent kinase

PGE_2 ……. Prostaglandin E2

PI3…….. Phospho inositol 3

PKG…… Protein kinase G

PDGF …….. Platelet-derived Growth Factor

PPAR-γ …….. Peroxisome proliferator activated receptor-gamma

PO…… pathologically obese

sGC…… Soluble Guanylyl cyclase

SNP…… Sodium nitroprusside

SNS …….. Sympathetic nervous system

SREBP …… Sterol regulatory element binding proteins

TF …… Tissue factor

TLR4……. Toll-like receptor 4

TNF-α…… Tumor necrosis factor-alpha

TGF…… Transforming growth factor

TXA2 …… Thromboxane A2

VEGF……. Vascular endothelial growth factor

VIP …… Vasoactive Intestinal Polypeptide

VOCCs …….. Voltage-operated Ca^{2+} channels

VSMC…… Vascular Smooth Muscle cell

WAT…… White Adipose Tissue

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Chapter 1

Introduction and Literature review

1.0 INTRODUCTION

1.1 Obesity:

Obesity is defined as an expansion and excessive accumulation of adipose tissue. It is often associated with changes in the structure and function of the adipose organ, as well as its distribution and the secretion of adipose derived factors, adipokines (1).

Obesity is also associated with susceptibility to certain complications such as, hyperglycemia, hyperlipidemia, chronic inflammation, insulin resistance and endothelial dysfunction, which contribute to various metabolic diseases and pathologies including type 2 diabetes, cardiovascular disease (CVD) and hypertension (2).

1.2 Obesity in Qatar

Over the last century there has been socioeconomic development in many countries, especially in those of the Co-operation Council for the Arab States of the Gulf (GCC). The discovery of oil in this region has resulted in a move away from traditional to a modern way of life in diet and life style habits which have led to an increase in the prevalence of obesity amongst the population. These changes have contributed to the increases in diseases such as, obesity, cardiovascular diseases, hypertension, diabetes and cancer (3). Qatar is a rapidly developing country that has seen a substantial increase in the prevalence of obesity in its population. The proportion of adult Qataris that were overweight and obese in 2005 was 34.6% for males and 45.3% for females (4), which puts Qatar sixth in the world for female obesity ranking according to WHO (2010). Globally more than 1.7 billion adults are overweight, and 312 million are obese. About 18 million people die every year from cardiovascular disease, diabetes and hypertension, major factors that are propelled by the increasing prevalence of overweight and obesity. When joined by underweight, malnutrition and infectious diseases they are the major problems threatening the world (5).

1.3 Adipose tissue:

The adipose tissue is composed of adipocytes and other cells within an endocrine, paracrine and autocrine organ secreting a host of factors referred to as adipokines which regulate many central and peripheral processes such as appetite, energy metabolism, glucose homeostasis, blood pressure, lipid metabolism, inflammation, angiogenesis and reproductive function (4, 6). Many of these adipokines are also known to regulate vascular tone. The vasoactive adipokines that have been described include: adiponectin, omentin and visfatin as vasorelaxants, while angiotensin II and resistin appear to act mainly as vasoconstrictors (7). Recently an unidentified adipocyte-derived relaxing factor (ADRF) was also shown to relax arteries (8). Leptin, tumour necrosis factor (TNF-α), interleukin-6 (IL-6) and apelin are reported to have both vasorelaxant and constricting properties (8). A failure of the regulation of the synthesis of these vasoactive and proinflammatory adipokines may underlie the compromised vascular reactivity in obesity and obesity-related disorders (2).

1.3.1 Adipose tissue Composition:

The adipose tissue is heterogeneous and made up of predominantly adipocytes (50%), stromal preadipocytes, progenitor cells, macrophages, fibroblasts, endothelial cells, vascular smooth muscle cells (as part of the blood vessels), neurons, as well as a host of different immune cells (9,10) as shown in figure 1. The tissue is located around blood vessels (perivascular), as well as around internal organs (visceral or omental) and in the subcutaneous compartments. The subcutaneous depot is the largest of the adipose tissue organs. Histologically and functionally adipose tissue consists of at least two distinct types; white and brown (1). In humans the white adipose tissue (WAT) constitutes the major type of this tissue and makes up most of the total body fat mass. Functionally WAT acts as an insulator, stores excess energy as triacylglycerol mediates glucose homeostasis and serves as an important endocrine/immune organ which secretes adipokines, that include inflammatory cytokines, acute phase proteins and complement-like factors (4, 11). In adult humans metabolically active brown depots are found in cervical, supraclavicular, axillary

and paraventral body's regions, which can be induced in response to cold and SNS activation (10, 12).

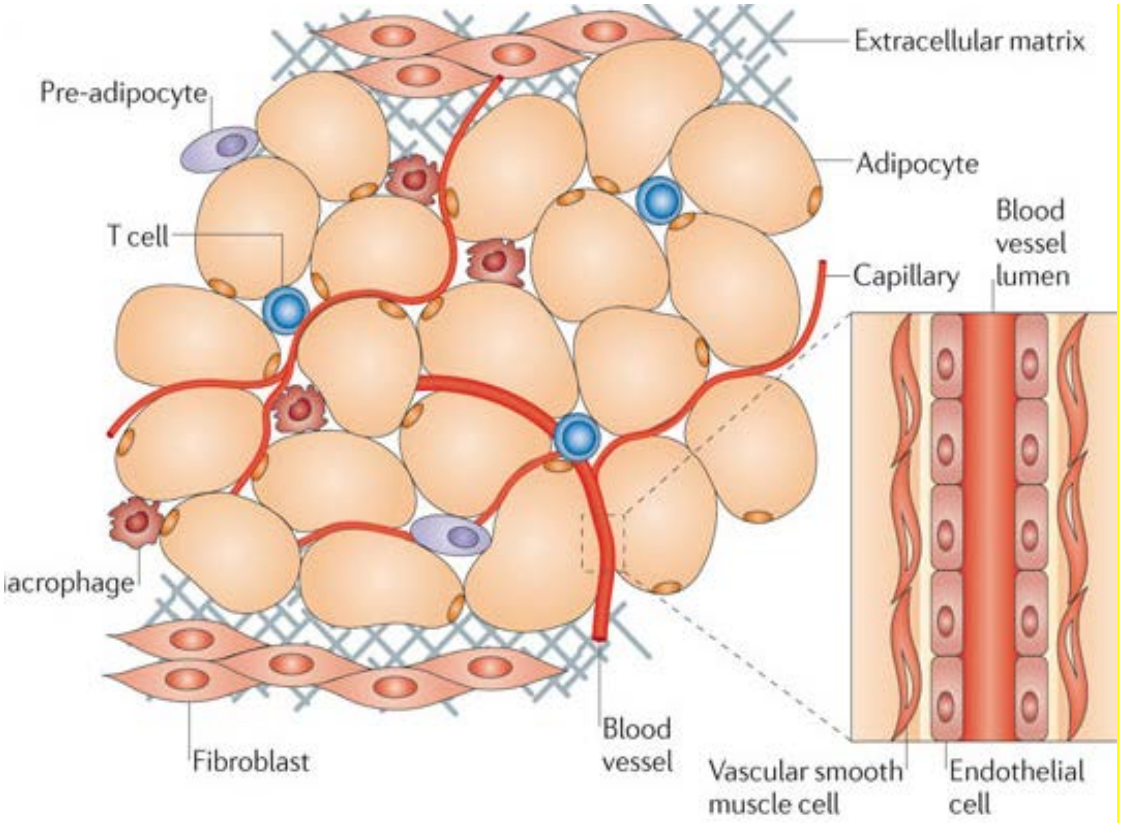


Figure 1: Adipose tissue composition. Adipose tissue as a heterogeneous mixture of cellular structures (adipocytes, precursor cells, macrophages, fibroblasts and endothelial cells) and tissue structures (small blood vessels and nerve tissue). Vascular cells include both endothelial cells & vascular smooth muscle cells present within the major blood vessels (Adapted from13).

Another type of adipose tissue is the brown adipose tissue (BAT) which is specialized in heat production and lipid oxidation; it consists of smaller number of fat cells, with richer vascular supplies and more abundant mitochondrial chromogens, responsible for the brown colour (14). BAT also responds more rapidly to sympathetic nervous system (SNS) stimulation that elicits heat, rather than ATP production (14).

Major locations for WAT are in the intraabdominal regions, around the omentum and perirenal areas and subcutaneous in the buttocks, thighs and abdomen. Adipocytes constitute about 50% of WAT, macrophages about 10% and the rest are preadipocytes, endothelial and epithelial cells. Both adipocytes and non-adipocyte cells secrete adipokines. Adipocytes mainly produce adiponectin, and to a lesser extent leptin. Cytokines such as MCP-1and IL-6 are predominantly secreted by the hypertrophied adipocytes or they along with many of the other inflammatory signals are produced by the non-adipocyte cells of the stromal vascular fraction (SVF). In obesity, macrophages infiltrate into WAT, and the number of these macrophages directly correlates to adipocyte size (15). More recently other, perhaps intermediate, types of adipose tissue, in addition to WAT and BAT, have also been described, such as ‘beige’ adipose tissue (16, 17). Data suggests that the two types of brown adipocytes have different developmental origins; classical brown adipocytes arise from precursors in the dermomyotome (15), while the ‘beige’ cells seem to originate from endothelial and perivascular cells within WAT depots (16). Brown fat is characterized by high mitochondrial content and elevated expression of mitochondrial uncoupling protein 1(UCP1) which functionally uncouples respiration and dissipates chemical energy as heat. Recent data has shown that the adult humans contain significant deposits of UCP1-positive brown fat in the supraclavicular and neck region which can be detected by positron emission tomography (PET) (17). Some WAT also contain these ‘beige’ cells that resemble white fat cells in having extremely low basal expression of UCP1, but, after prolonged stimulation by cold or pathways that elevate intracellular cyclic AMP, like classical brown fat, they can express high levels of UCP1 and take on a multilocular appearance (18). The physiological significance of the ‘beige’ fat is under investigation.

1.3.2 Adipokines

Adipose tissue is not only a storage organ, but now known to play a key role in the integration of systemic metabolic functions, mediated in part by its ability to secrete adipokines (19). These include leptin, adiponectin, TNF-α, resistin, IL-6, omentin, visfatin and apelin. The secretory status of an adipose tissue depot can be modified following the onset of obesity by changes in the cellular composition of the tissue, including alterations in the number, phenotype and localization of immune, vascular and structural cells. Also, the expression of adipokines can vary depending on the site of an adipose tissue depot and the two most abundant depots are visceral and subcutaneous adipose tissues, which produce unique profiles of adipokines (20, 21). The roles of some of the adipokines with known vascular effects are discussed in the following sections.

1.3.2.1 Adiponectin is an abundant, adipocyte-derived plasma protein with anti-inflammatory, insulin sensitizing, and antiatherogenic properties. It comprises 247-amino acid and plays an increasingly important role in energy homeostasis and insulin sensitivity, in contrast to many of the other known adipokines (22). Adiponectin levels in the plasma and adipose tissue are decreased in obese compared with the lean (23). This may at least partly be due to adiponectin production in adipocytes being inhibited by pro-inflammatory factors, like TNF-α and IL-6, as well as by hypoxia and oxidative stress (13). On the other hand, adiponectin secretion is stimulated in adipocytes by the activation of PPARγ, a nuclear receptor, and PPARγ agonists promote adipocyte differentiation, and also by weight loss. So adiponectin mRNA is reduced in adipose tissue from obese and diabetic humans probably as a consequence of inflammation in the adipose tissue, but restored to normal levels after weight loss or hypoglycaemic therapy. Circulating adiponectin levels correlate more with hyperinsulinemia and insulin resistance than obesity or body fat (24). It is however not clear if adiponectin plays a causative role or is just a marker of insulin resistance. Also the mechanisms by which adiponectin may cause insulin resistance are not fully elucidated (32). Adiponectin inhibits TNF-α production and other inflammatory pathways in adipocytes and macrophages, and plasma levels have been correlated with endothelium-dependent vasorelaxation in

humans (25). Other studies have shown an increase in NO production as well as NO and potassium channel-mediated vasorelaxation in rats by adiponectin (26). NO release from the endothelium may be stimulated by adiponectin binding to either the adiponectin type 2 receptor or T-cadherin on the endothelial surface (27). Increased NO production leads to inhibition of platelet aggregation, leucocyte adhesion to endothelial cells (5) and vascular smooth muscle cell proliferation. Moreover, adiponectin reduces oxidative stress by decreasing ROS production (although mechanism is unknown) in endothelial cells (5). All these effects protect the vascular system against endothelial dysfunction (2). The metabolic effect of adiponectin on insulin sensitivity appears to be mediated by AMP-activated protein kinase (AMPK) activation in skeletal muscle and liver. In skeletal muscle cells, adiponectin increased intracellular Ca^{2+} concentration and the activities of calcium/calmodulin-dependent protein kinase kinase (CaMKK), AMPK and sirtuin 1 (SIRT1), resulting in enhanced expression and activity of PPARγ co-activator 1α (PPARγC1α; PGC1α) (13). As AMPK activation leads to an increase in fatty acid oxidation and glucose uptake in muscle tissue, and inhibition of gluconeogenesis in the liver, this pathway is associated with insulin sensitivity. Also disruption of adiponectin receptor 1 expression, especially in muscle cells, has been shown to prevent these adiponectin-mediated changes and lead to insulin resistance (13).

1.3.2.2 Leptin is a 167-amino acid peptide hormone secreted by adipocytes in proportion to adipose tissue mass and circulates bound to a soluble form of its receptor, though evidence for this is poor. It is a signaling molecule relating the long-term nutritional and fat mass status to the brain (hypothalamus) (28). It exerts its effects through binding to the leptin receptor (Ob-R), a member of the cytokine family of transmembrane receptors (29). In monocytes leptin increases TNF-α and IL-6 production and stimulates the production of CC-chemokine ligands (CCL3, CCL4 and CCL5) by macrophages (13). Also, leptin stimulates the production of ROS and promotes cell proliferation and migratory responses. Leptin levels in the serum and adipose tissues are increased in response to pro-inflammatory stimuli, such as TNF-α and lipopolysaccharide (LPs) (13). Under normal conditions leptin contributes to blood pressure homeostasis by both its vasorelaxing and

vasocontractile effects, the latter often attributed to sympathetic nervous system activation (30). Various mechanisms may be responsible for leptin-induced vasorelaxation, some of them being endothelium-dependent through the release of NO, or by other mechanisms (31). The involvement of the endothelium-derived hyperpolarizing factor (EDHF) in leptin-induced vasorelaxation remains debatable (32). In obesity, endothelium-dependent vasorelaxation is likely to become less effective, if sustained hyperleptinemia, and thus leptin resistance, leads to endothelial dysfunction (29). While the basis of leptin resistance in humans is unclear and may involve multiple mechanisms, one of them may be the induction of the suppressor of cytokine signaling-3 that blocks the intracellular pathway of leptin (33). Another mechanism might be defects in transfer of leptin across the blood-brain barrier that may lead to central leptin resistance (32). Leptin production is augmented in large adipocytes, stimulated by insulin and affected by estrogens, FFAs and growth hormone, but not directly influenced by food uptake itself (34).

1.3.2.3 Resistin is a recently discovered (in 2001) adipocyte-secreted polypeptide that has been implicated in the development of insulin resistance and is a member of a family of tissue-specific signaling molecules, called resistin-like molecules (35) The resistin mRNA encodes a 114-amino acid polypeptide with a 20-amino acid signal sequence which is secreted as a disulfide-linked dimmer (32). Resistin mRNA and protein expression were found to be similar in adipose tissues from both the abdominal subcutaneous and omental depots. However expression in abdominal depots was increased compared with thigh fat. This suggests a potential link between central obesity and increased risk of diabetes (36). Resistin may not be a major adipokine in humans. Recent evidence suggests that it may come from circulating monocytes and macrophages (25). In human mononuclear cells, transcription of the resistin gene (RETN) is enhanced by proinflammatory cytokines, such as IL-1, IL-6 and TNFα, while in white adipose tissue it is inhibited by the PPARγ agonist rosiglitazone, suggesting that the anti-inflammatory effect of rosiglitazone is mediated in part by the attenuation of RETN transcription (13). On the vascular endothelial cells, resistin directly counters the anti-inflammatory effects of adiponectin by promoting the expression of the pro-inflammatory adhesion

molecules vascular cell adhesion molecule 1 (vCAm1), intercellular adhesion molecule 1 (ICAM1) and pentraxin 3 in these cells, thereby enhancing leukocyte adhesion (37). Although resistin does not directly affect the contractility of isolated blood vessels (38), coronary blood flow, mean arterial pressure or heart rate (39), it has been associated with endothelial dysfunction in coronary artery disease (40). Both TNF-α and IL-6 which are increased in obesity enhance resistin expression (41).

1.3.2.4 TNF-α is a proinflammatory cytokine mainly produced by monocytes and macrophages and has a central role in inflammatory and autoimmune diseases and has been implicated in the pathogenesis of insulin resistance. It is expressed as a 26-KDa cell surface transmembrane protein that undergoes cleavage to produce a 17-kDa soluble, biologically active form (42). Adipose tissue TNF-α mRNA correlates with the body mass index, percentage of the body fat, and hyperinsulinemia, and decreased by weight loss (43). In muscle and adipose tissues, TNF attenuates insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate 1 (IRs1), thus promoting insulin resistance (13). These data suggest that TNF functions as a pro-inflammatory cytokine that has a crucial role in obesity related insulin resistance (13). Potential mechanisms by which adipose tissue TNF-α increases insulin resistance includes increased release of FFA by adipocytes, reduction in adiponectin synthesis and impairment of insulin signaling (44). TNFα acts as potent inhibitor of adipogenesis, possibly through inhibition of PPARγ-dependent phenomena, as presented by the fact that thiazolidinediones prevent TNFα-induced anti-adipogenesis (45). TNF-α is a potent, time-dependent vasoconstrictor (46) and vasodilator (47). It is unclear what underlies the differential regulation of arterial contractility by TNF-α. (48). TNF-α is produced in the direct vicinity of the vascular endothelium and vasoregulation can occur through both endothelium-dependent and endothelium-independent mechanisms. Some studies have suggested that TNF-α promotes vasorelaxation by an increase of NO and prostaglandin production (47). On the other hand TNF-α is able to induce vasoconstriction by increasing endothelin-1 and angiotensinogen levels (49). In addition it impairs endothelium-dependent vasorelaxation in various vascular beds

as a result of a decrease in endothelial NO release or an increase in NO scavengers such as ROS (46). Much of the evidence of TNF from adipose tissue is based on mouse studies.

1.3.2.5 IL-6 is a proinflammatory cytokine associated with insulin resistance, and secreted by many cell types, including fibroblasts, immune cells, endothelial cells, skeletal muscle and adipose tissue, with visceral depots releasing more IL-6 compared with subcutaneous depots (50). It circulates as a variably glycosylated 22- to 27-kDa protein. Plasma IL-6 levels positively correlate with human obesity and insulin resistance (51). This is consistence with the observation of an increase in IL-6 at the mRNA, protein levels, and by its release in vivo by WAT in obesity (60,52). Elevated levels of IL-6 predict the development of type 2 diabetes (53). Weight loss significantly decreases IL- 6 levels in both adipose tissue and serum (54). IL-6 exerts its adverse effects, at least partly, by decreasing adiponectin secretion (55). IL-6 also inhibits differentiation and lipid accumulation of fibroblasts and affects the main adipogenic transcription factor C/EBPα, whereas earlier activation of C/EBPδ, C/EBPβ and PPARγ remain intact (56). A sustained increase in IL-6 plasma levels is associated with high blood pressure (57). IL-6 relaxes skeletal muscle resistance vessels (58). The vasorelaxing effect is likely regulated by an endothelium-independent pathway involving an increase in prostacyclin in vascular smooth muscle cells. IL-6 has been shown to be a predictor of future myocardial infarction and induces hepatic CRP production, which is now identified to be an independent major risk factor for cardiovascular complications (48). Certain studies have suggested that IL-6 is an indirect marker of vascular dysfunction, whereas others have suggested a more active role in vascular dysfunction (59).

1.3.2.6 Omentin, is adipose tissue-derived cytokine consisting of 313 amino acids and the expression is mainly in visceral rather than in subcutaneous adipose tissue (60). Secretion of omentin-1 is preferentially expressed by omental stromal vascular fraction cells, but not by adipocytes (61). Omentin consists of two isoforms (codified by two genes 1 and 2) in which omentin-1 appears to be the major isoform in human plasma (62) and is higher in women compared with men (62). Omentin-1

enhances the insulin mediated glucose uptake in human adipocytes through increased phosphorylation of AKT/PKB (63). In isolated rat aorta and mesenteric, omentin inhibited noradrenaline- induced concentration dependent contractions, and directly induced an endothelium-dependent vasorelaxation which is mediated by NO (60). Omentin is also capable of inducing vasorelaxation in an endothelium-independent way (60). Omentin-1 plasma levels and adipose tissue gene expression are decreased in obesity and correlates negatively with BMI, leptin, and insulin resistance, (62) and more so when overweight is combined with type 2 diabetes (64). Plasma omentin-1 correlates positively with plasma adiponectin and high-density lipoprotein (HDL) levels. (62). Circulating omentin-1 concentrations increases after weight loss-induced improvement of insulin sensitivity (65). Omentin-1 might act as an anti-inflammatory adipokine by attenuating C-reactive protein (CRP) and TNF-α induced NF-kB activation in human endothelial cells (66).

1.3.2.7 Visfatin is a 52 kDa newly identified adipokine, which is released from perivascular and visceral adipose tissue that has an insulin-mimetic effect (67). Some studies show an increase in visfatin levels in obesity and a positive correlation with BMI and percentage of body fat, but not with abdominal circumference or visceral fat estimated on the basis of computed tomography (68). Visfatin has multiple functions in the vasculature including; stimulation of growth of vascular smooth muscle cells (69) and endothelial angiogenesis via upregulating VEGF and matrix metalloproteinases (67). Visfatin also appears to mediate vascular endothelial inflammation by enhancing the expression of adhesion molecules (VCAM-1 and ICAM-1) through oxidative stress dependent NF-kB activation. It may mediate inflammatory responses in monocytes by induction of pro-inflammatory cytokines IL-1B, IL-6 and TNF-α. Visfatin with higher concentrations augment the expression of anti-inflammatory cytokines such as, IL-10 (70). Visfatin Plasma levels are negatively correlated with vascular endothelial function (71). Visfatin can directly affect vascular contractility and it has been shown in rat isolated aorta to induce endothelium-dependent vasorelaxation via NO production. It is also an effective dilator of resistance vessels such as mesenteric arteries of rats (72).

Table 1: Summery of adipokines

Adipokines	Sources	Functions
Adiponectin	Adipocytes	Anti-inflammatory, insulin sensitizing, and antiatherogenic properties. NO-dependent vasorelaxation mediated by opening of Kv channels.
Leptin	Adipocytes	Food intake and proinflammatory mediator. Vasorelaxation (EC dependent and EC independent) and vasoconstriction due to sympathetic nervous system activation.
Resistin	Adipocytes	Proinflammatory mediator. No direct effect on contractility of blood vessels.
TNF-α	Monocytes and macrophages	Proinflammatory cytokine. Vasorelaxation (EC dependent and EC-independent), and impairs EC dependent vasorelaxation by decreased NO or increased ROS production. Triggers ET-1- and Ang-induced vasoconstriction.
IL-6	Fibroblasts, immune cells, EC, skeletal muscle and visceral AT more compared with SAT	Proinflammatory cytokine. EC-independent vasorelaxation. Reduces vasorelaxing effect of PVAT by increased ROS production. Impairs endothelial function by increased ROS and decreased NO production.
Omentin	OM SVF, but not by adipocytes	Vasorelaxation (EC dependent and EC independent).
Visfatin	Perivascular and visceral AT	NO dependent vasorelaxation.

1.3.3 Adipose tissue distribution

The adipose tissue is distributed in numerous discrete anatomical depots (73) and the size of fat stored in the depots is highly variable, ranging from 5% to 60% of total body weight. Approximately 85% of total adipose tissue mass, either in lean or obese humans, is in the subcutaneous (SC) depots, including the abdominal, gluteal, mammary and femoral, while the remaining 15% constitutes intra-abdominal fat, including both retroperitoneal adipose and visceral depots, which encompass mesenteric and omental adipose depots (OM) (74). There are other smaller adipose depots such as epicardial and intermuscular which may serve specialized functions related to their neighboring tissues (75,76).

The SC & OM depots, which are of particular interest in the current study, show functional differences in the expression of genes, especially those regulating inflammation and vascular function, presence of complement factors and fatty acid binding protein, which are all expressed at higher levels in OM than in SC fat. Expansion of these depots may happen through adipocyte hypertrophy and/or hyperplasia. The accumulation of excess fat in the visceral compartment, while subcutaneous fat mass remain normal, carries the greater metabolic risk (28,77).

1.3.3.1 Subcutaneous (SC) depots:

Adipose tissue is deposited peripherally in subcutaneous depots, such as mammary and gluteofemoral regions. SC contains higher numbers of preadipocytes (78) and appears less metabolically active, secretes more leptin and less free fatty acids (79) than omental depot. Thus, excessive accumulation of subcutaneous adipose tissue is more likely to be associated with hyperleptinemia that may lead to central leptin resistance and greater susceptibility to further weight gain (79,80). Thus, the depot specific differences in adipose tissue secretory function are dependent on the anatomical location of this tissue (81). Recently it was shown that SC adipose tissue has a higher capacity to expand its capillary network than visceral tissue (82). But with increasing fat accumulation, this capacity decreases, and the decrease in SC tissue functional angiogenesis correlates with insulin resistance and suggests that impaired SC vascularization capacity may contribute to metabolic diseases (82). The

inability of healthy SC fat depots to store excess calories may represent a critical node in the development of subsequent ectopic fat deposition in visceral depots, the liver and other cell types (83).

1.3.3.2 Visceral adipose tissues (VAT): Intra-abdominal fat depots are associated with internal organs, and both intra and retroperitoneal depots represent 10–20% of total body fat in men and 5–10% in women. Intraperitoneal VAT which are associated with digestive organs, include the omental (OM) (hangs off the stomach), the mesenteric (associated with the intestine), and epiploic (along the colon) (75). VAT is also deposited internally in the thoracic compartments, and the epicardial region.

1.3.3.2.1 Omental (OM) depots;

The number of stromal cells (nonadipocytes) per gram of AT is greater in OM than SC (84) and OM secrete more of the proinflammatory cytokines, such as IL-6, visfatin and MCP-1, which may be responsible for the insulin resistance (85,86). OM depot is more heavily implicated in metabolic syndrome and more extensively infiltrated with immune-inflammatory cells, such as macrophages and T-lymphocytes, than the subcutaneous depot.

1.3.4 Obesity and Adipose tissue dysfunction:

Obesity is an enlargement of adipose tissue, which probably has a strong genetic predisposition that possibly regulates both excess energy intake and/or energy expenditure. AT dysfunction is characterized by mainly visceral (ectopic) fat accumulation, (87) changes in the intracellular matrix and cellular composition of adipose tissue (88), increased number of immune cells infiltration (89), enlarged adipocytes (87), increased autophagy (90), apoptosis (91), and changes in AT mRNA and protein expression patterns (87). Thus, with the development of AT dysfunction, adipokine secretion is significantly altered toward a proinflammatory, atherogenic and diabetogenic pattern (92). These changes link impaired adipose tissue function to insulin resistance and cardiovascular disease (87, 92). There are two possible mechanisms, adipose tissue hyperplasia and hypertrophy, that can lead to increased adipose tissue size, which are influenced by diet and genetics, as well as

by their interaction. Hyperplastic growth (increase in adipocyte cell number) appears in the early stages of adipose tissue development (92,93). Hypertrophy (increase in adipocyte volume), on the other hand, occurs prior to hyperplasia to meet the need for additional fat storage capacity in the progression of obesity (94,95,96). The latter is characterized by the presence of large, lipid-laden adipocytes that plays an important role in obesity-related cardiovascular and metabolic disorders. Thus, increased triglyceride storage was shown to be a major determinant of obesity development (87). The number of adipocytes appears to be set during childhood and adolescence, which suggests hypertrophy of adipocytes, is the major mechanism for adipose tissue expansion in obesity (93).

When adipocytes hypertrophy, local adipose tissue hypoxia may occur due to hypoperfusion, and as a result hypoxia-inducible transcription factors are expressed triggering the expression of angiogenic factors (VEGF, hepatocyte growth factor, PAI-1). These inhibit adiponectin gene transcription illustrated by decreased adiponectin promoter and peroxisome proliferator activated receptor gamma (PPAR-γ) activity, by reduced adiponectin mRNA stability and finally by a decline in adiponectin expression (97). Various unsaturated fatty acids and their metabolites have been shown to bind PPAR-γ and its activation results in adipocyte hyperplasia with a concomitant shift of TG from circulating lipoproteins and muscle tissue into adipocytes as shown in animal models (98). These changes lead indirectly to improved endothelial function and in decreased plasma levels of insulin, FFAs, and cytokines.

PPAR-γ and sterol regulatory element binding proteins (SREBP) are two transcription factors involved in the development and metabolism of adipocytes (99). PPAR-γ is expressed at high levels in adipose tissue and activates genes involved in adipocyte differentiation and fatty acid trapping such as fatty acid transport protein, lipoprotein lipase (LPL), fatty acid binding protein, adiponectin (as discussed above) and acyl-CoA synthase (100). SREBP-1c is the main isoform and highly expressed in most tissues including adipose tissue. Once it is activated by insulin it activates a cascade of genes required for endogenous lipogenesis and preadipocyte differentiation (101). The consequence of inflammation-induced

PPAR-γ down-regulation is directly through reduced adiponectin expression or alteration in adipogenesis (102). Adipogenesis is the process of preadipocyte differentiation into lipid-laden and insulin responsive adipocytes. This occurs through several stages involving a cascade of transcription factors, including PPAR-γ as well as enhancer binding proteins (C/EBPs), which are crucial determinants of adipocyte fate (103). Thus it could be envisaged that inflammation induces secretory changes in the tissue as a consequence of the changes in cellular make up.

1.3.4.1 Obesity and Inflammation:

Overall obesity may be considered a chronic, low grade inflammatory condition, with the cellularity and the secretions of adipose tissue reflecting these changes. Over-nutrition leads to adipocyte hypertrophy, followed by cell death that may act as a stimulus for immune cell infiltration into the tissue. An inflammatory process is simultaneously activated by increased adipose tissue mass in metabolically active sites, such as WAT, liver and immune cells. Monocyte infiltration and differentiation in particular has been shown to correlate with adipocyte hypertrophy, as well as body mass. Monocytes move by diapedesis from the blood to adipose tissue and differentiate into macrophages in the obese adipose tissue which switch their phenotype from one of a non-inflammatory resident macrophage to that of a lipid glutted foam cell, expressing dendritic cell markers, such as CD11c (4). M1-Macrophages, the ones with the proinflammatory phenotype, are more prevalent in adipose tissue and predominant in obesity, secreting TNFα and IL-6 thereby enhancing inflammation. TNF-α activates human adipocytes further to induce lipolysis and enhance the expression of various genes such as; ICAM-1, IL-6, MCP-1. The secretions from these macrophages (IL-6 and TNFα) along with those from the hypertrophied adipocyte (MCP-1 and leptin) regulate the pathological changes of obesity, such as insulin resistance and endothelial dysfunction (32).

1.4 Obesity and Insulin resistance:

Insulin resistance is known as a failure of target organs to respond normally to the action of insulin. It manifests as suppression of hepatic glucose output and impaired

insulin-mediated glucose uptake in skeletal muscle and adipose tissue, leading to increased insulin requirements. Obesity is a well-defined risk factor for the development of insulin resistance and the metabolic syndrome, which include reduction in the levels of endogenous insulin sensitizers, such as adiponectin, and elevation in insulin antagonists, such as resistin, TNF-α and IL-6 (104).

1.4.1 Normal insulin function and secretion

1.4.1.1 Insulin is a peptide hormone secreted from the β cells of the pancreatic islets of Langerhans to maintain normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects (105).

1.4.1.2 Mechanism of Insulin Secretion

Synthesis and secretion of insulin is regulated by both nutrient and non-nutrient secretagogues, as well as environmental stimuli and the interplay of other hormones. Increased levels of glucose induce the glucose-mediated insulin secretion as the first phase by release of insulin from secretory granules in the β cell. Once glucose entry into the β cell is sensed by glucokinase, the enzymes phosphorylate glucose to glucose-6-phosphate (G6P), and generate ATP. Increase in ATP leads to closure of KATP-channels which results in membrane depolarization and activation of voltage dependent calcium channels leading to an increase in intracellular calcium concentration, that triggers pulsatile insulin secretion (106,107). Glucose does not require insulin action to enter the β cell. At the same time cyclic AMP and other cellular energy intermediates are also augmented, further enhancing insulin release (108). Other mediators of insulin release involve activation of phospholipases and protein kinase C (e.g. by acetycholine) and by stimulation of adenylyl cyclase activity and activation of β cell protein kinase A that potentiates insulin secretion. This latter mechanism may be activated by hormones, like vasoactive intestinal peptide (VIP), PACAP, GLP-1, and GIP. These mediators appear as the second phase of glucose mediated insulin secretion, after refilling of secretory granules translocated from reserve pools (109).

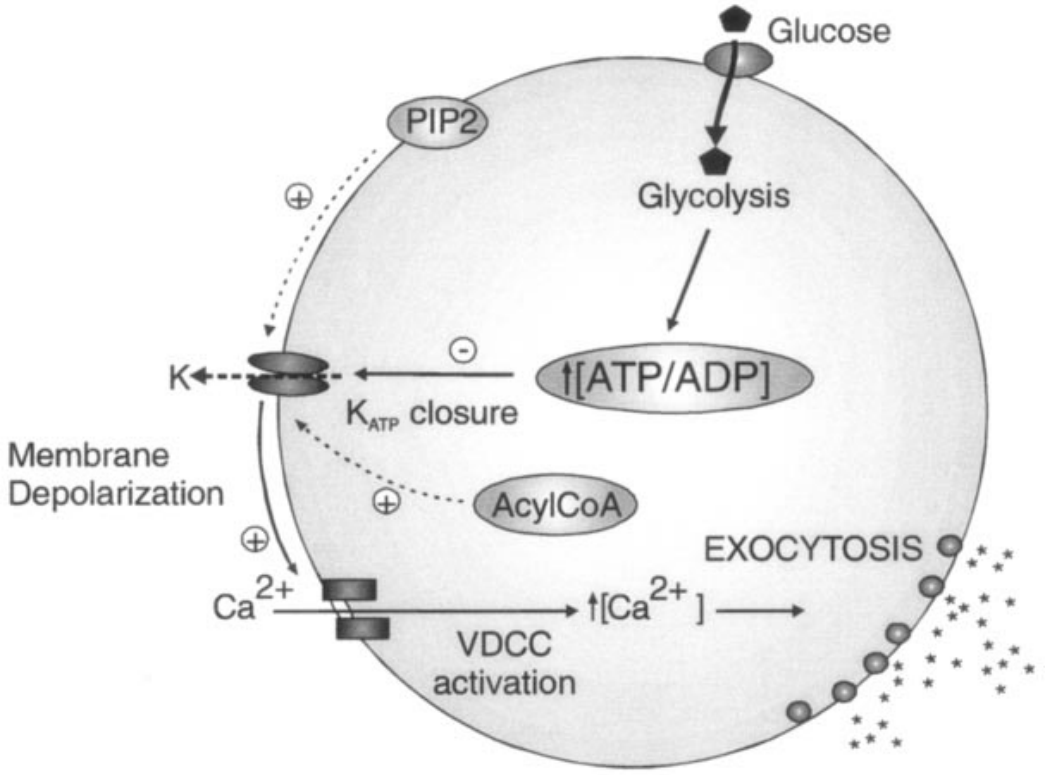


Figure 2 Pancreatic β-cell and KATP channel. The pancreatic β-cell, the ATP-sensitive K^+ channel (K_{ATP} channel) plays an essential role in coupling membrane excitability with glucose-stimulated insulin secretion. As increase in glucose metabolism leads to elevated intracellular [ATP]/ [ADP] ratio, closure of K_{ATP} channels, and membrane depolarization. The consequent activation of voltage-dependent Ca^{2+} channels causes a rise in Ca^{2+} that stimulates insulin release. The role of pancreatic K_{ATP} channel in insulin secretion, and elevated blood glucose increases glucose metabolism in the β cell and elevates [ATP]/ [ADP]. So this metabolic signal closes K_{ATP} channels, which causing depolarization, activation of voltage-dependent Ca^{2+} channels, leading to Ca^{2+} entry, and insulin exocytosis. Various additional effectors, involving phosphatidylinositol biphosphate (PIP2) and Acetyl coenzyme (A acyl CoAs), act to modulate ATP sensitivity of the channel and can thereby affect the coupling of metabolism to secretion (Adapted from110).

1.5 Obesity and vascular function:

In obesity, abnormalities in the size and distribution of fat mass are associated with insulin resistance, alterations in vascular function including; low-grade chronic inflammation, oxidative stress, endothelial dysfunction and a pro-thrombotic tendency (111). With central obesity AT synthesizes and releases increased amount of certain factors. These include proinflammatory chemokines and cytokines, such as MCP-1, macrophage migration inhibitory factor (MIF), IL-1β and IL-6 (112), procoagulant and proinflammatory mediators such as tissue factor (TF) and PAI-1 (112), vasoactive substances such as angiotensinogen and endothelin-1 (ET-1) (113), and molecules involved in the pathogenesis of insulin resistance, such as TNF-α and resistin (114).

The relationship between the adipokines and the vasculature is of increasing research interest, especially in obesity and its associated pathologies. Vascular smooth muscle (VSMC) receives numerous circulating mediators and hormones that exhibit vasoactive effects, some of which depend on the endothelium and others which act independent of the endothelium. (115).

1.5.1 Normal vascular function

1.5.1.1 Normal Endothelial function

Endothelial cells form the inner lining of the vasculature. The vascular endothelium, besides providing a physical barrier between the lumen and the vessel wall, also plays an important role in the control of vascular homeostasis; the endothelium actively regulates the basal vascular tone and reactivity in physiological conditions. It plays a central role in regulating circulating factors such as hormones, fatty acids and nutrients to vasodilators like NO, endothelium- derived hyperpolarization factor [EDHF], prostaglandins [PGI_2/PGE_2] and to vasoconstrictors, such as, endothelin-1 [ET-1] (116). Gaseous NO is the most important vasodilator generated by endothelial cells and freely diffuses into adjacent VSMC, where it activates guanylate cyclase to increase cyclic GMP and evoke vasorelaxation. Binding of vasodilators like acetylcholine to specific G-protein coupled receptors on the surface of endothelial cells leads subsequently to increase in intracellular Ca^{2+} levels (117)

and release of NO. The endothelium also produces vasoconstricting factors such as ET-1 which has both contracting and dilating properties that are mediated by at least two subtypes of receptors. The ETA receptor is located on vascular smooth muscle and mediates vasoconstriction, whereas ETB receptors are located on endothelium and mediate vasodilation (118). The normal regulation of vascular tone represents a finely tuned balance between the forces of vasoconstriction and vasorelaxation. This balance is regulated by both endothelium-dependent and endothelium- independent factors (Figure 3).

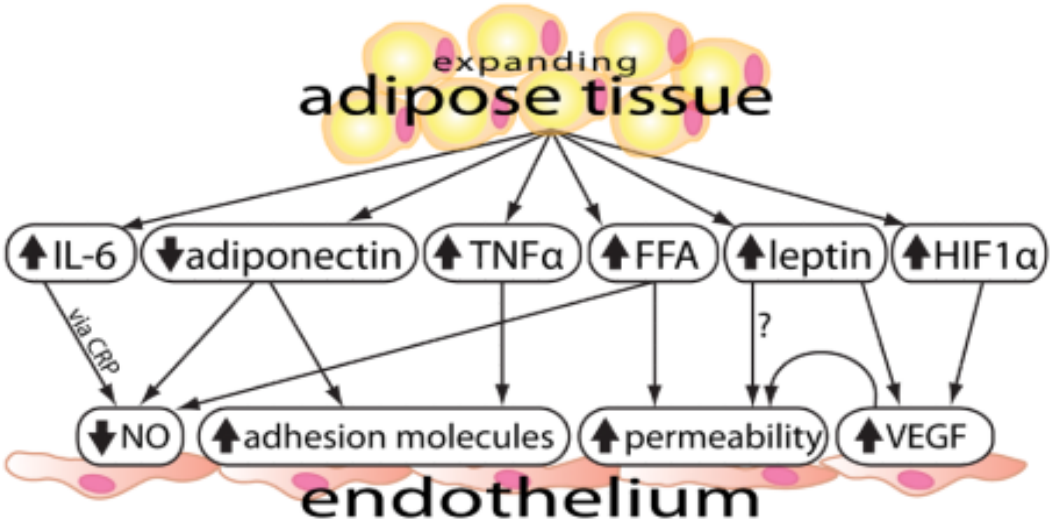


Figure 3 Adipokines & adipose tissue expansion. Adipose tissue expansion and its interaction with endothelium permeability and dysfunction induced by adipokines alter transendothelial transport and exclusion; which induce reduction in NO, hindering vasodilation, upregulation of adhesion molecules promoting immune infiltration and increase vessel permeability. Leptin: impair NO production and sensitivity and induce angiogenesis. TNF-α: increases endothelial-immune cell adhesion molecules and immune trafficking. Adiponectin: down regulates each of these responses. High concentrations of FFA: directly impair endothelial function, leading to further local metabolic instability. HIF1-α: induces fibrosis in response to hypoxia (Adapted from 6).

1.5.1.2 Vascular Smooth Muscle Cell (VSMC):

VSMC plays an essential role in providing structural integrity of the vessel wall and in controlling vascular tone and blood pressure (119). VSMC is the main target of the effects of endothelium-released NO that stimulates the synthesis of cGMP, therefore preventing the Ca^{2+} release from intracellular stores (120). Moreover the inhibition of Ca^{2+}- dependent Rho/Rho kinase pathway is a relevant mechanism involved in the modulation of VSMC relaxation induced by the NO/cGMP/PKG pathway (57). The surface receptors which regulate VSMC responses and modulate the contractile process include those for acetylcholine, catecholamines, serotonin, histamine, purinergic mediators, angiotensin II (Ang II), bradykinin, neuropeptide Y, vasopressin, vasoactive Intestinal Polypeptide (VIP), prostanoids, leukotrienes oxytocin, growth factors such as Epidermal Growth Factor (EGF), PDGF, TGF-β, Fibroblast Growth Factor (FGF) and Insulin-like growth factor (IGF-1) (121).

Vascular smooth muscle cells, endothelial cells and perivascular adipose tissues are known to contain reactive oxygen species (ROS) which might regulate ion channel functions. ROS are a class of oxygen- derived molecules including superoxide anion and hydrogen peroxide, both modulators of vascular tone (122). ROS may regulate cellular function by affecting ion channels, such as Ca^{2+}-dependent K^+ channels (123), ATP sensitive K^+ channel (124), and Ca^{2+} channels. ROS regulates KCa3.1 expression by modulation of the ERK and REST pathways, and influence KCa3.1 current production in human endothelial cells. Hydrogen peroxide donor tert-butyl hydroperoxide (TBHP) increases KCa3.1 expression by Phosphorylated extracellular signal-regulated kinase (pERK) upregulation and repressor element-1-silencing transcription factor (REST) downregulation. In contrast, the superoxide donors xanthine/xanthine oxidase mixture (X/XO,) and lysophosphatidylcholine (LPC) decrease KCa3.1 expression by pERK downergulation and REST upregulation. In addition, KCa3.1 current is augmented by TBHP, and inhibited by X/XO. This shows that ROS play a key role in both physiological and pathological processes in endothelial cells by regulating KCa3.1 which induces modulation of endothelial function (125).

Hydrogen peroxide accelerates the activation kinetics of Kv1.4 and Kv3.4, but not Kv1.3, Kv2.1 and Kv2.2 (126). It may also negatively shift the activation curve of Kv1.5 (127). This may explain why hydrogen peroxide is able to induce both vasorelaxation and vasoconstriction (128). Vasoconstriction by hydrogen peroxide is likely induced in a Ca^{2+}-dependent way, although Ca^{2+} sensitization and Ca^{2+}-independent pathways have also been reported (128). Superoxide anions can induce vasoconstriction through Ca^{2+} sensitization pathways. Oxidative stress occurs when the production of ROS exceeds the cell's capacity to detoxify these potentially injurious oxidants using antioxidant defense systems (129).

In obese subjects several studies have showed impaired arterial vasodilation, mostly involving cerebral, coronary, mesenteric, and skeletal muscle vasculature. The main cause is endothelial dysfunction by increased secretion of proinflammatory cytokines, reduced levels of circulating adiponectin, and increased release of free fatty acids (145). All these abnormalities change gene expression and cell signaling in vascular endothelium, cause vascular insulin resistance, change the release of endothelium-derived factors, and enhanced vascular oxidative stress (130). In central obesity, reduced adiponectin and elevated levels of resistin, leptin, TNFα, and IL-6, increases the production of superoxide anion (O_2^-), which interferes with NO availability, thus reducing vasodilation (130). Moreover insulin resistance that characterizes human obesity and involves the vascular effects of hormones can determine per se hemodynamic consequences by impairing the balance between the vasodilating insulin actions exerted via the NO/cGMP/PKG pathway and the vasoconstricting-ones (131). However, how diseases such as insulin resistance/ type 2 DM impact the microvasculature is still not well understood.

1.5.2 Adipose tissue expansion and angiogenesis:

The expansion of adipose tissue (AT) can occur through both adipocyte hyperplasia and hypertrophy. This expansion can lead to many effects, including hypoxia, adipocyte cell death, enhanced cytokine secretion, and dysregulation in fatty acid fluxes.

Human preadipocytes and capillary endothelial cells express αvβ3 integrin and plasminogen activator inhibitor 1 (PAI-1) that guide preadipocyte migration toward developing capillary networks to ensure the coordination of the development of both tissues at the same place (132). Also, PPAR-γ is essential mediator for preadipocyte differentiation, and regulation of adipose angiogenesis (133,134). Adipose tissue produces several matrix metalloproteinases (MMPs) including MMP-2 and -9 that can potentially affect preadipocyte differentiation and microvessel maturation by modulating extracellular matrix (ECM) (135). MMP-9 is able to release the matrix bound VEGF and indirectly induces angiogenesis (136). In adipose tissue that is rapidly expanding, hypoxia is an important factor for vascular growth and remodeling (137). In response to hypoxia, adipose tissues produce hypoxia inducible factor-1α (HIF-1α) induced angiogenic factors like VEGF, leptin, TNF-α, and PAI-1 that regulate angiogenesis and vasculogenesis. Thus adipose tissue expansion is associated with local hypoxia, which contributes to angiogenesis by induction of a number of growth factors (138).

In the obese state, adipocyte hypertrophy creates areas of local AT micro-hypoxia at the earliest stages of expansion which suggest that AT is poorly oxygenated. The master regulator of hypoxia and oxygen homeostasis is HIF-1α as shown in figure 4. Several important hypoxia-associated genes especially leptin and VEGF, are directly regulated by HIF-1α (139). Other adipokines such as basic fibroblast growth factor and IL-6 have also been shown to induce angiogenesis (140).

Adipose tissue contains adipocytes, adipose stromal cells (ASCs), endothelial cells, and inflammatory cells, which produce several angiogenic factors including leptin, VEGF, FGF-2, HGF, IGF, TNF-α, TGF-β, VEGF-C, resistin, tissue factor (TF), and neuropeptide Y (NPY) (141,142,143,144,145). ASCs also secrete high levels of GM-CSF (135). Recruitment of inflammatory cells also significantly contributes to adipose neovascularization. Activated macrophages produce potent angiogenic factors including TNF-α, VEGF, FGF-2, IL-1b, IL-6, and IL-8 (114).

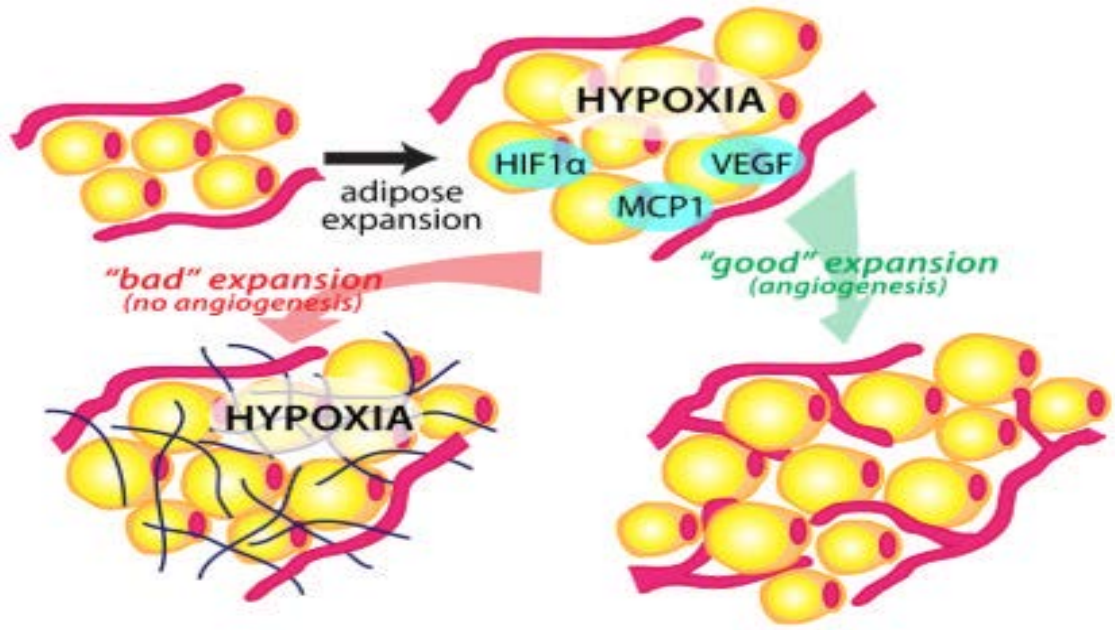


Figure 4: Adipose tissue & angiogenesis. Expanding adipose depots leading to hypoxia which induces the upregulation of an array of adipokines, among them HIF-1α, monocyte chemotactic protein (MCP)-1, & VEGF that necessitates angiogenesis for healthy tissue function. In depots where angiogenesis progresses slowly, the adipose matrix becomes fibrotic and induces further metabolic dysfunction (Adapted from 6).

Necrosis of adipocytes, driven by hypertrophy and accelerated by obesity, is a phagocytic stimulus that regulates AT macrophage infiltration and aggregation, forming crown-like structures (CLSs) that surround necrotic adipocytes in advanced obesity (146). Adipose tissue macrophages serve multiple functions including removal of necrotic adipocytes leading to lipid-engulfed foam cells, performing as proinflammatory mediators, and serving as angiogenic precursors (6).

Leptin is a potent angiogenic factor, and the expression of the functional long form of leptin receptor (OB-Rb) in endothelial cells has led to the discovery of its angiogenic activity (147). Leptin could also indirectly induce angiogenesis via upregulation of VEGF mRNA expression via activation of the Jak/Stat3 signaling pathway (148). Resistin is also defined as an angiogenic factor, which directly promotes endothelial cell proliferation, migration, and tube formation (145).

Fatty acids stored in the form of triglycerides, are released from hypertrophic adipocytes through lipolysis during fasting. Some of FFAs are shunted to the liver and stored in lipid droplets and many of them are locally re-esterified in adipocytes (149). Those FFAs that escape re-esterification play a critical role in several organs as a primary energy source during prolonged fasting (150). They also serve as ligands for the toll like receptor (TLR4) complex which activates the classical inflammatory responses by increasing local extracellular lipid concentrations, thereby ultimately driving AT macrophage accumulation (151). So either high rates of lipolysis or an influx of saturated FFAs into adipocytes can cause temporary inflammation within the tissue (152).

1.5.2.1 Healthy and pathologic angiogenesis:

Recent studies suggest that the balance between angiogenesis and hypoxia has a significant impact on the modulation of “good/healthy” versus “bad/unhealthy” tissue expansion, thereby involving the local microvasculature as a key modulator of the systemic impact of adipose depots (6). The vasculature serves to transport systemic lipids to their storage depot in the adipocytes and adipokines and nutrients such as FFAs from these cells to other tissues in time of metabolic need. So expansion and contraction of fat mass relies on the adipose tissue circulation (153). Therefore insufficient circulation results in local hypoxia which leads to up-

regulation of hypoxia inducible transcription factor. These in turn trigger the expression of angiogenic adipokines such as, VEGF, hepatocyte growth factor and PAI-1 (101), which promote vascular endothelial cell proliferation and the latter stages of new vessel formation. Also, increased levels of HIF1-α leads to an up-regulation of the inflammatory adipokines (IL–6, TNF-α, and MCP-1) (6). These pro-inflammatory secretory products enhance insulin resistance, and induce adipose tissue fibrosis that leads to further adipose dysfunction as shown in figure 5.

1.5.2.2 Microvascular dysfunction in Obesity and Insulin Resistance:

The vasodilator actions of insulin involve the endothelial insulin receptor, insulin receptor substrate 1 and receptor substrate 2 (IRS1 and IRS2), PI3-kinase, phosphoinositide-dependent kinase 1 (PDK-1), and protein kinase B (Akt) (154, 155). Insulin-induced stimulation of Akt directly increases endothelial NO production via endothelial nitric oxide synthase (eNOS). The metabolic action of insulin to stimulate glucose uptake in skeletal muscle and adipose tissue is mediated through stimulation of similar PI3-kinase–dependent signaling pathways beside these vasodilators actions. The vasoconstrictor effects are mainly mediated by the vasoconstrictor peptide ET- 1 (154). ET-1 is produced in the vascular endothelium through stimulation of the intracellular mitogen-active protein kinase (MAPK) signaling pathway and the extracellular signal-regulated kinase-1/2 (ERK1/2) as shown in figure 6 (59). The key feature of insulin resistance is that it is characterized by specific impairment in PI3K-dependent signaling pathways, while insulin's signaling through the MAPK pathways remains intact (Figure 6) (155).

The obese insulin-resistant individual suffers impairments in the microvasculature. The existence of microvessel endothelial dysfunction has been established by blunted NO-mediated vasodilator responses in skin and resistance arterioles to classic endothelium-dependent vasodilators. Impaired capillary recruitment to reactive hyperemia has also been reported (156).

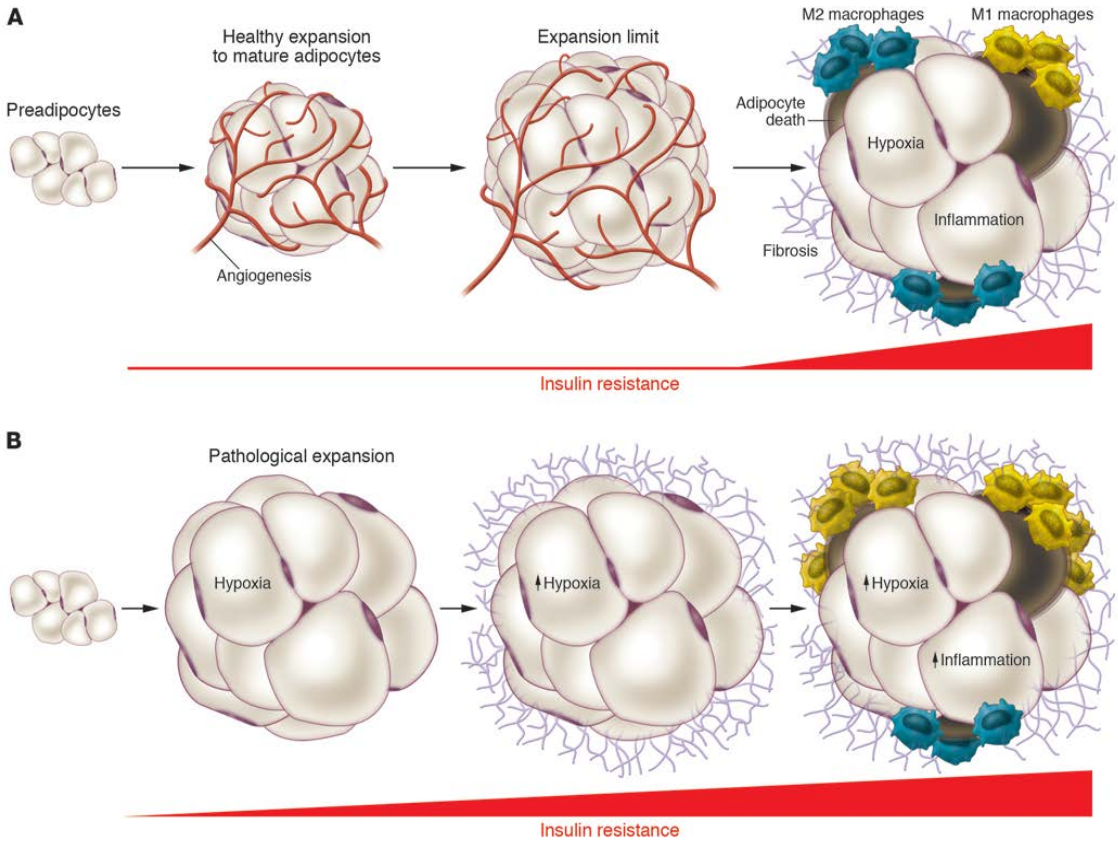
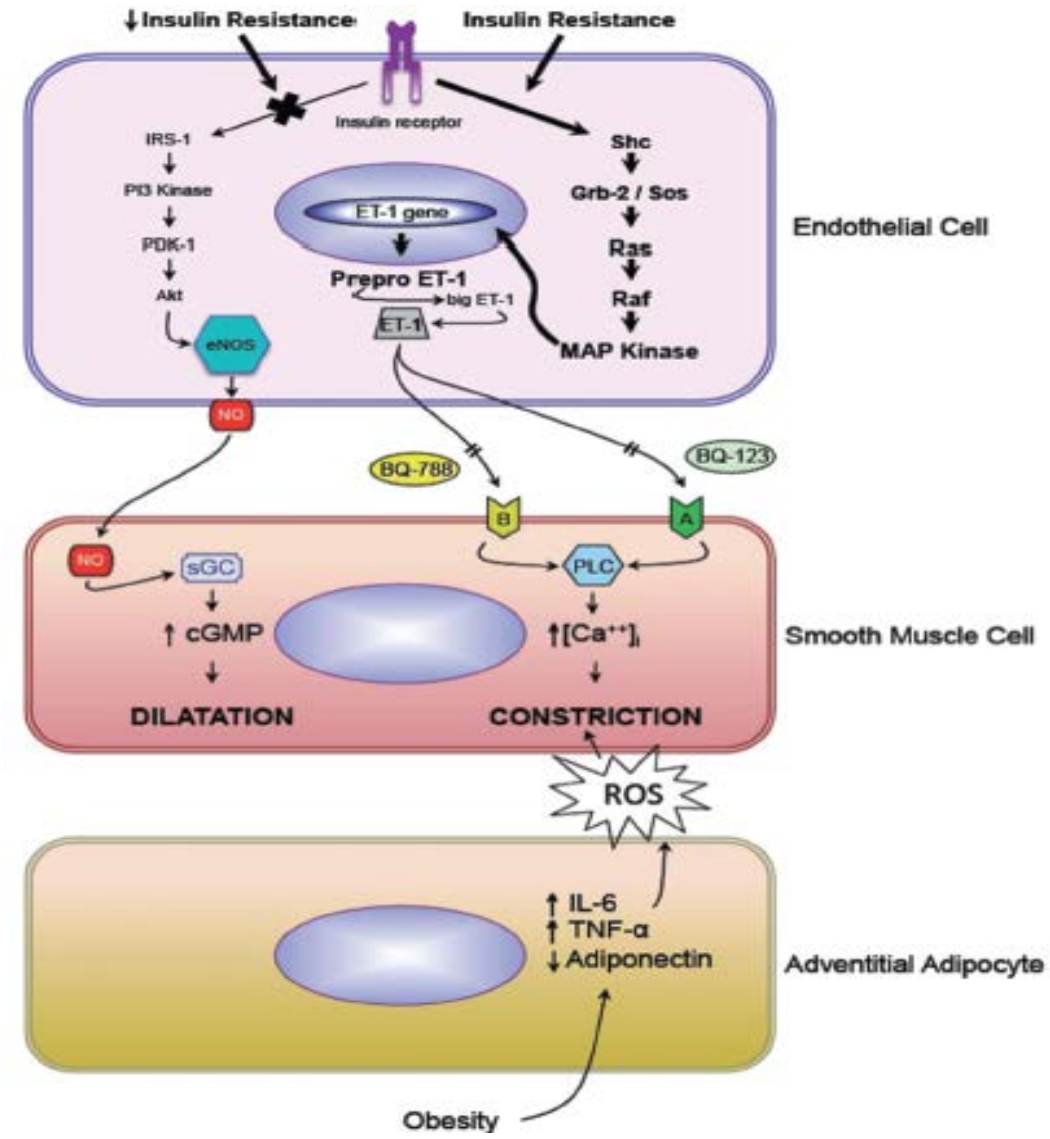


Figure 5: Adipose tissue expansion. Healthy adipose depot expansion comprises enlargement in tissues through effective and adequate angiogenic response and appropriate remodeling of the ECM. While, pathological adipose depot expansion comprises huge enlargement of existing adipocytes, limited angiogenesis, and hypoxia lead to induction of HIF-1α, which in turn can cause induction of fibrotic. Ultimately, M1 stage macrophage leading to an inflammation which is associated with systemic insulin resistance (Adapted from 23).



<u>Figure 6:</u> Insulin resistance mechanism. The interactions between endothelial, smooth muscle (SM) and adventitial cells. In conditions of insulin resistance the balance between vasoconstrictor and vasodilator pathways is shifted towards a predominant vasoconstriction. Moreover, the impairment of adipocyte function and production of inflammatory cytokines within the perivascular adipose tissue leads to increase ROS production and oxidative stress that, increases smooth muscle cell contractility (Adapted from 214).

1.5.2.3 Microvascular dysfunction and type 2 diabetes:

β-cell dysfunction was thought to be the most important risk factor for type 2 diabetes in normoglycemic subjects. However, more recent evidence suggests that a combination of both insulin resistance and relatively diminished insulin secretory function of pancreatic β-cells (157) play a part in the disease. Expansion of β-cell mass has been reported in obese subjects and is related to increased intake of nutrients (glucose and FFAs). As the insulin resistance increases, insulin production also increases in concert. However if these processes are not coordinated diabetes will ensue. Low adiponectin and elevated levels of other adipokines such as, leptin, TNF-α, and IL-6 are also associated with an increased risk of diabetes. This probably relates to their effects on both insulin sensitivity and the pancreas leading to β-cell failure (158,159).

Diabetes alters the function of various cell types, including the endothelium, smooth muscle cells and platelets. The disease impairs endothelium dependent vasodilation (NO mediated) by a number of fundamental mechanisms, such as hyperglycemia, dyslipidemia and insulin resistance, which lead to decreased endothelium derived NO, (160). Hyperglycemia inhibits production of NO by blocking eNOS synthase activation and inducing reactive O_2 production, in particular superoxide anion (O_2^-) in endothelial and VSMC (161). Circulating levels of free fatty acids are elevated in diabetes and insulin resistance due to their excess release from adipose tissue and diminished uptake by skeletal muscle (162,158). Free fatty acids impair endothelial function through increased production of oxygen-derived free radicals, activation of PKC, and exacerbation of dyslipidemia (163). Elevation of free fatty acid concentrations activate PKC and decrease insulin receptor substrate-1–associated phosphatidylinositol-3 kinase activity (164). These effects on signal transduction may decrease NOS. In insulin-resistant peoples, endothelium-dependent vasodilation is reduced (165). Abnormal endothelium-dependent vasodilation in insulin-resistant states is due to alterations in intracellular signaling that reduces the production of NO. In particular, insulin signal transduction through the Pl-3 kinase pathway is impaired, and insulin is less able to activate NOS and produce NO. Abdominal adipose tissue, which is prominent in type 2 diabetes, is more insulin resistant and

releases more free fatty acids compared with the type of adipose in other locations (98). Thus, free fatty acid–induced alterations in intracellular signaling also contribute to decreased NOS activity and reduced production of NO in insulin-resistance. In diabetes, endothelial cell dysfunction is not only by decreased NO but also by increased synthesis of vasoconstrictor prostanoids and endothelin (161). Endothelin-1 activates endothelin A receptors on VSMC to enhance vasoconstriction. Dysregulation of vascular smooth muscle function is exacerbated by impairments in sympathetic nervous system function (166). Diabetes also induces PKC activity, NF-κβ production, and generation of oxygen-derived free radicals in VSMC, similar to the effects in endothelial cells (155).

1.6 Ion Channels of adipose tissue

Ion channels are protein molecules present as pores in the plasma membrane and membranes of intracellular organelles of all cells. They play an important role in maintaining cellular integrity, excitation, smooth muscle contraction, secretion of hormones and neurotransmitters. These channels use the ionic gradient between the cytosolic and extracellular spaces to transfer sodium (Na^+), Potassium (K^+), and calcium (Ca^+) ions, and regulate the shape and frequency of action potentials in skeletal muscle, cardiac muscle, smooth muscle and neurons. They restore the balance of ions across a membrane and the difference in voltage occurs across that membrane creating a membrane potential, when ions are present in a higher concentration on one side of a membrane than the other. So ion channels open in response to a change in membrane potential, allowing the ions to move from the side of the membrane with the higher ion concentration to the side with the lower ion concentration (91).

Adipocytes are pivotal in imparting the endocrine organ of adipose tissue as well as metabolism, storage and effects of fatty acids. Electrophysiological and molecular evidence show that several ion channels can be found in adipocytes and adipose tissue. These include Ca^+ and K^+ channels as well as selective and non-selective ion transporters as represented in figure 7.

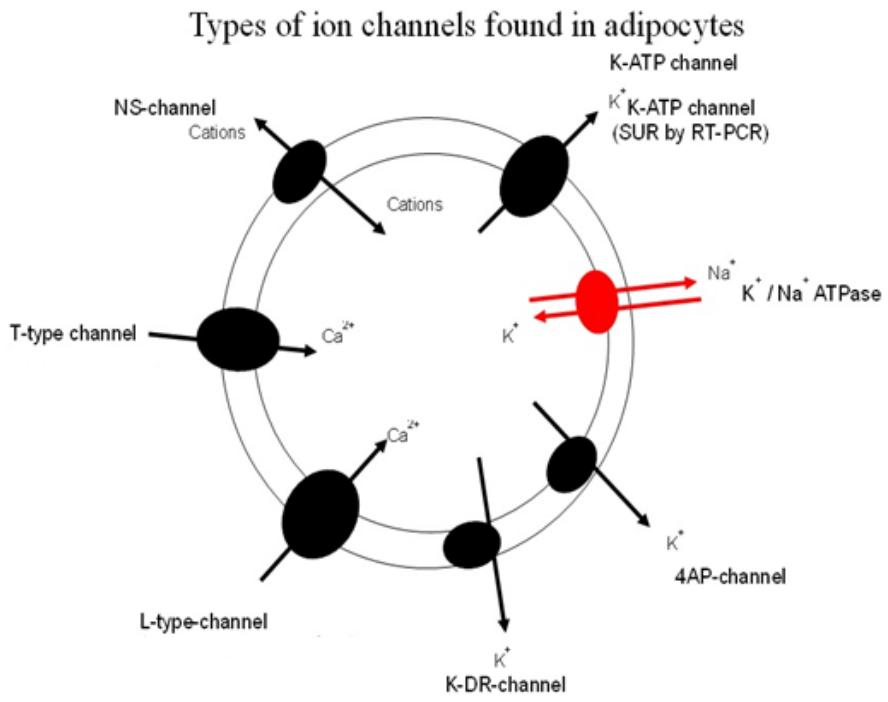


Figure 7: Types of ion channels described in adipocytes. From the literature these were the ion channels that have been described in vessels isolated from the adipose tissue. L: Long lasting-type Ca^{2+} channels. T: Transient-type Ca^{2+} channels. K_{ATP}: Adenosine-triphosphate-sensitive potassium channel. KDR: Delayed rectifier potassium channel. 4AP: Aminopyridine sensitive potassium channel. NS: Non selective channel. K^+/Na ATPase$^+$: Ion transport/pump systems.

The voltage–activated (Kv), adenosine-triphosphate-sensitive (K_{ATP}) and calcium-activated (K_{Ca}) have been described in adipose tissue. In a recent study Kv7.1 was identified as the active isoform of Kv found in vessels embedded in human adipose tissue (167). The inhibitory action of the newly described but yet unidentified adipocyte-derived relaxant factor (ADRF) is reportedly mediated by tyrosine kinase pathways and opening of K_{ATP} channels (8) in the aorta. In rat mesenteric arteries, the vasodilatory effect of perivascular fat involves the activation of Kv channels (168). Interestingly, the channels activated by fat in mesenteric arteries (Kv) differ from the channels proposed to be activated in the aorta (K_{ATP}) suggesting that there are vascular regional differences in the effects of perivascular adipose tissue on ion channels (Figure 6).

Potassium channels (K^+) is the most abundant, diverse one of these channels and its activity is regulated by voltage, calcium and neurotransmitters. This channel plays an important role in maintaining the normal physiological cellular process in the body, especially smooth muscle relaxation, immune function and insulin secretion (169,170).

1.6.1 Vascular ion Channel and insulin resistance/obesity:

Vascular ion channels generally contribute to the excitability and reactivity of blood vessels. In VSMCs, potassium (K^+) channels situated in the plasmalemma play a fundamental role in maintaining the membrane potential, which is a major determinant of vascular tone, particularly in systemic resistance vessels. The membrane potential is determined by membrane permeability to several ions, including K^+, Ca^{2+}, Na^+, and Cl^- ions. Ion transport systems such as the Na^+/K^+ pump or anion transporters can also contribute. The blockade of K^+ channels results in membrane depolarization and increased Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCCs), leading to vasoconstriction. On the other hand the activation of K^+ channels results in plasmalemmal K efflux, membrane hyperpolarization, and reduced Ca^{2+} influx through VOCCs, leading to vasodilation (171). Altered vascular K^+ channel function has been reported under pathological conditions and during major cardiovascular diseases like chronic hypertension, diabetes and

atherosclerosis. The vasoconstriction and the compromised ability of an artery to dilate are likely consequences of defective K^+ channel function in blood vessels and may be due to a change in the number, unitary conductance, and/or exposed probability of the channels (172).

Activation of calcium channels by adipokines has been described. Both L- and T-type plasmalemmal Ca^{2+} channels have been described for the human subcutaneous adipose vessels (173). Changes in cytosolic Ca^{2+} concentration are the principle mechanism that regulates the contractile state of VSMCs. In response to vasoconstrictor stimuli, calcium accumulation occurs by release from intracellular stores such as the sarcoplasmic reticulum and/or influx through various types of Ca^{2+} channels to increase the cytosolic Ca^{2+} concentration in VSMCs (174). The increases in intracellular calcium are also known to activate mitogen-activated protein kinases that are involved in smooth muscle contraction by increasing the sensitivity of the contractile apparatus to calcium (175). Initial rise in intracellular calcium inhibits Kv channels, which results in membrane depolarization and subsequent activation of voltage-gated calcium channels in which calcium influx then leads to myosin light chain phosphorylation and smooth muscle contraction (176).

The intermediate conductance calcium-activated potassium channel KCa3.1 (KCNN4 and IKCa), contributes to a variety of cell activation processes in pathologies like inflammation, carcinogenesis, and vascular remodeling (177). KCa3.1 is a member of the calcium-activated potassium channel (K_{Ca}) 4 family, tightly binds the Ca^{2+} sensor calmodulin near its C-terminal domain. KCa3.1 is opened when there is a small rise in free cytosolic Ca^{2+} ($[Ca^{2+}]i$) due to Ca^{2+}-calmodulin-mediated cross-linking in the subunits of the channel tetramer (178). Channel activation induces membrane hyperpolarization that promotes Ca^{2+} influx, and increase in KCa3.1 expression has been associated with cancer development, immune disorders, and vascular inflammation. The electrophysiological and transcriptional mechanisms of VSMCs; shows change as they proliferate and in the contractile form. Ca^{2+} influx through voltage-dependent Ca^{2+} channels causes

VSMC contraction and co-activation of large conductance K_{Ca} channels (BK) that in turn induces Ca^{2+} channel closure through repolarization (177).

Potassium and calcium channels play an important role in mediating the hypoxic response of blood vessels (176). Hypoxia has also been shown to block outward potassium current that results in membrane depolarization (179). Hypoxia increases reduced β-nicotinamide adenine dinucleotide levels that activates adenosine diphosphate-ribosyl cyclase and inhibits cyclic adenosine diphosphate-ribose hydrolase. This leads to accumulation of cyclic adenosine diphosphate-ribose that stimulates calcium release from the sarcoplasmic reticulum and subsequent events leading to vasoconstriction (85).

Insulin resistance in obesity and hypertension is associated with impairment of insulin-mediated ion exchange processes (Ca^{2+}-ATPase and Na^{+}, K^{+}-ATPase), leading to Ca^{2+} and Na^{+} accumulation on the vascular wall (180). This alteration facilitates the action of vasoconstrictor agents such as Ang II and norepinephrine (181). Insulin resistance is accompanied by endothelial dysfunction in experimental models of hypertension in resistance vessels with impaired PI3-kinase-dependent NO production and enhanced ET-1 secretion that may combine with elevated peripheral vascular resistance and contribute to hypertension in this model (182).

1.6.2 Vascular studies on the human vessels

Central obesity and accumulation of intra-abdominal visceral fat are linked to cardiometabolic risk, by increased elaboration of proatherogenic mediators from the visceral adipose microenvironment which are toxic to the vasculature due to upregulation of proinflammatory, oxidative-stress related, and hypoxia-induced adipocytokines that severely impair arteriolar endothelial vasodilator function in visceral compared to subcutaneous depots (183). A recent study demonstrated upregulated expression of transcripts involving the cyclooxygenase pathway, which is involved in the production of vasoconstrictive prostanoids and reactive oxygen species known to impair vascular tone and typical of obesity-related conditions such as diabetes, hypertension, and atherosclerosis. The imbalance between endothelium derived constrictive and relaxing factors that exists in visceral depot, is in part due to

cyclooxygenase mediated generation of vasoconstrictor mediators that impair vascular tone (184).

In healthy people, PVAT modulates the contractile tone of adjacent small arteries by secreting vasodilatory molecules that act independently of the endothelium and include adiponectin, NO, and hydrogen sulfide. The first human small artery study of PVAT was performed in 2009 and showed that subcutaneous gluteal PVAT from lean healthy individuals reduced adrenergic constriction in adjacent arteries, an "anticontractile" effect (175). In metabolic syndrome patients the vasodilatory effect of the PVAT was entirely lost, due to dual processes of adipose tissue hypoxia and inflammation, both of which are established sequel of obesity in fat depots (26). Recently it was shown that macrophage activation in adipose tissue contributes to the attenuation in PVAT anticontractile effect (185).

1.7 Effect of Bariatric surgery on human:

Bariatric surgery is performed to achieve both significant and sustained weight-loss in severely obese patients, improves cardiovascular risk profiles and reduces overall mortality. The improvements in weight, blood pressure, inflammation, and metabolism overall reverse the obesity-induced alteration to adipose tissue anticontractile function (186). These reversals are due to reductions in local adipose inflammation and oxidative stress with improved adiponectin and nitric oxide bioavailability (186). There are 3 types of bariatric surgical procedures: restrictive, malabsorptive, and combined operations (187). Gastric bypass surgery is a combination of restriction and malabsorption and achieves a significantly higher degree of weight loss than restrictive bariatric surgery as shown in figure 8 (188).

A study that investigated the effect of bariatric surgery on the vasodilatory properties of PVAT showed 3 main findings. Firstly, bariatric surgery reversed the damage induced by obesity to PVAT anticontractile function. Secondly the PVAT functional recovery was independent of the endothelium. Thirdly bariatric surgery restored PVAT function by reducing adipose inflammation and increasing local adiponectin and NO bioavailability (186).

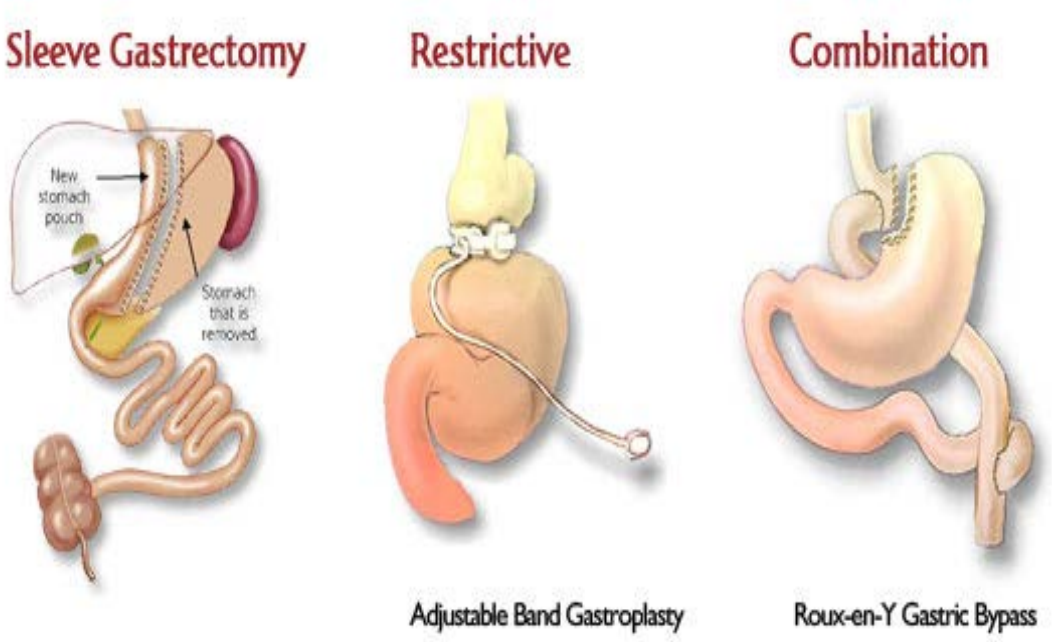


Figure 8: Types of bariatric surgery. Three types of bariatric surgery: Sleeve gastrectomy, band gastroplasty, and gastric bypass. Gastric bypass is a combination of restriction and malabsorption, this type of surgery achieved higher degree of weight loss than others (Adapted from: www.nationalbariatriclink.org).

1.8 Heterogeneity of the obese population

The majority of normal weight or lean individuals have normal metabolic function, normal adipokine levels, including adiponectin and their macrophages express markers of M2 (alternatively activated state) as shown in the figure 9. As obesity develops, adipocytes undergo hypertrophy owing to increased triglyceride storage. The expansion of adipose tissue promotes the transition to obesity accompanied by metabolic dysfunction, as well as generation of large amounts of pro-inflammatory factors such as, leptin, resistin, retinol-binding protein 4 (RBP4), lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), TNF-α, IL-6, IL-18, CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5) and nicotinamide phosphoribosyltransferase (NAMPT) (189, 190). Obesity, when associated with mild metabolic dysfunction exhibit improved metabolic parameters, diminished inflammatory marker expression and better vascular function, when compared with obesity associated with full metabolic dysfunction.

Therefore the presence of metabolic abnormalities varies even between equally obese individuals, with some of them being protected or resistant to the development of metabolic abnormalities (Figure 10;191) and others less so. The former individuals, defined as metabolically healthy but obese (MHO), have excessive body subcutaneous fat accumulation and display a favorable metabolic profile characterized by high levels of insulin sensitivity, no hypertension, as well as, a favorable lipid, inflammation, hormonal, liver enzyme and immune profile (192). Khittle et al found that about 20% of obese individuals do not have metabolic abnormalities and healthy obese appear to have less clustering of CVD risk factors (193). Overall, healthy obesity describes the absence of any metabolic disorder including type 2 diabetes, dyslipidemia and hypertension. The existence of distinct group MHO lead to more precise definition of healthy obesity which represents the continuous relationship between increasing BMI and by using specific parameters for different metabolic syndrome components such as blood pressure, fasting insulin levels, HDL and triglycerides. So this group remains insulin sensitive and metabolically healthy. In their study Stefan et al (194) divided their subjects into 3

groups – normal weight, obese-insulin sensitive, and obese-insulin resistant, and also examined cardiovascular risk factors. They found, the obese-insulin sensitive group had intimal medial thicknesses similar to that seen in the normal weight group and when compared to the insulin resistant group, had less skeletal muscle fat, less hepatic fat deposits, and lower intimal-media thickness of the common carotid artery, giving them a more favorable risk profile despite similar weights. For thus, it appears that in some limited circumstances, the adipose tissue has a greater ability to expand without the associated metabolic stress that triggers pro-inflammatory cytokine production and downstream effects such as insulin resistance (194).

This healthier metabolic profile may not necessarily translate into a lower risk for cardiovascular mortality. It might just reflect a transition stage, prior to conversion to the pathologically obese (PO) state. The mechanisms that could explain the differences between the metabolic profile of MHO and PO individuals are poorly understood. Preliminary evidence suggests that differences in visceral fat accumulation, birth weight, and adipose cell size and gene expression-encoding markers of adipose cell differentiation may explain the development of the MHO phenotype (195).

The growing incidence of obesity and type 2 diabetes mellitus is a worldwide epidemic problem and now recognized as one of the most challenging threats to public health (196). In the setting of obesity, the accumulation of fat tissue associated with excess fatty acid secretion, increased production of inflammatory cytokines and abnormal adipocyte hormone signaling leading to insulin resistance and chronic inflammation is postulated to play a role in development of type 2 diabetes and other obesity-related comorbidities (1,2). Elevated levels of fatty acids are commonly seen in obesity and diabetes and lead to diminished utilization of whole body glucose and cardiac muscle glucose and diminished skeletal muscle (197). In addition, increased circulating fatty acids levels lead to reduced beta cell function with diminished insulin secretion (198). In the pathology of diabetes, inflammation plays a role in disease development and progression in adipose tissue, liver, and skeletal muscle by provoking insulin resistance and beta cell dysfunction.

So the combination of insulin resistance and beta cell dysfunction, which characterizes diabetes. Several of inflammatory markers have been associated with diabetes including CRP, IL-6, MCP-1, IL-8, and PAI-1 (199).

Central obesity has been shown to have a greater association with diabetes or metabolic syndrome, as opposed to overall obesity (200). Visceral depot has more significant impact on diabetes related risk factors than that found in SC depots (200). Thus, uncontrolled diabetes leads to macrovascular and microvascular complications, like myocardial infarction, stroke, blindness, neuropathy, and renal failure in many patients. The main aim of current medical treatment is to halt disease progression by reducing hyperglycemia, hypertension, dyslipidemia, and other cardiovascular risk factors (201).

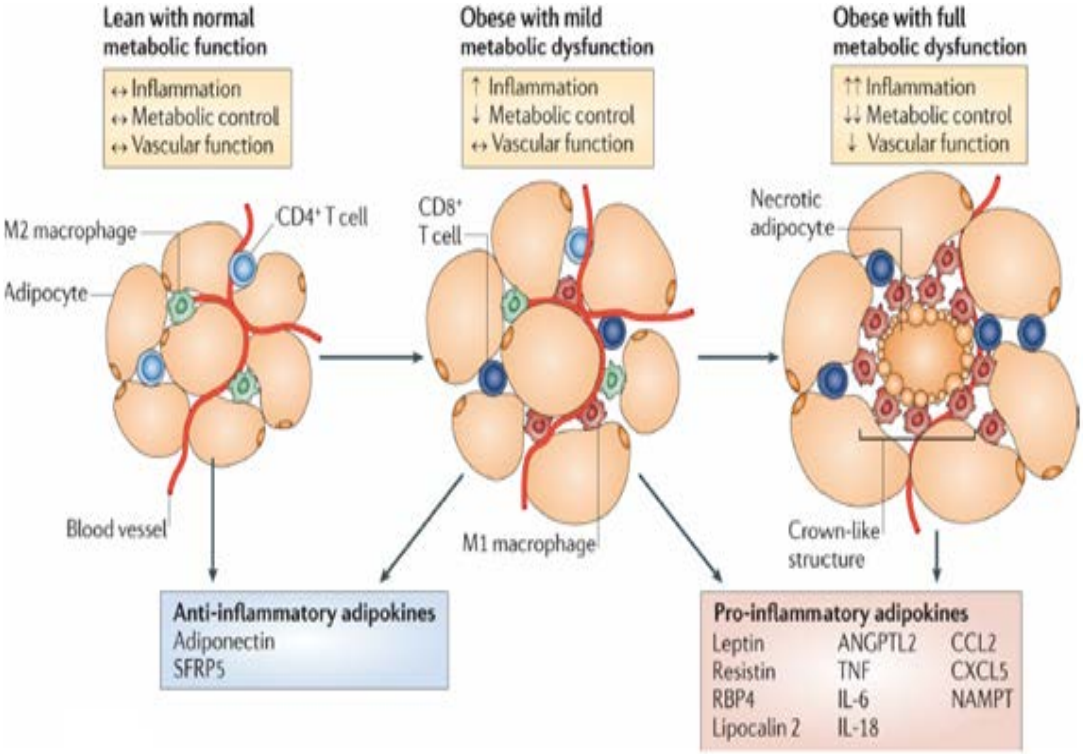
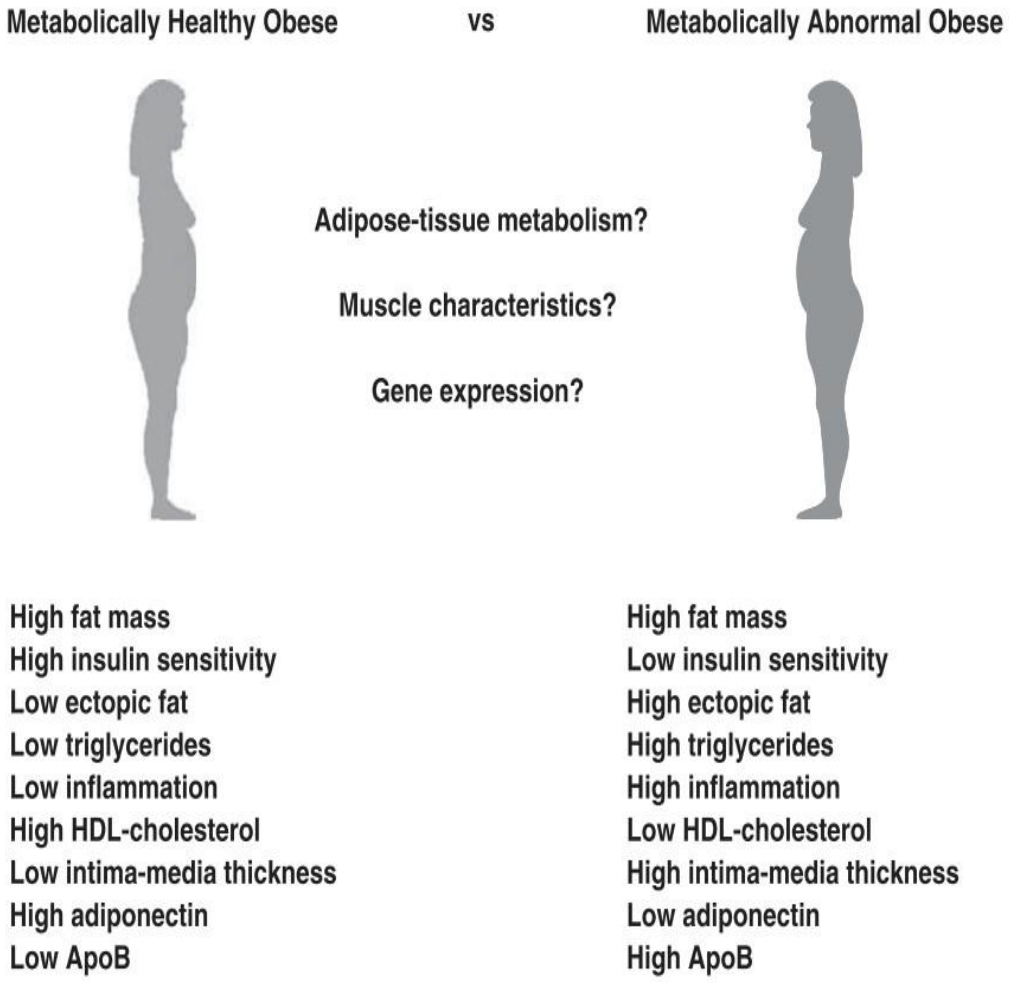


Figure 9: Heterogeneity of fat tissue. Adipose tissue can be described in three structural and functional classifications: lean with normal metabolic function, obese with mild metabolic dysfunction and obese with full metabolic dysfunction (Adapted from 13).



<u>Figure 10:</u> Clinical characteristics of MHO and PO patients

1.9 Hypothesis

The subcutaneous adipocyte dysfunction and inflammation have been reported along with adipocyte hypertrophy in pathological obese (PO) and diabetic obese (DO) subjects. The enlarged adipocytes are more susceptible to hypoxia due to changes in vascular tone and decreased capillary density of the depot. Hypoxia will leads to cell necrosis and the formation of immunological foci. The more hypoxic and inflamed fat depot secretes less adiponectin, which then may determine the increased systemic insulin resistance and dyslipidaemia seen in the PO and DO vs metabolically healthy obese (MHO).

1.9.1 Aim

Specifically, differences between MHO, PO and DO in relation to vascular function were determined.

1.9.2 Objectives:

- A cohort of MHO, PO and DO subjects were identified.
- Vascular differences in the adipose tissue of MHO versus PO versus DO by functional studies (myography) and histological assessment were carried out.
- Mechanisms that underlie these differences and dysfunctions were assessed.
- Different key pathways were investigated such as NO, COX, and EDHF.

Chapter 2

Materials & Methodology

2.0 Methods

2.1 Patient recruitment and classification:

The study was approved by the Ethics Committees of Shafallah Medical Genetics Center, Doha, Qatar and written informed consent was obtained from all participants. Sixty three Qatari patients with BMI>40kg/m^2 undergoing laparoscopic bariatric surgery for weight loss were recruited from the pre-operative clinic at Al-Emadi Hospital and Hamad medical corporation (HMC). Patients with known coronary artery disease, uncontrolled hypertension, malignancy or terminal illness, connective tissue disease or other inflammatory conditions likely to affect cytokine levels, compromise immune function or those with substance abuse or other causes for poor compliance were excluded.

Currently there are no WHO criteria defining the levels for hyperinsulinaemia. However previous studies, by Mohamed-Ali et al., (202) reported value of 5.9 to 6.7 µU/ml, and the Framingham study reported insulin levels of 7.5 µU/ml (203) in healthy people. 8 µU/ml of insulin was chosen as the cut-off in the current study as this produced two distinct metabolic popoulations. Thus, MHO subjects were defined as those with insulin < 8 µU/ml (normoinsulinaemia) and PO as those with insulin > 8.0 µU/ml (hyperinsulinaemia) (Table 1).

2.1.1 Anthropometric measurements:

Body mass index (BMI) was calculated as the weight (kg) divided by the square of 0the height (m^2). Blood pressure was measured with a digital blood pressure monitor. All these measurements were taken at Al-Emadi Hospital and HMC.

2.1.2 Blood and Adipose tissue collection:

Following an overnight fast, blood samples were obtained from an ante-cubital vein on the day of the operation immediately after anesthesia, separated into plasma and cells and stored at -80 ^{o}C until analysis. During the surgery, adipose tissues from the abdominal subcutaneous and intra-abdominal omental tissues were obtained (~5g each) and quickly transported in serum-free medium (Cellgro, Mediatech Manassas, VA) to the laboratory.

2.2 Determination of vascular reactivity by Myography

Micro-myography was used to investigate reactivity of arterioles/small arteries isolated from both SC and OM. This process consists of several steps; dissection, mounting, normalization to put the vessel under appropriate pre-tension & checking viability of the vessel prior to conducting experiments.

Adipose tissues surrounding the blood vessels were carefully removed under a dissecting microscope. Since arteries and veins commonly run parallel to each other, the arteries were distinguished from veins by their relatively smaller size, greater firmness, as well as V shaped branching (Figure 11B). The arteries were cut into segments (~2 mm long) ready for mounting in normal physiological salt solution (PSS). The normal PSS contained (in mM) 112 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 0.5 KH_2PO_3, 0.5 NaH_2PO_3, and 10 glucose (gassed with 95% O_2 / 5% CO_2 to pH 7.4).

2.2.1 Dissection:

The first step of vessel preparation was to distinguish artery from vein. There were 3 major differences between artery and vein in human AT: (1). the branch of the artery is seen as “V shaped: while the branch of the vein was “U-shaped”; (2) the lumen size of artery is apparently smaller compared to the vein; (3) the blood flow was faster in artery versus vein (Figure.11). After carefully dissecting the surrounding AT, arterioles were isolated under a dissecting microscope and cut into segments (~2 mm long).

2.2.2 Mounting:

A mounting wire with length of ~2.2 cm was 1st attached to the jaws of the myograph and clamped. Using forceps the vessel was held close to the proximal end, and pulled over the wire. A second wire was then inserted and aligned in parallel with the 1st within the vessel (Figure 12). The ends of the wires were then screwed onto the left and right nuts on the mounting jaws as shown in figure 12.

2.2.3 Normalization:

Normalisation procedure was carried out automatically under transmural pressure of 100 mmHg to put the vessel under appropriate pretension and determine the internal diameter (ID) for each vessel mounted on the myograph. The ID for OM vessels averaged 250±16 µm and for SC was 244±40 µm.

2.2.4 Equilibration period and test of viability:

Following normalization, each vessel was allowed an equilibration period of at least 1hr. During this time, the vessel was challenged with high KCl (90 mM) and NA (10 µM) to optimize its response. The ability of the isolated arterioles to contract (at least up to 1 mN) in response to the 90 mM KCl was used as an index of vessel viability. Vessels that generated less than 1 mN force were discarded.

2.2.5 Experiment protocol:

Following equilibration, vasoconstriction was assessed by constructing cumulative concentration-response curves for KCl (1 to 70 mM; non receptor depolarization-dependent contractile agent) or NA (10^{-9} to $10^{-4.5}$ M; sympathomimetic and adrenergic receptor agonist). Vasorelaxation was assessed by constructing cumulative relaxation response curves for Acetylcholine (Ach, 10^{-9} to $10^{-4.5}$ M; classical endothelium-dependent relaxant), Prostaglandin E_2 (PGE_2) -10^{-9} to $10^{-4.5}$ M or Sodium Nitroprusside (SNP, 10^{-9} to $10^{-4.5}$ M; NO donor and endothelium independent relaxant). Up to 2 curves were obtained per vessel segment separated by a washout period of 30–60 min. With this protocol, there was no apparent time-dependent change in the response to any of the vasoconstrictors or vasorelaxants. In most cases vessels from SC and OM depots from the same patients were tested in the same chamber at the same time.

To assess mechanisms of responses some agonists were tested in the presence of respective anatagonist. L-NAME (100µM) for mediators of endothelium NO, apamine (0.5µM) and charybdotoxin (0.1µM) for EDHF, indomethacin (10µM) for COX (COX1 & COX2), barium chloride (30µM) for Kir channel.

Figure 11A

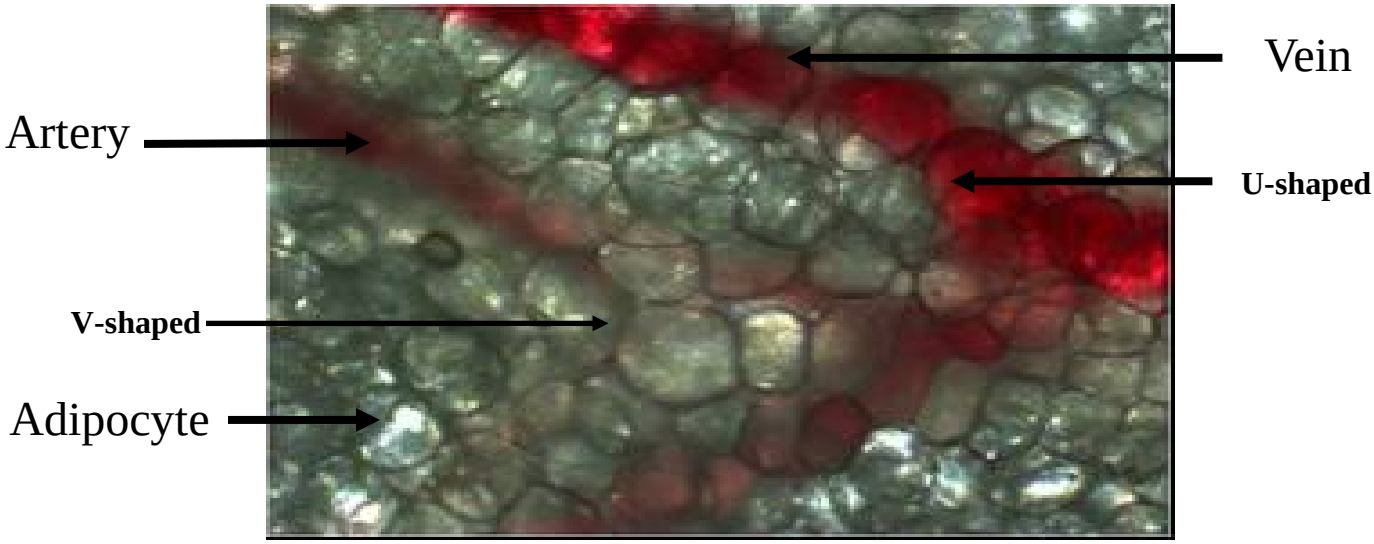


Figure 11B:

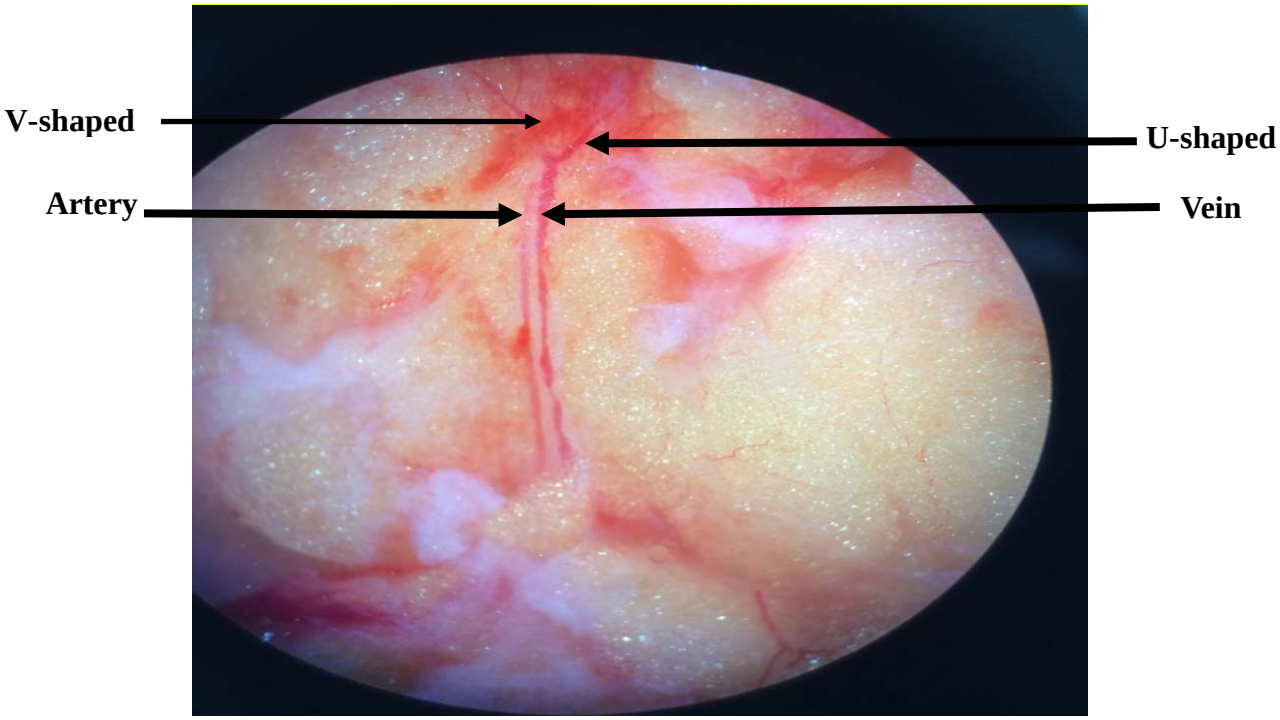
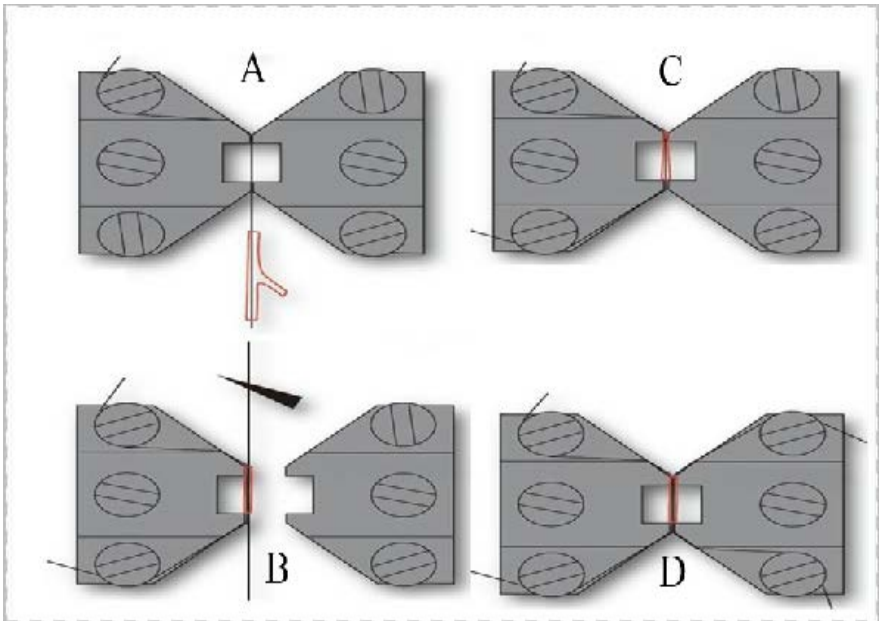


Figure 11: Identification of blood vessels within the omental adipose tissue. (A) Artery and vein as seen within the omental adipose tissue. (B) Typical "V" shaped branching for artery and "U" shaped for vein. These pictures were taken with camera on a microscope (A) and camera alone (B).



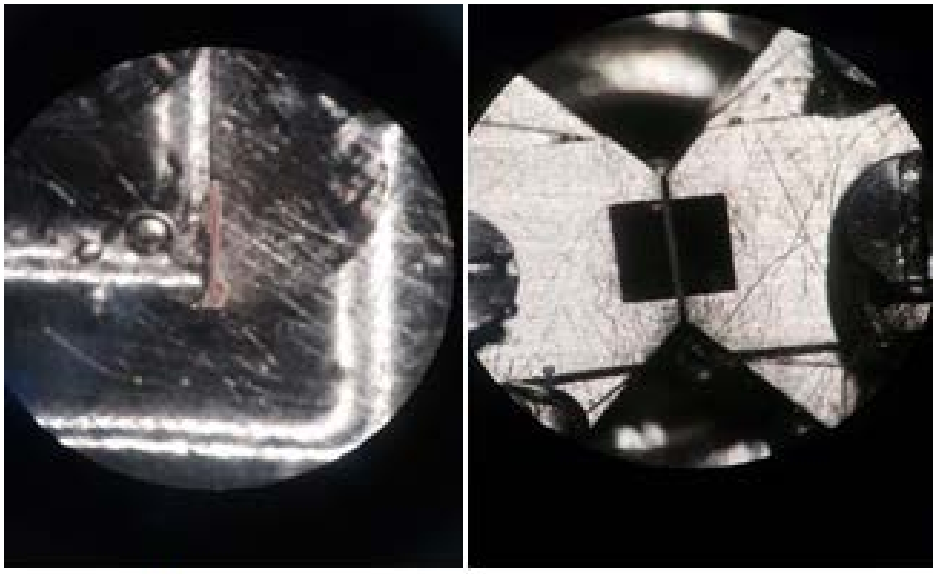


Figure 12: Mounting of vessels on a wire myography. A-D shows the main steps of vessel mounting (DMT User Manual). E and F pictures were taken from our preparation in our lab.

2.3 mRNA expressions

RNA extraction was performed using Trizol-Chloroform extraction method. Adipose tissue (0.5-1g) was used either directly or collagenase digested to produce separate fractions (stromal vascular fraction; SVF, and mature adipocytes) and then used for RNA extraction.

2.3.1 Collagenase digestion protocol:

Adipose tissue biopsies (1-2g) was washed with warm PBS containing 1% antibiotic solution then minced into small pieces. Tissues were then digested using freshly made collagenase solution (0.1% collagenase I/1% BSA in PBS). Adipocytes were separated from SVF after centrifugation at 400g for 10 min and mature adipocytes were collected for further analysis. Erythrocytes were removed by resuspension of SVF pellets in lysis buffer (2.06 mg/ml Tris base, pH 7.2, 7.49 mg/ml NH4Cl) for 10 min. After centrifugation, the pellets were resuspended in HBSS containing 2% fetal bovine serum (FBS) and filtered through a 70uM and 40uM sieves.

2.3.2 RNA extraction protocol:

Trizol (0.8 ml) was added to the pelleted SVF or mature adipocytes fraction that was obtained from 1g collagenase digested adipose tissue. This was followed by mixing on a vortex (10 cycles of 5 seconds each and separated by 5 seconds). Chloroform (1/10 of recorded volume) was then added, followed by 15 seconds of mixing on a vortex and incubation for 5 minutes on ice. This was followed by centrifugation for 15 minutes at 14,000 rpm and 4^{o}C. Aqueous phase was then collected and equal volume of isopropanol was added. Samples were left at -20 ^{o}C for 1.5 hours, then centrifuged for 15 minutes at 14,000 rpm at 4^{o}C. Isopropanol was then removed very carefully and the pellet washed three times with 0.8 ml of 75% ethanol, by centrifugation for 5 minutes at 14,000 rpm at 4^{o}C. After the last wash, ethanol was removed carefully and the pellet was air dried on ice with cling film on top and Eppendorf lid left open for 10 minutes. The pellet was resuspended in 45µl of DEPC/H_2O and left at room temperature for 10 minutes, then mixed by vortex and left on ice for 10 minutes. Purity was assessed and aliquots were stored at -80C (3x15µl aliquots).

From 200-500 ng RNA, cDNA was synthesized using Reverse Transcription Reagent Kit (Applied Biosystems, New Jersey, and USA) and followed by Real-time PCR (Taqman, Applied Biosystems, and Califonia, US). The mRNA expression of genes related to vascular function such as adrenoceptors (α 1), ion channels (K^+ & Ca^{2+} channels) and components of the NOS and COX pathway were investigated. Following assessment of RNA purity/quantity with a Nanodrop spectrophotometer, the expression of β-actin was used as the 'house-keeping' gene, as an additional indicator for RNA yield and quality and PCR efficiency. All samples showing β-actin CT higher than 25 were excluded.

2.3.3 cDNA Synthesis:

cDNA was synthesized from one microgram of RNA using Applied Biosystems Inc (ABI) high capacity cDNA RT kit according to manufacturer's instructions. Briefly, the amounts of buffer, MgCl2, dNTPs, Oligo $d(T)_{16}$ and RNase inhibitor required for the total reaction volume for all samples were calculated to make up the master mix. The required volume of master mix was then added to each tube with 200ng-1000ng of RNA per sample. Reverse transcriptase was finally added and reverse transcription was carried out by incubating samples for 2 hours at 37^oC on PCR machine. cDNA samples were then stored at -20^oC.

2.3.4 Real Time-PCR

RT-PCR was performed using RT² SYBR® Green qPCR (ABI, USA) using the following protocol.

1. The following components were added in a PCR tube: (RT² SYBR® Green qPCR Mastermix, ddH2O, template cDNA (up to 250 ng), gene-specific 10 µM PCR primer pair stock).
2. RT-PCR was performed using 95ºC, 10 min; 40 cycles of (95ºC, 15 sec; and 60ºC, 60 sec) on ViiA7 (ABI, USA).

2.3.5 R^2 Profiler PCR Array

In order to assess the expression of hypertension-related genes, R^2 Profiler PCR Array (Human hypertension PAHS-037Z) was utilized according to manufacturer's instructions (Qiagen, UK).

2.3.5.1 Procedure:

Reagents were thawed at room temperature and briefly centrifuged (10– 15 s) to bring the contents to the bottom of the tubes. The genomic DNA elimination mix for each RNA sample was prepared, mixed gently and centrifuged briefly according to the Table below:

Genomic DNA elimination mix

The genomic DNA elimination mix (RNA 25 ng–5 µg, Buffer GE 2 µl, RNase-free water to be added to reach 10 µl the total volume) was incubated for 5 min at 42°C, then immediately placed on ice for at least 1 min. The reverse-transcription mix (5x Buffer BC3 (4 µl), Control P (2 1 µl), RE3 Reverse Transcriptase Mix (2 µl), RNase-free water (3 µl)) multiplied according to the number of the reactions was prepared.

10 µl reverse-transcription mix was added and mixed gently to each tube containing 10 µl genomic DNA elimination mix, incubated at 42°C for exactly 15 min and then the reaction stopped immediately by incubating at 95°C for 5 min. 91 µl of RNase-free water was added to each reaction. The reaction mixture was placed on ice and then analysed with the real-time PCR protocol.

2.3.5.2 RT^2 PCR Procedure:

Briefly, the RT^2 SYBR Green Master mix (10–15 s) was centrifuged to bring the contents to the bottom of the tube. The PCR components mix was prepared in a 5 ml tube or a loading reservoir depending on the RT^2 Profiler PCR Array format, as described below:

Formats A, C, D, or F (96-well). The RT^2 Profiler PCR Array was removed carefully from its sealed bag. 25 µl PCR components mix of 2x RT^2SYBR Green Mastermix (1350 µl), cDNA synthesis reaction (102 µl), and RNase-free water (1248 µl) was added to each well of the RT^2 Profiler PCR Array using an 8-channel pipette, or a

12-channel pipette using only 8 tips. The RT^2 Profiler PCR Array was sealed carefully with Optical Thin Wall 8-Cap Strips (Formats A and D) or Optical Adhesive Film (Formats C, E, F, and G) and centrifuged for 1 min at 1000 g at room temperature (15–25°C) to remove bubbles. The plates were visually inspected from underneath to ensure no bubbles were present in the wells. The RT^2 Profiler PCR Array was placed on ice while setting up the PCR cycling program. The real-time cycler was programmed according to the ViiA7 (ABI, USA).

The array plate format and the list of genes studied are shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACE	ACE2	ACTA2	ADM	ADRA1B	ADRA1D	ADRB1	AGT	AGTR1	AGTR2	ALOX5	ARG2
B	ATP2C1	ATP6AP2	AVP	AVPR1A	AVPR1B	BDKRB1	BDKRB2	BMPR2	CACNA1C	CALCA	CAV1	CHRNA1
C	CHRNB1	CLIC1	CLIC4	CLIC5	CNGA1	CNGA2	CNGA3	CNGA4	CNGB1	CNGB3	CPS1	DRD3
D	DRD5	ECE1	EDN1	EDN2	EDNRA	EDNRB	EPHX2	GCH1	GCHFR	GUCY1A3	GUCY1B3	HIF1A
E	ITPR1	ITPR2	KCNJ8	KCNMA1	KNG1	MYLK	MYLK2	MYLK3	NOS3	NOSIP	NOSTRIN	NPPB
F	NPPC	NPR1	NPY1R	P2RX4	PDE3A	PDE3B	PDE5A	PLCG1	PLCG2	PRKG1	PRKG2	PTGIR
G	PTGS1	PTGS2	REN	S1PR1	SCNN1A	SCNN1B	SCNN1G	SLC7A1	SPHK1	SPHK2	UTS2	UTS2R
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Position	UniGene	GenBank	Symbol	Description
A01	Hs.654434	NM_000789	ACE	Angiotensin I converting enzyme
A02	Hs.178098	NM_021804	ACE2	Angiotensin I converting enzyme 2
A03	Hs.500483	NM_001613	ACTA2	Actin, alpha 2, smooth muscle, aorta
A04	Hs.441047	NM_001124	ADM	Adrenomedullin
A05	Hs.368632	NM_000679	ADRA1B	Adrenergic, alpha-1B-, receptor
A06	Hs.557	NM_000678	ADRA1D	Adrenergic, alpha-1D-, receptor
A07	Hs.99913	NM_000684	ADRB1	Adrenergic, beta-1-, receptor
A08	Hs.19383	NM_000029	AGT	Angiotensinogen
A09	Hs.728754	NM_031850	AGTR1	Angiotensin II receptor, type 1
A10	Hs.405348	NM_000686	AGTR2	Angiotensin II receptor, type 2
A11	Hs.89499	NM_000698	ALOX5	Arachidonate 5-lipoxygenase
A12	Hs.708024	NM_001172	ARG2	Arginase, type II
B01	Hs.584884	NM_014382	ATP2C1	ATPase, Ca++ transport, type 2C, 1
B02	Hs.495960	NM_005765	ATP6AP2	ATPase, H+ transporting, AP2
B03	Hs.89648	NM_000490	AVP	Arginine vasopressin
B04	Hs.2131	NM_000706	AVPR1A	Arginine vasopressin receptor 1A
B05	Hs.1372	NM_000707	AVPR1B	Arginine vasopressin receptor 1B
B06	Hs.525572	NM_000710	BDKRB1	Bradykinin receptor B1
B07	Hs.654542	NM_000623	BDKRB2	Bradykinin receptor B2
B08	Hs.471119	NM_001204	BMPR2	Bone morphogenic protein RII
B09	Hs.118262	NM_000719	CACNA1C	Calcium voltage-dependent, L, α1C
B10	Hs.37058	NM_001741	CALCA	Calcitonin-related polypeptide alpha
B11	Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22kDa
B12	Hs.434479	NM_000079	CHRNA1	Cholinergic receptor, nicotinic, α1
C01	Hs.330386	NM_000747	CHRNB1	Cholinergic receptor, nicotinic, β 1
C02	Hs.414565	NM_001288	CLIC1	Chloride intracellular channel 1
C03	Hs.440544	NM_013943	CLIC4	Chloride intracellular channel 4
C04	Hs.485489	NM_016929	CLIC5	Chloride intracellular channel 5
C05	Hs.1323	NM_000087	CNGA1	Cyclic nucleotide gated channel α1
C06	Hs.447360	NM_005140	CNGA2	Cyclic nucleotide gated channel α2
C07	Hs.234785	NM_001298	CNGA3	Cyclic nucleotide gated channel α3
C08	Hs.434618	NM_001037329	CNGA4	Cyclic nucleotide gated channel α 4
C09	Hs.147062	NM_001297	CNGB1	Cyclic nucleotide gated channel β1
C10	Hs.154433	NM_019098	CNGB3	Cyclic nucleotide gated channel β3
C11	Hs.149252	NM_001875	CPS1	Carbamoyl-phosphate synthase 1,
C12	Hs.121478	NM_000796	DRD3	Dopamine receptor D3
D01	Hs.380681	NM_000798	DRD5	Dopamine receptor D5
D02	Hs.195080	NM_001397	ECE1	Endothelin converting enzyme 1
D03	Hs.511899	NM_001955	EDN1	Endothelin 1
D04	Hs.1407	NM_001956	EDN2	Endothelin 2
D05	Hs.183713	NM_001957	EDNRA	Endothelin receptor type A
D06	Hs.82002	NM_000115	EDNRB	Endothelin receptor type B
D07	Hs.212088	NM_001979	EPHX2	Epoxide hydrolase 2, cytoplasmic
D08	Hs.86724	NM_000161	GCH1	GTP cyclohydrolase 1
D09	Hs.631717	NM_005258	GCHFR	GTP cyclohydrolase I FR
D10	Hs.24258	NM_000856	GUCY1A3	Guanylate cyclase 1, soluble, α3
D11	Hs.77890	NM_000857	GUCY1B3	Guanylate cyclase 1, soluble, β3
D12	Hs.597216	NM_001530	HIF1A	Hypoxia inducible factor 1, α subunit
E01	Hs.567295	NM_002222	ITPR1	Inositol 1,4,5-trisphosphate receptor1
E02	Hs.512235	NM_002223	ITPR2	Inositol 1,4,5-trisphosphate receptor2
E03	Hs.102308	NM_004982	KCNJ8	Potassium inwardly-rectifying c J8
E04	Hs.144795	NM_002247	KCNMA1	Potassium large conductance Ca^{2+}activated c
E05	Hs.77741	NM_000893	KNG1	Kininogen 1
E06	Hs.477375	NM_053025	MYLK	Myosin light chain kinase
E07	Hs.86092	NM_033118	MYLK2	Myosin light chain kinase 2
E08	Hs.130465	NM_182493	MYLK3	Myosin light chain kinase 3
E09	Hs.707978	NM_000603	NOS3	Nitric oxide synthase 3
E10	Hs.7236	NM_015953	NOSIP	NOS interacting protein
E11	Hs.189780	NM_052946	NOSTRIN	Nitric oxide synthase trafficker
E12	Hs.219140	NM_002521	NPPB	Natriuretic peptide B
F01	Hs.247916	NM_024409	NPPC	Natriuretic peptide C
F02	Hs.490330	NM_000906	NPR1	Natriuretic peptide receptor A
F03	Hs.519057	NM_000909	NPY1R	Neuropeptide Y receptor Y1
F04	Hs.321709	NM_002560	P2RX4	Purinergic receptor P2X, channel, 4
F05	Hs.591150	NM_000921	PDE3A	Phosphodiesterase 3A, cGMP-i
F06	Hs.445711	NM_000922	PDE3B	Phosphodiesterase 3B, cGMP-i
F07	Hs.647971	NM_001083	PDE5A	Phosphodiesterase 5A, cGMP-s
F08	Hs.268177	NM_002660	PLCG1	Phospholipase C, gamma 1

F09	Hs.413111	NM_002661	PLCG2	Phospholipase C, gamma 2
F10	Hs.654556	NM_006258	PRKG1	Protein kinase, cGMP-dependent,I
F11	Hs.570833	NM_006259	PRKG2	Protein kinase, cGMP-dependent,II
F12	Hs.458324	NM_000960	PTGIR	Prostaglandin I2 receptor (IP)
G01	Hs.201978	NM_000962	PTGS1	Prostaglandin-synthase 1 (COX1)
G02	Hs.196384	NM_000963	PTGS2	Prostaglandin- synthase 2 (COX2)
G03	Hs.3210	NM_000537	REN	Renin
G04	Hs.154210	NM_001400	S1PR1	Sphingosine-1-phosphate receptor 1
G05	Hs.591047	NM_001038	SCNN1A	Sodium channel, nonvolt-gated 1 α
G06	Hs.414614	NM_000336	SCNN1B	Sodium channel, nonvolt-gated 1, β
G07	Hs.371727	NM_001039	SCNN1G	Sodium channel, nonvolt-gated 1, γ
G08	Hs.14846	NM_003045	SLC7A1	Solute carrier family 7
G09	Hs.68061	NM_021972	SPHK1	Sphingosine kinase 1
G10	Hs.528006	NM_020126	SPHK2	Sphingosine kinase 2
G11	Hs.715862	NM_006786	UTS2	Urotensin 2
G12	Hs.192720	NM_018949	UTS2R	Urotensin 2 receptor
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate DH
H04	Hs.412707	NM_000194	HPRT1	Hypoxan.phosphoribosyltransferas 1
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	N/A	SA_00105	HGDC	Human Genomic DNA
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

2.4 Assays:

Serum insulin and proinsulin levels were measured by specific insulin and proinsulin ELISA (Mercodia, Sweden). Insulin sensitivity was determined by the Homeostasis Model Assessment Index-Insulin Resistance (HOMA-IR). HOMA-IR was calculated by using the equation HOMA-IR= (insulin (mU/L) * fasting glucose (mmol/l)) /22.5. Serum lipids profile was measured by conventional methods (COBAS, Roche, Switzerland). Adiponectin was measured in the serum, while TNF-α, IL-6 and leptin were measured in EDTA-plasma samples using commercially available 2-site ELISAs (R & D Systems, Oxon, UK). The IL-6 measures were carried out using the high sensitivity kit (R & D Systems, Oxon, UK). In brief, samples and standards were pipetted into 96 wells of microplates precoated with specific antibody against the target protein. After a specified incubation period to allow 'capturing' of the target protein the unbound material was then washed away. A 'detecting' enzyme-linked antibody specific for the target protein was then added and incubated. This was followed by washing to remove the unbound material. Then the substrate was added and the colour developed in proportion to the concentration of bound target protein. The reaction was stopped at set times as per manufactures instructions and the intensity of the colour measured on a microplate reader (TECAN, Infinite 2000) as absorbance at the specified wavelengths for each assay.

2.5 Statistical Analysis:

Myography data were recorded and analyzed using the Lab Chart software (DMT, Denmark). In response to each dose, peak values of vessel tension for contractile agent and lowest value for relaxing agents were recorded; tension increase was calculated by peak value for each dose minus basal vessel tension (mN) divided by vessel diameter. Since it was difficult to obtain vessels from healthy lean people (as control) comparison was made between Ach and SNP responses in each segment to determine endothelial dysfunction.

Data were analysed using SPSS version 15 for Windows (Statistical Package for the Social Sciences, SPSS UK Ltd, Chertsey, UK) and GraphPad Prism software version 5.04 (GraphPad Inc. La Jolla, Ca USA). Normality of distributions was tested with

the Kolmogorov-Smirnov test. Data are shown as mean (±SEM or SD), or for non-normally distributed data as median (interquartile range), in text and in tables. Agonist log –concentration response curves were constructed on log scale and data fitted using the sigmoidal fitting routine in Prism. The concentration of agonist causing 50% of maximum response is expressed as the mean log EC50 (pEC50), as measure of sensitivity to the agonist. Comparisons were made using student paired and unpaired t-tests or two way ANOVA as appropriate. Significance was defined as $p<0.05$. n= number of samples or experiments.

Chapter 3

Depolarization & Noradrenaline mediated vasoconstriction

3.1 Introduction

3.1.1 Regulation of arterial smooth muscle tone

Arterial tone is the state of contraction of arteries which is a major determinant of peripheral vascular resistance and blood pressure. The contractile force of arterial smooth muscle is regulated by the intracellular concentration of Ca^{2+} (204). This occurs by way of the dependence of myosin light chain phosphorylation on a kinase activated by the calcium/calmodulin complex. Then phosphorylation leads to actin-myosin interaction and on to force development (205). Transient contractions of vascular smooth muscle involve the release of Ca^{2+} from intracellular stores by inositol trisphosphate and/or by Ca^{2+} itself (206). Thus maintained contraction (i.e., tone) depends on the long-term balance between the entry of external Ca^{2+} (207) and its extrusion from the cell. Many pathways for membrane Ca^{2+} entry involve voltage-dependent Ca channels (VDCCs), receptor-operated channels (ROCs), and Na^{+}-Ca^{2+} exchange. Ca^{2+} is extruded from smooth muscle by a plasma membrane adenosine triphosphatase (ATPase) and Na^{+}-Ca^{2+} exchange (208).

3.1.2 Role of Potassium channel

Smooth muscles cells in arteries and arterioles have stable membrane potentials, which measured in vivo are in the range of -40 to -55 mV (209), with resting potentials mainly permeable to K^{+}. When subjected to physiological transmural pressures, they depolarize and develop tone (210). Sympathetic nerve stimulation depolarizes arteries and rarely causes action potential generation (210). Also, vasoconstrictors such as norepinephrine (NE) and serotonin appear to depolarize arterial smooth muscle (211). Vascular smooth muscle cell functions are regulated by membrane ion channels, mainly K^{+} and Ca^{2+} channels (212). In general, K^{+} channels regulate the membrane potential in these cells. Opening of K^{+} channels increases K^{+} efflux, which causes membrane hyperpolarization (the membrane potential becomes more negative as a result of loss of intracellular positive ions). This, in turn, closes the voltage sensitive L-type Ca^{2+} channels. In contrast, closing of K^{+} channels causes membrane depolarization, opening of the L-type Ca^{2+} channels, increase in the intracellular Ca^{2+}, and constriction. Thus, K^{+} channels are

involved in the regulation of intracellular Ca^{2+}, in addition to playing a role in the smooth muscle cell excitability and arterial tone (213).

In vascular smooth muscle cells there are four types of K^+ channels (213): (1) The delayed rectifier channel (KDR) opens with membrane depolarization, (2) The Ca^{2+}-dependent K^+ channel (K_{Ca}) opens with membrane depolarization and with increases in the intracellular Ca2+. K_{Ca} plays an important role in the regulation of myogenic tone and it is a major target for cGMP that activates it**.** (3) The inward rectifier channel (Kir) is the least studied channel which is opened by membrane hyperpolarization and, in contrast with the other K^+ channels, by increases in the extracellular K^+ and, (4) The ATP-dependent channel (K_{ATP}) opens when ADP levels rise and closes when ATP levels rise. Thus, K_{ATP} openers are vasodilators which play an important role in the control of basal tone and autoregulation of several vascular beds and in metabolic regulation of blood flow (214).

3.1.3 NA sympathetically mediated vasoconstriction

The sympathetic nervous system controls the total peripheral vascular resistance and blood flow, by controlling the contraction of small arteries (215). This control involves multiple neurotransmitters and receptors, as well as complex patterns of nerve fiber activity. There are 3 different sympathetic neurotransmitters: ATP, noradrenaline (NA), and neuropeptide Y (NPY), which are released from sympathetic varicosities, in which their effects vary with the pattern of nerve fiber activity (216). These neurotransmitters activate several cell signaling systems, including the ubiquitous intracellular Ca^{2+} signaling systems (217). The 3 sympathetic co-transmitters, NA, ATP, and NPY contribute to sympathetically mediated vasoconstriction.

NA binds to adrenoceptors. There are at least 9 subtypes of vascular adrenoceptors that have been identified: α1A, α 1B, α 1D, α 2A/D, α 2B, α 2C, β1, β2, and β3 (218). α1A adrenoceptors are found in various vascular beds and are located post-junctionally on vascular smooth muscle and have a primary role in controlling arterial tone, particularly in small resistance arteries (219). Endothelial cells have 5 subtypes at least of adrenoceptors: α2A/D, α2C, β1, β2, and β3. Recently adrenergic

signaling mechanisms in arterial smooth muscle have been reviewed (220), with the emphasis on Ca^{2+} activation of contraction and Ca^{2+}-sensitizing mechanisms activated by bath-applied phenylephrine. Sympathetic nerves in isolated blood vessel preparations can be selectively stimulated causing ATP and NA release. ATP is the predominant sympathetic effector in response to 'single' action potentials, while at high frequencies of activation, NA is the predominant sympathetic effector (221). The vasocontractile actions of ATP released from sympathetic nerves is mediated via P2X1 receptors (222). P2X receptors are ionotropic receptors, and their activation by ATP leads to a rapid response involving calcium entry directly via P2X cation channels, membrane depolarization, and calcium influx through voltage-activated calcium channels, while NA acts more slowly due to adrenoceptor coupling to G proteins and the involvement of second messengers. ATP and NA can act synergistically, resulting in a rapid onset augmented contraction (223).

3.1.4 Sympathetic activation in obesity:

In obesity there is over activity of the sympathetic nervous system due to overeating that leads to increased sympathetic outflow to the heart, kidneys, and vasculature, which may elevate BP (224), and increase insulin secretion (225).

Glucose utilization by skeletal muscle under the influence of insulin largely determines the process measured as insulin resistance, and is dictated by muscle blood flow (226). Therefore the reduction of skeletal muscle blood flow in hypertension resulting from neural vasoconstriction could be the primary cause of the insulin resistance and the attendant hyperinsulinemia (226). Also sympathetic nervous activation can directly contribute to the lipid change which is part of the metabolic syndrome (227). Landsberg's (225) explanation is that the sympathetic nervous activation of obesity might be driven by high plasma levels of leptin. Leptin, a protein derived from adipose tissue, has an effect attributed to both suppression of appetite and sympathetically mediated thermogenesis (228). Experimentally, rats after intravenous infusion of leptin, show activation of the sympathetic outflows to the kidneys and hindlimb vasculature, with no increase in heart rate, suggesting that the cardiac sympathetic nerves are not stimulated, these

effects have shown close parallels in the pattern of sympathetic nervous change seen in human obesity (229).

Vascular function was assessed by wire myography. Cumulative concentration-response curves were generated for various vasoconstrictors (e.g. noradrenaline, potassium chloride).

3.2 Method

3.2.1 Vascular reactivity:

Obese patients who were undergoing laparoscopic bariatric surgery for weight loss were recruited and adipose tissue samples collected during surgery. Patients with serious illness were excluded such as coronary artery disease, malignancy and any other inflammatory conditions that might affect cytokine levels as previously mentioned in section 2.1. Two AT samples was obtained during surgery from the abdominal subcutaneous and intra-abdominal omental depots and immediately transported in serum-free medium (Cellgro) to the lab. Arteries embedded in the tissues were then isolated and assessed for reactivity by wire myography.

3.2.2 Myography protocol:

Adipose tissues were dissected carefully under a dissecting microscope to isolate the vessels and differentiate artery from the vein. The artery was cleaned and cut into segments (2 mm long), and mounted on wires in an isometric myograph containing NPSS. Vessels were continuously aerated at 37 C in PSS and pre-tensioned to an equivalent of 100 mmHg. The normalized luminal diameter of segments was obtained and equilibration period of at least 1 h was allowed during which time tissues were contracted with KCl (100 mM) and NA (10 μM) to optimize tissue response. Following equilibration period, vasoconstriction was assessed by constructing cumulative concentration-response curves for KCl (1 to100 mM) and NA (10^{-9} - $10^{-4.5}$ M). The dose–response curves were obtained in the same preparation separated by a washout period of 30–60 min. With this protocol, there was no apparent time-dependent change in the response to any of the

vasoconstrictors. Data were recorded with a Power Lab/LabChart data acquisition system (ADinstrment/Myotech, Denmark) as described previously in section 2.2.

3.3 Results:

3.3.1 Patient characteristics.

Patients' characteristics are shown in Table 2. The MHO and PO patients were matched for BMI and did not differ in age or systemic blood pressure. The T2DM patients were marginally older. The plasma glucose levels were in the normal range in all groups. However, in order to maintain euglycaemia the PO and T2DM patients were hyperproinsulinaemic and hyperinsulinaemic compared to the MHO. The former two groups were also significantly insulin resistant compared to the MHO. However plasma leptin was released in PO vs MHO as well as in diabetic obese (DO) vs Non diabetic obese. There was no evidence of dyslipidaemia in the different groups. All other measured systemic markers were comparable.

Table 2: Patient characteristics for Vasoconstriction studies

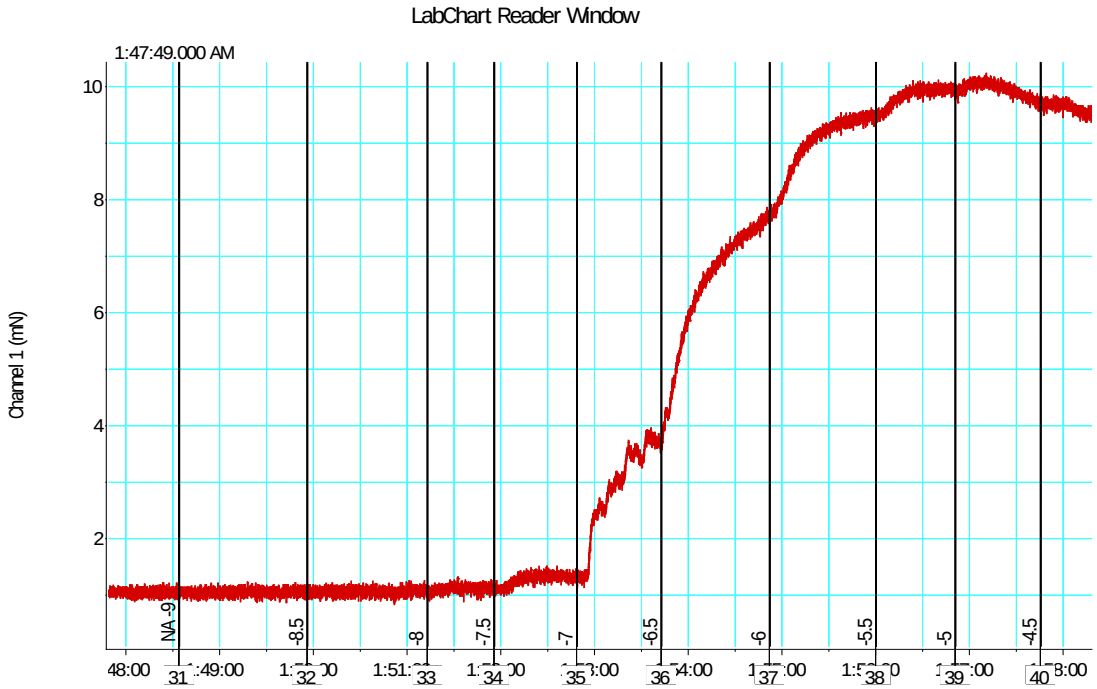
Variables	MHO (n=6)	PO (n=22)	*P* MHO vs PO	DO (n=6)	*p* DO.vs Nondiab.
Age (years)	28 (4.8)	33 (9.9)	0.28	40 (13)	0.05
BMI (kg/m2)	43 (5.15)	48 (22)	0.60	42 (6.8)	0.55
SBP (mmHg)	120 (13)	127 (16.5)	0.34	129 (7.9)	0.60
DBP (mmHg)	70 (8.3)	73 (9.4)	0.53	75 (5.7)	0.46
MAP (mmHg)	87 (9.3)	86.3 (22)	0.97	93 (5.7)	0.43
Proinsulin (miU/ml)	1.5 (1.2-2.5)	2.81 (1.6-15.4)	0.06	9.5 (6.6-29)	0.01
FBG (mmol/L)	4.8 (1.02)	5.8 (1.3)	0.10	8.32 (4.26)	0.17
Insulin (miU/ml)	7.1 (5.6-8.3)	15.1 (11-19.3)	0.001	16 (10-162)	0.47
HOMA	1.4 (0.9-1.6)	3.9 (2.7-4.7)	0.001	4.0 (3-103)	0.26
HDL (mmol/L)	1.4 (0.44)	0.90 (0.33)	0.01	0.8 (0.225)	0.21
LDL (mmol/L)	2.6 (0.8)	2.3 (0.78)	0.31	2 (0.5)	0.30
TG (mmol/L)	0.83 (0.7-0.9)	0.9 (0.63-1.4)	0.52	1.4 (0.83-3)	0.08
Cholesterol (mmol/L)	4.4 (0.9)	3.6 (1.09)	0.10	3.5 (0.9)	0.56
IL6 (pg/ml)	4.1 (1.7)	4.26 (2.3)	0.85	4.1 (2.1)	0.91
MCP-1 (pg/ml)	224.8 (175.8-352.1)	166 (160-276.6)	0.34	338.44	0.23
Leptin (ng/mL)	93.7 (21.6)	65.5 (32)	0.05	38 (31.1)	0.03
VEGF (pg/mL)	64 (50)	101.4 (64)	0.34	102.9	
Adiponectin (µg/mL)	3.01 (1.05)	3.54 (2.2)	0.62	6.81	

Data are shown as mean (SD) or median (interquartiles). * P<0.05, ** P<0.01, *** P<0.001.

3.3.2 Responses to vasoconstrictors

To test the contractile properties of the adipose microvessels, two vasoconstrictors with different mechanisms of action were applied. Cumulative concentration-response curves were constructed for KCl (depolarizandtion dependent constrictor) and NA (sympathomimetic and α- adrenergic receptor agonist) on both SC and OM vessels. For comparison between SC and OM only data from the same patients were considered. Tension recorded in each vessel was also normalized to vessel size to reduce the impact of size.

KCl, like NA, produced concentration-dependent contractions of both SC and OM vessels. A typical recording of force changes over time in response to cumulative addition of NA is shown in (Figure 13).



<u>Figure 13:</u> Typical tracing of contractile responses of a vessel segment (sub-cutaneous micro vessel) to increasing concentrations of NA (10^{-9}-$10^{-4.5}$M).

3.3.2.1 KCl contraction

3.3.2.1.1: Impact of adipose depot

To determine the impact of adipose depots on the contractile response to KCl, data obtained from same patients were used. Generally, OM vessels generated greater maximum force to KCl compared with SC vessels. However, when separated into the 3 metabolic groups of MHO (Fig 14), PO (Fig 15) and diabetic obese (DO, Fig 16), significantly greater maximum force was only recorded in OM vessels from PO group compared to SC from same patients (Emax 0.028±0.005 vs 0.019±0.003 mN/μm for OM vs SC respectively, $p<0.05$). Comparable maximum forces were recorded MHO (Emax 0.021±0.006 vs 0.017±0.004 for OM vs SC, $p= 0.4595$) and DO (0.028±0.004 vs 0.022±0.007 for OM vs SC $p= 0.5655$) groups. In the same vein, there were no differences in the sensitivities of both vessel types from any of the metabolic groups, to KCl.

MHO

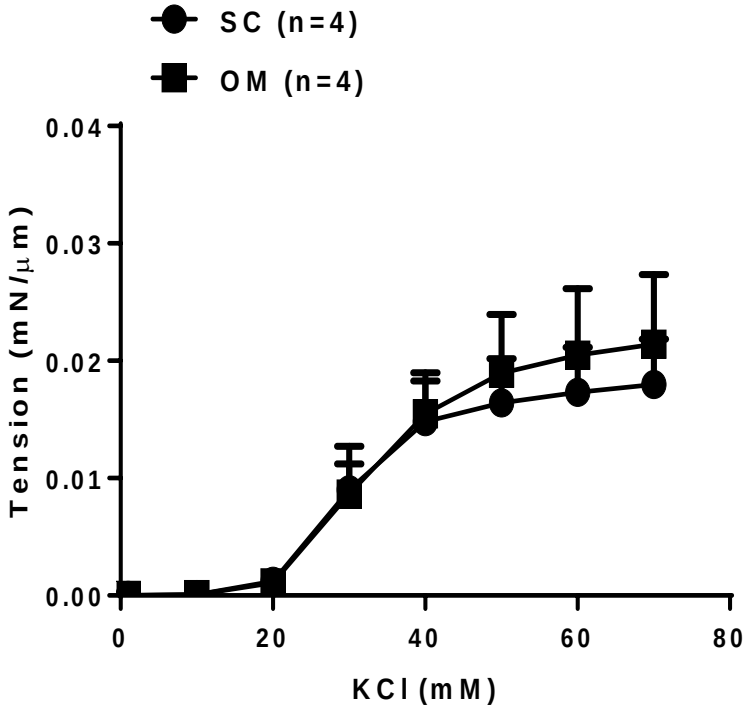


Figure 14: KCl contractions of SC and OM vessels obtained from same MHO individuals. Contractions by both vessel types were comparable at all KCl concentrations. Data points are Mean ± SEM of n=4.

PO

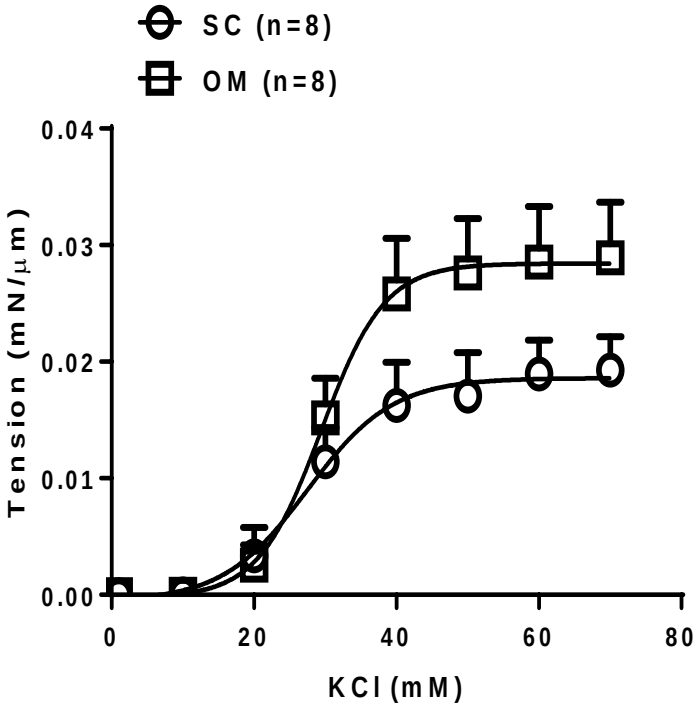


Figure 15: KCl contractions of SC and OM vessels obtained from same PO individuals. OM vessels developed greater force compared to SC vessels with increasing KCl concentrations (≥40 mM). The left ward shift of OM curve was significant ($p<0.05$). Data points are Mean ± SEM of n=8.

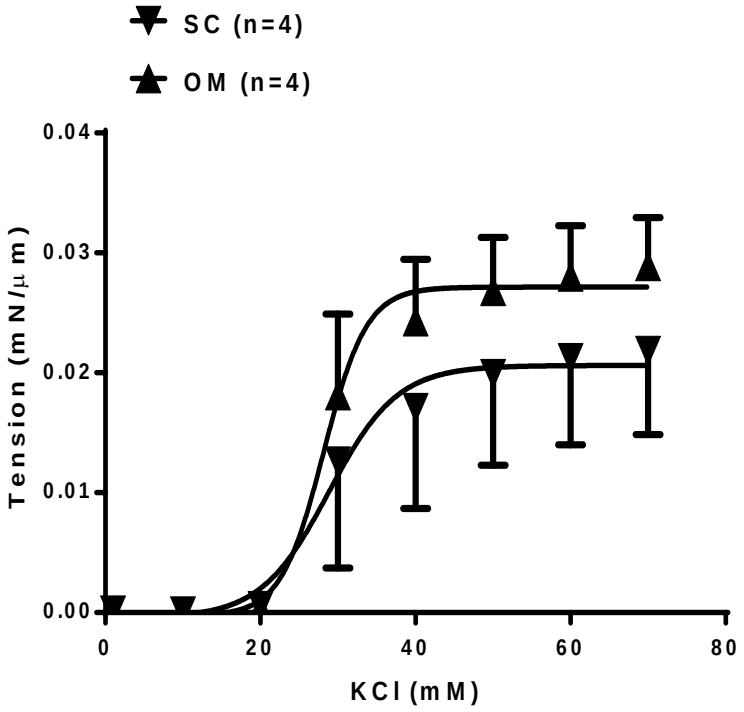
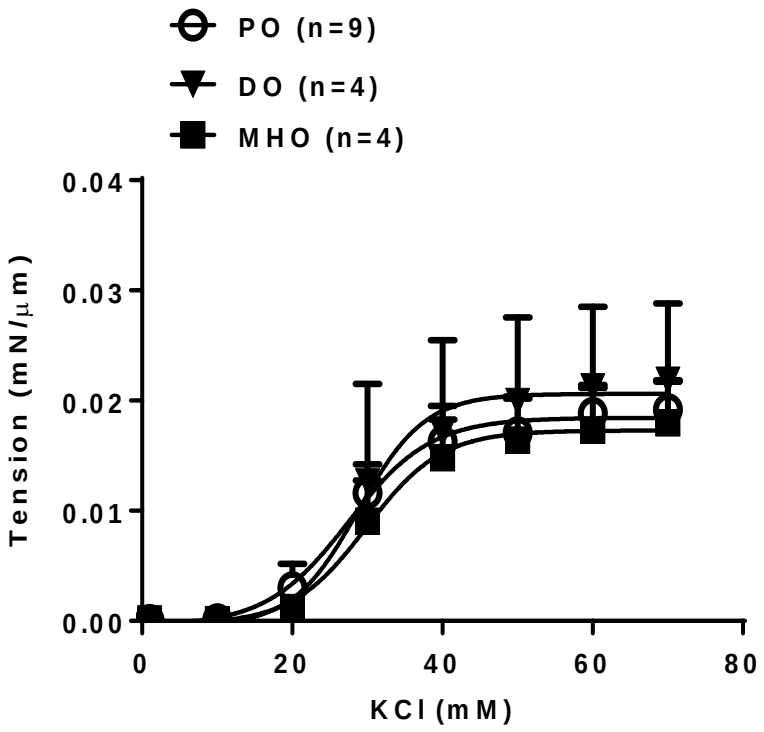


Figure 16: KCl contractions of SC and OM vessels obtained from same DO individuals. OM vessels developed greater force compared to SC vessels with increasing KCl concentrations (≥40 mM), although the curves were not statistically different. Data points are Mean ± SEM of n=4.

3.3.2.1.2: Impact of metabolic status and diabetes

To determine the impact of patients' metabolic status and of diabetes on the contractile response to KCl, data obtained from all OM and SC vessels from all patients were used. The SC vessel curves for the 3 metabolic groups of MHO, PO and DO were comparable (Fig 17 A). The Emax values (0.022±0.007 vs 0.019±0.003 vs 0.017±0.004 mN/μm for diabetic vs PO vs MHO) and sensitivities to KCl (EC50 34.22±4.42 vs 30.05±3.64 vs 29.52±2.60 mM for diabetic vs PO vs MHO) of the SC vessels from the 3 groups were also similar. In contrast, the OM vessel curves for the 3 metabolic groups were different ($p<0.05$). In particular, the OM curve for the DO group was significantly shifted to the left of both MHO ($p<0.01$) and PO ($p<0.05$) groups (17 B). Although the OM curve for PO lies slightly to the left of the MHO curve, the two were not statistically different. In the same vein, the Emax values for MHO (0.019±0.004 mN/μm), PO (0.022±0.003 mN/μm) and DO (0.028±0.004 mN/μm) OM vessels were not statistically different. Similarly, the EC50 values of 31.95±1.37, 30.36±1.48 and 30.14±2.59 mM for OM vessels from MHO, PO and DO groups respectively were also not different.

A



<u>Figure 17A:</u> Summary of the impact of metabolic status and diabetes on KCl contraction of SC from all patients studied. The curves for the 3 metabolic groups were comparable. Data are Mean ± SEM of n=4-15.

B

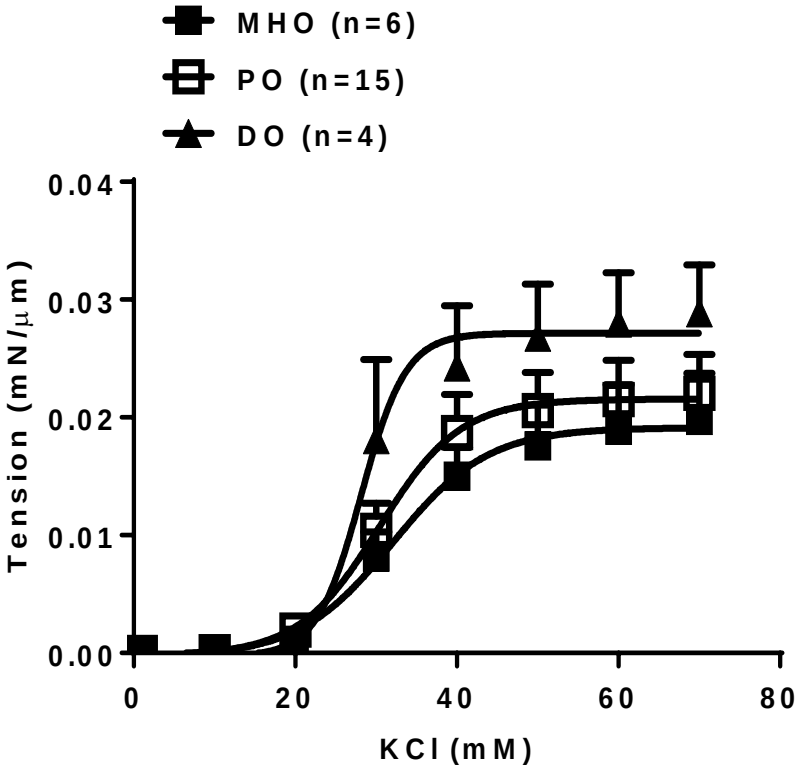


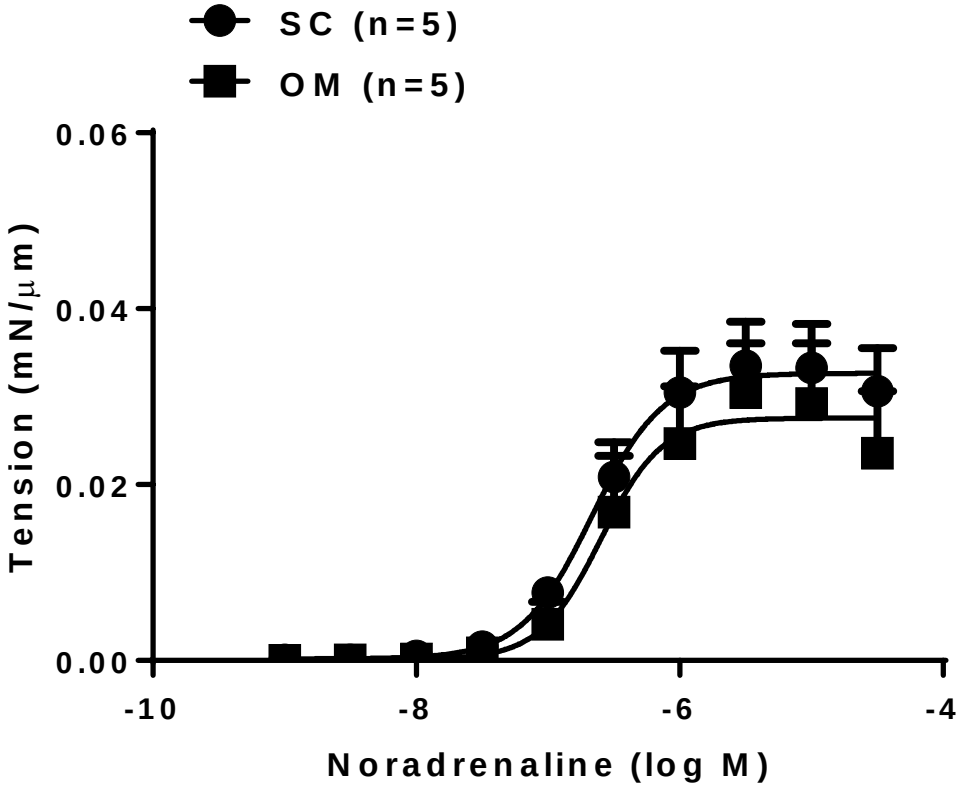
Figure 17B: Summary of the impact of metabolic status and diabetes on KCl contraction of OM vessels from all patients studied. The curve for the DO group was shifted to the left of the curves for both MHO and PO groups. The curves for MHO vs PO were comparable. Data are Mean ± SEM of n=4-15.

3.3.2.2 Noradrenaline (NA) Contractions

3.3.2.2.1: Impact of adipose depot

To determine the impact of adipose depots on the contractile response to NA, data obtained from same patients were used. For the MHO, comparable curves were generated for SC and OM vessels (Emax 0.028±0.006 vs 0.017±0.004 for OM vs SC, p= 0.418, Fig 18). For the PO group, the SC vessels were significantly more sensitive to NA compared with OM vessels (pEC50 = 7.08±0.002 for SC vs 6.29±0.158 for OM, $p<0.001$). Although maximum contraction was slightly greater in OM compared with SC vessels, the difference was not statistically significant (Emax 0.032±0.002 vs 0.027±0.002 mN/μm for OM vs SC respectively, p=0.097, Fig 19). The data for the DO group followed the pattern for the PO group: The curve for SC vessels was shifted to the left of the curve for OM vessels ($p<0.05$, Fig 20), with pEC50 values of 7.147±0.162 vs 6.429±0.157 respectively ($p<0.05$), representing greater sensitivity of the SC vessels to NA. However, there was no difference in the maximum contractions of both vessels (Emax values 0.036±0.005 vs 0.042±0.008 for OM vs SC p= 0.3105).

M H O



<u>Figure 18:</u> NA contractions of SC and OM vessels obtained from same MHO individuals. Contractions by both vessel types were comparable at all NA concentrations. Data points are Mean ± SEM of n=5.

PO

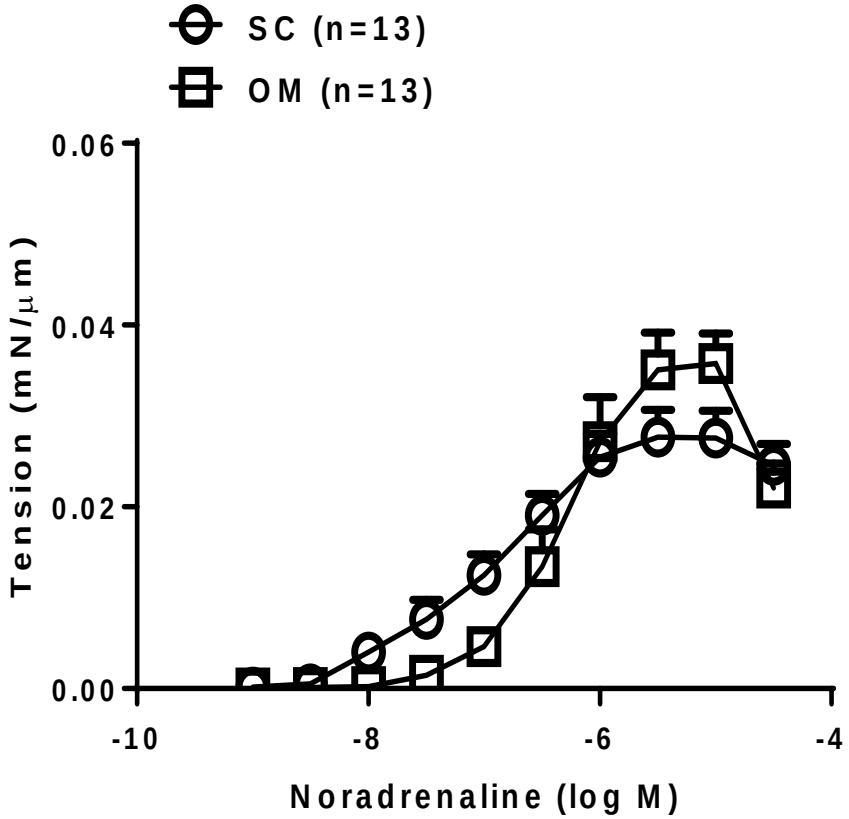
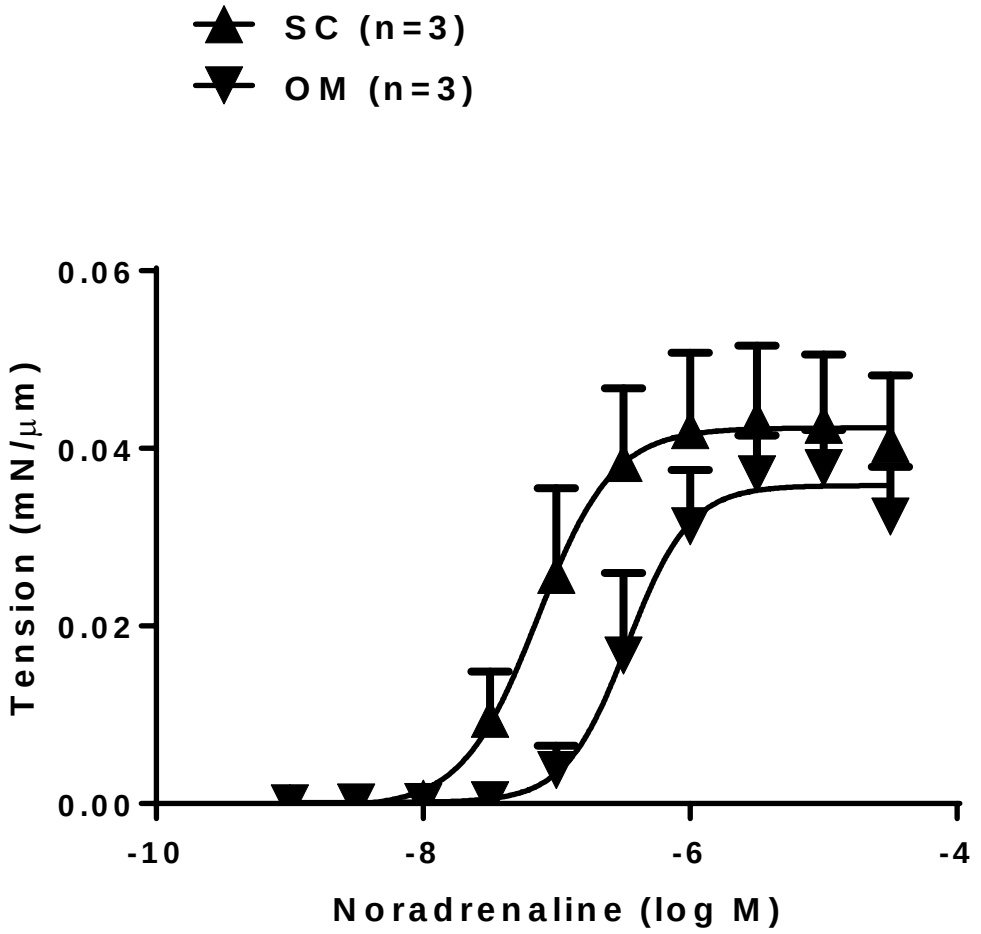


Figure 19: NA contractions of SC and OM vessels obtained from same PO individuals. First half of the curve for SC vessels was shifted to the left of the OM curve (reflecting greater sensitivity to NA compared with the OM vessels, $p<0.001$). Although maximum contractions were slightly higher in the OM compared to SC vessels, the differences were not significant. Data points are Mean ± SEM of n=13.

D O



<u>Figure 2018:</u> NA contractions of SC and OM vessels obtained from same DO individuals. The curve for SC vessels was shifted to the left of the OM curve (reflecting greater sensitivity to NA compared with the OM vessels, $p<0.05$). However, maximum contractions for both vessel types were comparable. Data points are Mean ± SEM of n=3.

3.3.2.2.2: Impact of metabolic status and diabetes

To determine the impact of patients' metabolic status and of diabetes on the contractile response to NA, data obtained from all OM and SC vessels from all patients were used. Whereas, the SC curves for the 3 metabolic groups (MHO, PO and DO) were different ($p<0.0001$, Fig 21A), the OM curves were statistically similar (Fig 21 B). In particular, the SC curve for the DO group was significantly shifted to the left of the SC curves for both MHO ($p<0.001$) and PO ($p<0.001$) groups. Similarly, the SC vessels from DO were more sensitive to NA compared with SC vessels from MHO (pEC50 = 7.148±0.162 vs 6.648±0.044, $p<0.01$) but not with SC vessels from PO (pEC50 = 7.048±0.156). The SC curves for MHO and PO groups were not statistically different. In the same vein, the maximum NA contraction of the SC vessels from the 3 groups were not statistically different (Emax = 0.042±0.008, 0.022±0.003 and 0.027±0.003 mN/µm for DO, PO and MHO respectively).

A

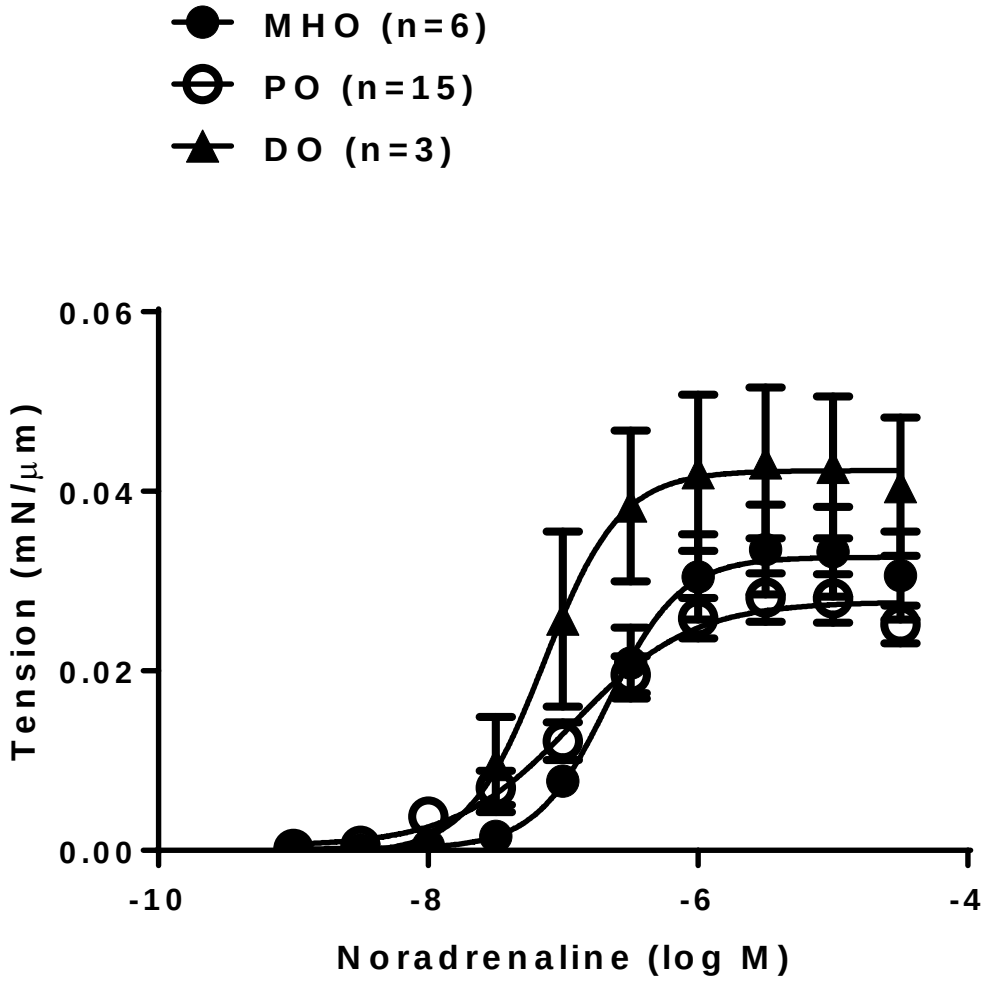


Figure 21A: Summary of the impact of metabolic status and diabetes on NA contraction of SC vessels from all patients studied. The curve for the DO group was shifted to the left of the curves for both MHO and PO groups. The DO was more sensitivity to NA compared with MHO ($p<0.01$) but not with PO. Data are Mean ± SEM of n=3-16.

B

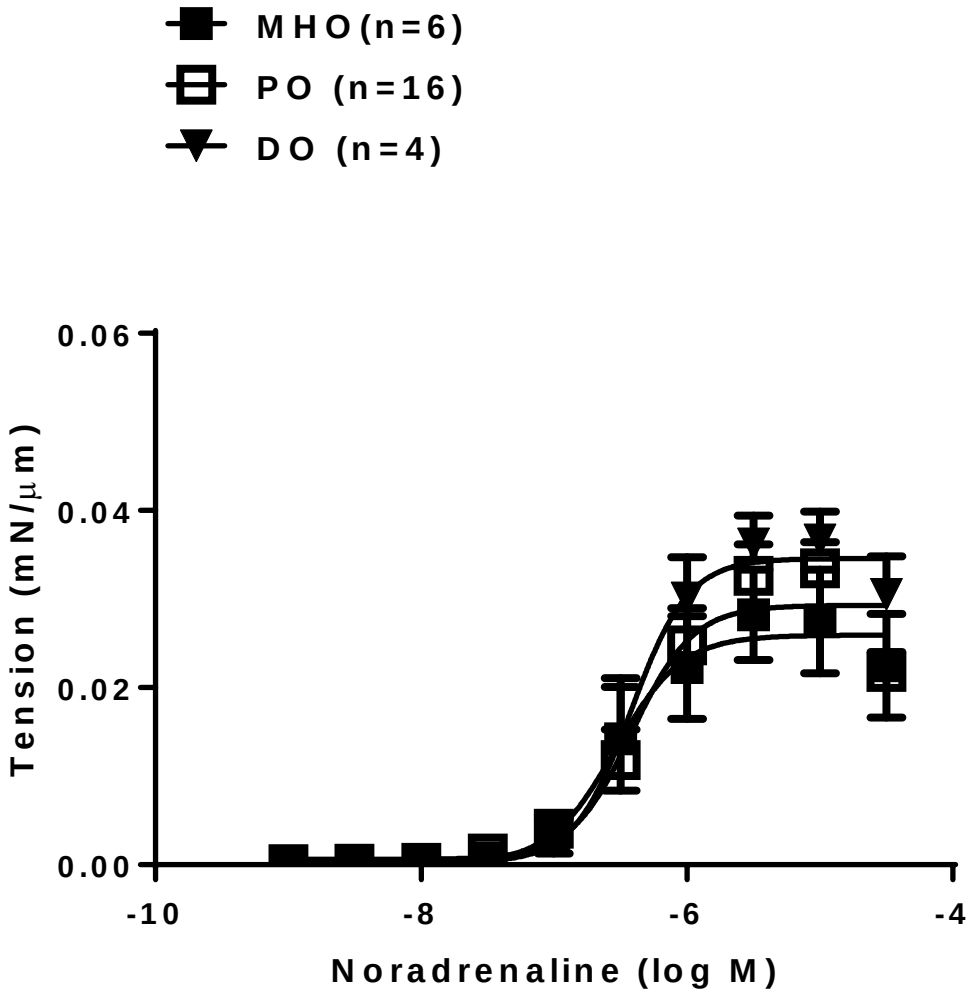


Figure 21B: Summary of the impact of metabolic status and diabetes on NA contraction of OM vessels from all patients studied. The curves from the 3 metabolic groups were comparable. Data are Mean ± SEM of n=3-16.

3.4 Discussion:

The results show that depolarization-induced (KCl) contractions of the more visceral OM vessels were generally more powerful compared with SC vessels from same patients. This was particularly so with the pathologically obese group. This suggests either greater amounts of contractile proteins or greater ability to maximally activate these proteins in these vessels. The former appears unlikely since the force generated by each vessel segment was normalized to size. More so, the mRNA expression arrays show no difference in the expression of α actin between the vessel types. Although the sensitivities of the 3 OM vessel groups to KCl were not statistically different, differences in their sensitivities to cytosolic calcium (Ca^{2+} sensitization) cannot be ruled out. Variation in local release of adipokines can indeed affect the later (165). This is consistent with the metabolic status of the patients having an effect on the OM KCl response with the PO and DO groups showing greater maximum force compared with the MHO group.

Noradrenergic activation also produced dose-dependent contractions of both SC and OM vessels from all patients. With respect to depot effect, SC vessels from PO and diabetic displayed greater sensitivity to NA compared to OM vessels. This is probably a reflection of differences in α1-adrenergic receptor populations of these vessels. The α1A receptor primarily controls the tone of small resistance arteries (201). It is however possible that other adrenoceptors present in both endothelium and vascular smooth muscle (217) of the 2 vessel preparations could have also influenced the sensitivity to NA.

The maximum NA contraction of all 3 SC and OM vessel groups was comparable, suggesting comparable calcium mobilization mechanisms and activation of the contractile proteins. When separated into the 3 metabolic groups, the impact of the metabolic status of the patients on their response to NA was much more pronounced in the SC vessels. The order of sensitivities to NA were DO > PO > MHO. This again may be explained by the impact of the metabolic changes in the SC depots on α1 adrenoceptor expression.

Chapter 4

Endothelium-dependent and independent vasorelaxation

4.1 Introduction

4.1.1 Endothelial dysfunction in obesity:

Endothelial dysfunction is a condition characterized by an impairment of endothelium-dependent relaxation (for instance, to acetylcholine) caused by a loss of NO bioactivity in the vessel wall which contributes to several pathological conditions (230). Increased pro-oxidant cell activity (oxidative stress), reduced expression/activity of eNOS, or unbalanced production/release of vasoconstrictor mediators are among mechanisms responsible for impaired NO availability (117). Besides impairment in NO bioavailability endothelial dysfunction is characterized by an enhanced bioactivity of vasoconstrictor and proatherogenic factors. ET-1, the most potent vasoconstrictor peptide synthesized by the endothelial cells, appears to play a central role in the pathophysiology of the vasomotor abnormalities associated with endothelial dysfunction and in the formation and progression of the atherosclerotic plaque (231). Increased production or activity of other vasoconstrictor substances, such as angiotensin II (Ang II), is also implicated in endothelial dysfunction in obesity and hypertension (232). In addition thromboxane A2 and prostaglandin H2 (TXA2/PGH2) receptor expression is enhanced in obesity-associated hypertension (233). This alteration suggests on association with increased conversion of arachidonic acid to prostaglandins by cyclooxygenase-2 (COX2), an isoform of COX described generally to be induced specifically under inflammatory conditions (234), though may be constitutively expressed in adipose tissue (235).

Endothelial dysfunction, secondary to impaired nitric oxide (NO) availability due to increased reactive oxygen species (ROS) generation, is a common alteration of the major cardiovascular risk factors (236). TNF-α is involved in reducing vascular NO availability; this action is exerted both indirectly, by induction of ROS generation via nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase or inducible nitric oxide synthase (iNOS) activation, and by direct inhibition of endothelial nitric oxide synthase (eNOS) activity (237).

4.1.2 Obesity and endothelial dysfunction:

In obesity endothelial dysfunction is known as an important factor in the pathogenesis of vascular disease. AT is an important modulator of endothelial function via secretion of a variety of hormones, including adiponectin, leptin, resistin, PAI-1, angiotensin, estradiol, and the cytokines TNFα and IL-6 (238). Obesity independently has been associated with a condition of chronic inflammation secondary to an abnormal production of pro-inflammatory mediators, in particular TNF-α that might negatively influence vascular reactivity (239). Moreover, the local inflammation of perivascular fat might exert a detrimental action on the vascular tone of adjacent small vessels in obese patients (175). A study showed that small resistance arteries, isolated from visceral adipose tissue from severely obese patients are characterized by a marked endothelial dysfunction caused by reduced NO availability. This result was attributed to an increased vascular production and biological activity of TNF-α that promotes superoxide generation via both NAD(P)H oxidase and iNOS activation. Thus, obesity is an inflammatory condition, in which small vessels of visceral fat are important sources of low-grade inflammation and oxidative stress that together with the perivascular adipose tissue could directly contribute to the local development of insulin resistance (240).

The responses to vasodilators (e.g. acetylcholine, Sodium nitroprusside (SNP), and prostaglandin E2) were ascertained.

4.2 Method:

The general protocol for wire myography described in 3.2 was used. Adipose tissues were dissected under a dissecting microscope to isolate the vessels and differentiate artery from the vein. The cleaned artery was cut into segments (2 mm long), and mounted on wires in an isometric myograph containing normal PSS. Vessels were continuously aerated at 37 C in PSS and pre-tensioned to an equivalent of 100 mmHg. The normalized luminal diameter of segments was obtained and equilibration period of at least 1 h was allowed during which time tissues were

contracted with KCl (90 mM) and NA (10 µM) to test viability and optimize tissue response.

Endothelial function was assessed by measurement of relaxation to acetylcholine. Cumulative concentration-response curves were constructed for acetylcholine on initial tone built with 1-5 µM NA. Curves were also generated for sodium nitroprusside to measure the responsiveness of segments to direct NO application. To test for relaxation to other endogenous dilators, curves were constructed for prostaglandin E2. The magnitude of drop in tone due to each dose of agonist was expressed as percentage relaxation of the initial tone.

4.3 Results:

4.3.1 Table 3: Patient characteristics for Vasodilatation studies.

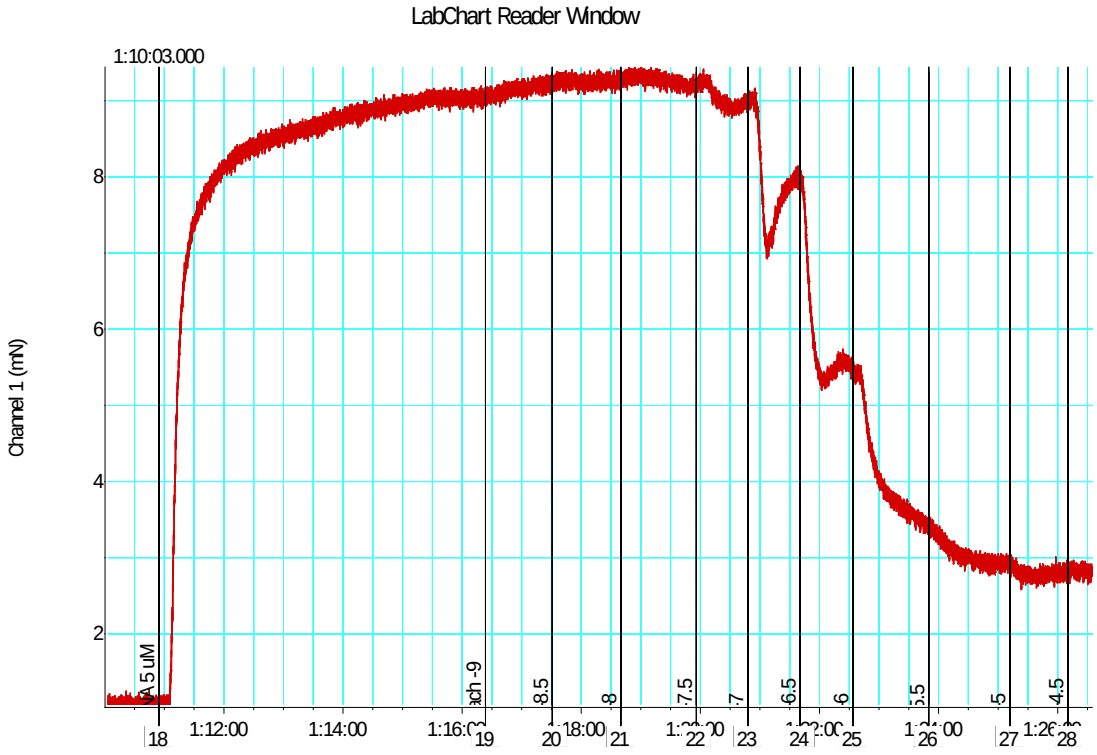
Variables	MHO (n=5)	PO (n=19)	*P* MHO vs PO	DO (n=5)	*P* DO vs Nondiab
Age (years)	38 (6.1)	31 (8.3)	0.11	38 (11.9)	0.25
BMI (kg/m2)	42 (5.9)	48 (23)	0.57	43 (7.3)	0.67
SBP (mmHg)	116 (11)	129 (16.2)	0.11	129 (8.8)	0.72
DBP (mmHg)	67 (11)	73 (10.6)	0.26	74 (5.7)	0.71
MAP (mmHg)	83.5 (8.2)	92 (11.7)	0.15	92 (6)	0.56
Proinsulin (miU/ml)	1.8 (0.9-3.03)	3.6 (1.6-10.4)	0.05	8.73 (5.9-22.3)	0.07
FBG (mmol/L)	5.6 (1.1)	6.1 (1.34)	0.44	9.1 (4.3)	0.19
Insulin (miU/ml)	5.6 (3.3-6.1)	15.1 (12-22)	0.002	17.6 (8.5-17.6)	0.35
HOMA	1.2 (0.3-1.8)	3.52 (2.3-6)	0.02	3.8 (2.6-3.8)	0.27
HDL (mmol/L)	1.5 (0.5)	1.12 (0.30)	0.07	1.04 (0.41)	0.43
LDL (mmol/L)	2.84 (0.55)	2.7 (1.1)	0.78	2.3 (0.62)	0.36
TG (mmol/L)	0.9 (0.6-2.1)	0.94 (0.7-1.5)	0.68	1.43 (1.1-3.4)	0.08
Cholesterol (mmol/L)	5.5 (0.7)	4.62 (1.3)	0.59	4.14 (0.8)	0.36
IL6 (pg/ml)	3.8 (1.8)	4.4 (2.2)	0.55	4.73 (2)	0.69
MCP-1 (pg/ml)	223.3 (160-500)	176.3 (160-342)	0.46	338.44	0.53
Leptin (ng/mL)	57 (25.04)	59.5 (30.9)	0.84	34.63 (35)	0.15
VEGF (pg/mL)	78.5 (36.3)	147.7 (78.2)	0.14	102.89	0.85
Adiponectin (µg/mL)	4.2 (2.3)	3.3 (1.84)	0.47		

Data are shown as mean (SD) or median (interquartiles). * P<0.05, ** P<0.01, *** P<0.001.

4.3.2 Responses to vasodilators

4.3.2.1 Relaxations to Ach:

Ach produced concentration-dependent relaxation of both SC and OM vessels (A typical tracing is shown in figure 22.



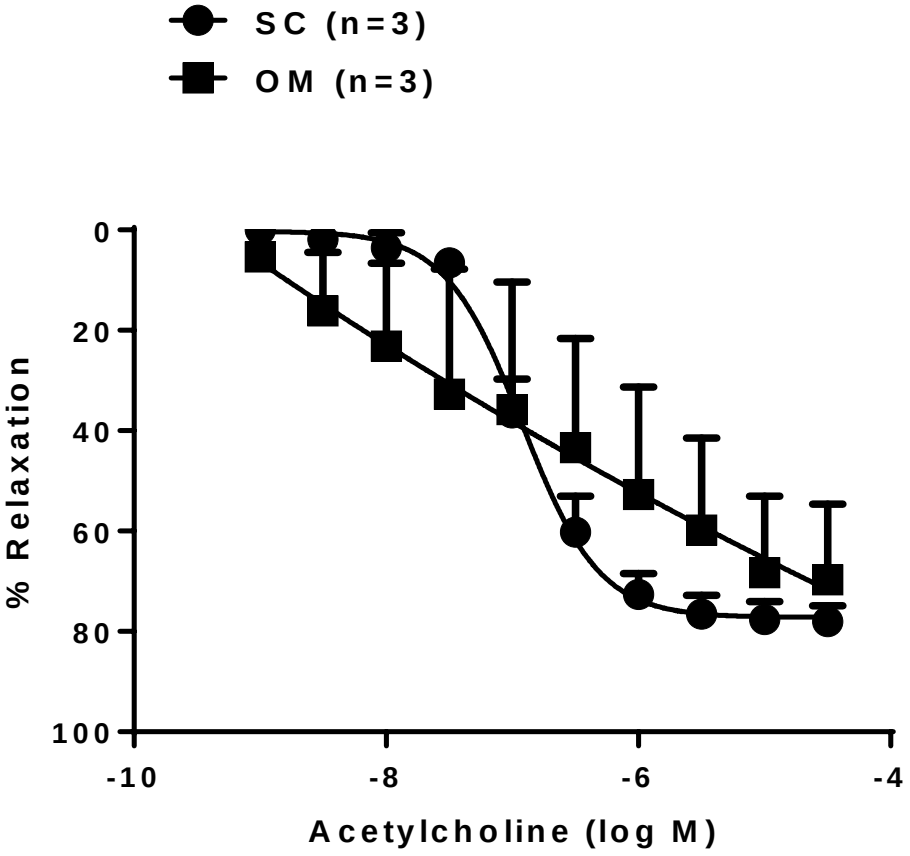
<u>Figure 22:</u> A typical relaxation response of a sub-cutaneous micro vessel to Acetylcholine. The vessel tone decreased with increasing doses of Ach (10^{-9}-$10^{-4.5}$M).

4.3.2.2 Ach relaxation

4.3.2.2.1 Impact of adipose depot:

To determine the impact of adipose depots Ach relaxation was recorded in both SC and OM vessels from the same patients. Generally, both vessel types responded dose-dependently to Ach. The OM and SC curves for MHO (n=3, Fig 23) and DO (n=4, Fig 24) groups did not show any depot- specific differences. The Imax values for OM vs SC vessels for MHO were 72.03±13.99 vs 77.113±3.922 % and for DO group were 71.95±12.72 vs 68.01± 15.18). In contrast, the curve for OM vessels from the PO group was significantly shifted to the right compared with the curve for SC vessels from same patients ($p < 0.01$, Fig 25). The Imax values were 64.978± 6.69 vs 80.74±4.66 mN/μm for OM vs SC vessels ($p= 0.067$). Both vessels from each of the 3 groups were equally sensitive. For the MHO group the pIC50 values were 6.804±0.888 vs 6.913±0.121 for OM vs SC vessels respectively. For the PO group the pIC50 values were 5.701±1.289 vs 7.032±0.140 and for the DO group 6.841± 0.376 vs 7.178± 0.067 for OM vs SC vessels respectively.

M H O



<u>Figure 23:</u> Ach relaxations of SC and OM vessels obtained from same MHO individuals. Relaxations by both vessel types were comparable at all Ach concentrations. Data points are Mean ± SEM of n=3.

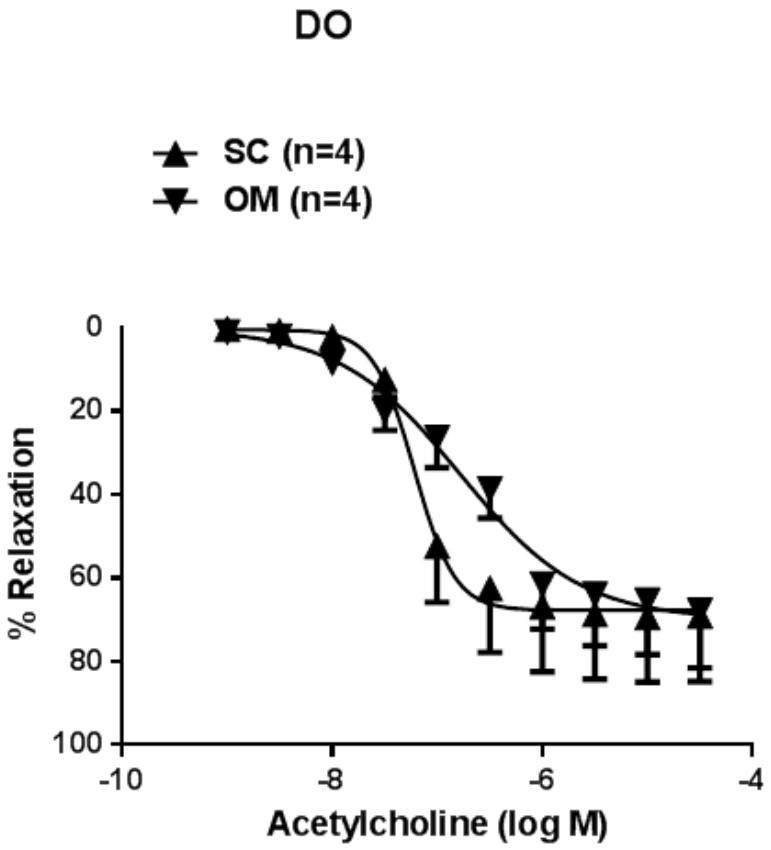


Figure 24: Ach relaxations of SC and OM vessels obtained from same DO individuals. Relaxations by both vessel types were comparable at all Ach concentrations. Data points are Mean ± SEM of n=4.

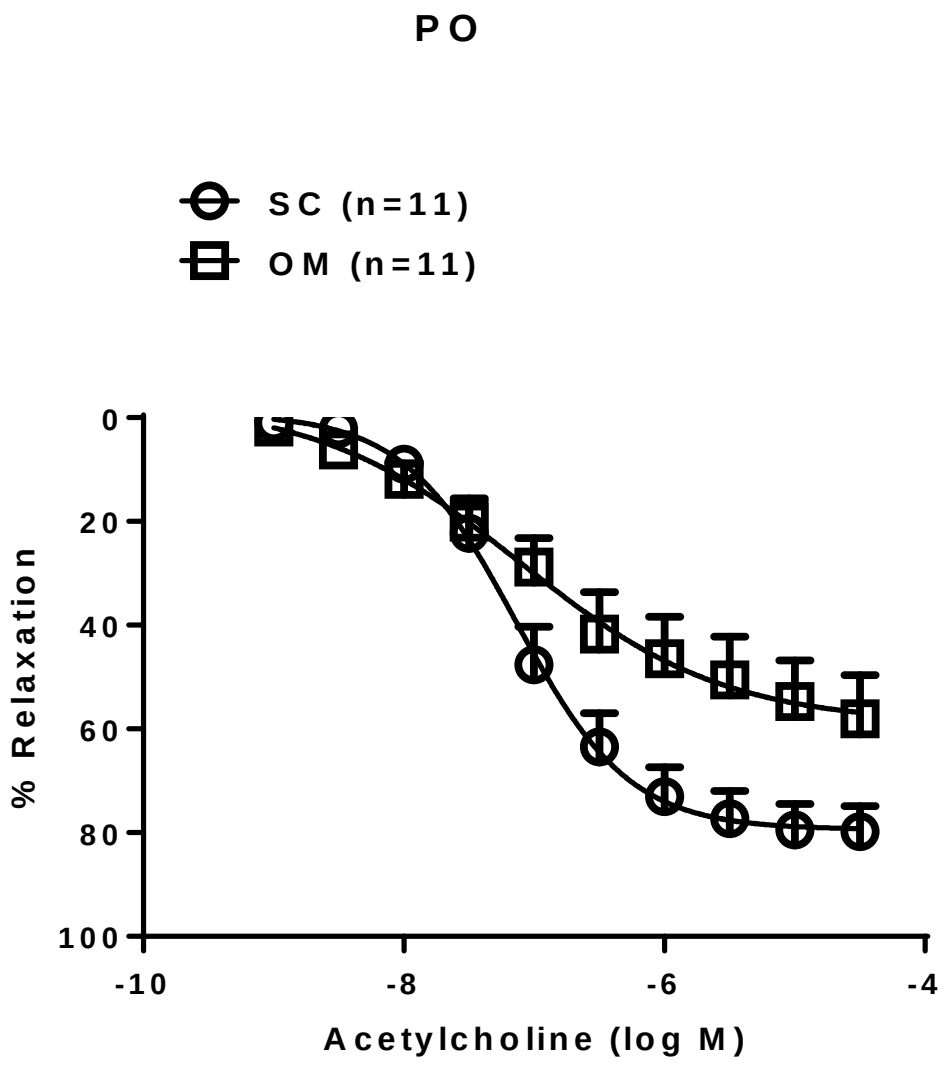


Figure 25: Ach relaxations of SC and OM vessels obtained from same PO individuals. Relaxation was attenuated in OM vessels compared to SC vessels with increasing Ach concentrations. The right ward shift of OM curve was significant ($p<0.01$). Data points are Mean ± SEM of n=11.

4.3.2.2.2 Impact of metabolic status and diabetes

To determine the impact of patients' metabolic status and of diabetes on the relaxation response to Ach, data obtained from all OM and SC vessels from all patients were used. The 3 SC curves for the 3 metabolic groups were not statistically different (Fig 26A); Their Imax values were 74.14±4.06 vs 82.57±2.68 vs 68.01±15.18 % for MHO vs PO vs DO respectively. Similarly, their pIC50 values were 7.11±0.216 vs 6.96±0.094 vs 7.18±0.067 mM for MHO vs PO vs diabetic respectively. In contrast, the Ach curves for OM vessels from the 3 metabolic groups were different ($p<0.001$). In particular, the curves for OM vessels from the DO ($p<0.01$) and PO ($p<0.0001$) groups were significantly shifted to the right of the curve for the MHO group (Fig 26B). The OM curves for DO and PO groups were comparable. The Imax values for OM vessels from DO, PO and MHO groups were 71.21 ±9.88, 64.06±3.84 and 80.21±6.58 % respectively, and the pIC50 values for these vessels were 6.845 ± 0.029, 7.163±0.266 and 7.167± 0.441 respectively. When values were compared between groups, only the Imax values for PO vs MHO were statistically different ($p<0.05$).

SNP is a direct donor of NO and dilates blood vessels independent of the endothelium. For this reason it was used as a control for Ach release of NO from the endothelium. Overall SNP produced greater relaxation of OM vessels compared with SC vessels ($p<0.05$, Fig 27). When SNP and Ach curves were compared for each vessel type, SNP curve was significantly shifted to the left of the Ach curve for OM vessels ($p<0.01$, Fig 28 A), with pIC50 values of 7.072± 0.312 for SNP vs 7.829±.847 for Ach ($p=0.8795$) and, maximum response of OM vessels to both drugs were higher for SNP vs Ach (Imax = 93.068±2.77 vs 55.19±7.716). In contrast, the positions of the curves were reversed for SC vessels with SNP curve on the right of the Ach curve ($p=0.0002$, Fig 28 B) and pIC50 values of 6.107±0.294 vs 6.88±0.194 for SNP vs Ach respectively ($p=0.0579$). Again, the Imax values (90.762±5.131 vs 87.586±2.537 for SNP vs Ach) for these vessels in response to the 2 drugs were comparable.

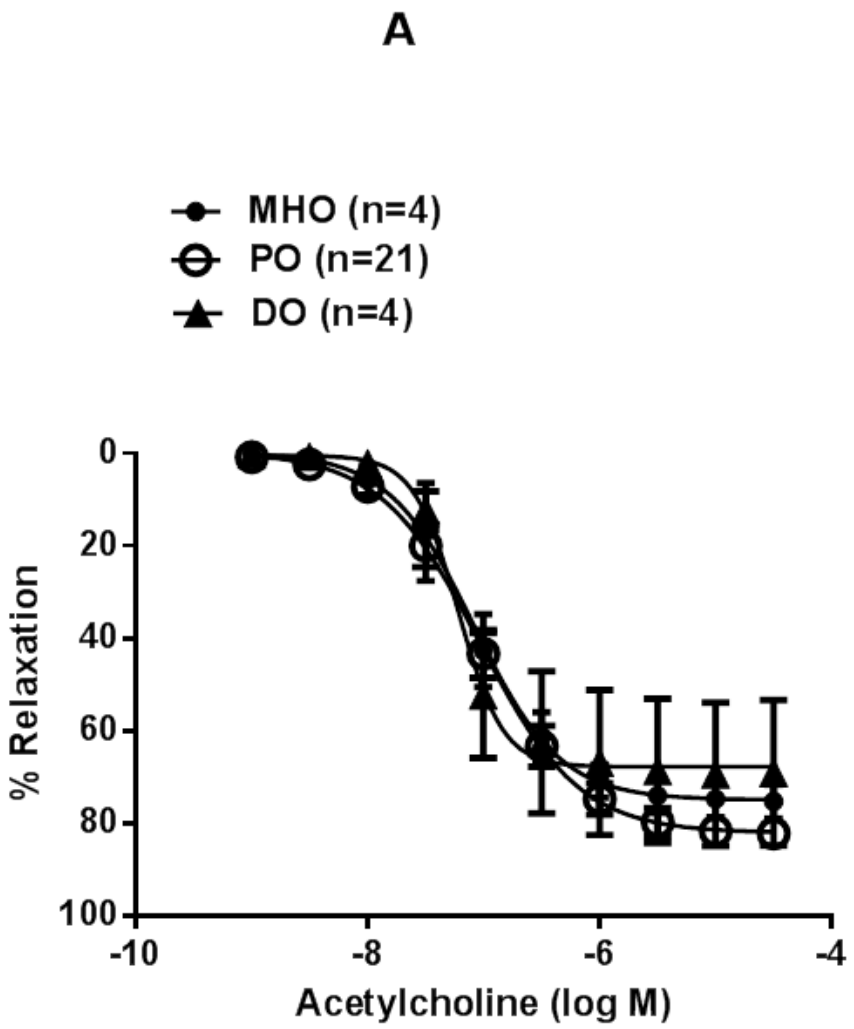


Figure 26A: Summary of the impact of metabolic status and diabetes on Ach relaxation of SC vessels from all patients studied. The curves from the 3 metabolic groups were comparable. Data are Mean ± SEM of n=4-21.

B

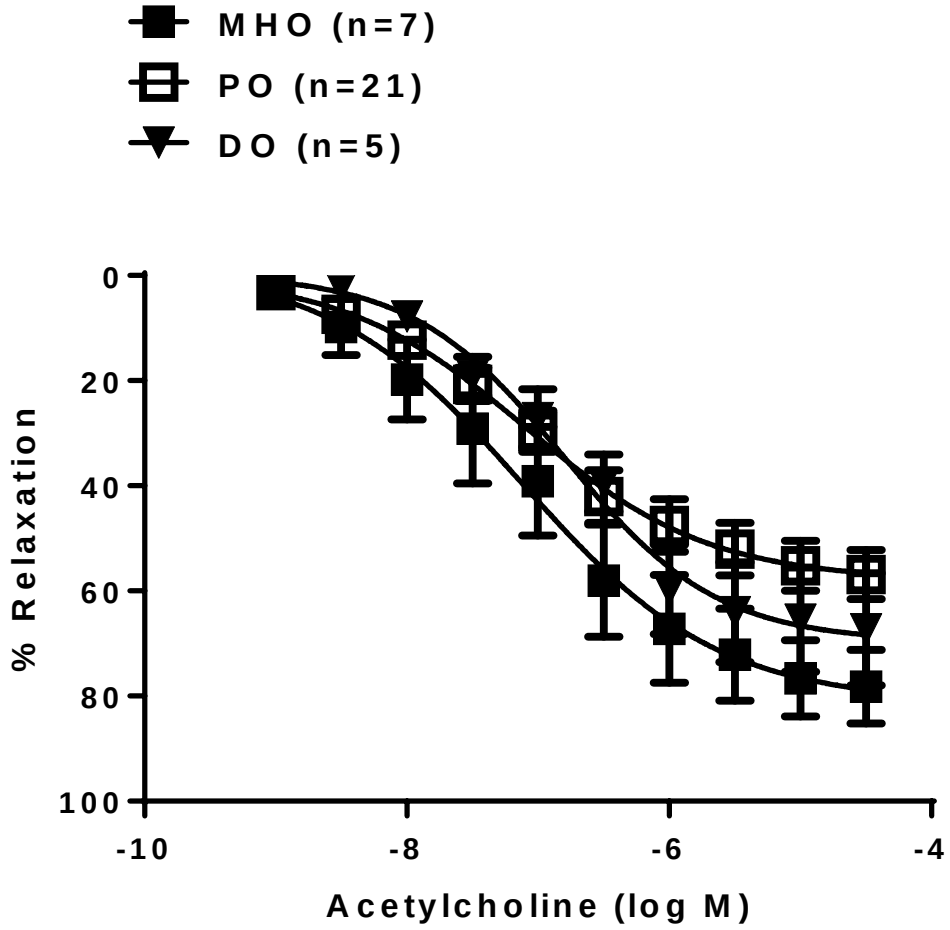


Figure 26B: Summary of the impact of metabolic status and diabetes on Ach relaxation of OM vessels from all patients studied. The curve for the PO was shifted to the right of the curves for both MHO (($p<0.0001$) and DO ($p<0.01$). Data are Mean ± SEM of n=5-21.

4.3.2.3 Relaxation to SNP:

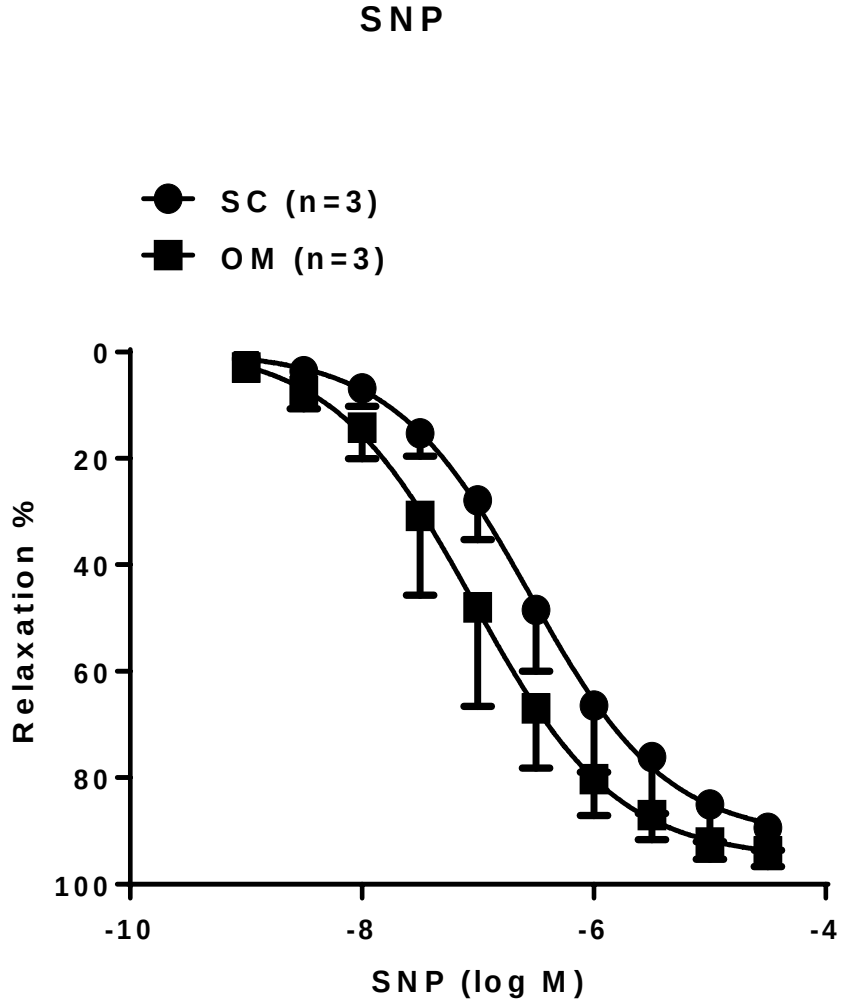


Figure 27: SNP relaxations of SC and OM vessels obtained from same individuals. The SC curve was shifted to the right of the OM curve ($p<0.05$). The Imax and IC50 were comparable. Data points are Mean ± SEM of n=3.

A

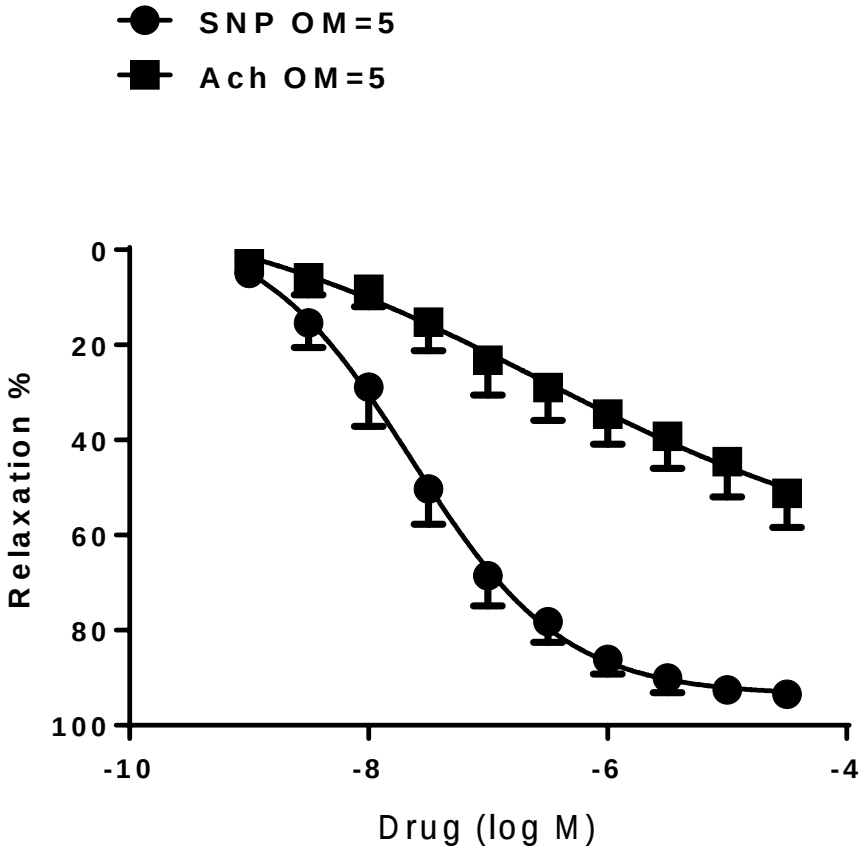


Figure 28A: Comparison between SNP and Ach relaxation of OM vessels. The relaxation to Ach was significantly attenuated (with significant rightward shift) compared with SNP ($p < 0.0001$). Data points are Mean ± SEM of n= 5.

B

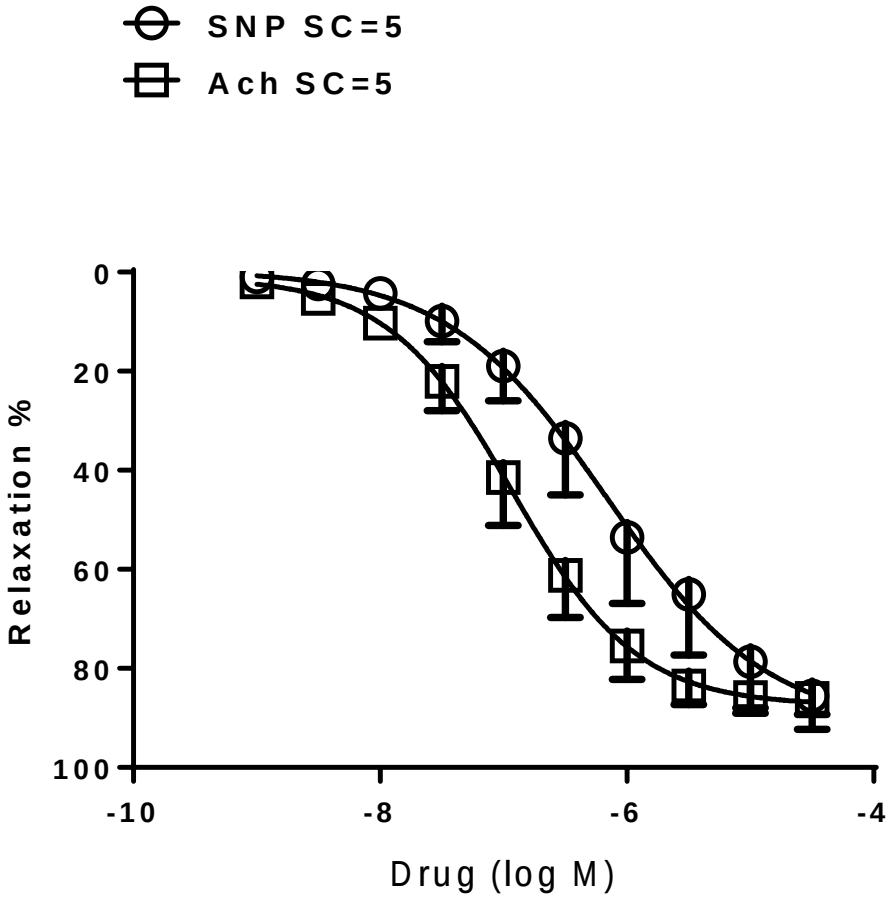


Figure 28B: Comparison between SNP and Ach relaxation of SC vessels. The relaxation curve for Ach was slightly shifted to the left of the SNP curve. The maximum responses to both drugs were comparable. Data points are Mean ± SEM of n= 5.

4.3.2.4 Relaxation to PGE_2:

PGE_2 like SNP, but unlike, Ach produced greater relaxation in OM compared with SC vessels, with SC to the right of OM curve ($p<0.05$, Fig 29). However, Imax was 84.48±6.75 for OM vs 65.99±13.55 for SC ($p= 0.2677$). There was no difference in sensitivity between SC and OM (pIC50 values were -6.34± 0.22 vs -7.06±0.45 for SC vs OM respectively ($p= 0.2094$).

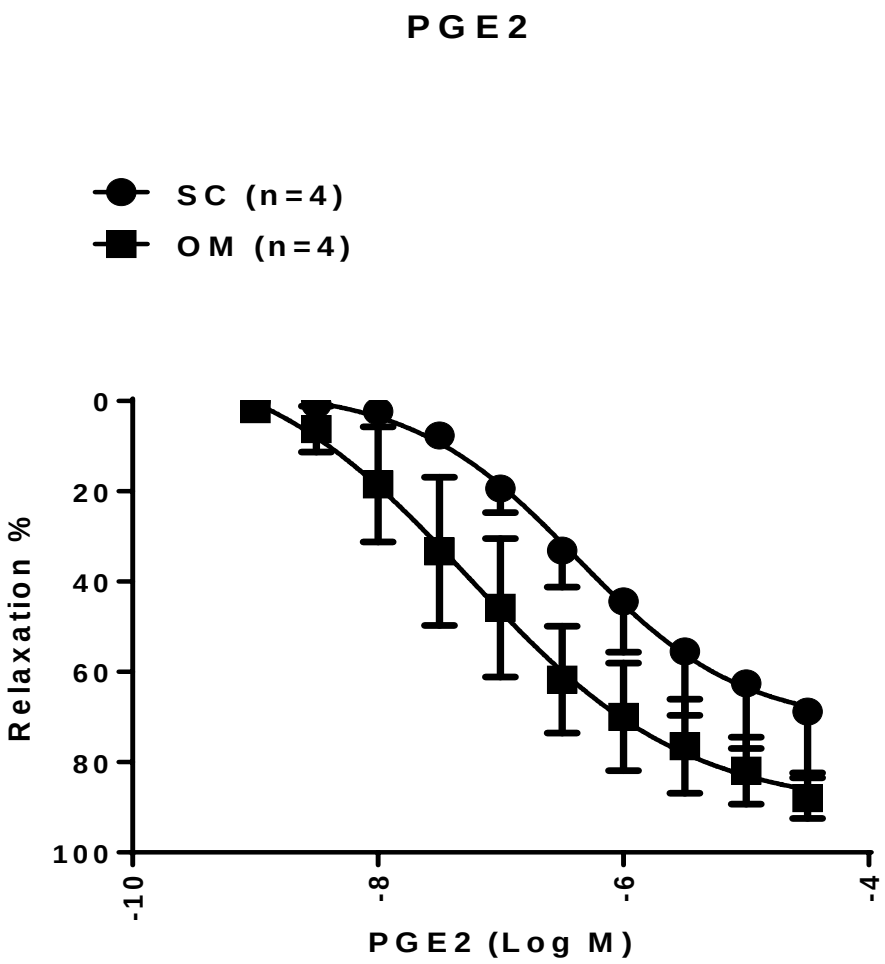


Figure 29: PGE2 relaxations of SC and OM vessels obtained from same individuals. PGE2 produced greater relaxation of OM compared with SC vessels ($p= 0.39$) with increasing PGE2 concentrations. Data points are Mean ± SEM of n=4.

4.4 Discussion

The data clearly demonstrate endothelial dysfunction in OM, but not in SC, vessels from PO and DO groups. SC vessels from the 3 metabolic groups appeared to have apparently normal and similar endothelium-dependent dilator profile. The depot specific differences in endothelial function of these vessels suggest differential impact of local adipose tissue environment on these vessels. This is consistent with the reported more pathogenic milieu of the OM compared with the SC adipose tissues (81, 82). The SC & OM show functional differences in the expression of genes, especially those regulating inflammation and vascular function, presence of complement factors and fatty acid binding protein that are all expressed at higher levels in OM than in SC fat (85, 86). Expansion of these depots might happen via adipocyte hypertrophy and/or hyperplasia that accumulate of excess fat in the visceral compartment, while subcutaneous fat mass remain normal, carries the greater metabolic risk (77).

Even though the numbers for the DO group was low, the data showed a trend consistent with reported vascular impact of the disease (160). Type 2 diabetes is characterized by insulin resistance which is associated with elevations in free fatty acid levels (165) and endothelial dysfunction. Abdominal adipose tissue is particularly dysfunctional, in type 2 diabetes, is more insulin resistant and releases more free fatty acids compared with the type of adipose in other locations (155). Diabetes also induces PKC activity, NF-κβ production, and generation of oxygen-derived free radicals in VSMC, similar to these effects in endothelial cells that might contribute to endothelial dysfunction.

Chapter 5

Mechanisms of endothelial function and dysfunction

5.1 Introduction

5.1.1 Role of Nitric oxide (NO)

Nitric oxide is generated from L-arginine by the action of endothelial nitric oxide synthase in the presence of cofactors, such as tetrahydrobiopterin. When NO is produced, it diffuses to vascular smooth muscle cells and activates guanylate cyclase, which leads to cGMP-mediated vasodilatation (241). Also NO is involved in several protective functions of the endothelium by limiting vascular inflammation, vascular smooth muscle proliferation, platelet aggregation, and production of tissue factors (242). So, modifications of normal endothelial physiology result in endothelial dysfunction that is characterized by decrease in the bioavailability of vasodilators, especially NO (243).

Endothelium functionally regulates vascular homeostasis by elaborating a number of paracrine factors responsible for maintaining normal vascular tone, and blood fluidity, and limiting vascular inflammation and smooth muscle cell proliferation (244, 245). Therefore endothelial dysfunction is a systemic pathological state of the endothelium, in which there is a reduction in the bioavailability of vasodilators, especially nitric oxide, leading to impaired endothelium-dependent vasodilation, as well as disarrangement in vascular wall metabolism and function. Also, another key factor in endothelial dysfunction is overproduction of reactive oxygen species which has been implicated in the development of hypertension, atherosclerosis, diabetes, cardiac hypertrophy, heart failure, ischemia-reperfusion injury, and stroke. Thus impaired endothelial activity is believed to have a major causal role in the pathophysiology of vascular disease, hypertension, and heart failure (246).

NO can hyperpolarize, or repolarize vascular smooth muscle cells by activating, in either a cyclic-GMP–dependent or cyclic-GMP–independent manner, potassium channels like K_{ATP}, BK_{Ca}, Kir, and/or K_V. NO interactions with other ionic channels of the smooth muscle, including chloride and cationic channels, also influences the membrane potential of the smooth muscle cells indirectly in an autocrine fashion (247). Inhibitions of NO synthesis do not necessarily fully inhibit the production of NO, thus residual NO can be produced by the endothelial cells and contribute to the

relaxation and/or hyperpolarization of the underlying vascular smooth muscle. NO can also contribute in another way to responses resistant to inhibitors of cyclooxygenases and NO synthases, because NO can be stored and released independently of the activation of NO synthase. Two such stores have been described, the first store is the so-called photosensitive store that involves the release of NO by ultraviolet light and the second one is located in the adventitia and generated by the formation of protein-bound dinitrosyl non-heme iron complexes and S-nitrosated proteins (248). Therefore, low-molecular-weight thiols displace NO from these stores and transfer it to various target membrane proteins including potassium channels and may therefore function as hyperpolarizing factors (249).

5.1.2 Role of Cyclooxygenase (COX)

Cyclooxygenase (COX) is the key enzyme required for the conversion of arachidonic acid to prostaglandins (PG) G2 and PGH2. PGH2 subsequently converts to a variety of prostanoids that constitute PGE2, PGD2, PGF2α, PGI2, and thromboxane (TX) A2 which interact with prostanoid (P) receptors. Prostanoid receptors belong to the G-protein-coupled seven transmembrane domains family, and are classified into five subtypes (DP, EP, FP, IP and TP receptors) as a function of their preferential affinity towards those PGs (250). The array of PGs produced varies according to the downstream enzymatic machinery present in each cell type (251). Prostanoids are involved in control of human vascular tone (252) and remodeling the vascular wall as well as in platelet aggregation and thrombosis (253,254). Prostaglandin F2α (PGF2α) and thromboxane A2 (TXA2) induce vasoconstriction, prostaglandin D2 (PGD2) and prostacyclin (PGI2) cause vasodilatation, while prostaglandin E2 (PGE2) may produce both effects depending on the receptors. PGE2 may activate 4 receptor subtypes (EP1, EP2, EP3 and EP4). Once they are expressed in smooth muscle cells; EP1or EP3 receptors are associated with vasoconstriction, whereas the activation of EP2 or EP4 receptors is associated with vasorelaxation (255).

Two cyclooxygenase isoforms have been identified known as COX-1 and COX-2 encoded by two different genes. Under many circumstances the COX-1 enzyme is

produced constitutively and many of the 'housekeeping' effects of COX appear to be mediated by COX-1 (e.g. in gastric mucosa) whereas COX-2 is inducible (e.g. at sites of inflammation). The functional role for each isoform is consistent according to their tissue expression patterns, although nearly all normal tissues express COX-1 with low to undetectable levels of COX-2. Other differences between COX-1 and COX-2 include differences in utilization of arachidonic acid substrate pools as well as in mRNA stability (256). In healthy blood vessels (both endothelial and, to a lesser extent, vascular smooth muscle cells) express the two COXs, COX-1 being the predominant isoform (257), however, COX-2 appears to be the major contributor of the overall systemic generation of prostacyclin, in endothelial cells of both healthy and diseased blood vessels, while COX-1 is also an important source of PGs (258,259).

5.1.2.1 COX-1: has been found in nearly all tissues under basal conditions (260) and the major function for COX-1's is to provide PG precursors for homeostatic regulation. The important site of COX-1 function is the blood platelet, where the enzyme is responsible for providing precursors for thromboxane synthesis (261). While, in the adjacent vascular endothelium PGs play a different role to release eicosanoids by activated platelets to provide both a substrate and stimulus for the generation of prostacyclin (PGI2) by the endothelium. The latter compound stimulates vasodilatation, counteracting the vasoconstrictor, thromboxane. Thus, COX-1 appears to function in two physiological systems leading to vasodilatation in the presence of contractile conditions (262).

5.1.2.2 COX-2: is mainly an inflammatory, inducible enzyme, and is the major generator of the vasodilator PGI2, which functions especially when NO bioavailability is diminished. Endothelial dysfunction is reversed by a complementary up regulation of COX-2 expression and activity as shown in the mesenteric vascular bed of mice with streptozotocin-induced diabetes (263), whereas attenuated NO-dependent vasodilatations in diabetic patients may be compensated for by the emerging vasodilator effect of prostacyclin (264). Compared with patients, non-documented as diabetic, coronary arterioles from diabetic patients

show a significant up-regulation of COX-2 expression, that contributes to enhance bradykinin-induced vasodilatations (265). The availability of selective COX-2 inhibitors now allows a more in-depth investigation on the emerging role of COX-2 as an inflammatory mediator releasing vasoconstrictors in morbidly obese.

COX is also involved in the endothelial generation of reactive oxygen species, which is a key factor in the generation of endothelium-dependent contractions (266,267). Reactive oxygen species cause decrease in NO bioavailability (268) and as a positive feedback loop, the formation of hydroperoxides lead to further activation of COX (269). Also, since the reactive oxygen species diffuse towards the vascular smooth muscle cells, they can stimulate COX in these cells and produce more contractile prostanoids. Then the generated PGs directly activate TP receptors (270,271). TP receptors are also expressed in endothelial cells and their stimulation induces the Rho kinase–dependent inhibition of NO production (272). The isoform α of the human TP receptor is negatively and independently regulated either by NO or prostacyclin, following the phosphorylation of serine residues by protein kinases G and A respectively (273). Also NO can inhibit the activity of thromboxane synthase (274), which indicates that a decrease in NO bioavailability may facilitate the TP receptor–dependent signalling pathway. Endothelial dysfunction could also include a marked attenuation of the endothelial dependent hyperpolarizing factor (EDHF)-mediated component of the endothelium-dependent relaxations (247). TP receptor stimulation induces a loss in the activity of endothelial small conductance calcium activated potassium channels (275), that is an essential component of EDHF-mediated responses (259). Conversely, the impairment of EDHF-mediated responses also can favour the development of endothelium-dependent contractions (276).

5.1.3 Prostaglandins (PGs) regulate physiological responses involved in diverse functions such as; blood clotting, wound healing, kidney function, blood vessel tone, immune response etc. So, they play more role than just the central role in inflammation. PGs are synthesized in a broad range of tissue types and mediate via autocrine or paracrine, signal changes within the immediate environment. PGs

act either via their receptors coupled to G protein or directly bind to transcription factor or, the nuclear PPAR receptor (PPARα, PPARγ, PPARδ) (277).

In the human body PGE2, the most abundant PG, is involved in multiple physiological effects including inflammation, regulation of kidney functions and blood pressure. Its effect on kidney functions includes regulation of glomerular filtration rate, tubular salt and water transports, renin release, renal blood flow and vascular tone (278). PGE2 can produce both relaxation and contraction of vascular smooth muscle depending on the expression of its four receptor subtypes, namely EP1, EP2, EP3 (of which eight splice variants exist in the human) and EP4, which are coupled to different signaling pathways (279, 280). Prostacyclin is the other major metabolite of arachidonic acid produced by COX and is closely associated especially with COX-1 and highly expressed in endothelial cells, (257,281), also expressed in vascular smooth muscle cells, neurons, oviducts, intestinal epithelial cells and embryonic cells (282). Prostacyclin is the favored IP receptors ligand and its effects often, but not always, include activation of adenylyl cyclase and subsequent elevation of intracellular cyclic AMP produces relaxation (283,284). Depending on the artery and/or the species, a hyperpolarization can occur that involves the opening of one or more of types of potassium channels. Thus, ATP-sensitive potassium channels (K_{ATP}), large conductance calcium-activated potassium channels (BK_{Ca}), inwardly rectifying potassium channels (Kir) and/or voltage activated potassium channels (K_V) can be associated with the prostacyclin-induced relaxation (247). Thus, in numerous vascular beds, prostanoids can be referred to as endothelium- derived hyperpolarizing substances. Due to most of the available inhibitors of cyclooxygenase abolish the production of prostaglandins in vascular tissues; any endothelium dependent hyperpolarization observed in the presence of one of these inhibitors is unlikely to involve prostacyclin (247).

5.1.4 Endothelium Derived hyperpolarizing factor (EDHF) and vascular function

EDHF: The endothelium controls vascular tone of the underlying smooth muscle cells, not only by releasing nitric oxide (NO) and prostacyclin, but also by other

pathways causing hyperpolarization (245, 285). The so called endothelium derived hyperpolarizing factor (EDHF), therefore endothelial hyperpolarization is likely due to several factors that are site- and species specific, causing vascular smooth muscle hyperpolarization and relaxation. EDHF-mediated responses are characterized by: increasing in the intracellular calcium concentration, the opening of calcium-activated potassium channels of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance, and the hyperpolarization of the endothelial cells (286,287). Such EDHF-mediated responses, in most arteries, involve the activation of SK_{Ca} (blocked by apamin, scyllatoxin, tubocurarine, or UCL 1684) and/or IK_{Ca} (blocked by charybdotoxin, TRAM-34 or TRAM-39) (288,289,290), 23,24,26 but not that of BK_{Ca} (blocked by charybdotoxin and iberiotoxin) (291). Both SK_{Ca} and IK_{Ca} channels are present in freshly isolated endothelial cells from various arteries, the combination of apamin (a specific inhibitor of SK_{Ca}^{+} channels) plus charybdotoxin (a nonselective inhibitor of BK_{Ca}^{+}, IK_{Ca}^{+} channels), and of some voltage-dependent K^{+}(K_{V}^{+}) channels, abolish EDHF mediate responses (289,292).

Increasing intracellular free calcium in endothelial cells opens K_{Ca}^{+} channels and allow efflux and accumulation of K^{+} into the myoendothelial space which triggers several processes that explain the EDHF phenomena; involving (i) synthesis of cytochrome P450 (CYP450) metabolites, a family of epoxides, (ii) transmission of endothelial cell hyperpolarization to the vascular smooth muscle via gap junctions, and (iii) K^{+} released from the endothelial cells via K_{Ca}^{+} channels induces smooth muscle hyperpolarization by activating Kir channels and/or Na^{+}-K^{+}-ATPase on vascular smooth muscle cells (293).

5.1.5 Cytochrome P450 (CYP450):

These are membrane-bound, heme-containing terminal oxidases in a multienzyme system. CYP450-dependent EDHF plays a significant role in the regulation of coronary arteriolar tone by KCa+ channel activation and smooth muscle hyperpolarization (294). Epoxyeicosatrienoic acids (EETs) are arachidonic acid derived products of cytochrome P450 (CYP450) epoxygenases (295, 296). EETs (in particular 11, 12- and 14, 15-EET) activate several intracellular protein kinases

which including tyrosine kinases, the p38 MAP kinase, and extracellular-regulated protein kinases 1 and 2 (Erk1/2) and increase the proliferation of various cell types, such as vascular smooth muscle cells and endothelial cells (296). The arachidonic acid metabolizing CYP450 enzymes with prominent roles in vascular regulation are the epoxygenases of the CYP 2 gene family (e.g., CYP 2B, 2C8, 2C9, 2C10, and 2J2 in humans) which generate a series of region-specific and stereo-specific epoxides (5,6-, 8,9-, 11,12-, and 14,15-EETs) (297).

5.1.6 Gap junctions:

Gap junctions are formed by the docking of two connexons present in adjacent cells leading to the creation of an aqueous central pore permitting the transfer of ions and polar molecules that provides an electrical continuity allowing a uniform membrane potential among coupled cells (298). These are myoendothelial and heterocellular and they couple endothelial cells to other endothelial cells and to smooth muscle cells, providing a low-resistance electrical pathway between the cell layers (299,300). The number of myo-endothelial gap junctions increases with diminution in the size of the artery (301, 299), a phenomenon that parallels the contribution of EDHF-mediated responses in endothelium-dependent relaxations to vessel size with a greater influence in the resistance than in the conductance vessels (302). Although, many questions remain unanswered to date, what flows through myo-endothelial gap junction is basically unknown. That a single layer of endothelial cell can drive the hyperpolarization of multiple layers of smooth muscle cells suggests the involvement of an undefined active membrane process in the conducted hyperpolarization (303). Few EDHF-mediated responses are associated with a small but significant early and transient endothelium-dependent increase in the cyclic-AMP content of the smooth muscle cells. Stimulation of a calcium-sensitive adenyl cyclase isoform results in an increase in the production of cyclic-AMP with subsequent enhancement of gap junctional communication (304,305).

5.1.7 Potassium (K^+):

K^+ is another mechanism for achieving hyperpolarization and relaxation of the underlying vascular smooth muscle cell linked directly to the hyperpolarization of

the endothelial cells. The activation of endothelial IK_{Ca} and/or SK_{Ca} causes an efflux of potassium ions from the intracellular compartment toward the extracellular space (306). A moderate increase in the extracellular (myo-endothelial) K^+ concentration (1 to 15 mmol/L) induce hyperpolarization (relaxation) of vascular smooth muscle by activating the inwardly rectifying K^+ (Kir) channels and the Na^+/ K^+ pump. Endothelium is known as thin monolayer and any efflux of potassium from this small cell mass into the lumen of the blood vessel is removed by the flowing blood. However, an efflux of potassium in the abluminal direction can lead to accumulation of these ions in the small intercellular space between endothelial and smooth muscle cells and to reach levels sufficient to activate Kir and the Na^+/ K^+ ATPase on the latter. Thus, even if K^+ is not EDHF it could contribute to EDHF-mediated responses (213,307).

In order to further understand the mechanisms the relaxation to acetylcholine was recorded in the absence or presence of N_ω-Nitro-L-arginine methyl ester (L-NAME), indomethacin, diclofenac, $BaCl_2$, apamin+charybdotoxin, and arginine.

mRNA expression of hypertension associated genes in stromal vascular fractions (SVFs) of both depots was assessed by real time RT-PCR. Paraffin-embedded tissues were used for histological studies.

5.2 Methods

5.2.1 Myography:

The general protocol for wire myography described in 3.2 was used.

To determine whether changes in endothelial NO release contributed to the changes in Ach relaxation recorded, vessels were pretreated with L-NAME (100μM) for 30 minutes before Ach concentration-response curves were constructed. For changes in EDHF role, vessels were pretreated with combination of apamine (0.5μM) and charybdotoxin (0.1μM) for 15 minutes before Ach curves were generated. Vessels were also exposed to barium chloride (30μM) for 15 minutes to determine whether the Kir channels were involved in Ach-induced hyperpolarization. To test for the role of prostanoids, vessels were pretreated for 30 minutes with the COX inhibitor

indomethacin (10μM) before Ach curves were constructed. % relaxation to Ach in the presence of these interventions was calculated as described above.

5.2.2 Hypertension arrays:

RNA samples were extracted from SC and OM tissues as described in section 2.4.2 in methods. RNA concentrations were determined using nano-drop. Table 4 indicates samples concentration and purity as determined by A260/A280 ratio. Samples with satisfactory concentrations and good purity were converted into cDNA as described in 2.4.3 methods. In order to confirm whether yield and purity were suitable to run hypertension expression arrays, B-Actin expression was assayed using RT-PCR as described in 2.4.4 methods. Table 4 shows CT corresponding to each sample. All samples showed b-actin CT lower than 21 and therefore were used for hypertension PCR arrays. Table 5 shows selected RNA samples for hypertension expression arrays using RT-PCR Applied biosystems ViiA7 as described in section 2.4.5 in methods.

5.3 Results

5.3.1 Table 4: Patient characteristics for mechanisms of endothelial dysfunction studies.

Variables	MHO (n=5)	PO (n=20)	*P* MHO vs PO	DO (n=4)	*P* DO vs Nondiab
Age (years)	32 (12)	37 (8.3)	0.28	40 (7)	0.41
BMI (kg/m2)	43 (2.9)	47 (6.9)	0.27	46 (8.2)	0.87
SBP (mmHg)	123 (11.50	126 (15.5)	0.62	132 (16.6)	0.46
DBP (mmHg)	68 (7.45)	73 (11.32)	0.33	79 (12.6)	0.26
MAP (mmHg)	86.2 (7.3)	82 (30.12)	0.76	96.5 (13.7)	0.33
Proinsulin (miU/ml)	1.83 (1.1-5.3)	8 (3.1-19)	0.03	14.3 (2.9-13.5)	0.49
FBG (mmol/L)	5.92 (1.3)	6.22 (1.8)	0.73	12.6 (5.8)	0.19
Insulin (miU/ml)	7.4 (6.1-7.6)	15.6 (11.1-21.7)	0.006	15.41 (8-15)	0.44
HOMA	2.1 (1.8-2.5)	4.08 (2.6-6.2)	0.08	9.2 (6.2-9.2)	0.01
HDL (mmol/L)	0.93 (0.44)	0.93 (0.40)	0.98	0.97 (0.4)	0.88
LDL (mmol/L)	1.8 (0.98)	2.3 (0.8)	0.19	2.7 (0.74)	0.36
TG (mmol/L)	0.91 (0.73-1.1)	1.2 (1-1.7)	0.10	1.72 (1.4-1.7)	0.05
Cholesterol (mmol/L)	3.1 (1.41)	3.8 (1.13)	0.27	4.3 (1.2)	0.38
IL6 (pg/ml)	3.5 (1.14)	6 (1.9)	0.02	3.60 (0.6)	0.24
Leptin (ng/mL)	60 (26.4)	55.8 (25.8)	0.75	34.06 (13.05)	0.14

Data are shown as mean (SD) or median (interquartiles). * P<0.05, ** P<0.01, *** P<0.001.

5.4 Mechanisms of endothelial dysfunction

To investigate further the endothelial dysfunction reported in chapter 4, more experiments were carried out to determine changes in the roles of NO, prostaglandins/COX and EDHF. The role of the Kir channel was also investigated.

5.4.1 Effect of eNOS inhibition:

To test for changes in NO contribution to the Ach responses, curves were generated in the presence of the eNOS inhibitor, L-NAME. Following incubation with 100 μM L-NAME for 30 minutes, the Ach curves for both SC and OM were shifted to the right ($P < 0.0001$ for SC vessels, Figure 30 A, and $p < 0.0001$ for OM Fig 30 B). The Imax values for SC vessels dropped from 87.586± 2.537% to 55.102± 55.102% ($P = 0.0942$) and for OM vessels dropped from 67.566±3.633% to 49.391±6.631% ($P=0.1071$). There were no changes in the sensitivities of both vessels to Ach: The pIC50 values were 7.171±0.240 vs 6.994±0.245 control vs L-Name-treated OM vessels and 6.885±0.194 vs 6.732±0.367 for SC vessels.

A

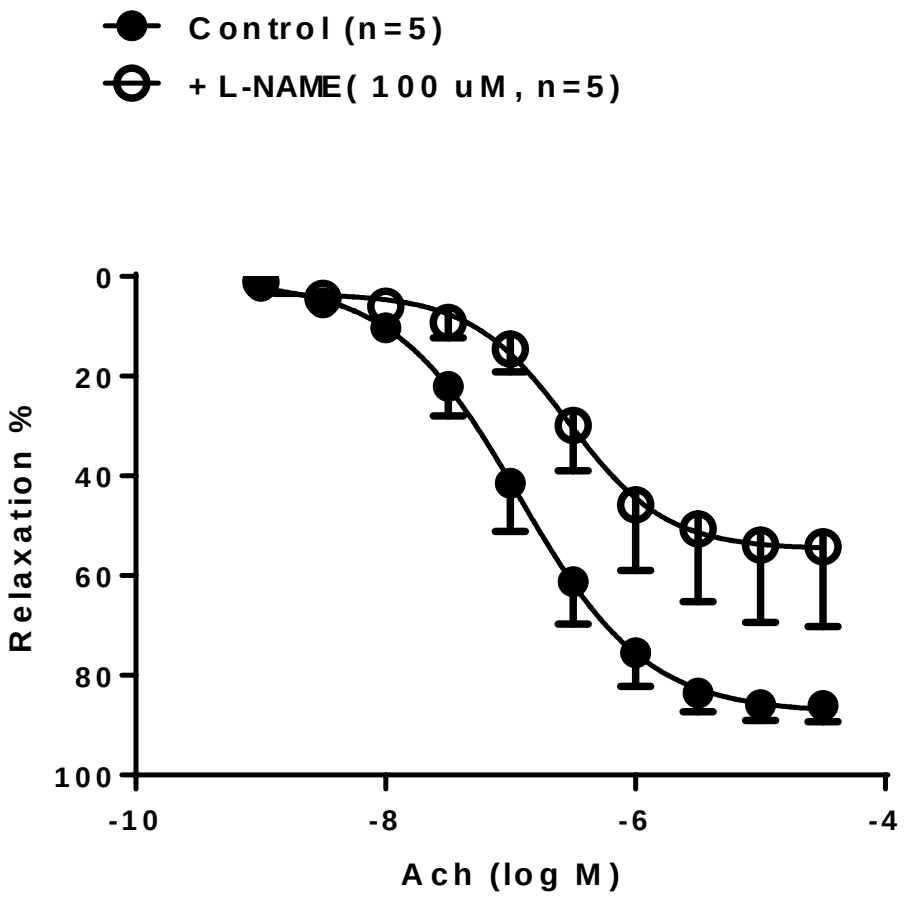


Figure 30A: Inhibition of Ach relaxation of SC vessels by L-NAME. L-NAME caused a rightward shift of the Ach curve ($p < 0.0001$), but with no change in sensitivity. Data points are Mean ± SEM of n=5-7.

B

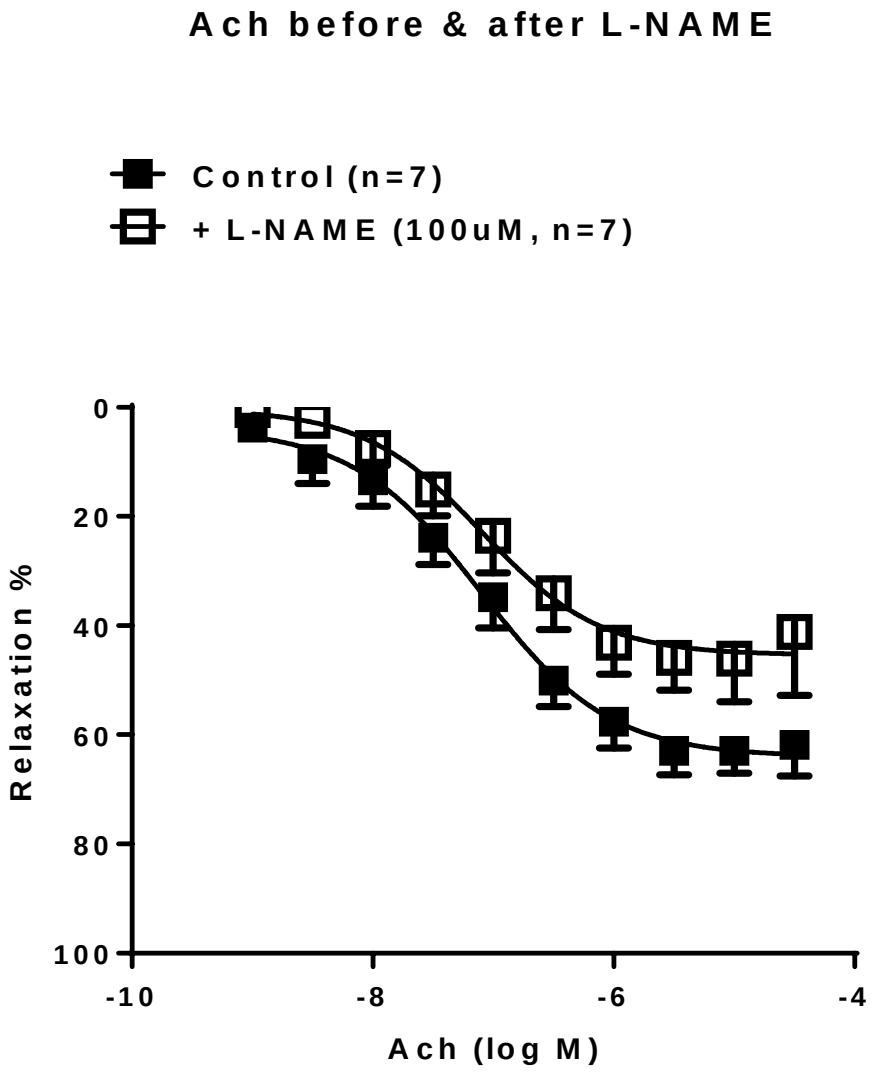
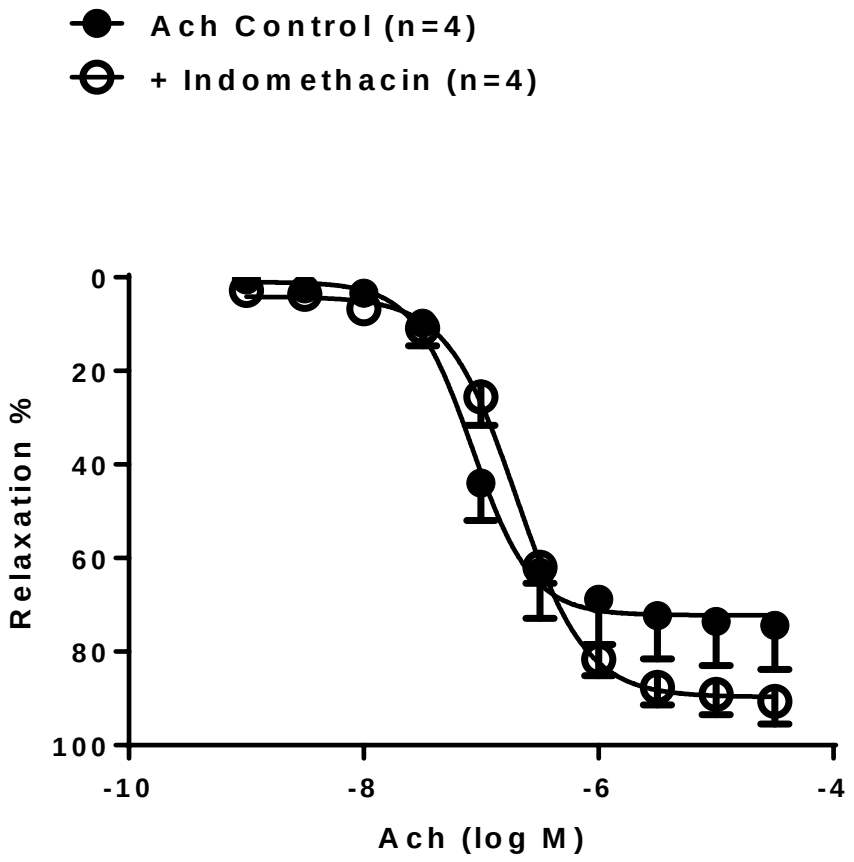


Figure 30B: Inhibition of Ach relaxation of OM vessels by L-NAME. L-NAME caused a rightward shift of the Ach curve ($p < 0.0001$), but with no change in sensitivity. Data points are Mean ± SEM of n=5-7.

5.4.2 Effect of COX inhibition:

To test for changes in the contributions of prostaglandins, Ach curves were generated in the present of 10 μM indomethacin (COX inhibitor). Surprisingly Ach curves were potentiated (shifted to the left) under this condition, particularly in the OM vessels (Fig 31 B, $P<0.0001$). The shifts in the curves for SC vessels were not statistically significant (Fig 31 A). Imax values increased from 72.067±7.329 to 86.630±2.174 in OM vessels ($P=0.1608$), and from 72.530 ± 9.036 to 89.765±4.424% in SC vessels ($P= 0.2563$). Sensitivities of both vessels to Ach were not changed by pretreatment with Indomethacin. The pIC50 values before vs after Indomethacin were 7.287± 0.964 vs 7.763±0.227 for OM vessels and 7.050±0.051 vs 6.715±0.069 respectively for SC vessel.

A



<u>Figure 31A:</u> Effect of Inhibition of COX by Indomethacin in SC vessels. Indomethacin had no significant effect on Ach relaxation of the SC vessels. Data points are Mean ± SEM of n=4.

B

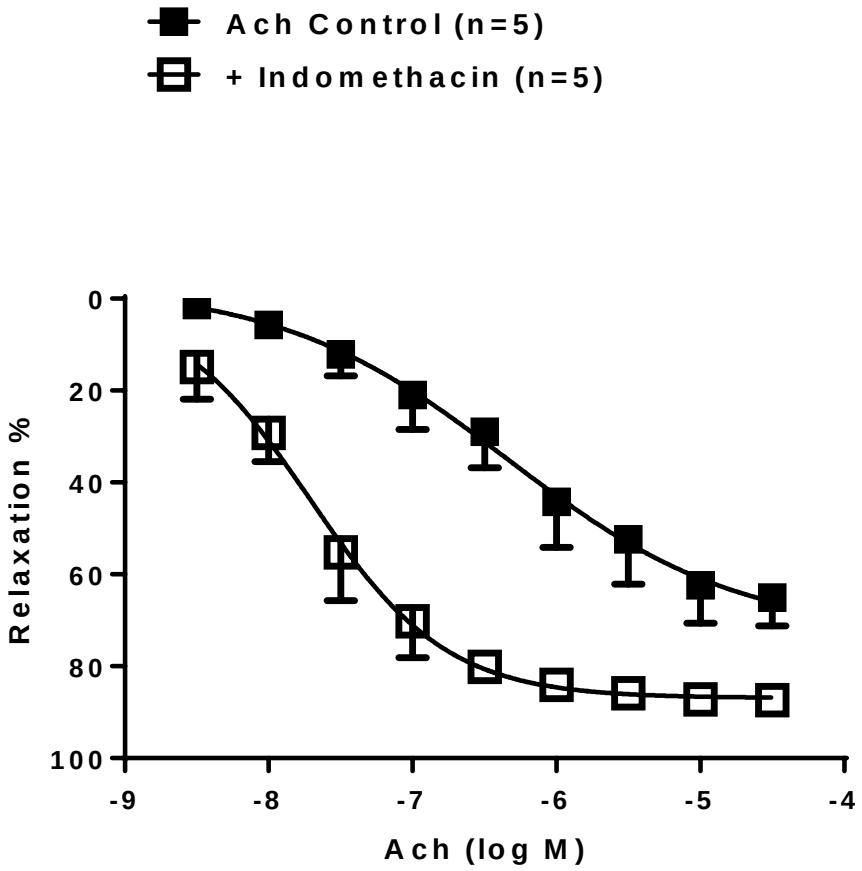


Figure 31B: Effect of Inhibition of COX by Indomethacin in OM vessels. Indomethacin caused a leftward shift in the Ach curve of the OM vessels ($p < 0.0001$) -indicating an improvement in Ach relaxation. Data points are Mean ± SEM of n=5.

5.4.3 Effect of EDHF inhibition:

EDHF is another mediator of Ach relaxation released from the endothelium. To test for any changes in EDHF contributions, Ach curves were constructed in the presence of a combination of 0.5 μM apamin (SK_{ca} blocker) and 0.1 μM charybdotoxin (IK_{ca} blocker). Under these conditions, Ach curves for both SC and OM were shifted to the right. The curve for SC vessels was shifted to the right (Fig 32 A; $p< 0.0001$) with Imax reduced from 84.057±5.214 to 28.213±7.581 ($p=0.0004$). The curve for OM vessels was significantly shifted to the right (Fig 32 B; $p< 0.0001$) with Imax value reduced from 71.095± 5.881 to 42.797±12.6675. Sensitivities of both vessel types to Ach were unchanged by pretreatment with apamin and charybdotoxin. The pIC50 values were 6.727±0.382 vs 6.290±0.145 for SC and 7.104±0.593 vs 5.294±1.454 for OM before and after EDHF blokade respectively.

A

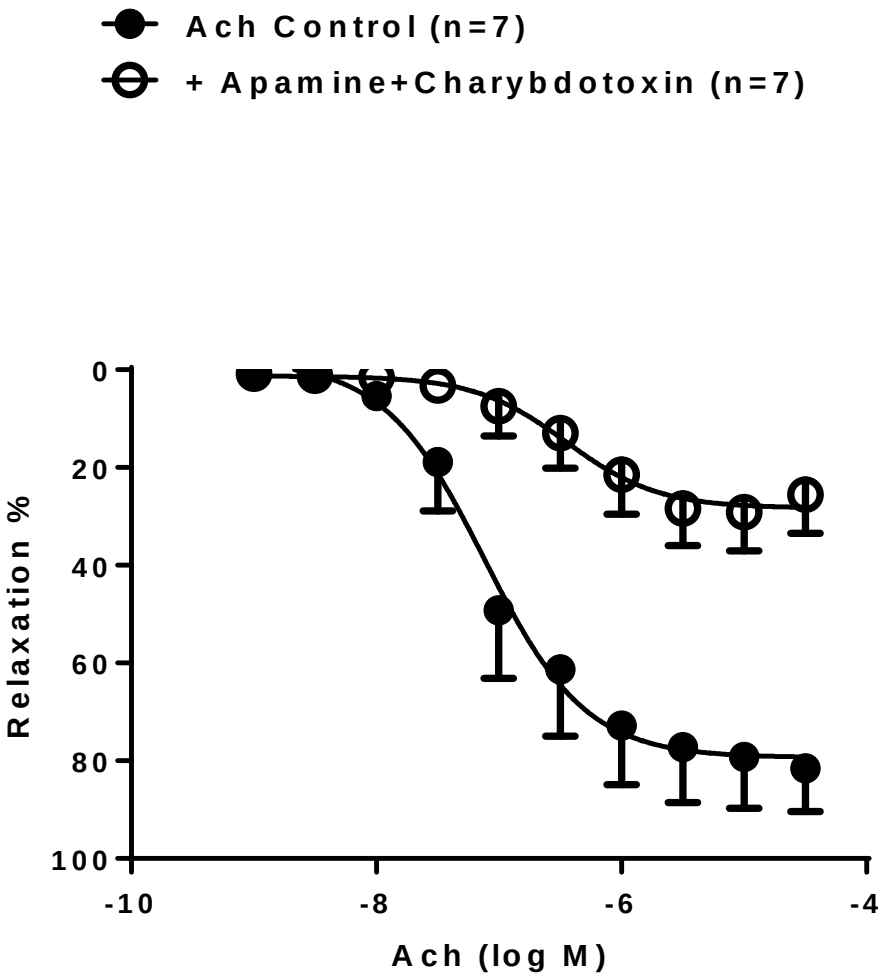


Figure 32A: Effect of blocking EDHF release in SC vessels: Ach curve was significantly shifted to the right by Apamin & Charybdotoxin – blockers of EDHF release ($p < 0.0001$). Data points are Mean ± SEM of n=7.

B

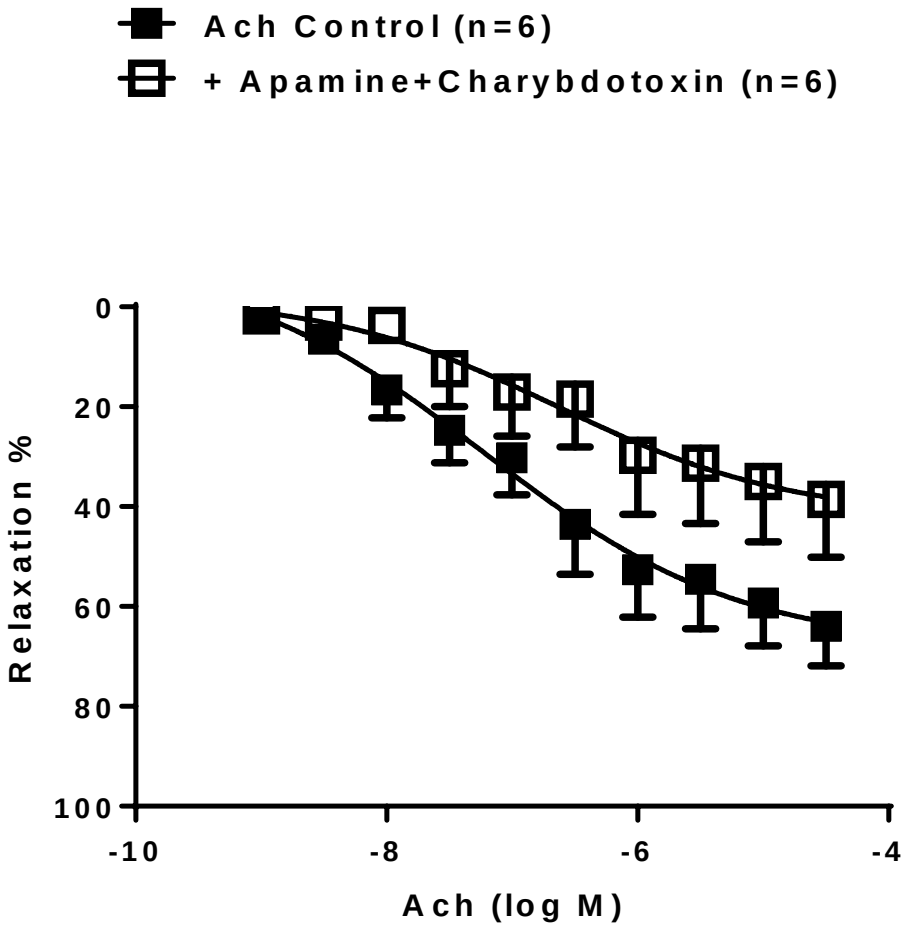
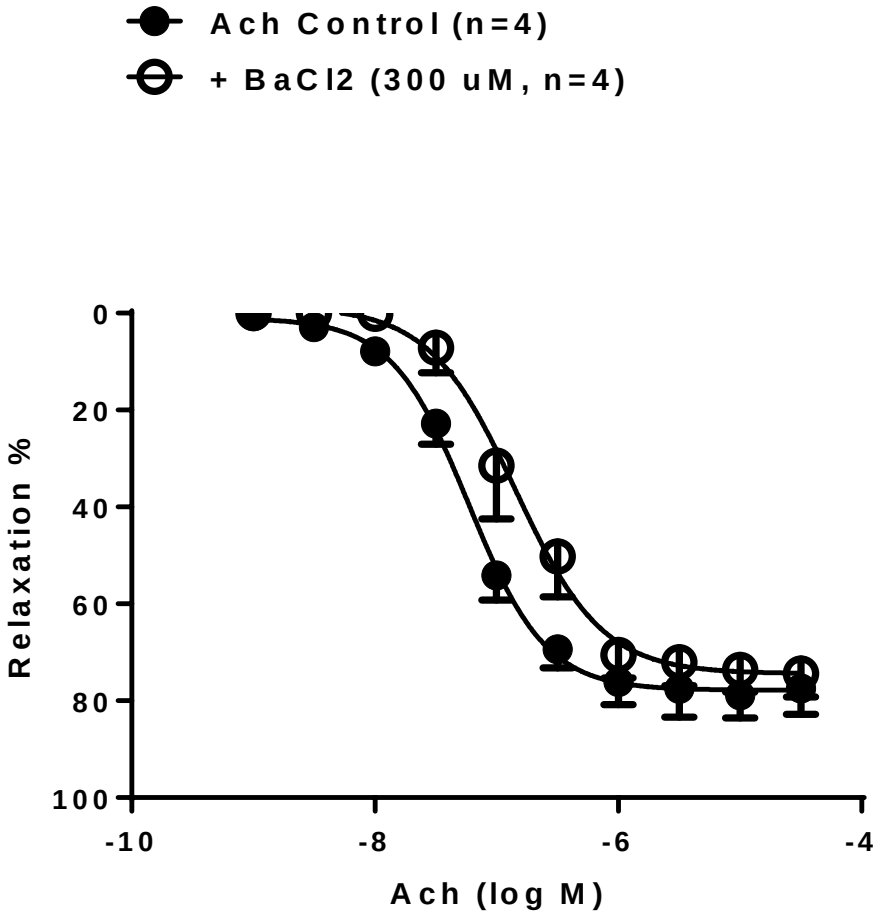


Figure 32B: Effect of blocking EDHF release in OM vessels: Ach curve was significantly shifted to the right by Apamin & Charybdotoxin – blockers of EDHF release ($p < 0.0001$). Data points are Mean ± SEM of n=6.

5.4.4 Effect of Kir channel inhibition:

Following the observation of a significant EDHF role in the Ach response, tests were carried out to determine whether the Kir channel activated by EDHF was involved. For this, Ach curves were generated in the presence of 30 μM BaCl2 (a relatively selective blocker of this channel). Under this condition, Ach curves were shifted to the right in both SC (p=0.0001, Fig 33) and OM (p=0.0001). However, significant reduction in Imax was only recorded in OM vessels (from 73.400± 11.9600 % to 58.934± 27.589 %, p= 0.5752). On the other hand, the sensitivities of the SC vessels dropped (pIC50 values 7.233± 0.096 to 6.867± 0.211) in the presence of BaCl2 (p= 0.0874).

A



<u>Figure 33A:</u> Effect of blocking K_{IR} in SC vessels. 30 μM $BaCl_2$ (Kir blocker) caused a rightward shift of Ach curve (p=0.001 value) but without affecting the sensitivity and maximum relaxation to Ach. Data points are Mean ± SEM of n= 4.

B

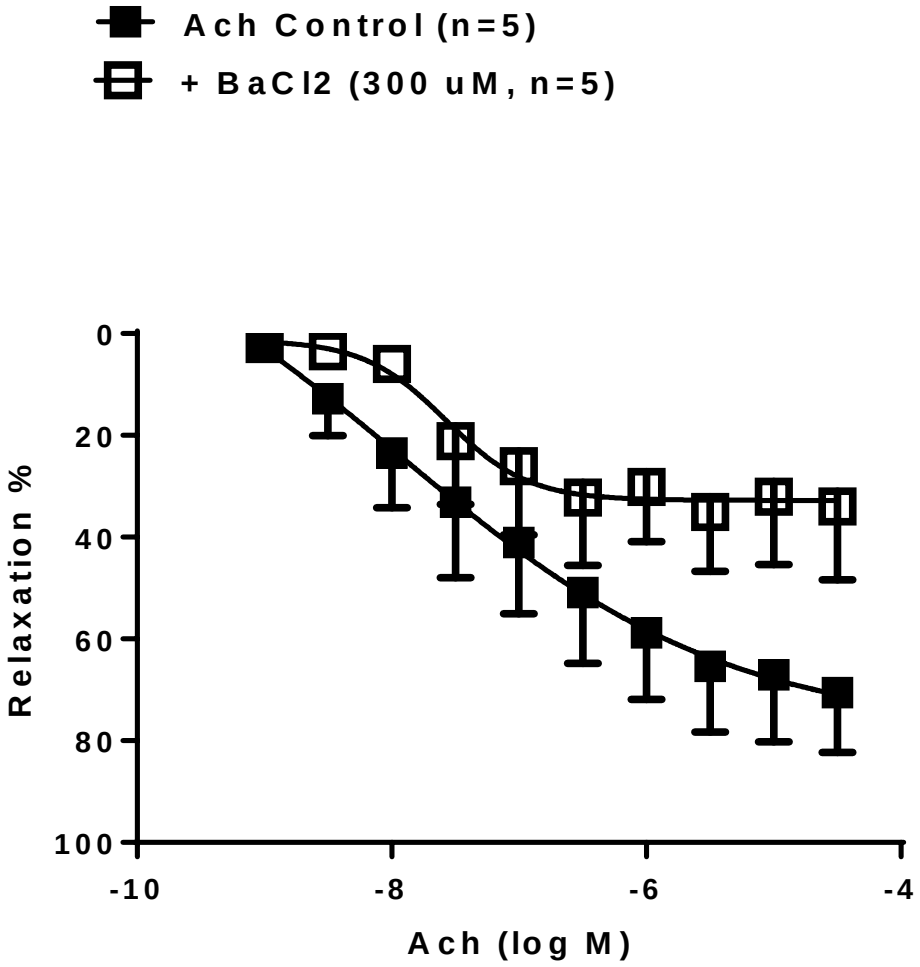


Figure 33B: Effect of blocking K_{IR} in OM vessels. 30 μM $BaCl_2$ (Kir blocker) caused a rightward shift of Ach curve (p=0.001) with slight change in the maximum relaxation to Ach (p=0.085). Data points are Mean ± SEM of n= 5.

5.5 Hypertension associated gene arrays

Comparisons were made between the SVF of the depots, SC and OM, of the MHO patients and the PO patients separately. Further comparisons were also made between the SC and OM SVF of the MHO compared to the PO.

In the MHO patients the OM SVF compared to the SC SVF expressed significantly greater levels of mRNA for AGT (3.7 [1.6, 5.8], $p<0.0005$), ARG2 (6.7 [0.0001, 20.5], $p=0.02$), CLIC5 (5.5 [0.63, 10.3], $p=0.02$), EPHX2 (2.0 [1.0, 3.1], $p=0.05$), ITPR1 (2.2 [1.3, 3.1], $p=0.01$) and PRKG1 (3.0 [0.61, 5.3], $p=0.004$).

In the PO patients the OM SVF compared to the SC SVF expressed significantly greater levels of mRNA for only CLIC5 (8.4 [0.33, 16.5], $p=0.04$) and PDE3B (2.5 [0.32, 4.7], $p=0.04$).

In the overall analysis of the PO compared to the MHO only COX 2 mRNA was significantly up-regulated (7.2 [3.61, 15.3], $p=0.008$).

5.6 Histological analysis

The results from most of the histology samples were very poor and therefore not analyzed further.

5.7 Discussion

The data showed that NO and EDHF are the key mediators of Ach endothelium-dependent relaxation in both OM and SC vessels from obese humans (n=29), with a greater role for EDHF. The big surprise in the current data was the absence of a role for dilator prostaglandins. COX inhibition resulted in a potentiation instead of a reduction in Ach relaxation. This suggests increased activity of contractile prostanoids in these vessels, particularly in the OM vessels where such activities appear to largely account for the attenuation in Ach relaxation. That the role of EDHF in the SC vessels which showed no apparent endothelial dysfunction was greater than in the OM vessels with endothelial dysfunction points to lack of a link between EDHF contribution and endothelial dysfunction in these vessels. Thus, the suggestion from animal vessel experiments that EDHF role increases in obesity as a compensatory response to reduction in NO bioavailability is not the case in these human adipose micro vessels. Moreover, EDHF has been shown in apparently healthy humans to play a significant part in the vasodilator mechanism of SC vessels. EDHF mediated response involves an increase in endothelial cell calcium concentration, the opening of Ca^{+2} activated K^{+} channels of small (SKca) and intermediate (IKca) conductances and the hyperpolarization of the endothelial cells leading to transfer of the signals to vascular smooth muscle cells and relaxation (289,290).

The differential response by OM and SC vessels to Ach in the presence of $BaCl_2$, suggests greater involvement of kir channel in the OM compared with the SC vessels. The EDHF response in SC vessels probably involves another type of K channel such as BKCa or Kv channels which are other known candidates for EDHF response in blood vessel (289, 292).

Chapter 6

Discussion

6.1 Patients Characteristics:

Blood vessel functions of patients (n=63) who were undergoing surgery for weight loss were studied. Although all the patients were morbidly obese (BMI >40 Kg m-2) they were further categorized into 3 groups, MHO (n=13), PO (n=42) and diabetic (n=8) based on their metabolic profiles. This represents a prevalence of MHO of 20.6%, PO of 66.7% and T2DM of 12.7% in this study cohort. As these were not samples collected from consecutive patients unintended selection bias may be likely. Therefore any observation on the prevalence of the different phenotypes in a morbidly obese population only refers to this cohort. The prevalence of MHO and PO in this cohort appears comparable to those reported by other groups from 10 and 40% (195), however, the prevalence of T2DM in this study population seem significantly lower than those reported, which range from 22-50% (308,309,310,311). As the overall prevalence of T2DM in the Qatari general population is currently 16.7% this figure of 12.7 % in our morbidly obese cohort may just reflect selection bias (312,313).

The metabolic profiling was based on the patient's plasma insulin level, insulin sensitivity, plasma glucose and blood pressures. There were no noticeable differences in lipid profiles between these groups. These results suggest that although obesity does not necessarily translate into insulin resistance and increased risk of metabolic comorbidities (314) in all subjects, as demonstrated by the small group of MHOs, most patients studied were at risk of future health complications.

6.2 Vascular reactivity:

The impact of obesity on blood vessels accounts for much of the morbidity of the condition. The endothelium is usually among the first targets of the changing milieu with the potential to change both vasodilator and vasoconstrictor mechanisms. Due to technical difficulties in the direct measurement of vascular reactivity in humans, most studies have relied on indirect assessment of vascular functions in obese individuals such as the forearm flow mediated dilatation technique (315,316) with variable results. This current study is one of the few that have used wire myography

to directly measure reactivity of OM and SC vessels from obese individuals to provide a clearer picture of the impact of surrounding fat on microvascular function.

6.2.1 Response to vasoconstrictors:

Two vasocontractile mechanisms were tested in this study – depolarization-induced with KCl and adrenergic receptor activation with noradrenaline. Maximum contractions in response to depolarization were greater in the OM compared to SC vessels. This was particularly so with the pathologically obese group and probably reflects differences in the impact of the local adipose environment on the contractile mechanisms of these microvessels. The lack of any measurable difference in the responses of SC vessels from the 3 metabolic groups to KCl suggests a comparable SC profile for the groups. KCl acts by depolarizing vascular smooth muscle membrane leading to opening of voltage sensitive Ca^{2+} channels which allow for Ca^{2+} influx and activation of contractile proteins. Since maximum contractions are functions of cytosolic calcium and the contractile proteins, the results suggests greater mobilization of calcium and/or contractile proteins in the OM compared to SC. Differences in the amount of contractile proteins is less likely to explain the KCl data since the force generated by each vessel segment was normalized to size. More so, the mRNA expression arrays show no significant difference in the expression of α actin between the two vessel types. Although the sensitivities of the 3 OM vessel groups to KCl were not statistically different, differences in their sensitivities to cytosolic calcium (Ca^{2+} sensitization) cannot be ruled out. Variation in local release of adipokines can indeed affect the later (156, 157).

In contrast to depolarization-induced contractions, there were no differences in both maximum contractions induced by and sensitivity to adrenergic receptor activation in OM vessels from the 3 metabolic groups. The picture was however different for the SC vessels, which showed greater sensitivity for PO and DO vessels compared to MHO vessels to noradrenaline. The reasons for these differences in noradrenergic sensitivities are not clear but may relate to impact of the local adipose tissue on adrenergic receptor activity. NA contraction involves activation of α-adrenergic receptors which are coupled to both Ca^{+} influx and generation of inositol triphosphate (IP3) which signals intracellular Ca^{+} release from sarcoplasmic

reticulum. Simultaneous activation of β adrenoceptor (which is rare on the vascular smooth muscle (317) would theoretically dampen the effect of α adrenergic activation. Over activity of the sympathetic nervous system in obesity may lead to increased sympathetic outflow which may elevate BP (224), and insulin secretion (225). Egan et al, demonstrated that overweight subjects, most of them hypertensive, exhibit increased α-adrenergic vascular tone (318). Due to reduction of skeletal muscle blood flow in hypertension resulting from neural vasoconstriction could be the primary cause of the insulin resistance and the attendant hyperinsulinemia (226), as well as sympathetic nervous activation can directly contribute to the lipid change which is part of the metabolic syndrome (227).Chronic metabolic diseases including type 2 diabetes and metabolic syndrome are widespread in those with obstructive sleep apnoea (OSA). Furthermore, obesity is the main risk factor and a common characteristic of OSA (319). The latter may worsen the effect of obesity on cardiometabolic risk, through mechanisms which it may trigger several pathological mediating pathways including; sympathetic activation, neurohumoral changes, glucose homeostasis disruption, inflammation and oxidative stress via chronic intermittent hypoxia (CIH), and these may ultimately cause deterioration in the metabolic function (320). South Asians have a higher prevalence of type 2 diabetes, dyslipidemia, and CVD, and it is possible that South Asians are at greater risk of OSA than white Europeans (319).

6.2.2 Response to vasodilators:

The current data reveal both depot-specific and metabolic impact of obesity on endothelium-dependent micro vascular dilator mechanisms in humans. The OM vessels from PO and DO groups were particularly affected, suggesting a local impact that is exaggerated by the metabolic status of the individuals. Thus, OM depot is more heavily implicated in metabolic syndrome and more extensively infiltrated with immune-inflammatory cells, such as macrophages and T-lymphocytes (86), whereas, the subcutaneous depot appears less metabolically active, secrete more leptin and less free fatty acids (79). The depot specificity of the endothelial dysfunction is consistent with previous report (86) and attributable to the more pathogenic changes reported for OM compared with the SC depot (86).

However, unlike a previous report, the current data showed continuing NO involvement (27 %) in Ach relaxation of these vessels, albeit reduced by about 10 % compared with SC vessels. Reactivity in the previous report referred was measured by imaging, and so, the difference in technique might be a factor in the difference in NO involvement in OM vessel relaxation reported. Differences in the pathogenicity of the OM depot of the two obese populations (Caucasian vs Qatari) might also explain the differences observed. Typically Ach vasodilatation is attributed to NO release from the endothelium (116), although additional mediators are known to be involved in the small vessels (117). NO relaxes blood vessels by activating soluble Gauanylyl cyclase (sGC) in the VSMC leading to the generation of cyclic guanine monophosphate (cGMP) and the activation of protein kinase G (PKG) which among other things will activate K+ channels leading to relaxation (241, 292).

Since NO alone could not fully account for Ach relaxation of these vessels, the role of 2 other known endothelium-dependent dilator mechanisms (EDHF and prostanoids) were explored. In small vessels, the contribution of EDHF becomes more apparent when endothelium-dependent vasodilation persists in the face of inhibition of NOS (292**,**321). EDHF accounted for 44% of maximum relaxation to Ach in the OM vessels and 64 % in the SC vessels. This suggests even a greater contribution by EDHF to endothelium-dependent dilatation of these vessels compared with NO. Since EDHF played a bigger role in the SC compared with OM vessels, its contribution could not be linked to endothelial dysfunction in these vessels. Therefore the current data shows that the widely held suggestion (based on animal vessels) that EDHF may act as a back-up vasodilator system when NO production or bioavailability is impaired (294,322,323,324) is not the case in human adipose microvessel. Moreover, similarly large EDHF contribution to dilatation of SC vessels from apparently healthy humans has been reported (293). However, it could not be determined if EDHF contribution to OM vessel dilatation was altered in this study as there is no normal reference, although this could not be ruled out. Interestingly the EDHF responses in SC and OM vessels appear to activate different K channel mechanisms to hyperpolarize the vascular smooth muscle. Whereas, Kir was demonstrably involved in OM response, it was not as clear in the SC vessel

where a different K channel appears to be involved. Other K channels that can be involved in EDHF response include BK_{Ca} and K_V (325) The EDHF response in SC vessels probably involves another type of K channel such as BK_{Ca} or Kv channels which are other known candidates for EDHF response in blood vessel). The term endothelium-derived hyperpolarizing factor (EDHF) was coined (247) for molecule (s) of yet unknown identity, released by the endothelium which then hyperpolarizes the underlying vascular smooth muscle to produce vasodilatation. A number of candidates have been reported as EDHF in different vascular beds, including K^+, H_2S, EET, CytP450, PGI2, and even NO (325). The EDHF candidate (s) released by the adipose micro vessels in this study are not known but EDHF-mediated hyperpolarization of human vessels has been shown to involve the release of K^+ from endothelial cells, and the subsequent activation of Kir or Na-K-ATPase leading to relaxation (326). Even though the candidates in this study were not identified, they share the characteristic of requiring the activation of both SK_{Ca} and IK_{Ca} channels of endothelial cells prior to their transfer to vascular smooth muscle cells. Hence, the responses were sensitivity to the combination of charybdotoxin and apamin (327) which inhibit IK_{Ca} and SK_{Ca} respectively and typically used to block EDHF response. Overall, the data show that NO and to a greater extend EDHF, are involved in endothelium-dependent dilatation of both OM and SC vessels from obese individuals. While NO and EDHF could fully account for Ach relaxation of SC vessels, this is not the case with OM vessels that clearly show endothelial dysfunction. Since prostanoids such as prostacyclin (PGI2) and PGE2 could mediate endothelium-dependent dilatation, the role of COX pathway was explored to see whether that would explain the residual Ach relaxation recorded. COX inhibition with indomethacin resulted in an enhancement instead of a reduction in Ach relaxation. This was particularly profound in OM vessels where hitherto the response to Ach was attenuated. The data following COX inhibition suggested that increased contractile prostanoid activity was largely responsible for the attenuation in Ach relaxation of these vessels. This is consistent with recent report which suggested that endothelial dysfunction in visceral fat is driven in part via increased activity of the eicosanoid/cyclooxygenase pathway in human obesity. As with

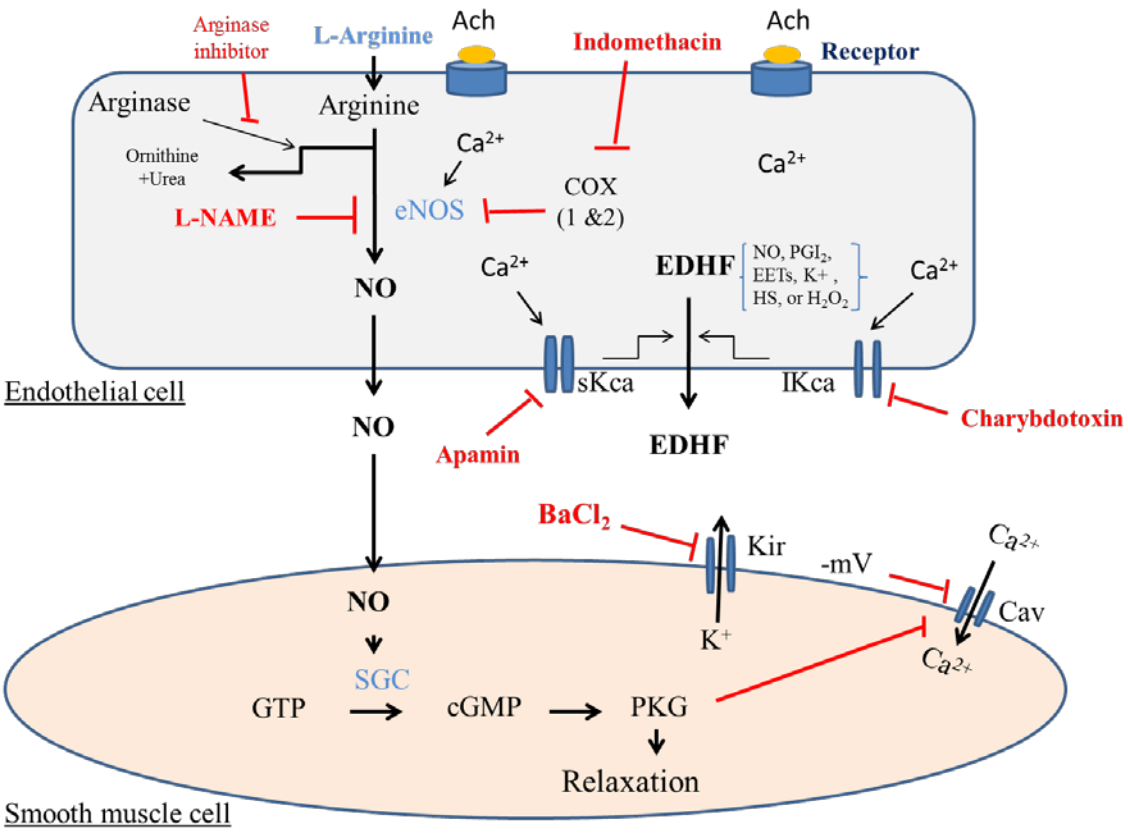
current data, COX inhibition did not change Ach response in the SC vessels where vasodilator function was relatively preserved. COX products have also been shown to impair NO vascular activity in other human diseases such as hypertension (328) and chronic renal failure (329). Although the COX isoform responsible for heightened contractile prostanoid activity in this study was not determine, COX1 was suggested in previous human studies (184). Both COX1 and COX2 isoforms have been implicated in mechanisms of endothelial dysfunction via generation of vasoconstrictor prostanoids in animal models with isoform specificity relating to disease phenotype (325,327,330). In diabetes, due to increased synthesis of vasoconstrictor prostanoids and endothelin (161). Endothelin-1 activates endothelin A receptors on VSMC to enhance vasoconstriction. Diabetes also induces PKC activity, NF-κβ production, and generation of oxygen-derived free radicals in VSMC, similar to these effects in endothelial cells (165). However, dysregulation of vascular smooth muscle function is exacerbated by impairments in sympathetic nervous system function (166).

6.3 Conclusion

The data so far show clear differences between OM and SC vessels obtained from morbidly obese Qataris in respect of endothelium-dependent relaxation and noradrenergic sensitivity, with more marked alteration in the vascular mechanisms in the OM compared with SC. The results also show altered noradrenergic (density/function) sensitivity and attenuated endothelium-dependent relaxation compared to SC arterioles and abnormal endothelial function appears to be due to decreased NO bioavailability, which may underlie these depot specific responses. The latter appears to be at least in part due to increase in COX antagonistic activity.

6.4 Limitation of the study:

i. The major limitation, perhaps due to cultural reasons, was that it took longer to recruit patients into the various studies as many were unwilling to provide signed consent for participation in any research.
ii. It was difficult to determine how much the various surgical procedures would have impacted on the responsiveness of the tested vessels as the numbers were too small to carry out such comparisons, and
iii. Due to small number of diabetic patients some of the data skewed.



<u>Figure 34:</u> Proposed mechanisms of endothelium-dependent relaxation of adipose tissue micro vessels. Activation of the endothelial cell by a dilator agonist such as Ach, leads to release of NO or EDHF which is transferred to the underlying smooth muscle cell to cause its relaxation. NO activates soluble guanylyl cyclase which generates cGMP which in turn activate PKG leading to relaxation of the vascular smooth muscle cell. On its side, EDHF activates K channels (e.g Kir) causing efflux of K+ and hyperpolarization of the smooth muscle cell. Hyperpolarization of the smooth muscle cell leads to closure of voltage sensitive Ca^{2+} channels on its membrane and leading to relaxation. The current data suggests that COX activity is increased in the favour of contractile prostanoids which appear to work against these dilator mechanisms particularly the NO mechanism thereby contributing to the endothelial dysfunction recorded. Drugs used to study these pathways and their sites of actions are indicated in red.

6.5 Future studies

- Continue the study for endothelium dependent relaxation especially for EDHF mechanisms between vessels from the 2 depots and the groups of patients.
- To assess changes in the involvement of ion channels for K+, Ca+ channels.
- Reprocess the embedded paraffin block for histology to look the differences between the MHO and PO patients.
- Assessment of changes in proteins related to vascular functions by western blot.
- Confirming hypertension gene array results by checking the validity for single PCR.

6.5.1 Immunohistochemical analysis.

6.5.1 .1Estimation of adipocyte number, size and capillary density

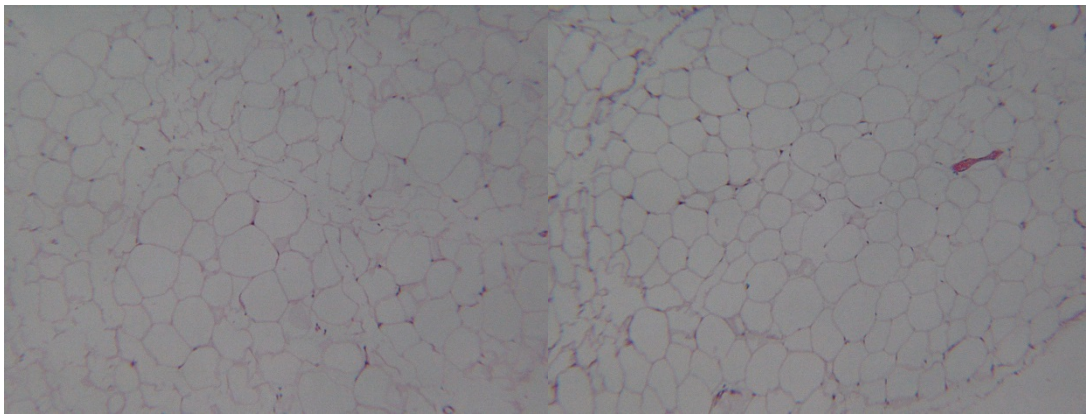
Paraffin processing was performed by the Histology Department at HMC., Doha, Qatar. Briefly 0.3g tissue from each depot was fixed in 10% formalin for 24h at room temperature and then transferred to 50% ethanol at +4^{o}C prior to being embedded in paraffin. 3 μm sections were deparaffinized and dehydrated by incubation in Xylene (Sigma-Aldrich, UK) for 20 min followed by incubation with 100, 90, 80, 70, and 60% ethanol for about 2 min for each step.

Lectin staining (Mahrokh Nahadani, UCL, UK) was used as a marker of capillaries. Slides were rinsed with 0.1M sodium phosphate buffer (PBS, pH 7.4) for 20 min. The sections were incubated for 30 min in a dark moist container with the staining solution containing lectin−fluorescein isothiocyanate (FITC)-GS conjugate (from Griffonia simplicifolia; Sigma-Aldrich), 25 μg/ml, lectin tetramethylrhodamine isothiocyanate conjugate (TRITC)-UEA conjugate [from Ulex europaeus (UEA), Sigma-Aldrich], 10μg/ml, and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) 0.3μg/ml (Sigma-Aldrich, UK). The GS-lectin stains the endothelium and generally used as a marker of capillary; UEA stains the capillaries and DAPI stains

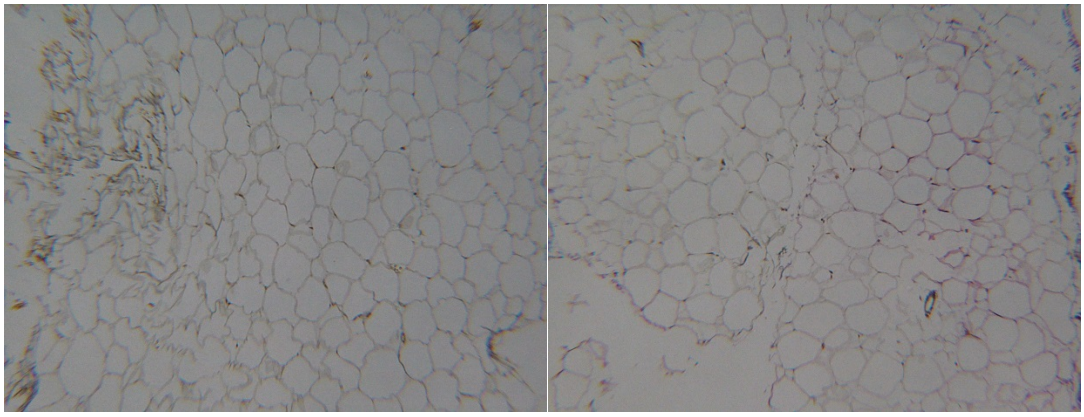
the nuclei. The sections were rinsed with PBS (40 min) and then mounted on microscope slides with water-soluble mounting medium (Cardinal Health, Dublin).

Images of the stained sections were captured with a Zeiss Axioplan 2 upright microscope (Intelligent Imaging Innovations, Denver, USA) using a Photometrics CoolSnap HQ CCD camera and a Sutter Lambda LS 175W Xenon arc lamp. A planar Apochromat 20x/0.75 objective lens, three filters sets (DAPI-EX 360/40, FITC-EX HQ487/25, and CY3-EX HQ535/50) and Slidebook Software version 2.0 were used for image capture.

Samples were processed and embedded at histology department in HMC. Images were stained and captured as shown in the figure 35.



H & E stain for SC & OM



EVG stain for SC & OM

<u>Figure 35:</u> A typical example of the various stained sections of SC and OM adipose tissue shows poorly stained adipocytes.

Chapter 7

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