Insulin transcytosis across adipose microvascular endothelial cells: Mechanism and physiological implications

by

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Abstract

Transport of insulin across the microvasculature is necessary to reach its target organs (e.g. adipose and muscle) and is rate-limiting in insulin action. We report an approach to study insulin transcytosis across primary human adipose microvascular endothelial cells (HAMEC), involving insulin uptake followed by vesicle-mediated exocytosis visualized by total internal reflection fluorescence microscopy. In this setting, fluorophore-conjugated insulin exocytosis depended on its initial binding and uptake, which was saturable. Unlike its degradation within muscle cells, insulin was stable within HAMEC and escaped lysosomal colocalization. Insulin transcytosis required dynamin but was unaffected by caveolin-1 knockdown or cholesterol depletion. Instead, insulin transcytosis was significantly inhibited by both a clathrin-mediated endocytosis inhibitor and siRNA-mediated clathrin-depletion. Accordingly, insulin internalized for 1 minute in HAMEC colocalized with clathrin far more than with caveolin-1. This study constitutes the first evidence of vesicle-mediated insulin transcytosis and highlights that its initial uptake is clathrin-dependent and caveolae-independent.

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Contributions

Unless otherwise indicated, all experiments were performed by Paymon M. Azizi.

Paymon M. Azizi and Roman E. Zyla performed the experiments in Figure 4-4 and Figure 4-5.

Sha Guan performed the experiments in Figure 4-9, Figure 4-13, and Figure 5-6.

Paymon M. Azizi and Changsen Wang were responsible for isolating human adipose microvascular endothelial cells (HAMEC) from donor adipose tissue obtained from Steffen-Sebastian Bolz's lab.

Jun Liu produced the ICAM image in Figure 4-1.

Data from Figure 5-8 was obtained in the lab of Wolfgang Kuebler with the help of Arata Tabuchi.

Bryan Heit was responsible for writing the mathematical scripts that quantify the number of exocytosis events.

Paymon M. Azizi, Amira Klip, and Warren L. Lee were responsible for designing the experiments and directing the project.

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List of abbreviations

AF555	Alexa Fluor 555
AF568	Alexa Fluor 568
ANG	Angiopoietin
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
Cav1	Caveolin-1
DAG	Diacylglyceride
DAPI	4',6-diamidino-2-phenylindole
DN	Dominant-negative
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
FPS	Frames per second
GFP	Green fluorescent protein
GLUT	Glucose transporter
HAEC	Human aortic endothelial cell
HAMEC	Human adipose microvascular endothelial cell
HEV	High endothelial venules
HRP	Horseradish peroxidase
HSC70	Heat shock cognate 7
HUVEC	Human umbillical vein endothelial cell
ICAM1	Intercellular adhesion molecule 1
ICAM-1	Intercellular adhesion molecule 1
IDE	Insulin degrading enzyme
IGF1R	Insulin like growth factor 1 receptor
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
MBCD	Methyl-beta-cyclodextrin
MEK	Mitogen-activated protein kinase
MEM	Minimum essential media
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PA	Palmitate
PDGF	Platelet-derived growth factor
PDK	Phosphoinositide-dependent kinase
PECAM1	Platelet endothelial-cell adhesion molecule 1
PHLPP	PH domain and leucine rich repeat protein phosphatases
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PO	Palmitoleate
PP2A	Protein phosphatase 2A
PSLG1	P-selectin glycoprotein ligand 1
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-tyrosine phosphatase 1B
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SOS	Son of Sevenless
TEER	Transendothelial electrical resistance
TIRF	Total internal reflection fluorescence
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VCAM1	Vascular cell-adhesion molecule 1
VEGF	Vascular endothelial growth factor
VENIRKO	Vascular endothelial cell IR knockout
VLA4	Very late antigen 4
VVO	Vesiculo-vacuolar organelles
vWF	von willebrand factor
ZO-1	Zona occulden 1

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Chapter 1

Literature Review

1 LITERATURE REVIEW

1.1 Insulin action

1.1.1 Insulin

Insulin is an anabolic hormone that serves to regulate the metabolism of carbohydrates, lipids, and proteins. The active hormone consists of two short peptides, totaling 51 amino acids and weighing 5808 Daltons. Insulin is produced in the pancreas by the β cells of the islets of Langerhans. Initially, it is synthesized as a single 110 amino acid polypeptide called preproinsulin [1]. The N-terminus of preproinsulin contains a signal peptide that directs it to the rough endoplasmic reticulum [2]. As preproinsulin translocates across to the lumen of the rough endoplasmic reticulum, the signal peptide at the N-terminus is cleaved yielding proinsulin [3]. Proinsulin then folds and forms three disulfide bonds with itself before moving to the trans-golgi network [4]. In the golgi, proinsulin is cleaved in two positions to yield a mature insulin and short peptide referred to as C-peptide [1]. Both insulin and C-peptide are stored into concentrated granules. The concentrated nature of the granules causes the crystallization of insulin into hexamers around a zinc atom [5].

In response to the appropriate stimuli (i.e. elevated blood glucose), the β cells of the pancreas release insulin granules into the interstitial space of the pancreas. The insulin hexamers break apart into monomers, and then enter into the microvasculature of the pancreas through fenestrations in the endothelium. From there, insulin enters first the portal circulation followed by its dissemination to the systemic circulation.

1.1.2 Metabolic actions of insulin

Insulin is an important hormone in regulation of energy homeostasis. It has a broad range of actions, influencing metabolism of carbohydrates, lipids, and proteins. Insulin shifts the metabolic axis of cells such that carbohydrates are become primary fuel while discouraging the use of lipids. Moreover, insulin encourages anabolic activities by increasing storage of

energy. Here, we briefly describe the actions of insulin on metabolism of carbohydrates, lipids, and proteins.

Carbohydrate metabolism

Insulin is best known for its effects on the metabolism of carbohydrates. Insulin signalling in muscle and adipose cells increases the uptake of glucose. This is achieved by the translocation of the glucose transporters to the plasma membrane, thus allowing glucose from the interstitial fluid to enter the cell [6]. In addition, insulin stimulates hexokinase and 6-phosphofructokinase in the muscle and adipose cells to promote glycolysis [7, 8]. Concurrently, insulin increases glycogen synthesis in muscle and liver by inducing expression of glycogen synthase [9]. Finally, insulin acts on the liver to inhibit gluconeogenesis by suppressing expression of phosphoenolpyruvate carboxykinase [10].

Lipid metabolism

Insulin has many effects on lipid metabolism on the adipose, liver, and muscle. In general, insulin discourages the use of lipids as an energy source. Insulin decreases the rate of lipolysis by inhibition of hormone-sensitive lipase [11]. Insulin also inhibits fatty acid oxidation in many tissues including the adipose and muscle. This is achieved by inhibition of carnitine palmitoyltransferase-1, which is responsible for transporting fatty acids into mitochondria [12]. Finally, insulin signalling in the liver also influences cholesterol metabolism. Synthesis of cholesterol and very-low-density lipoprotein in the liver are increased in response to insulin [13].

Protein metabolism

Insulin also has a role in regulating the body's protein levels [14]. Insulin decreases the rate of protein degradation in a number of tissues, particularly the skeletal and cardiac muscle [15, 16]. Moreover, stimulation with insulin has been shown to increase rates of protein synthesis [17]. Finally, insulin can increase the transport of some amino acids into tissues [13].

1.1.3 Insulin signalling

The insulin receptor is a tetrameric complex, containing two alpha subunits that are completely extracellular, and two beta subunits that each contain one intracellular, transmembrane, and extracellular domains [18]. Once insulin is bound to the insulin receptor, it induces a conformational change in the beta subunit, causing autophosphorylation of tyrosine residues on the receptor, thus putting it into the active state [18]. The insulin receptor, now activated, phosphorylates a number of substrates including insulin receptor substrate (IRS) family of proteins. Activated IRS proteins activate phosphoinositol 3 kinase (PI3K) by interacting with its p85 regulatory subunit [19]. The regulatory subunit then heterodimerizes with a catalytic subunit (of which there a few isoforms) to form functional PI3K. Activated PI3K is then recruited to the plasma membrane where it catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 in turn, activates phosphoinositide-dependent protein kinase 1 (PDK). PDK is responsible for activating a number of kinases, particularly Akt which is responsible for mediating many of insulin's downstream actions [20].

Akt is an important regular of many cell processes including cell growth, survival, proliferation and metabolism. All three isoforms of Akt (Akt1, Akt2, and Akt3) are activated in response to insulin, but their actions differ. Akt1 is suggested to have a role in regulating cell survival, as knockdown of Akt1 in mice retards growth and increases the rates of apoptosis without having much of an effect on glucose homeostasis [21, 22]. Mice lacking Akt2 are insulin resistant, indicating Akt2 has an important role in metabolism [23, 24]. The third isoform of Akt has been implicated to have a role in neural development, as mice lacking this isoform exhibit normal growth and metabolism, but have a smaller brain size [25].

One of the most important functions of insulin in regulating metabolism is the stimulation of glucose uptake from the circulation into the tissues. Insulin-stimulated Akt mediates glucose uptake in tissues such as the muscle and the adipose by increasing the abundance of glucose transporter type 4 (GLUT4) at the plasma membrane [26]. GLUT4, like other GLUT proteins, is a transmembrane protein responsible for facilitating passive transport

of glucose into cells [27]. GLUT4 is the primary component by which insulin mediates its effects to lower blood glucose. Under basal conditions, there is a relatively large intracellular component of GLUT4 in storage vesicles that is mobilized to the membrane in response to insulin signals emanating downstream of Akt [28, 29].

A major Akt target is the Rab GTPase activating protein AS160, which targets Rabs 2A, 8A, 10, 13 and 14. Rab family proteins are members of the Ras superfamily of monomeric G proteins that regulate many aspects of membrane traffic including the intracellular mobilization of GLUT4. Under basal conditions, AS160 inactivates Ras GTPase by promoting their self-mediated hydrolysis of GTP to GDP [30]. However, under insulin stimulation, Akt phosphorylates AS160 and prevents its inhibitory actions on Rabs, thus leaving them in the active state. Rabs 8A and 13 have major roles in GLUT4 translocation in muscle cells, whereas Rab10 has a dominating role in adipose cells [31, 32].

In addition to mediating glucose uptake in the muscle and adipose tissues, insulin has many other tissue-specific effects. In both muscle and liver, insulin functions to promote glucose uptake and storage in the form of glycogen by activating hexokinase and glycogen synthase [10]. In the liver specifically, insulin inhibits gluconeogenesis via inhibition of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. In the adipose tissue, insulin decreases the rate of lipolysis. This is achieved by Akt activation of phosphodiesterase 3B which ultimately leads to reduction of hormone-sensitive lipase [33].

One of the important actions of insulin are is effects on the vasculature by mediating vasodilation. Insulin induces vasodilation of arteries and arterioles via Akt mediated phosphorylation and activation of endothelial nitric oxide synthase (eNOS). eNOS is a very important enzyme in regulating vasomotor tone by producing nitric oxide (NO), which acts on surrounding smooth muscle cells in the wall of the blood vessel (the *tunica media*; see Figure 1-2) to induce a vasodilatory response. Vasodilation of the arteries and arterioles increases blood flow to the insulin sensitive tissues and increases perfusion of the microvasculature, ultimately increases the insulin delivered.

Insulin exerts some of its effects, for example gluconeogenesis, through the regulation of gene expression. In this case, signalling is independent of the PI3K-Akt pathway and instead involves the mitogen-activated protein (MAP) kinase ERK [34]. A key element in this signalling pathway is the protein Grb2 which contains an SH2 domain, allowing it to bind to phosphorylated IRS and recruit the guanine nucleotide exchange factor Son-of-sevenless (SOS). Alternatively, Grb2-SOS be activated through an IRS independent pathway involving the insulin receptor through activation of the protein Shc. Grb2-SOS then recruits Ras, activating it initiating the Ras–ERK cascade. Ras activates mitogen-activated protein kinase kinase (MEK) which then activates ERK. Activated ERK can now phosphorylate a number of targets. In addition to regulating gluconeogenesis, some of these targets regulate cell growth. The insulin-ERK signalling also has important roles in endothelial biology as it induces expression of the vasoconstrictor endothelin-1 [35].

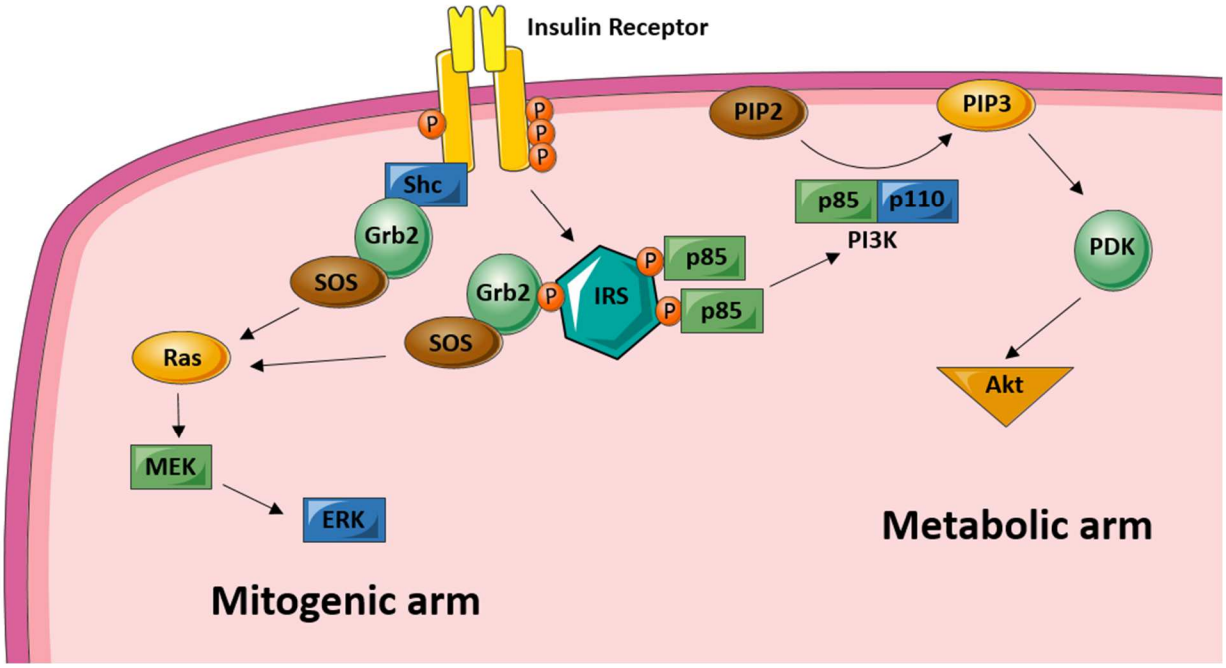

Figure 1-1 Schematic for insulin signalling

Insulin signalling pathway in many cells involves two signalling cascades, the metabolic and mitogenic arms. The metabolic arm is responsible for activating Akt, which in turn is responsible for increasing glucose uptake in most cells. The mitogenic arm activates ERK, which is responsible for regulating various aspects of cell growth. Figure created using templates from Servier Medical Art.

1.1.4 Termination of insulin signalling

Insulin signalling needs to be a transient process to ensure blood glucose levels do not decline too far as that would have serious side effects. There are a few mechanisms in place to ensure termination of the insulin signalling cascade. The first level of control is at the level of insulin. As insulin is cleared by the kidney and the liver, the circulating insulin concentration decreases. Moreover, interstitial insulin is removed as the interstitial fluid is removed by the lymphatic circulation [36]. The decline in insulin concentrations results in decreased activation of the insulin signalling cascade.

The second level of control occurs at the level of the signalling cascade and is mediated by proteins that inhibit actions of key targets in the insulin signalling cascade (i.e. insulin receptor, PI3K, and Akt). A number of phosphatases play an important role in regulating the activity of the insulin receptor, and thus terminate the signal when insulin levels decline. One important phosphatase identified for the insulin receptor is the tyrosine phosphatase protein-tyrosine phosphatase 1B (PTP1B). PTP1B removes phosphotyrosine residues off of the insulin receptor, thereby terminating the insulin signalling cascade [37]. PTP1B's role in the insulin signalling cascade is highlighted in mice that lack this phosphatase. These mice are much more insulin sensitive and are resistant to weight gain [38].

Phosphatase and tensin homolog (PTEN) is a phosphatase that regulates insulin signalling at the site of PIP3 [39]. PTEN hydrolyzes a phosphate on PIP3 to generate PIP2 thus preventing further activation of PDK and its downstream effectors. PTEN is very important in tumor progression, as loss of the gene is found in many cancers [40]. Moreover, is required for embryonic development, as knockout mice die at E9.5 [41, 42].

Protein phosphatase 2A (PP2A) and PH domain and leucine rich repeat protein phosphatases (PHLPP) are two phosphatases important in regulating insulin signalling at the level of Akt. PP2A removes phosphate groups from the residue Thr308 Akt [43]. PHLPP dephosphorylates Akt at Ser473 [44]. PHLPP has two isoforms, PHLPP1 and PHLPP2, which are

responsible for dephosphorylating different Akt isoforms. PHLPP1 dephosphorylates Akt2 and Akt3 while PHLPP1 removes phosphates from Akt1 and Akt3 [45].

A final regulator of the signalling of insulin also occurs at the level of the receptor and its abundance. Upon binding of insulin to the insulin receptor, the complex may be internalized by and degraded. The complex has been shown to enter cells through a caveolar process, which is expected as the insulin receptor often resides in caveolae [46]. The degradation of the complex is responsible for not only reducing circulating insulin levels, but also down regulating the abundance of insulin receptor at the plasma membrane, thereby decreasing the sensitivity of the cell to insulin.

1.1.5 Insulin resistance

Insulin is an important regulator of metabolism and as described earlier, has vast implications on many organs. Impairments in insulin function (termed insulin resistance), is one of the precursors to type 2 diabetes. Although the exact cause for insulin resistance is not known, many different *in vitro and in vivo* models for insulin resistance have been developed. The nature by which many different conditions (a couple are described below) can induce the insulin resistance phenotype suggests that there are many different etiologies for the pathology. Individually interfering with the function of the muscle, adipose, pancreas, endothelium, immune cells, etc. can cause insulin resistance in an animal model.

1.1.5.1 *Factors inducing insulin resistance*

Inflammation

Insulin resistance and type 2 diabetes has also been associated with a chronic low-grade inflammatory response. Individuals with type 2 diabetes often have elevated inflammatory markers in their plasma including TNF-α, C-reactive protein, IL-6, plasminogen activator inhibitor-1 [47, 48]. Moreover, the inflammatory IκB kinase (IKK) and c-Jun N-terminal kinase

(JNK) signalling pathways have been shown to be activated in adipose tissue and liver of obese mice [49]. These signalling pathways can be activated through a number of stimuli including TNF-α, IL-6, free fatty acids, diacylglyceride (DAG), ceramide, and reactive oxygen species [50]. Stimulation of IKK and JNK both inhibit the insulin signalling pathway.

Stimulation of cells through an inflammatory pathway (i.e. TLR4) activates IKK, which is composed of three subunits: IKKα, IKKβ, IKKγ. Active IKK phosphorylates IRS1 on serine residues inhibiting the insulin signalling cascade [51]. Moreover, IKK activates the inflammatory NFκB signalling pathway [52]. During unstimulated states NFκB is localized to the cytoplasm, bound to inhibitory IκB proteins. Active IKK targets IκB for degradation via phosphorylation on two serine residues (Ser32 and Ser36). The phosphorylated IκB is then ubiquitinated and sent for proteasomal degradation, allowing NFκB to translocate to the nucleus and initiate transcription. Similarly, JNK inhibits IRS1 by phosphorylating IRS1 at Ser307 [53].

Although it is evident that there is a relationship between inflammation and insulin resistance, targeting inflammation through anti-TNF, anti-IL-1, and anti-IL-6 treatments have not shown to be viable as a treatment option for insulin resistance [54].

Mitochondrial dysfunction

Mitochondria are organelles responsible for generating most of the useable energy of the cell in the form of adenosine triphosphate [55]. In terms of energy conversion, mitochondria are the site of the citric acid cycle and the electron transport chain. Many fatty acids undergo β oxidation in the mitochondria as well. Moreover, mitochondria have a role in regulation of apoptosis [56] and maintaining calcium homeostasis [57].

Mitochondrial dysfunction has been implicated in pathogenesis of insulin resistance. Genetic mutations altering mitochondrial function have been associated with insulin resistance and glucose intolerance [58, 59] suggesting that impairments in mitochondrial function may lead to diabetes. Moreover, oxidative capacity of muscle from type 2 diabetes is impaired for

both glucose and lipids [60]. Many animal insulin resistant models also show impaired mitochondrial function in many tissues including adipose, liver, and heart [61-63].

There are a number of potential mechanisms by which mitochondrial dysfunction may cause insulin resistance. It has been suggested that impaired lipid oxidation at the level of the mitochondria results in accumulation of ectopic lipid [64]. Metabolites of the lipid such as DAG and ceramides may then inhibit insulin signalling via activation of various protein kinase C (PKC) isoforms [65]. Elevated DAG can activate PKCθ which in turn inhibits IRS1 via serine phosphorylation [66]. Ceramides on the other hand can activate PKCζ that bind to and inhibit Akt to impair insulin signalling.

Another potential link between mitochondrial dysfunction and insulin resistance is at the level of reactive oxygen species (ROS). This was initially suggested when antioxidants administration decreased insulin resistance in cell culture and animal models [67]. Later studies noted that insulin resistant and obese mice and humans have elevated levels of ROS [68]. Moreover, insulin resistance is lessened when these mice are administered mitochondrial specific antioxidants. This suggests that nutrient oversupply is elevating ROS and thereby inducing insulin resistance. Although ROS has been implicated in insulin resistance, the mechanism by which ROS induce insulin resistance is not yet known [69].

1.2 Metabolism of insulin

1.2.1 Cellular degradation of insulin

Almost all insulin sensitive tissues have the capacity to degrade insulin. Degradation of insulin begins shortly after endocytosis in the early endosome by the insulin-degrading enzyme (IDE) [70, 71]. IDE is active at pH values of 6.0 - 8.5 so it becomes inactive once the endosome becomes too acidic. IDE is a zinc metalloprotease that is responsible for the degradation of a number of short peptides. For example, IDE has been shown to degrade glucagon, TGF-α, and amyloid beta (whose accumulation is a hallmark of Alzheimer's disease) [72]. In the endosomes, the B chain of insulin is cleaved a number of times and the disulfide bond is cleaved [73]. Insulin's by-products are further degraded as the endosome matures into a lysosome [74]. The acidic pH, alongside other proteases supplements the degradation of insulin [75].

1.2.2 Insulin clearance by liver

Before insulin enters systemic circulation it first moves through the hepatic portal circulation and undergoes the first-pass metabolism by the liver. Approximately 50% of insulin produced by the pancreas is removed before it reaches systemic circulation [76]. Insulin destined for degradation is primarily taken up by a receptor-mediated process and targeted to degradative compartments. Insulin that is destined for degradation follows the classical route whereby it is initiated in the endosomes by IDE, and further continued in the lysosome. However, not all insulin taken up is degraded as some is released back into the interstitium by the hepatocytes.

The overall functional ability of the liver is correlated with its capacity to remove insulin. Early on, an association was found between obesity and diabetes with impaired insulin clearance in humans [77]. Moreover, lower insulin clearance rates were measured in subjects with diseased livers [78]. More specifically, fat content in the liver is inversely proportional to insulin clearance and even circulating free fatty acids are related to impaired insulin clearance [79].

1.2.3 Insulin clearance by kidney

Once insulin is in the systemic circulation, the primary route by which it is cleared is via the kidney. At this point, the kidney is responsible for removing 50% of the remaining circulating insulin (i.e. approximately 25% of the total insulin produced) [80]. Initially, insulin in the blood enters the nephron as it is filtered through the glomerulus. As the insulin moves through the nephron, it is reabsorbed by the cells of the proximal tubule by an endocytic process [81]. Only small amounts of insulin are found in urine, indicating that most of it is taken up by the kidney. Most of the internalized insulin is degraded (approximately 85%) but the rest is transported to the other side of the cell where it is then released [82]. The proximal tubule cells of the kidney degrade insulin similarly compared to other tissues. The role of the kidney in insulin removal is much more important in diabetic patients that take insulin, as injected insulin (and its analogs) evades first pass removal by the liver [83].

1.2.4 Insulin clearance by other tissues

Almost all insulin-sensitive tissues have the ability to degrade insulin. Once insulin is endocytosed, likely with the insulin receptor, its degradation begins in the early endosomes with the function of IDE. The by-products of endosomal degradation move to the lysosome where degradation is completed.

1.3 **Endothelial cells**

The circulatory system is an organ system used to transport nutrients while collecting wastes from all parts of the organism. One fundamental component of the circulatory system is the endothelium, which spans each and every tissue. The endothelium represents a monolayer of endothelial cells that comprise the inner cellular lining of blood and lymphatic vessels.

1.3.1 Vascular Beds

The circulatory system contains three categories of vessels: arteries, veins and capillaries (Figure 1-2). Arteries are defined as vessels that carry blood away from the heart while veins are vessels that move blood towards the heart. In most cases oxygenated blood flows through arteries and deoxygenated blood flows through the veins. The only exceptions are in the pulmonary and umbilical circulation, where the opposite occurs.

The various components of the circulatory system have specific and unique functions that are reflected by their differing structures. The outer layer of both arteries and veins, termed the tunica externa (or tunica adventitia), is composed of connective tissues [84]. In arteries, the tunica externa is supported by the external elastic lamina that as its name suggests is a layer of elastic tissue. The middle layer, termed tunica media, contains layers of smooth muscle cells and elastic tissue. Arteries contain a much larger tunica media, enabling it to withstand the high pressure experienced in arterial blood flow. The inner most layer of arteries and veins is the endothelium, which is separated from the tunica media by an elastic membrane. A unique property of veins is the presence of valves which prevent the backflow of blood. Both arteries and veins have smaller counterparts, called the arterioles and venules respectively. Arteries and arterioles play an important role in directing blood flow as they relax and constrict. The final component of the circulatory system are the capillaries. They are the smallest component of the circulatory system and contain a single layer of endothelial cells. Capillaries are the primary site of nutrient exchange, as will be described below.

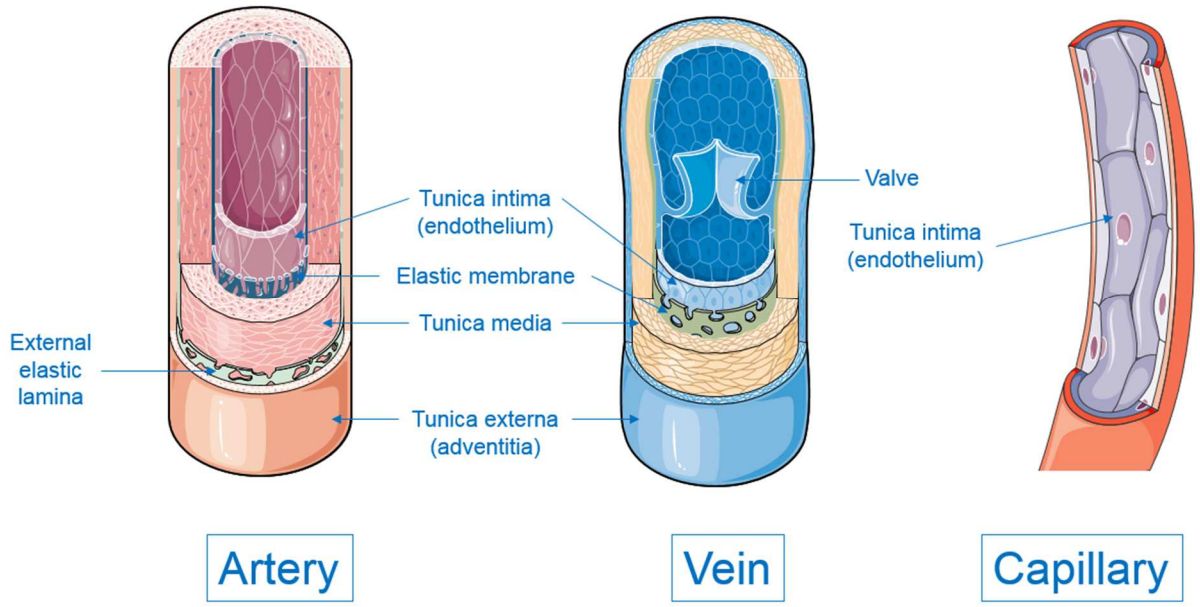

Figure 1-2 Structure of arteries, veins, and capillaries

Arteries, veins, and capillaries greatly vary in structure. Arteries and veins are large vessels containing many different layers of cells. A major component of arteries are the smooth muscle cells found in the tunica media that contribute to the elasticity and vasomotor tone of the vessel. Veins are thinner structures, containing fewer smooth muscle cells. Capillaries are the smallest component of the vasculature and contain only a layer of endothelial cells and their basement membrane. Note: Vessels are not drawn to scale. Figure created using templates from Servier Medical Art.

1.3.2 Endothelial heterogeneity

The diverse cellular compositions of different parts of the body create various niches with differing functions and metabolic requirements. The endothelium must be heterogeneous in structure and function in order to provide for the needs of the different parts of the body. For example, endothelial cells found in the capillaries are very important regulators of both paracellular and transcellular nutrient exchange, the two primary processes whereby material can cross the endothelium. Paracellular leak describes the passive diffusion between cells while transcellular transport is an active process where cells transport material from one side of the cell to the other. Both paracellular leak and transcellular transport will be described in greater detail below. In contrast, endothelial cells found in the arteries are often not involved in nutrient exchange, but instead are more important in regulating vasomotor tone. Understandably, the development of specialized organ-specific properties by endothelial cells is crucial in the function of the different organs.

1.3.3 Mechanisms maintaining endothelial heterogeneity

Arising from a common precursor called angioblast, endothelial cells differentiate and acquire organ-specific properties depending on their interaction with their microenvironments. Biomechanical and biochemical factors are crucial determinants for endothelial cell differentiation. These factors culminate to differences in endothelial cell phenotype, including variances in cell morphology and gene expression.

In vivo, endothelial cells are constantly subjected to shear stress acting on its apical surface as a result of blood flow [85]. Shear stress results in distinct phenotypes through alteration of gene expression profile, including production and secretion of growth factors (e.g. PDGF) [86], vasoactive substances (e.g. endothelin-1) [87], and adhesion molecule expression (e.g. ICAM-1). Shear stress also promotes actin cytoskeleton rearrangement [88]. For example,

aortic endothelial cells, which experience greater pressure and shear stress from blood flow originating from the heart when compared to any microvasculature, have a more spindle-shaped appearance as F-actin filaments rearrange in order to align with the direction of blood flow. The integrity of the actin cytoskeleton is crucial in resisting fluid-imposed shear stress [89].

The interaction of endothelial cells with their microenvironments also affects their differentiation mainly due to the secretion of growth factors, cytokines, and hormones by surrounding cells. For example, cells that comprise the blood brain barrier receive biochemical support from closely associated cells including perivascular endfeet, astrocytic glia, pericytes, microglia, and neuronal processes. These differing cell types synergistically induce changes in endothelial cell phenotype, especially given the complexity of the barrier properties of the blood brain barrier. Studies have shown that astrocytes can regulate many aspects of the blood brain barrier, causing tighter tight junctions to provide a stronger physical barrier [90] and an increase in expression of transporters such as Pgp [91] and GLUT1 [92]. Astrocytes mediate these changes in blood brain barrier features through their secretion of growth factors such as glial-derived neurotrophic factor [93] and angiopoietin-1, which promote tight junction formation [94].

An additional factor regulating endothelial cell heterogeneity is their attachment site, which can include other cells or extracellular matrix, materials which have a wide range of elastic moduli. Cell growth, survival, and morphology depend on substrate stiffness. Cellular response to substrate or matrix stiffness differ between cell types and to be highly dependent on the adhesion receptors by which the cell binds its substrate [95].

Alterations in substrate stiffness can also be a determinant for endothelial cell phenotype. As endothelial cells respond to changes in their mechanical surroundings, they rearrange their cytoskeleton in order to adjust the contractile forces exerted. They further modify their biological behaviour, differentially secreting various factors to aid in withstanding their environment. The effect of substrate stiffness on the mechanical and functional behaviour of endothelia ultimately determines their barrier, with enhancing or disruptive responses [96].

Birukova et al. [97] showed that both microvascular and macrovascular endothelial cells increase their expression of F-actin stress fibers as substrate stiffness increases. Alterations in matrix stiffness are associated with various pathologies such as cardiovascular disease, diabetes [98], aging [99], and tumor progression [100]. One outstanding example is seen in lung fibrosis, where the stiffness of the lung parenchyma increases 6 – 8 fold relative to the normal range of 0.5 – 3 kPa [101]. Therefore, under pathological conditions, alterations in vascular barrier function due to changes in the endothelial microenvironment contribute to the severity of endothelial dysfunction. This notion is clearly evident in the given example of lung fibrosis where there is an increase in both lung stiffness and vascular permeability.

Overall, it is increasingly recognized that the microenvironment by which specific endothelial cells are found contribute to endothelial heterogeneity. Factors including biomechanical (shear stress) and biochemical (adjacent cells) all contribute to the resulting phenotype and function endothelial cells display. This dynamic heterogeneity of endothelial cells not only allow them to conform to the specific needs of their underlying tissues, but also confer a fitness advantage as they are more capable of adapting to diverse environments.

1.3.4 Structural and functional heterogeneity

1.3.4.1 *Shape*

The phenotypic differences between endothelial cells from different vascular beds reflect their various functions and specific interactions with their microenvironments. Endothelial cells are typically flat, but their thickness can vary from less than 0.1 µm to 1 µm in capillaries and the aorta, respectively [102]. Furthermore, their general shape can also differ. As stated before, biophysical factors can also impose changes in cellular structure. Endothelial cells are aligned with the direction of blood flow, but of course, this cannot be the case in branch points within the vasculature [103, 104]. Endothelial cells restructure their actin cytoskeleton in response to hemodynamic shear stress. For example, endothelial cells comprising the pulmonary artery are broad and short when compared with endothelial cells of the pulmonary

vein which are larger and round in shape, while endothelial cells of aorta, which receives the highest degree of shear stress, are long, narrow, and rectangular [105].

1.3.4.2 *Fenestrated and sinusoidal endothelium*

The endothelium must restrict or limit access of compounds in the blood to the tissues. The endothelium can partition the two by acting as a physical barrier. Aside from the composition of their junctions, endothelium may also be continuous or discontinuous. Continuous endothelium is further subdivided into fenestrated or non-fenestrated. Fenestrations are transcellular pores (70 nm in diameter) that extend from the luminal side to the basolateral side of the endothelial cell, allowing for more rapid diffusion of larger molecules [84]. Fenestrated continuous endothelium is found in organs with high filtration and transendothelial transport such as in glands, intestinal mucosa, and glomeruli. Conversely, non-fenestrated continuous endothelium is found in the muscle, adipose, brain, lung, heart, and skin. Discontinuous endothelium is found mainly in sinusoidal vascular beds, such as in the liver which requires a less restricted access to whole blood [84]. These possess larger fenestrations relative to fenestrated continuous endothelium and also contain gaps to facilitate diffusion of solutes as the liver plays a critical role in the filtration of blood.

1.3.4.3 *Junctions*

The proteins that hold endothelial cells together, termed junctional proteins, are also uniquely organized along the vasculature in an organ-specific manner. Two main types of junctions recognized in the endothelium are adherens and tight junctions. Adherens junctions are responsible for cell contact and signalling while tight junctions are primarily responsible for mediating paracellular permeability. However, there is considerable overlap in their functions. However, despite having common features, adherens and tight junctions are formed by different proteins [106]. Adherens and tight junctions will be discussed in some detail in the paracellular permeability section below.

1.3.4.4 *Hemostasis*

Hemostasis refers to an intricate system maintaining blood in a fluid state. Platelets play a critical role in maintaining hemostasis as they play a pivotal role in clot formation in lieu of damage to the vascular wall. However, unregulated clot formation in cases of thrombosis is detrimental as it may cause vascular occlusion. Under quiescent conditions, endothelial cells prevent thrombus formation through the release of various anti-coagulants, anti-platelet, and fibrinolytic factors [107]. Endothelial heterogeneity allows for differential hemostasis in the different parts of the vasculature, where different vascular beds express distinct repertoires of anti- and pro-coagulants [108].

Prostacylin is one of the major anti-platelet factors produced and secreted by endothelial cells. As an inhibitor of platelet activation, prostacyclin induces the relaxation of vascular smooth muscle cells and decreases leukocyte-endothelial cell interaction [109]. Similarly, nitric oxide (NO) is an important vasodilator and inhibitor of platelet activation [110]. NO and prostacyclin act together to additionally reverse platelet aggregation [111]. Anticoagulant factors produced by endothelial cells include thrombomodulin [112] and heparin sulfate [113], providing a non-thrombogenic surface along the length of the endothelium. Endothelial cells also produce and secrete fibrinolytic factors such as tissue plasminogen activator as well as its inhibitor plasminogen activator inhibitor-1, thereby controlling the balance between inefficient and excess blood clot formation [114].

The endothelium plays additional roles in hemostasis. In response to trauma resulting in injury to vascular wall, endothelial cells produce vasoconstrictors (e.g. endothelins) to limit blood flow to the area [115]. Hemostatic plug formation is mediated by von Willebrand factor (vWF), produced by endothelial cells, which acts as a ligand for platelet binding in sites of endothelial injury [116]. Interestingly, insulin resistance is a prothrombotic condition due to endothelial dysfunction resulting in upregulation of vWF (i.e. increased platelet aggregation) and PAI-1 (decreased fibrinolysis) [117].

1.3.4.5 *Leukocyte interaction*

The endothelium plays a critical role in the regulation of immune responses, namely through the exit and re-entrance of leukocytes to and from various organs. Leukocyte extravasation refers to the movement of immune cells from circulation to the underlying tissue towards sites of tissue damage or infection (Springer, 1994). This process, critical to the innate immune response, requires a succession of specific cell-cell contacts between the immune and endothelial cell. It involves an initial attachment of the leukocytes to the endothelium, subsequent rolling, arrest, and transmigration [118, 119]. Leukocyte-endothelial cell-specific adhesion are largely determined by the hemodynamic shear stress present in the microvasculature and the expression of adhesion glycoproteins on the surface of both leukocytes and endothelial cells [120]. Given these two crucial determinants, leukocyte extravasation mainly occurs in post-capillary venules [121] at specialized sites called high endothelial venules (HEVs) [122]. Endothelial cells composing HEVs are cuboidal in shape [123] – a stark contrast from the typical flat morphology of endothelial cells. This difference in shape of HEVs result in increased turbulence, minimizing shear stress [124] and allowing more contact between leukocytes and the endothelium [123, 125]. Furthermore, HEVs have increased capacity to respond to inflammatory mediators resulting in the upregulation of both basal and induced adhesion receptor molecules [126]. However, leukocyte extravasation is not limited to HEVs. In cases of acute inflammation, the endothelium of the inflamed site have induced adhesive properties that allow for local leukocyte extravasation [127].

The initial step of the leukocyte extravasation cascade involves an initial adherence and rolling of leukocytes along the length of the endothelium [128]. Endothelial and platelet (E- and P-) selectins expressed by activated endothelial cells, as well as leukocyte (L-) selectin expressed solely by leukocytes synergistically mediate rolling of leukocytes along the apical surface of the endothelium [129]. Interactions of each selectin with their ligands (e.g. P-selectin glycoprotein ligand 1 (PSLG1) for all three selectins [130, 131], E-selectin ligand 1 and CD44 for E-selectin, and CD34 and podocalyxin for L-selectin [132]) allow leukocytes to adhere to the activated

endothelium despite constant blood flow. Aside from selectins, integrins also play a role in the initial attachment and subsequent rolling of leukocytes along the endothelium surface. For example, lymphocytes expressing the cell surface ligand very late antigen 4 (VLA4) adhere to endothelial cells expressing vascular cell-adhesion molecule 1 (VCAM1) [133], while $\alpha_1\beta_2$-integrin on lymphocytes adheres to intercellular adhesion molecule 1 (ICAM1) [134]. Notably, tumor necrosis factor (TNF) induction of E-selectin and ICAM1 decreased the speed of neutrophil rolling along HEVs *in vivo* [135, 136], indicating that strong adhesion of leukocytes to the endothelium is synergistically mediated by both selectins and integrins. Current evidence shows that the activation of spleen tyrosine kinase upon engagement of PSLG1 by E- or P-selectins [137]. Furthermore, selectin activation induces activation of signalling pathways involved in dynamic cortical actin cytoskeleton reorganization [reviewed in [138]], such as the p39 mitogen-activated protein kinase (MAPK)-dependent pathway [139].

Firm adhesion and subsequent arrest is mediated by the presence of chemoattractants and the binding of leukocyte integrins to adhesion molecules ICAM-1 and VCAM-1 [140]. As stated previously, activated endothelial cells have increased expression of surface adhesion molecules in their apical membrane. However, the activated endothelial cell also synthesize and express lipid chemoattractants and chemokines (e.g. interleukin-8) on their luminal membrane [141] in order to facilitate the transendothelial movement of immune cells. Strengthened binding of VCAM-1 and ICAM-1 to the integrins VLA4 and lymphocyte function-associated antigen 1 (LFA1) allows for rapid leukocyte rolling arrest [142, 143]. This also activates reorganization of cortical actin to form a docking site composed of adhesion receptor aggregates and other focal adhesion proteins such as talin [144]. This prevents the unbinding of the adhered leukocyte in lieu of physiological flow.

Migration through the endothelium is the final step of leukocyte extravasation, which is mainly thought to undergo a paracellular route. Activated endothelial cells reorganize their junctional proteins in order to favor cell migration. For example, VE-cadherin are moved away from junctional regions [145], while junctional proteins with ligands found on leukocytes such as platelet endothelial-cell adhesion molecule 1 (PECAM1) are mobilized to the membrane

[146]. Many studies suggest that leukocytes are able to transmigrate transcellularly (through endothelial cells) in addition to the widely accepted notion of passing through the endothelium paracellularly (in between endothelial cells). [147, 148]. Continuous membrane-associated passageways termed vesiculo-vacuolar organelles (VVOs) are gateways with which immune cells can traverse the body of an endothelial cell [149]. Leukocytes may also be transcellularly transported with caveolin-1, as mediated by ICAM-1 binding [150, 151]. Whether or not different vascular beds employ one route over the other has not yet been answered. A prominent example where this may be the case is with the blood brain barrier. The endothelium composing the blood brain barrier has an intricate network of junctional proteins that may make it difficult for leukocytes to transmigrate through the paracellular route. Therefore, in such a case, leukocyte may elect to pass through transcellularly, much like how most solutes and fluids pass through the blood brain barrier.

Leukocyte extravasation may also occur in other segments of the vascular tree apart from the post-capillary venules. For example transmigration and sequestration of leukocytes occur in alveolar capillaries during lung injury and/or inflammation [152, 153]. Furthermore, the cascade of leukocyte transmigration does not involve the rolling mechanism when occurring in the lung and liver capillary endothelium, suggesting that leukocyte-endothelial interactions are microvasculature- and organ-specific.

Importantly, increased leukocyte extravasation into adipose tissue is associated with adipose tissue dysfunction. Inflammation of the adipose tissue has been implicated in the development of insulin resistance, as obese individuals with relatively less visceral fat inflammation remain partially insulin-sensitive [154]. Increased leukocyte recruitment, namely macrophages, to adipose tissue is widely viewed to be due to hyperproliferation of adipocytes which produce chemotactic signals [155]. However, the role of the microvasculature in the development of adipose tissue inflammation cannot be dismissed. Specifically, angiogenesis of the adipose microvasculature, due to expanding adipose tissue, through the activation of hypoxia-inducible factor 1α (HIF-1α) has been implicated in visceral fat inflammation [156]. Additionally, nutrient-overload by free fatty acids (FFA) and glucose as seen in obese individuals

is associated with adipose microvascular dysfunction [157, 158]. Reduced AMPK activity in endothelial cells due to high exposure to FFA can cause upregulation of P-selectin through decreased endothelial NO levels, as witnessed in both animal models [159, 160] and in humans [161].

1.3.4.6 *Vasomotor tone*

Arteries and arterioles have the capability to constrict and relax, thereby altering the diameter of the vessel and thus altering blood flow. Vasomotor tone is important in directing blood flow to desired organs, but it is also responsible for regulating the perfusion of the microvasculature.

Vasomotor tone is modulated through a number of mechanisms including sympathetic innervation and endothelial interactions [162]. The tunica media of many vessels are innervated by the sympathetic nervous system. Release of neurotransmitters have the capability of both relaxing and constricting the vessel through activation of α and β adrenergic receptors.

Endothelial cells have an important role in regulating the vasomotor tone of arteries and arterioles as they are a source of various regulatory substances that interact with the smooth muscle cells of the vessel. Endothelial cells can release vascular relaxants such as nitric oxide (NO) and prostacyclin [163]. Opposing this, endothelial cells also release factors that induce constriction of the vessel such as endothelin-1 [115] and angiotensin-II [164].

Role of eNOS in regulating vasomotor tone

Endothelial nitric oxide synthase (eNOS) is a critical component of the endothelium in maintaining vasomotor tone. This is especially important in the context of insulin, as one of the downstream targets of the insulin signalling cascade in endothelial cells is eNOS. eNOS is able to regulate vasomotor tone by producing NO, which relaxes the surrounding smooth muscle. In

addition to regulating vasomotor tone, NO is also important in preventing infiltration of leukocytes and thrombus formation [165].

eNOS is one of the three isoforms of nitric oxide synthase. The other two isoforms are inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) and they are very similar in structure and function. The primary enzymatic reaction by eNOS is the production of NO and L-citrulline from L-arginine. To function, eNOS forms homodimers [166] and requires a number of cofactors: tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide [167]. Moreover, eNOS function is modulated by calmodulin as it can increase the rate of the reaction.

eNOS is activated in response to many different signalling pathways, flow induced shear stress, and changes in oxygen levels [168]. Moreover, eNOS localizes to the plasma membrane, which may assist in its activation from stimuli such as shear stress [169]. eNOS can be activated at a number of serine residues to become active Ser116, Ser617, Ser635, or Ser1179 [170]. Different signalling pathways are responsible for phosphorylating specific sites (i.e. Akt phosphorylates Ser617 and Ser1179).

1.3.5 Paracellular permeability

As mentioned previously, endothelial cells are paramount in maintaining a barrier between surrounding tissues and the circulation. The endothelium is semipermeable and regulates the transport of solutes and fluids from the blood to tissues. The major site for continuous flux of materials through the endothelium takes place in capillaries. Fluids and smaller molecules are thought to passively diffuse through paracellular gaps between individual endothelial cells, while larger molecules pass through transcellularly. Transcellular transport may be receptor-dependent or independent [171]. Endothelium from different parts of the body have varying degrees of permeability, owing to their structure (continuous, fenestrated, or discontinuous), the composition of their junctional properties, and their differing capacities to mediate transcellular transport. For example, mice lacking claudin-5 have increased vascular

leakage in their blood brain barrier [172], showing that permeability is dependent on the complexity of the endothelial junctional properties. Endothelial permeability can also be induced, such as by inflammation [173]. The post-capillary venule is most susceptible to induced permeability as it is the main site for leukocyte extravasation [174]. The regulation of paracellular permeability involves the participation of the molecular elements descried on the following sections (1.3.5.1 – 1.3.5.3).

1.3.5.1 *Adherens junctions*

Adherens junctions are mainly composed of cadherins. A major constituent of the adherens junction is the adhesion protein VE-cadherin (vascular endothelial cadherin), which is only expressed in endothelial cells [175, 176]. VE-cadherin is believed to be responsible for the architectural integrity of endothelium which is a necessary for proper function of tight junctions [177]. This is supported by the fact that adherens junctions formation precedes the formation of tight junctions [178]. In addition, VE-cadherin is suggested to regulate contact inhibition, the process whereby endothelial cells stop dividing when they become confluent. Human umbilical endothelial cells expressing a mutant form of VE-cadherin proliferate even after forming a confluent monolayer [179, 180].

VE-cadherin, like most cadherin proteins, contain a cytoplasmic tail, a single transmembrane domain, and five extracellular domains [181]. VE-cadherin interacts with other VE-cadherins molecules from both the same cell (cis) and neighboring cells (trans) [182]. This process is mediated by the extracellular domains and is calcium dependent [183]. The cytoplasmic tail of VE-cadherin binds to Armadillo-repeat gene family proteins and are responsible for tethering and anchoring VE-cadherin to the cytoskeleton.

The Armadillo-repeat gene family includes a number of catenins, and are responsible for the correct structural and signalling functioning of VE-cadherin. The c-terminus of VE-cadherin

binds a number of proteins from the armadillo repeat family including p120-catenin, β-catenin and plakoglobin [184]. β-catenin binding to the cytoplasmic tail of VE-cadherin induces the cytoplasmic tail to develop a more organized structure [185]. Moreover, it masks an epitope that can be ubiquitylated, thus preventing VE-cadherin's degradation [186]. Plakoglobin, also known as γ-catenin, binds to the same location on VE-cadherin as β-catenin at a later stage to strengthen the junction [187, 188]. Moreover, plakoglobin has a role in stabilizing adherens junctions (9374777). p120-catenin also binds to the cytoplasmic tail of VE-cadherin as well, but much closer to the plasma membrane [189]. p120-catenin is responsible for regulating and recruiting kinases, phosphatases, and GTPase-activating proteins [190]. Together these are responsible for maintaining the structural integrity of the adherens junction and regulating the interaction with other cadherins. α-catenin is another protein involved in protein complex on the cytoplasmic tail of VE-cadherin. α-catenin is responsible for linking the entire VE-cadherin complex to the cytoskeleton as it can bind both β-catenin and actin filaments [191, 192].

VE-cadherin's role in maintaining endothelial barrier integrity is underscored by studies impairing its function. Genetic deletion of VE-cadherin causes mice to die during development at stage E9.5 [193]. Moreover, injection of anti-VE-cadherin antibodies into mice to disrupt the junctions induced a significant increase in vascular permeability especially in the heart and lung [194], suggesting that the relevance of VE-cadherin mediated regulation of vascular permeability varies along the vascular tree.

1.3.5.2 *Tight junctions*

Tight junctions are considered the key regulators of maintaining vascular barrier integrity in arterial beds under high pressure, while adherens junctions have been shown to play a critical role in the microvasculature [195]. Tight junctions, whose major constituents include claudins, and occludins, separate the membrane into apical and basolateral regions and restricts permeability of solutes to the interstitium [106]. Tight junctions display considerable variability along the vascular tree, where they are better developed and organized in arteries

and veins relative to veins and post-capillary venules [196]. Tight junctions are looser in post-capillary venules, reflecting its role in the dynamic trafficking of immune cells and plasma proteins following an inflammatory episode. A stark contrast is seen in the blood brain barrier where endothelial cells are well developed and rich in tight junctional proteins in order to protect the brain from any fluctuations in blood composition. In general, tight junctions are less organized in organs where there is high rate of trafficking solutes [197].

Occludins

The occludin family of proteins were the first tight junctional proteins to be discovered. The intracellular component zonula occludens-1 (ZO-1) was first described in 1986 in epithelial cells, while its transmembrane counterpart, occludin, was found in 1993 [198]. Occludin, is a 65kDa transmembrane protein. It contains 4 transmembrane domains, three intracellular domains, including the N and C terminus, and two short extracellular domains that are 46 and 48 amino acids in length [199]. The C terminal portion of the protein has been shown to mediate associations with occludin's adaptor proteins [200]. Occludin interacts with a number of adapter proteins (ZO-1, ZO-2, and ZO-3) that tether it to the actin cytoskeleton [201]. Interfering with either its adapter proteins or truncating the C-terminus of the occludin impairs its traffic to the plasma membrane. The extracellular domains of occludin are responsible for maintaining the stability of the tight junction [202]. Intriguingly, occludin knockout mice are viable, however they suffer from a number of disorders including "chronic inflammation and hyperplasia of the gastric epithelium, calcification in the brain, testicular atrophy, loss of cytoplasmic granules in striated duct cells of the salivary gland, and thinning of the compact bone" [203].

Claudins

Claudins are a major constituent of tight junctions. Similarly to occludin, most claudins form a barrier that prevents paracellular leak. However, some claudins are unique in that they create pores to allow small charged molecules to diffuse across. Currently, only claudins 2, 10, 15, and 17 have been shown to form pores [204].

Like occludin, claudins contain four transmembrane helices and contain two extracellular domains, but only a single intracellular domain [204]. The first extracellular domain is considerably larger than the other, and is primarily responsible for the regulation of permeability of the tight junction [205]. The second extracellular domain is primarily responsible for mediating interactions between claudins [206]. Claudin's function also heavily relies on its many adaptor proteins. Claudin contains a PDZ binding motif at its C terminus that allows it to bind to a number of adapter proteins including ZO-(1, 2 and 3), MAGI-(1, 2, and 3), and MUPP-1 [204]. These adapter proteins connect claudins to the cytoskeleton.

Claudins are found in a number of cell types, but are enriched in epithelial and endothelial cells. Claudin 5, is the only claudin specific to endothelial cells, and is the most abundant claudin expressed [207]. Other claudins, however, are also found in endothelial cells, such as claudin-1, 2, 3, 10, and 12. Claudins generally form homodimers with claudins from neighboring cells, however, claudin 3 has been reported to interact with other claudin proteins [208].

The role of claudins in regulating paracellular permeability are highlighted in knockout studies. Claudin-5 deficient mice die shortly after birth due to increased permeability of the blood brain barrier [172]. Mice lacking claudin-1 also die shortly after birth due to dehydration as a result of impaired tight junction function in the epithelium [209]. Claudin-3 has an important role in regulating permeability at the blood brain barrier. Many pathologies that disrupt the blood brain barrier also result in a loss of claudin-3 expression [210].

1.3.5.3 *Size restriction of paracellular leak*

The endothelial barrier is highly permeable to small molecules, but highly impermeable to large macromolecules. This is regulated by the complex interplay of adherens and junctional proteins holding endothelial cells together. The molecular radius of compounds is one factor that determines whether a molecular is capable of diffusion across paracellular gaps. This has been demonstrated using endothelial cells cultured onto transwells [211-213]. Molecules with

known molecular radii were labelled and added to the top chamber of the transwell while appearance into the bottom chamber was measured. The authors saw an inverse relationship between permeability and size. For instance, Inulin, which has a molecular radius of 15Å has a capacity to diffuse across the endothelium that is 30% of mannitol (3.9Å radius that can freely diffuse. The capacity to diffuse stops as molecules reach a radius of around 36Å which is reflected by the observation that albumin (36Å radius), plasminogen (45Å radius), and fibrinogen (106Å radius) all had the same permeability.

An important factor to note is that many of these studies have been performed using macrovascular endothelial cells. However, microvascular endothelial cells have been shown to form tighter junctions and restrict diffusion via paracellular leak [96] indicating that these studies may be overestimating the size selectivity of paracellular leak. Moreover, it is important to note that endothelial cells grown in culture do not achieve the same level of paracellular tightness or integrity as in vivo.

1.3.6 Endothelial dysfunction

The endothelium is a complex organ system responsible for many different functions. Proper functioning of the endothelium is necessary to maintain homeostasis and as a result, many diseases are associated with impaired endothelial function. Peripheral vascular disease, stroke, hypertension and atherosclerosis, diabetes, chronic kidney failure, and some forms of cancer are influenced by diseased endothelium [214]. Impaired endothelial function, termed, endothelial dysfunction, is characterized by a state in which the endothelium has impaired vasodilation, is pro-inflammatory, and has pro-thombotic properties.

Healthy endothelium has the capacity to respond to vasodilators such as prostacyclins and NO and they are present in relatively high amounts. Moreover, under normal physiological conditions, the endothelium is anti-inflammatory and anti-thrombotic. As a result, it has no or very little expression of adhesion molecules such as ICAM, E-selectin, and P-selectin. Healthy endothelium also does not release many pro-inflammatory cytokines such as TNF-α and IL6.

Diseased endothelium, on the other hand, behaves the opposite. It becomes much less responsive to vasodilatory compounds. There is an increase in the amount of reactive oxygen species and other free radicals. Endothelial cells will begin to express adhesion molecules and they will produce pro-inflammatory cytokines. Finally, the endothelium has more difficulty repairing itself, especially since there are reduced circulating endothelial progenitor cells.

1.3.6.1 *Endothelial function and dysfunction in diabetes*

Insulin action on peripheral tissues such as the muscle and adipose relies on the vasculature and proper endothelial function. The microvasculature is responsible for delivering insulin efficiently. Under basal conditions, the entire capillary bed responsible for supplying a particular organ is not perfused [215]. However, after insulin is released into circulation, it can act on areas of the vasculature to increase perfusion of the microvasculature and thus increase the surface area available for nutrient exchange.

The process of insulin mediated capillary recruitment is dependent on both arms of the insulin signalling pathway (metabolic and mitogenic) in endothelial cells. Insulin signalling in endothelial cells through the metabolic pathway activates eNOS via Akt. Active eNOS then begins to produce NO, which diffuses into its neighbouring smooth muscle, relaxing it. On the mitogenic arm, insulin signals through ERK to increase expression of endothelin-1, a potent vasoconstrictor. Intriguingly, the interplay of insulin's vasodilatory and vasocontrictive actions on different aspects of a vascular network actually increases the surface area available for perfusion. NO release on terminal arterioles increases blood flow to the capillaries and increases microvascular perfusion. This is demonstrated in experiments where eNOS inhibition impedes insulin mediated microvascular perfusion [216]. Endothelin-1 also acts to increase microvascular perfusion, but by a completely different mechanism. Endothelin-1 is thought to act on non-nutritive vascular networks, essentially shunting blood to the capillaries, thereby increasing the blood available to the nutritive microvascular network [217].

Endothelial cells and the vasculature clearly have a very important role for insulin delivery that is important for its action. This relationship has been known for quite some time as obesity and insulin resistance were shown to be associated with endothelial dysfunction in the 1990's [218]. It has been suggested that endothelial dysfunction precedes development of type 2 diabetes [219].Moreover, many insulin resistant animal models also display impaired endothelial function. For instance infusion of rats with lipid [220] or TNF-α [221] impairs capillary recruitment. In addition, knocking out key regulators of vasomotor tone, such as eNOS, causes insulin resistance [222].

1.4 Transcytosis

Continuous endothelial cells are held together with many adherens and tight junctions that limit paracellular leak of large molecules such as insulin, albumin, and low density lipoprotein. These molecules, however, need to cross the endothelium to gain access to their target tissues and they do so via transcytosis. Transcytosis is the process whereby a cell selectively moves cargo inside a vesicle from one side of the cell to the other side. Transcytosis integrates endocytosis, traffic, and exocytosis. It is a fundamental cellular process that occurs in multiple cell types (i.e. endothelial cells, epithelial cells, and neurons) [223]. Transcytosis is necessary to transport large material across a layer of cells while maintaining the general compositions of the two environments. Here we describe two mechanisms of endocytosis, clathrin- and caveolae-mediated, and how they are implicated in transcytosis.

1.4.1 Clathrin-mediated endocytosis and transcytosis

Clathrin-mediated endocytosis is the most extensively studied route of receptor-mediated endocytosis by which eukaryotic cells internalize extracellular material. The endocytic machinery relies on the main component clathrin and its associations with various adaptor proteins culminating in the formation of a lattice-like coat around vesicles. This coat mediates cargo internalization and subsequent delivery to various organelles. Clathrin is composed of a heavy and light chain, assembling into a trimer or triskelion where the heavy chains of the three clathrin monomers are linked through their C-terminus to form a triangular shape [224]. This formation of clathrin into a triskelion allows it to form a lattice-like network around vesicles [225]. The process of clathrin-mediated endocytosis includes initiation and pit formation, coat assembly, scission, and eventual disassembly of the clathrin cage.

Clathrin triskelia are not capable of binding directly to the membrane [226]. Other factors known as adaptor proteins are required to mediate the binding of clathrin to the membrane and are also responsible for initiating membrane curvature formation [227]. The adaptor protein acting as the master initiator for clathrin-mediated endocytosis is yet to be

elucidated, but many proteins to fulfill this role have been proposed including adaptor protein 2 (AP-2) and FCH domain only 1 and 2 (FCHo1/2). AP-2 a heterotetramer [228], consisting of α, β_2, σ_2, and μ_2 subunits, which binds to clathrin as mediated by its α and β_2 appendages [229]. AP-2 associates clathrin with the cytosolic side of the plasma membrane by binding to membrane-bound PIP2 as mediated by its core domain composed of the σ_2 and μ_2 subunits [230]. Evidence suggesting that AP-2 initiates clathrin-mediated endocytosis is largely from depletion studies. siRNA knockdown of AP-2 in mammalian cells resulted in a decrease in membrane-associated clathrin [231, 232] and a complete blockage of transferrin uptake [231]. Another study suggested that clathrin-mediated endocytosis is initiated by the membrane association of two AP-2 molecules with one clathrin triskelion determined by single-molecule tracking [233]. However, there are some studies against the role of AP-2 as the initiator for clathrin-mediated endocytosis. Studies in yeast cells show that AP-2 is not necessary for killer toxin uptake, nor does its deletion have any effect on endocytosis dynamics [234]. Furthermore, AP-2 was not found to be essential in the uptake of EGFR [235] and LDLR [236]. Another adaptor protein that was found to mediate membrane-association of clathrin are FCHo1 and 2. Both FCHo1/2 contain F-BAR domains that are capable of binding to the membrane [237]. Depletion of FCHo1/2 by siRNA decreased both membrane bound clathrin-AP-2 complexes and transferrin uptake [238] indicating that it may arrive to endocytic sites before recruitment of AP-2 [237]. In support of this, increased transferrin uptake and endocytic sites were observed after overexpression of FCHo1/2 (176204409). Aside from both AP-2 and FCHo1/2, epsins, clathrin assembly lymphoid myeloid leukemia (CALM), and AP-180 have also been implicated in the initiation of clathrin-mediated endocytosis [239-241]. These cases, however, are cargo-specific. For example, CALM and epsin, but not AP-2, is necessary for Notch ligand uptake [239]. This suggests that the initiator for clathrin-mediated endocytosis may include multiple clathrin adaptor proteins in a cargo-specific manner.

Clathrin-mediated endocytosis is dynamin-dependent [242]. Dynamin is a mechanochemical enzyme which polymerizes, through GTP binding [243], and coats the membrane neck of the invaginated clathrin-coated vesicle [244]. Utilizing the GTPase domain in its N-terminus, dynamin provides a torsional resulting in the scission of the clathrin-coated

vesicle. Although the precise mechanism is not known, dynamin is thought to undergo a conformational change upon polymerization favouring GTP hydrolysis [245, 246]. This notion is supported by the use of non-hydrolyzable GTP analogues allowed for the formation of invaginated coated pits, but did not allow mature vesicle formation [247]. Furthermore, pharmacological inhibition of dynamin blocks clathrin-mediated endocytosis at the level of vesicle scission [248, 249]. However, this also blocks other endocytic processes that depend on dynamin-mediated membrane fission, including caveolae-dependent endocytosis [246].

After dynamin-mediated scission, clathrin-coated vesicles roughly 50 – 100 nm in size are subsequently uncoated before the vesicle is allowed to fuse with its target endosome. Disassembly of the clathrin coat to individual triskelia is reliant on heat shock cognate 7 (HSC70), an ATPase [250], and its cofactor auxilin [251]. Auxilin is capable of binding to clathrin and AP-2 [252]. It recruits HSC70 to the site of clathrin-coated vesicle formation [253] and stimulates HSC70's ATPase activity [254]. Notably, an increase in fully assembled clathrin-coated vesicles has been observed in auxilin-deficient cells [255]. Through the use of single-particle tracking, Böcking and colleagues [255] elegantly showed that auxilin binding to clathrin causes displacement of the triskelion legs, allowing HSC70 to access its target segment within the C-terminus of the clathrin heavy chain. Binding of a single HSC70 to its target segment stimulates its ATPase activity, locking the protein in place and distorting the clathrin lattice. Accumulation of clathrin-bound HSC70 further destabilizes the lattice-like formation of clathrin resulting in disassembly [256]. Resolution of clathrin-mediated endocytosis, namely by the release of AP-2 and other endocytic accessory proteins associated to membrane-bound PIP2 is performed by the lipid phosphatase synaptojanin [257].

1.4.1.1 *Role of clathrin in transcytosis*

The role of clathrin has also been implicated in transcytosis. For example, the cerebral endothelium has been identified to transcytose iron bounded to transferrin receptor, from the apical to basolateral side of the endothelium, through a clathrin-mediated route [258]. Clathrin-mediated transcytosis also plays a role in immunological protection. Antigen sampling by which antigens are delivered from the apical to basolateral surface of M cells to the mucosal-

associated lymphoid tissue underneath is crucial in mucosal immune responses [259]. These sampled antigens are transcytosed through clathrin-coated vesicles [260]. Furthermore, dimeric IgA, another important mediator of the mucosal immune response, is transcytosed (basolateral to apical) by epithelial cells in the digestive tract and liver and secreted into the gut lumen and bile, respectively, in a clathrin-dependent manner by binding to its receptor pIgA-R [261-263].

1.4.2 Caveolar endocytosis and transcytosis

Caveolae are small 50 - 80nm vesicles that extend from cholesterol rich areas of the plasma membrane [264]. Caveolae have been implicated in the endocytosis and transcytosis of a number of compounds including insulin [265], albumin [266], low-density lipoprotein [267], and some chemokines [268].

Caveolae are found on almost all cell types but some cell lineages will have more than others. For instance, endothelial cells, adipocytes, and skeletal muscle cells have much higher abundance of caveolae compared to other cell types [269-271]. Moreover, there can be a great deal of heterogeneity of caveolar abundance within a cell type. Microvascular endothelial cells have many more caveolae than macrovascular endothelial cells [272]. Also, microvascular endothelial cells from the muscle have almost 10 fold more caveolae than what is found in the lung microvasculature [223].

1.4.2.1 *Role of caveolins*

Formation of caveolae are mediated by its coat protein caveolin. There are three isoforms of the caveolin protein: caveolin-1, caveolin-2, and caveolin-3 [273, 274]. Caveolin-1 is the most important component of caveolae and thus has been investigated the most. Knockdown of caveolin-1 is sufficient to deplete cells of caveolae [275] while expression of caveolin-1 in cells devoid of caveolin-1 is enough to induce formation of caveolae [276]. Caveolin-1 has been shown to homo-oligomerize, which is thought to be important in formation of the caveolae [277]. Caveolin-1 is predominantly expressed in endothelial cells and adipocytes [278]. Two isoforms of caveolin-1 are expressed endogenously: α and β. Caveolin-2

is a non-essential caveolin isoform, as caveolae still form after deletion of the protein [279]. Caveolin-2 forms hetero-oligomers with caveolin-1 [280] and requires expression of caveolin-1 to localize to the appropriate cellular compartments [275]. Consequently, caveolin-2 expression profiles parallel those of caveolin-1. Coexpression of both caveolin-1 and caveolin-2 results in more uniform and abundant caveolae [281, 282]. The final isoform of the caveolin proteins, caveolin-3 is only found in muscle cells [273]. Like caveolin-1, expression of caveolin-3 in cells lacking caveolin proteins is sufficient to cause formation of caveolae [283].

Caveolin proteins undergo many posttranslational modifications. Caveolin-1 is palmitoylated on a few cysteine residues near the C-terminus [284]. The role of palmitoylation of caveolin-1 is not yet known. Intriguingly it is not required for targeting of the protein to the plasma membrane or for its incorporation into caveolae. Caveolin proteins are also phosphorylated on tyrosine and threonine residues. Caveolin-1 can be phosphorylated on tyrosine 14 in response to multiple signals including oxidative stress [285], growth factor and hormonal stimulation [286] and integrin activation [287]. Phosphorylation of caveolin-1 on tyrosine 14 is important in caveolar endocytosis [288]. Caveolin-1 can also be phosphorylated on serine 80, however less is known about the biological role of this modification. Phosphorylation on serine 80 has been shown to be important at the level of the endoplasmic reticulum [289].

1.4.2.2 *Role of cavins*

Caveolae also require a number of adaptor and scaffolding proteins to function correctly. Four scaffolding and adapter proteins, cavin-1, cavin-2, cavin-3, and cavin-4 have been identified so far. Cavin-1, also known as polymerase transcript release factor (PTRF), was the first cavin to be described. Cavin-1 has been shown to interact with caveolin-1 in lipid rafts at the membrane [290]. Cavin-1 is also capable of regulating levels of caveolin-1, as overexpression of cavin-1 increases caveolin-1 levels, while knockdown of cavin-1 decreases caveolin-1 levels [291]. Cavin-2 is also known as serum deprivation-response protein (SDPR). It

is required for caveolae formation by regulating both cavin-1 and caveolin-1 levels [292]. Cavin-2 interacts directly with cavin-1 and recruits it to the plasma membrane. Cavin-3 is has been shown to mediate budding of caveolae and also influences traffic of caveolae [293]. Cavin-4 is the most recently discovered cavin. It is found primarily in the muscle and interacts with caveolin-3 [294].

1.4.2.3 *Mechanism of caveolar endocytosis*

Mechanisms of cargo selection of caveolar endocytosis has not been clearly identified. Compared to clathrin-mediated endocytosis, there are no adapter proteins that link receptors to components of caveolae at the membrane. Instead is thought that the cargo specificity for caveolae arises from receptors that localize to caveolar microdomains at the plasma membrane [295]. At these microdomains, interactions between proteins (e.g. Ubiquitin interacting motif) and lipids (e.g. clustering of lipid-tethered proteins) may mediate the formation of the caveolae. Many receptors have been shown to live in caveolar microdomains including the albumin receptor (Gp60) [296], insulin receptor [297] and many G-protein coupled receptors [298].

Caveolar membrane budding requires the interplay of a few components including actin and Src kinases. Local actin polymerization has been shown to be a necessary step of caveolar endocytosis. This is demonstrated when depolymerization of actin with the agent cytochalasin D blocks caveolar endocytosis [299, 300]. Src kinases are a family of tyrosine kinases that have been shown to mediate caveolar endocytosis. They are responsible for phosphorylating a number of proteins involved in the caveolar endocytic pathway including caveolin-1, caveolin-2, and dynamin [288]. The significance of Src kinases is exhibited by studies that show inhibition of Src prevents caveolar formation.

A final step in the caveolar endocytic pathway involves scission of the vesicle from the plasma membrane. This is mediated by the GTPase dynamin which is also responsible for vesicle scission in clathrin-mediated endocytosis [301].

1.4.2.4 *Role of caveolae in transcytosis*

To Date, most work on endothelial transcytosis has been focused on albumin. Albumin is the major protein component in the blood and is responsible for facilitating the systemic transport of many solutes. Albumin is too large to diffuse across endothelial cells [302], so instead moves across the endothelium via transcytosis. Transcytosis of albumin has been demonstrated to move via caveolae. Caveolin-1 deficient mice, which lack caveolae, have impaired transport of albumin [171, 303]. Moreover, interfering with caveolar formation using the pharmacological agent filipin reduces the transcytosis of albumin in culture [304]. Finally, gp60 has been implicated in the receptor responsible for initiating transcytosis of albumin [305].

1.4.3 Evidence for insulin transcytosis

Once in the vascular circulation, insulin needs to cross the vasculature one more time to get access to its target tissue. In the liver, this is not a problem since the vascular bed is discontinuous, allowing whole blood to exit. However, the microvasculature of the muscle and adipose consists of continuous endothelial cells with no fenestrations. As with other macromolecules moving across the endothelium, insulin delivery to the target tissues can happen by passive diffusion between cells (paracellularly) or by active transport through the cells (transcellularly) [306]. Although there is some debate regarding the route through which insulin cross the endothelium, most evidence suggests that it moves by a transcellular route. Firstly, circulating plasma insulin levels are 2-3 fold greater than insulin concentrations in the interstitial fluid or lymph indicating that the endothelium is acting as a barrier [307-309]. Moreover, these studies reported a high correlation between glucose uptake and interstitial or lymphatic insulin levels, while observing much weaker relationships with plasma insulin concentrations [310, 311]. Most importantly, data obtained from dogs using euglycemic clamps and radiolabelled insulin and inulin show insulin kinetics differ from inulin that moves

paracellularly. Instead the data suggest that insulin entry to the tissue is saturable and rate-limiting in insulin-mediated glucose uptake.

It has also been shown that endothelial cells have the capability to internalize and concentrate insulin. Additionally, endothelial cells do not degrade a significant portion of the insulin, which differs from other insulin sensitive cell types. Finally, early work by Johnson and King showed that endothelial cells grown on transwells have the capability to transport insulin and that this process is likely receptor-mediated as serum from type 2 diabetic patients inhibits the transport of insulin.

Chapter 2

Research Aims & Hypotheses

2 RESEARCH AIMS & HYPOTHESES

Given the high prevalence of Type 2 diabetes, there has been extensive research on the mechanisms of insulin resistance. Classically, this has focused on impaired insulin signalling in downstream tissues such as muscle and fat. However, this approach carries the underlying assumption that circulating insulin has unimpaired access to its target tissues and can freely bind its receptor on target cells. In fact, after its secretion into the bloodstream by the beta cells of the pancreas, insulin must first cross the endothelial barrier in order to exit the vasculature. Key physiological studies, performed mostly in dogs, show a delay between injected insulin levels and their appearance in interstitial fluids [307]. Moreover, insulin action in muscle correlates more closely with lymph concentrations of insulin rather than with those in the circulation [311]. Together, these observations suggest that transfer across the endothelium is a rate-limiting step in insulin availability.

In theory, the transit of insulin across the endothelial barrier can occur by passive diffusion between cells (paracellular) or by actual transport through individual cells (transcytosis) [306]. Insulin's size *vis-à-vis* the tight nature of the microvascular endothelium supplying metabolically-relevant tissues like muscle and fat suggest that transcytosis may be the dominant route for its extravasation [309, 312].

Understanding the regulation of insulin transcytosis is important since it may be related to the pathogenesis of insulin resistance [313]. Indeed, reductions in NO production by the endothelium are characteristic of insulin-resistant states; thus it is intriguing that NO was recently found to stimulate insulin permeability across aortic endothelial cells grown on transwells [314].

Despite its importance, surprisingly little is known of the cell biology of insulin transport across the microvasculature, possibly due to lack of an appropriate and physiologically relevant cellular system. Most of the literature on insulin transcytosis has been performed in endothelia from large vessels (e.g. aorta) [314, 315], despite the fact that passage of insulin to tissues *in vivo* occurs selectively in the *micro*vasculature. This is a critical distinction, since endothelial

cells from different tissue beds exhibit numerous important phenotypic and functional characteristics [84, 316]. Furthermore, almost all *in vitro* assays for transcytosis to date have been performed with cells seeded on transwells (Boyden chambers). Unfortunately, in this setting, pharmacologic or molecular manipulation of endothelial monolayers often induces paracellular gaps [306], potentially confounding the measurement of actual transcytosis. In this Thesis, I report on the characteristics of microvascular endothelial cells derived from human adipose tissue; I then describe our approach to measure insulin transit across these cells; thirdly, I describe our results on the mechanism of this transcytosis; finally, I describe our initial approach to understand the impact of saturated fatty acids on insulin transcytosis.

Aim 1 Compare and contrast insulin handling in microvascular endothelial cells with skeletal muscle cells

Given that insulin must arrive at its target tissues (i.e. muscle and adipose) where it is then degraded, it must pass through endothelial cells largely intact. This suggests that endothelial cells handle insulin differently compared to other cells. Here we compare the degradation and localization of insulin in HAMEC and L6 myoblasts through immunofluorescence and biochemical assays. We hypothesize that a majority of insulin is not degraded in HAMECs while it is in L6 myoblasts. Moreover, we suspect that most of insulin internalized by endothelial cells is subsequently released through the process of transcytosis.

Aim 2 Develop assay to measure insulin transcytosis live in single cells

Here, we report a novel single-cell assay for the quantification of insulin transcytosis across primary adipose microvascular endothelial cells (HAMEC). This approach avoids the potential contribution of paracellular leak and, unlike studies with cell populations, is not affected by poor transfection efficiency as individual cells can be selected for study. Using this

method, we are able to intimately investigate the mechanisms whereby insulin transcytosis occurs.

Aim 3 Identification of the mechanism by which insulin is internalized in microvascular endothelial cells

Transcytosis for other compounds such as albumin have been described to require caveolae [317] and furthermore, insulin transcytosis has been shown to require caveolae in macrovascular endothelial cells [265]. We hypothesize that insulin transcytosis across microvascular endothelial cells also occurs by a caveolae dependent pathway. We investigate the role of caveolae in HAMEC by interfering with its function by a number of means. We use pharmacological agents, overexpression of mutant constructs, and knock down to identify if insulin transcytosis is caveolar.

Chapter 3

Methods

The text presented in this chapter is adapted from Paymon M. Azizi, Roman E. Zyla, Sha Guan, Changsen Wang, Jun Liu, Steffen-Sebastian Bolz, Bryan Heit, Amira Klip, and Warren L. Lee (2014). Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. Mol Biol Cell.

3 METHODS

3.1 Cell Culture

Human adipose microvascular endothelial cells (HAMEC) were isolated from human visceral fat of non-diabetic patients undergoing abdominal surgery unrelated to this study at St. Michael's Hospital, Toronto. Written informed consent for tissue utilization was obtained and the study was approved by the institution's Research Ethics Board (REB#11-198). To isolate HAMEC, human visceral adipose tissue fragments were digested using collagenase II and endothelial cells were sorted out using Dynabeads CD31 (beads loaded with antibody to CD31) (Invitrogen, Carlsbad, CA). After isolation, approximately 95% of these cells expressed von Willebrand factor (vWF), VE-cadherin and exhibited typical cobblestone morphology under phase contrast microscopy, confirming their endothelial phenotype (Figure 1). Cell cultures were expanded and used for experiments between the 5^{th} and 8^{th} passage. Primary HAMECs were also purchased from ScienCell (Carlsbad, CA); in pilot experiments, no morphological or functional differences could be detected between commercially obtained HAMEC and those isolated in our lab from adipose tissue, and hence they were then used interchangeably throughout this study. Primary human aortic endothelial cells (HAEC) were purchased from Lonza (Allendale, NJ). Endothelial cells were cultured in EGM-MV media (Lonza, Allendale, NJ) in an incubator at 37°C and 5% CO_2. Cells were always plated on gelatin-coated glass coverslips and were used within 48 hours of reaching confluence. L6 myoblasts were cultured in α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum. In some experiments, cells were seeded on gelatin-coated 12-well transwell inserts with a membrane pore size of 0.4 µm (Costar 3460, Corning, NY) and grown until confluency as assessed by measurement of the transendothelial electrical resistance (TEER) using the Endohm-12 (WPI, Florida). In other experiments, to induce ICAM-1 expression, HAMEC were exposed to lipopolysaccharide (LPS, 100ng/mL; L2880, Sigma) for 18 hours.

3.2 Electroporation and Transfection

HAMECs were electroporated as previously described [318]. Prior to electroporation, cells were resuspended in Opti-MEM (Life Technologies, Grand Island, NY) with 10% FBS at a concentration of $3*10^6$ cells/mL. A 400uL cell suspension with 20ug of plasmid was chilled at 4°C for 10 minutes prior to electroporation. To transfect plasmid constructs, cells were electroporated using ECM 830 (BTX, Holliston, MA) at a setting of 200V for 45msec. 18 hours after electroporation, media was changed and cells were imaged 6-30 hours afterwards. Cells were transfected with pEGFP-N1 (Clontech, Mountain View, CA), Cav1-GFP (Addgene, plasmid 14433), or Cav1 DN-GFP (Addgene, plasmid 27708).

siRNA was delivered using Lipofectamine RNAiMAX transfection reagent in accordance with the manufacturer's instructions (Life Technologies). Cells were treated with siRNA 24 hours after plating. 24 hours later, a second dose of siRNA was delivered. The cells were used 48 hours later; effective knockdown was confirmed by immunoblotting. The following functionally verified siRNAs from Qiagen (Valencia, CA) were used: AllStars Negative Control siRNA (SI03650318), Hs_CAV1_10 FlexiTube siRNA (SI00299642), and Hs_CLTC_10 FlexiTube siRNA (SI00299880).

3.3 Confocal Fluorescence Microscopy

For colocalization experiments, cells were pulsed with 500nM insulin-FITC (I2383; Sigma, St. Louis, USA) for 5 minutes at 37°C and LysoTracker Deep Red (Life Technologies) was added 10 minutes prior to fixation; alternatively, cells were incubated with transferrin conjugated to Alexa Fluor 555 (transferrin-AF555, Life Technologies) for the duration of the experiment. For insulin uptake experiments, cells were pre-treated with either 30µM dyngo 4a (Abcam, Cambridge, MA), 1mM methyl-β-cyclodextrin (Sigma, St. Louis, USA), 50ug/mL nystatin (Bioshop Canada, Burlington, ON), or 10µM Pitstop 2 (Abcam). Afterwards cells were treated with 500nM insulin-FITC for 10 minutes prior to fixation. As a control for cholesterol depletion,

membrane cholesterol was assessed with a recombinant GFP-tagged D4 probe kindly provided by Dr. Greg Fairn [319]. Cells were treated with recombinant GFP-D4 (15ug/mL) for 10 minutes prior to fixation. Cells were fixed with 4% paraformaldehyde for 30-60 minutes and afterwards incubated with 0.15% glycine for 15 minutes. For immunofluorescence, cells were permeabilized post-fixation with 0.1% Triton X-100 for 20 minutes. The following antibodies were used: Anti-caveolin-1 (N-20; Santa Cruz Biotechnology, Santa Cruz, CA), Anti-clathrin (ab2731, Abcam), Anti-occludin (F-11; Santa Cruz Biotechnology), Anti-von Willebrand factor (ab6994; Abcam), Anti-VE-cadherin (C-19; Santa Cruz Biotechnology), Anti-ZO-1 (N-19; Santa Cruz Biotechnology), Goat anti-mouse Cy3 (115-166-003, Jackson ImmunoResearch), Rabbit anti-goat Alexa Fluor 488 (305-546-003, Jackson ImmunoResearch), and Donkey anti-rabbit Alexa Fluor 555 (A-31572, Invitrogen). Anti-ICAM1 was from R&D Systems (BBA4; Minneapolis, MN). Primary antibodies were incubated at a dilution of 1:100 for 1 hour at room temperature. Secondary antibodies were incubated at 1:1000 for 1 hour at room temperature. Coverslips were mounted in fluorescent mounting medium (Dako, Carpinteria, CA) supplemented with DAPI (1ug/mL). Images were acquired with an Olympus IX81 spinning disc confocal microscope with a 60×/1.35 NA oil immersion objective with settings kept constant between conditions. Images were deconvolved using Volocity 6.3 (PerkinElmer, Waltham, MA). The fluorescence intensity of images was assessed by ImageJ (NIH, Bethesda, MD). Image colocalization analysis was performed using Manders' colocalization measurements via JACoP plugin for ImageJ [320].

3.4 Total Internal Reflection Fluorescence Microscopy

To visualize insulin arriving at the ventral membrane we utilized total internal reflection fluorescence (TIRF) microscopy. As insulin-FITC photobleaches rapidly, we generated insulin-AF568 by conjugating untagged insulin (Sigma) with Alexa Fluor 568 Succinimidyl Ester as outlined in the Molecular Probes manual (A-20003, Life Technologies). Free, unbound fluorophore was separated using Amicon Ultra-0.5 mL 3k centrifugal filters with multiple washes (Millipore, Bedford, MA). Retention of biological activity of both forms of fluorophore-

tagged insulin was verified by immunoblotting for phosphorylated Akt (Ser473; see Supplemental Figure S3). For transcytosis experiments, cells were pulsed with 500nM insulin-AF568 at 4°C for 10 minutes. Unbound insulin was washed off with PBS (containing Mg^{2+} and Ca^{2+}) and then imaged on a heated stand (37°C) in RPMI 1640 media supplemented with HEPES buffer. To determine if transcytosis of fluid-phase markers also occurs, HAMEC were allowed to internalize 25 μg/mL dextran tetramethylrhodamine (70 kDa, Life Technologies) at 37°C for 5 minutes followed by assessment of transcytosis by TIRF microscopy. Non-internalized dextran was removed by rinsing with PBS prior to imaging.

Total internal reflection fluorescence (TIRF) microscopy images were acquired on an Olympus cell TIRF Motorized Multicolor TIRF module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Samples were imaged using a 150×/1.45NA objective with 561nm excitation and an 110nm TIRF field depth using Volocity software for acquisition. Images (150 per cell) were taken at 10 FPS. Quantification of transcytotic events was performed in a blinded fashion using a vesicular detection and tracking algorithm using custom-written MATLAB scripts developed by Dr. Bryan Heit. This algorithm first applies a 0.5 pixel Gaussian filter to remove sub-resolution noise, followed by a local background subtraction with a local area of 324 $pixels^2$. Putative vesicles are identified by applying a threshold 10% above the mean image intensity. Vesicles thus identified were then separated from other cellular structures by filtering the threshold-fitted image for objects of the expected size (16-81 $pixels^2$) and circularity (>0.2). The moving vesicles were then tracked using a maximum-probability assessment of how closely potential tracks resemble free and super-diffusive Brownian diffusion [321], followed by quantification of the diffusivity (mean-squared displacement) of each vesicle:

$$MSD = \left(\sum_{k=1}^{N} |{x_k}^2| \right)^{\gamma}$$

Although the traffic of intracellular vesicles is actin/microtubule-dependent, the plasma membrane fusion requires that this transport cease and the vesicle stably dock with the plasma

membrane before releasing their cargo (Becherer et al., 2007). Thus, by excluding diffusive or super-diffusive (vectorial motion), we restrict our analysis to stationary (and therefore potentially docked) vesicles. As such, we limit our analyses to vesicles displaying sub-diffusive behaviour ($\gamma < 0.8733$) that deviates significantly from free Brownian diffusion ($\gamma = 1$) or super-diffusive/vectorial motion ($\gamma > 1$). Vesicle traces are segmented in order to capture any vesicles that transition from diffusive or super-diffusive motion to sub-diffusive motion. Exocytosis events were then identified in this subdiffusive population by identifying vesicles which undergo a decrease in fluorescence intensity at least 2.5 standard deviations greater over the final 2 time points compared to the rate of fluorescence decrease over the duration of the vesicle's track.

The TIRF-based transcytosis assay overcomes the limitations of more conventional transwell and microscopy assays, as it has the capacity to differentiate true transcytotic events from both permeabilization of endothelial junctions and transient interactions of vesicles with the basolateral plasmalemma [322].

3.5 **Immunoblotting**

Lysates were collected in a lysis buffer containing 50mM Tris and 150mM NaCl and 1% Triton X-100. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Hudson, NH). Samples were prepared at equal protein concentrations and were reduced and denatured via Dithiothreitol (DTT) and boiling for 5 minutes. Samples were run on an SDS-PAGE using 8% to 12% polyacrylamide gels at 100V for the first 15 minutes, followed by 140V for the remainder. Proteins were transferred onto nitrocellulose at 100V for 90 minutes. Membranes were subsequently and blocked for 1 hour with 5% milk in TBST. The following primary antibodies were used: Anti-caveolin-1 (N-20; Santa Cruz Biotechnology), Anti-clathrin heavy chain (C-20; Santa Cruz Biotechnology), Anti-phospho Akt Ser473 (9271, Cell Signalling Technology) at a dilution of 1:1000. Primary antibodies were incubated overnight at 4°C and secondary antibodies conjugated with horse radish peroxidase were incubated for 1

hour at room temperature (dilution 1:10000). Membranes were visualized using Amersham enhanced chemiluminescence as per the manufacturer's recommended procedure.

3.6 ELISA

Cells were pulsed with either 500nM insulin for 5 minutes, or 10nM insulin for 10 minutes at 37°C. Cells were collected in a lysis buffer containing 50mM Tris and 150mM NaCl and 0.25% Triton X-100, sheared using a 27 gauge needle, and freeze thawed once. Protein concentration was assessed using the BCA assay (Thermo Scientific) and equal concentrations were loaded in each well. The ELISA for insulin was performed in accordance with the manufacturer's protocol (RAB0327; Sigma, St. Louis, USA). Samples were incubated on the ELISA plate at 4°C overnight. The samples were washed off four times, then the wells were incubated with a biotinylated insulin antibody for 1 hour. After washing four more times, the wells were incubated with a streptavidin-HRP conjugate for 45 minutes. The wells were washed and then TMB substrate was added to the wells for 30 minutes to begin the colourimetric reaction. Finally the reaction was suspended using acid and imaged immediately at 450nm using a spectrophotometer.

3.7 Statistical Analyses

All experiments were performed at least three times. Analyses were performed using Prism software (Graphpad, La Jolla, USA). Multiple comparisons were performed using one-way ANOVA with a Dunnett's post hoc test for comparisons to control. Normalized data were assessed with a one sample t-test using Graphpad's website: http://graphpad.com/quickcalcs/oneSampleT1/?Format=C. Data are presented as mean ± standard error.

Chapter 4

Results

The text presented in this chapter is adapted from Paymon M. Azizi, Roman E. Zyla, Sha Guan, Changsen Wang, Jun Liu, Steffen-Sebastian Bolz, Bryan Heit, Amira Klip, and Warren L. Lee (2014). Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. Mol Biol Cell.

4 RESULTS

4.1 Insulin is taken up and rapidly secreted by microvascular endothelial cells

We first established that HAMECs in culture express the endothelial cell specific markers von Willebrand factor and VE-cadherin, and exhibit apical/basolateral polarity. HAMEC monolayers display typical cobblestone morphology and exhibit continuous rings of junctional proteins such as ZO-1 and occludin. Furthermore, as is the case for polarized epithelia [323, 324], HAMEC express ICAM-1 in a polarized fashion (Figure 4-1). Thus, morphologically these cells are a suitable microvascular endothelial model and were used next to study insulin transcytosis.

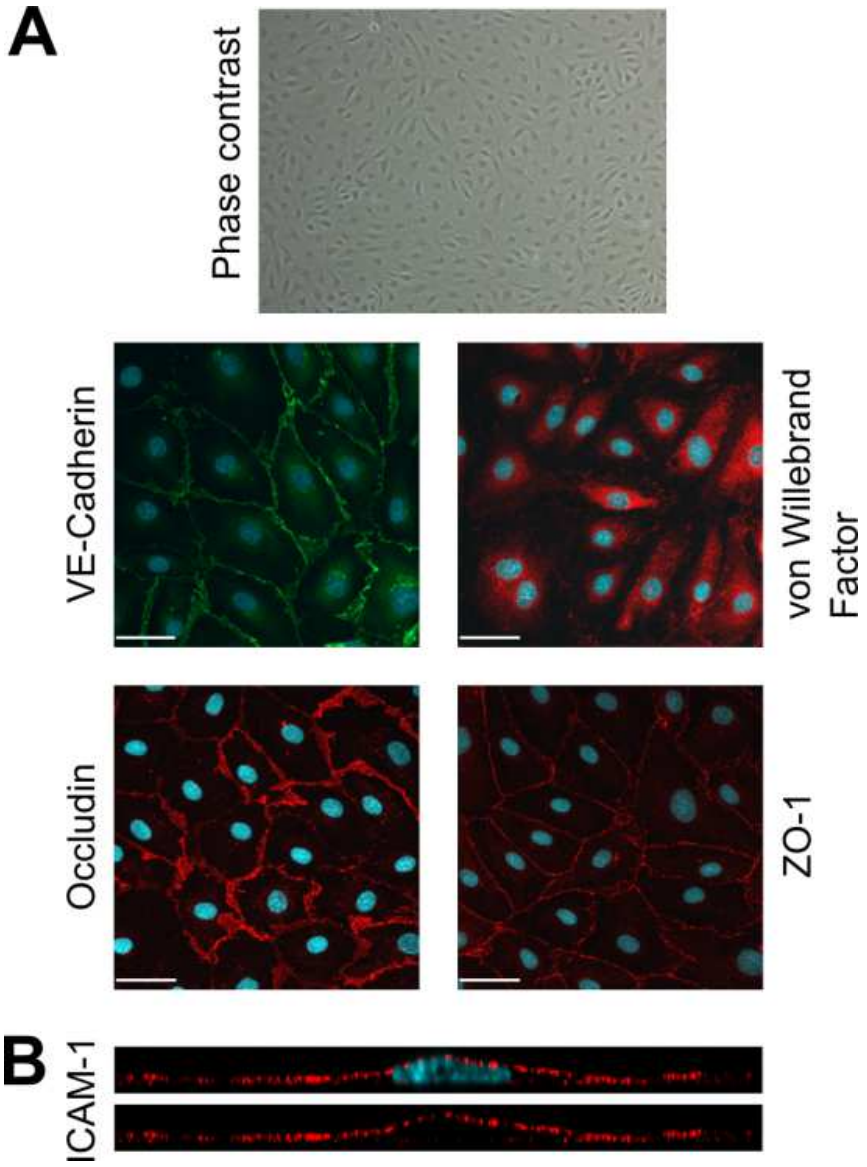

Figure 4-1 Isolation of human adipose microvascular endothelial cells.

(A) Phase contrast image of isolated HAMECs showing typical cobblestone morphology. (B) Expression of VE-cadherin, von Willebrand Factor, Occludin and ZO-1 detected by indirect immunofluorescence, in HAMECs. White scale represents 45µm. (C) Expression of ICAM-1 in LPS-treated cells, detected by immunofluorescence, in HAMEC. White scale represents 10µm. Note absence of ICAM-1 (red; shown by itself for clarity in the lower panel) on the basal membrane; ICAM-1 expression was undetectable in unstimulated cells (not shown).

To begin to analyze the fate of insulin in microvascular endothelial and muscle cells, a pulse of 500nM native insulin was delivered to monolayers of HAMEC (cells illustrated in Figure 1) or L6 myoblasts. After 5 minutes, insulin was washed off, and over time, the amount of insulin within cells and that appearing in the supernatant was measured by ELISA. Under these conditions, HAMEC took up about 10-times more insulin than L6 myoblasts (Figure 4-2A). Moreover, insulin taken up by myoblasts progressively disappeared and there was no concomitant, detectable insulin in the overlying media (Figure 4-2B). In contrast, insulin internalized by HAMEC decreased over time by about 20% in parallel with a progressive recovery of insulin in the supernatant (Figure 4-2, A and B). To ensure that this behaviour of insulin in HAMEC was not the result of saturation of the insulin-processing cellular machinery due to the high levels of insulin used, these experiments were repeated using 50-fold lower insulin concentration in the pulse, with correspondingly similar results (Figure 4-3).

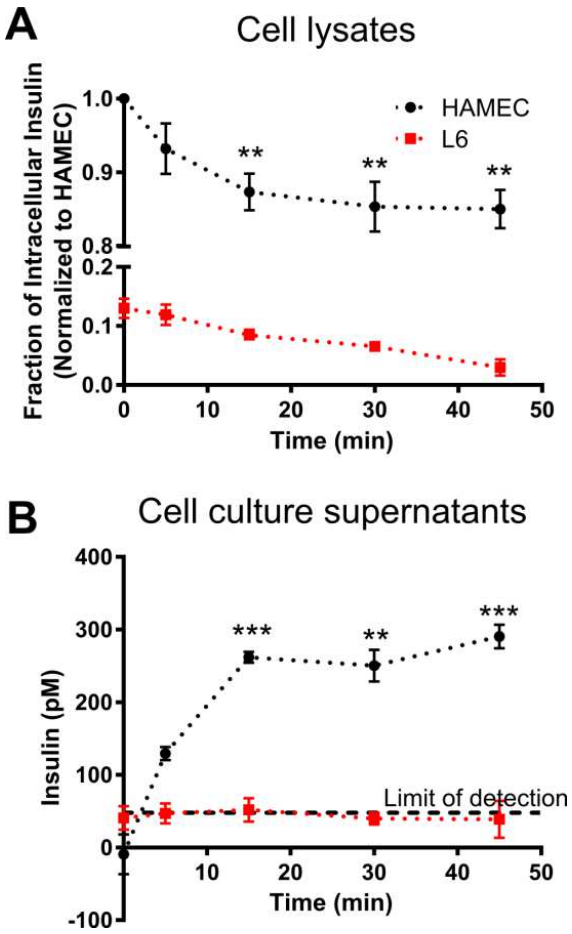

Figure 4-2 Insulin is stored and secreted in HAMECs but degraded in L6 myoblasts.

*(A) Insulin levels in lysates after a 5 minute insulin pulse; data are normalized to initial levels in HAMEC. *p < 0.05, **p < 0.01 compared to initial time point. (B) Insulin levels in cell culture supernatants after a 5 minute insulin pulse. **p < 0.01, ***p < 0.001 compared to initial time point.*

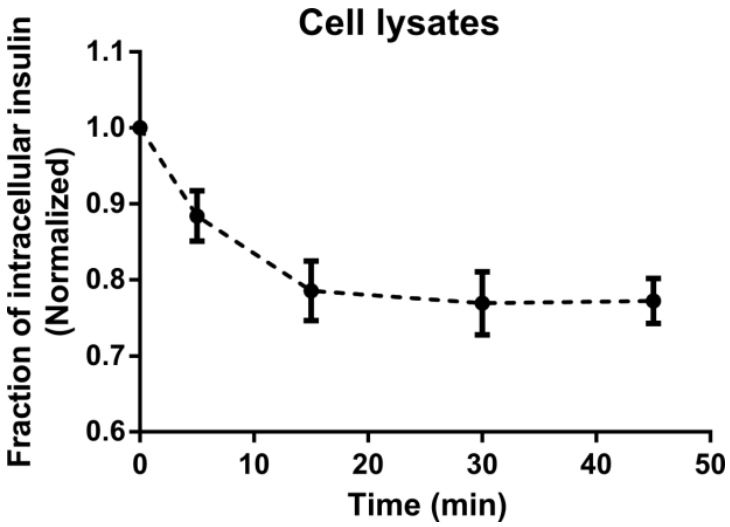

Figure 4-3 Insulin is stored in HAMECs even at lower insulin doses.

(A) Insulin levels in HAMEC lysates after a 10 minute insulin pulse with 10nM insulin; data are normalized to initial levels. Levels in supernatants were too low for consistent detection.

4.2 Insulin is not targeted to lysosomes in microvascular endothelial cells

The permanence of a large fraction of internalized insulin within HAMEC and its contrasting loss within myoblasts is in keeping with the physiological handling of the hormone in the corresponding tissues *in vivo*. Indeed, circulating insulin should be transported intact across the microvascular endothelium to access its target tissues (e.g., fat, muscle) in order to initiate signalling, where it is eventually degraded through the combined action of insulin-degrading enzyme and muscle/fat lysosomal hydrolysis [325, 326]. Accordingly, we examined whether internalized insulin is routed differentially inside microvascular and muscle cells. FITC-conjugated insulin (insulin-FITC) internalized by myoblasts accumulated progressively in lysosomes denoted by the colocalization of the FITC signal with that of LysoTracker, an acidotropic probe that concentrates in lysosomes [327](Figure 4-4, B and C). In contrast, there was little colocalization of internalized Insulin-FITC with lysosomes in HAMEC for the duration of the analysis (Figure 4-4, A and C). A significant fraction of the insulin-FITC internalized by HAMEC colocalized with transferrin, suggesting their joint retention in early or recycling endosomes (Figure 4-5). Insulin-FITC retained its bioactivity as determined by the activation of Akt assayed in muscle cells (Figure 4-6).

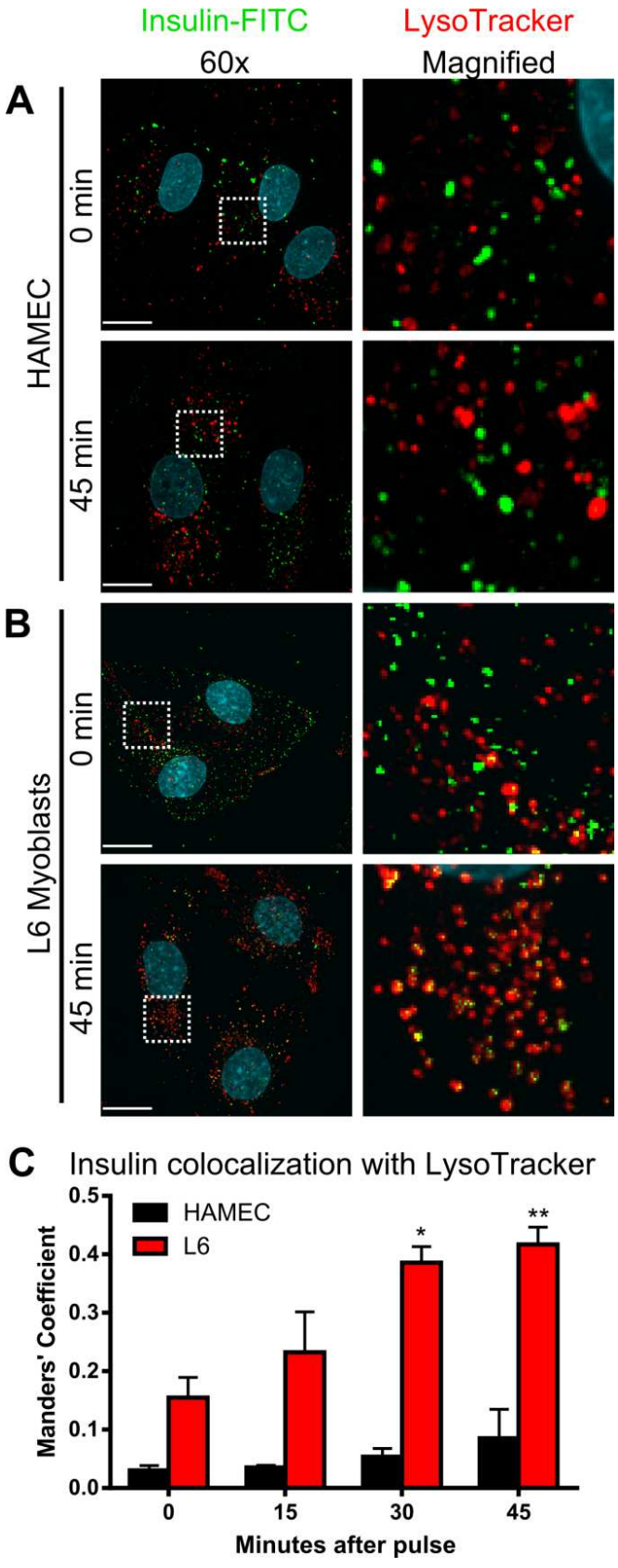
Insulin-FITC
LysoTracker
60x
Magnified
A
HAMEC
0 min
45 min
B
L6 Myoblasts
0 min
45 min
C
Insulin colocalization with LysoTracker
Manders' Coefficient
0.5
0.4
0.3
0.2
0.1
0.0
HAMEC
L6
*
**
0
15
30
45
Minutes after pulse

Figure 4-4 Insulin is not targeted to lysosomes in microvascular endothelial cells.

*(A) Insulin-FITC (green) does not colocalize significantly with LysoTracker (red) at early or late time points. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm (B) Insulin and LysoTracker colocalization increases over time in L6 myoblasts. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm. (C) Quantification of insulin-FITC colocalizing with LysoTracker over time using Manders' coefficient. *p < 0.05, **p < 0.01 compared to initial time point.*

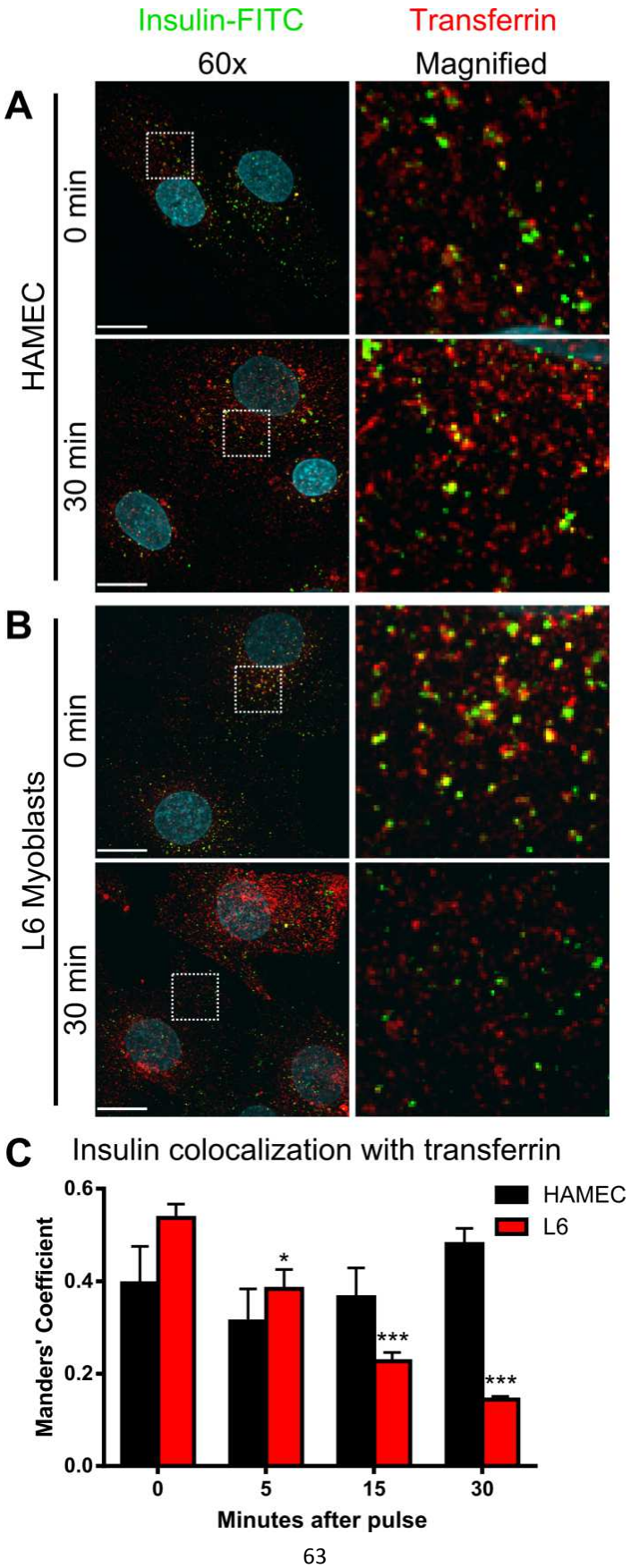
Insulin-FITC
Transferrin
60x
Magnified
A
HAMEC
0 min
30 min
B
L6 Myoblasts
0 min
30 min
C
Insulin colocalization with transferrin
HAMEC
L6
Manders' Coefficient
0.6
0.4
0.2
0.0
*
***
***
0
5
15
30
Minutes after pulse

Figure 4-5 Insulin in microvascular endothelium is retained in a transferrin-positive compartment.

*(A) Insulin-FITC (green) colocalizes moderately with transferrin (red) at early and late time points in HAMECs. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm. (B) Insulin-FITC colocalization with transferrin-AF555 decreases over time in L6 myoblasts. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm. (C) Quantification of insulin colocalizing with transferrin over time using Manders' coefficient. *p < 0.05, ***p < 0.001 compared to initial time point.*

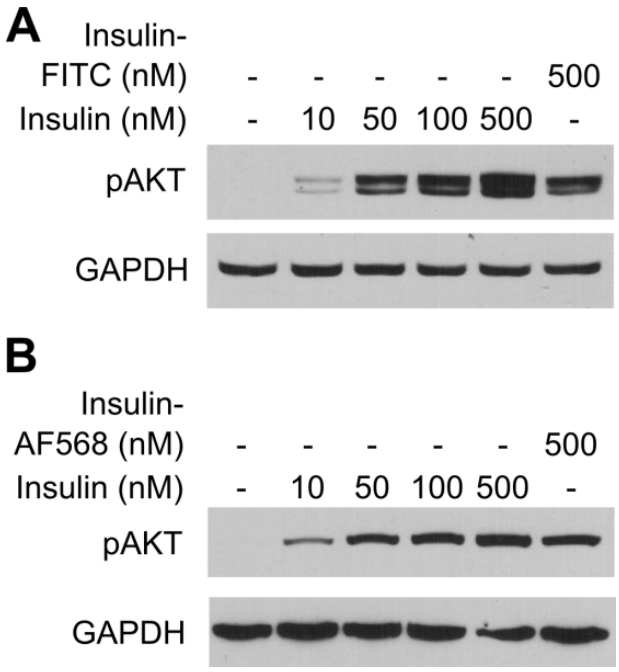

Figure 4-6 Fluorescent insulin molecules retain biological activity.

(A) Immunoblot for phosphorylated Akt (Ser473; pAkt) in L6 myoblasts after treatment with 10nM, 50nM, 100nM, and 500nM unlabelled insulin or 500nM Insulin-FITC. (B) Immunoblot for phosphorylated Akt (Ser473; pAkt) in L6 myoblasts after treatment with 10nM, 50nM, 100nM, and 500nM unlabelled insulin or 500nM Insulin-AF568.

4.3 Development of an assay to quantify insulin transcytosis by individual endothelial cells

The rapid appearance of insulin in the supernatant of microvascular endothelial cells shown in Figure 2 is consistent with the secretion of internalized insulin (as would be expected for its transcytosis). To date, mechanistic studies of insulin transcytosis have focused on the endothelium from large vessels even though these cells do not constitute physiological route of insulin extravasation, and further have relied on transwell assays, where insulin delivery from the upper to the lower chamber by transcytosis may be confounded by paracellular leak [306]. To overcome this potential confounder, and more importantly to obtain information on the actual events of insulin secretion at the exit membrane, we used total internal reflection fluorescence (TIRF) microscopy (Figure 4-7A). The assay was combined with the internalization of a pulse of fluorescently conjugated insulin so that only insulin that binds and is taken up into the cell is then imaged at the ventral membrane. Thus, this assay represents a direct assessment of insulin transcytosis. Briefly, a pulse of Alexa Fluor 568 (AF568)-tagged insulin was added to a confluent HAMEC monolayer at 4°C for 10 minutes, then insulin-AF568 was washed off and the temperature shifted to 37°C. Immediately thereafter, the ventral membrane was imaged by TIRF microscopy. The live-cell videos were then analyzed in a blinded, automated fashion as described under *Materials and Methods*, to quantify the number of individual fusion events (vesicle exocytosis) evinced by the abrupt disappearance of individual fluorescent particles (Figure 4-7B) versus photobleaching of trafficked but non-exocytosed vesicles (Figure 4-7C).

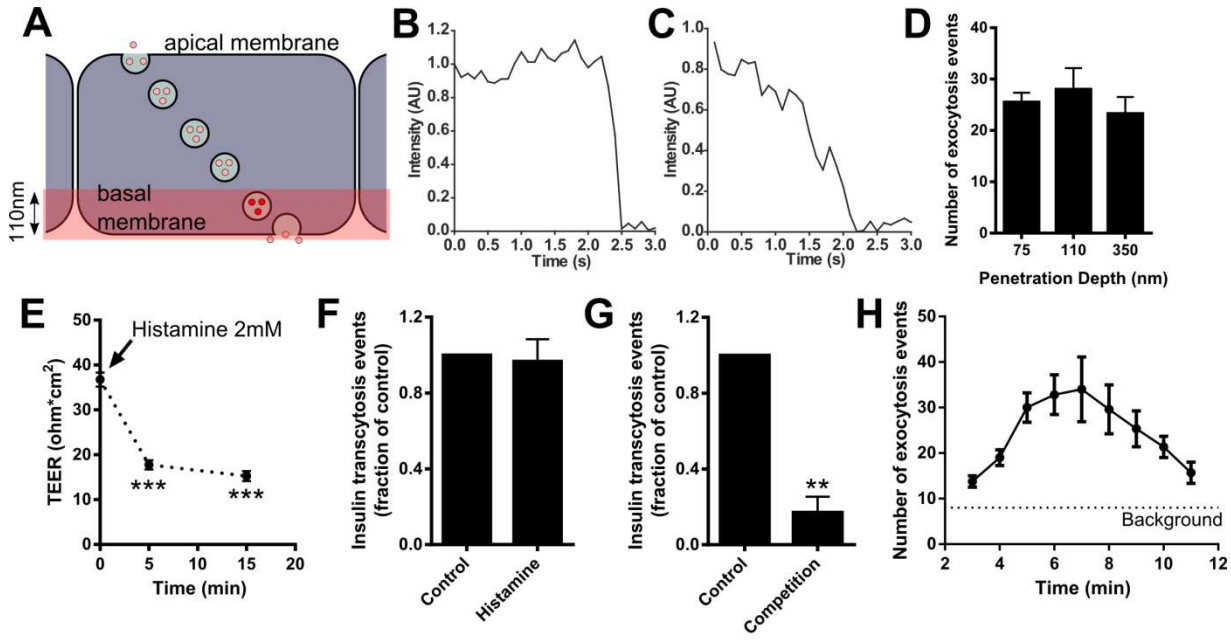

Figure 4-7 Development of a novel single-cell assay to measure insulin transcytosis.

*(A) Schematic depicting the TIRF microscopy assay. A vesicle bearing fluorescent insulin is visualized as it enters the excitation zone of the endothelial cell and its signal is lost upon fusion with the basal plasmalemma. (B) Intensity profile of a tracked particle that undergoes exocytosis causing a rapid loss of signal. (C) Intensity profile of a tracked particle undergoing Brownian diffusion (and photobleaching) but not exocytosis. (D) Varying penetration depth of the TIRF laser does not affect detection events. (E) Trans-endothelial electrical resistance (TEER) drops after addition of histamine (2mM) to top and bottom chambers of endothelial cells grown on transwells (***$p < 0.001$ compared to initial time point) but (F) does not affect the average number of transcytosis events (data are normalized to control cells). (G) Addition of excess unlabelled insulin (50-fold) to the membrane binding step essentially abrogates insulin-AF568 transcytosis, consistent with a receptor-mediated process. **$p < 0.01$ by one sample t-test; data are normalized to control cells. (H) Time course of insulin transcytosis in single cells using the TIRF assay.*

To ascertain that the criterion of sharp disappearance of individual particles (vesicles) of insulin-AF568 from the TIRF zone is due to insulin exocytosis and not to vesicles trafficking out of the TIRF field, we varied the depth of the TIRF field and recorded the number of insulin-AF568 exocytosis events. Under conditions where this field is deeper, the probability of vesicle diffusion out of the TIRF field would be expected to diminish, while the number of observed exocytic events should remain constant. As hypothesized, the number of detected putative exocytosis events remained the same regardless of the TIRF field depth, confirming that we are detecting exocytic events and not vesicular trafficking out of the TIRF field (Figure 4-7D).

To validate that the assay measures *bona fide* transcytosis and is not affected by paracellular leak, we tested the effect of the pro-inflammatory mediator histamine, a known destabilizer of endothelial barrier integrity [328]. While rapidly increasing endothelial paracellular permeability (Figure 4-7E), histamine had no effect on the number of detected insulin-AF568 exocytic events (Figure 4-7F).

To explore whether binding of insulin-AF568 delivered through the pulse involves a saturable step limiting exocytosis, we tested the effect of an excess unlabelled insulin delivered simultaneously with the fluorescent ligand during the binding at 4°C, followed by insulin-AF568 internalization at 37°C and imaging of exocytic events by TIRF microscopy (Figure 4-7G). Under these conditions, the TIRF microscopy signal of fluorescent vesicles was essentially abrogated, consistent with competition for a binding site at the uptake step. Hence, insulin enters HAMEC through a saturable mechanism rather than by fluid phase endocytosis or micropinocytosis. To confirm the directional movement of insulin by a second method, we acquired z-series images of the intracellular localization of fluorescent insulin, taken at 1 and 10 min following insulin binding. Consistent with a transcytotic event, insulin was visible only at/near the apical membrane at 1 min and localized to the bottom of the cell (lower z-axis section) by 10 min (Figure 4-8). The latter is consistent with the TIRF results showing that insulin bound at the apical membrane arrives as vesicles at the basolateral surface within 5-15 min.

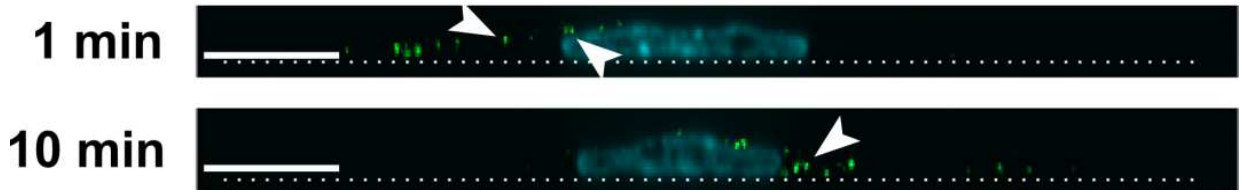

Figure 4-8 Insulin-FITC z-series images in HAMEC.

Cells were pulsed with insulin-FITC for 1 minute at 37°C and then fixed 1 or 10 minutes later. White scale represents 10µm. Note that insulin is located apically at 1 minute but can be visualized more basally in the cell at 10 minutes (arrowheads). Dotted line indicates the base of the cell; nucleus is stained with DAPI (blue).

Having thus validated the assay, we used this approach to establish the time course of insulin transcytosis. Following the insulin-AF568 pulse and internalization, the number of insulin exocytic events recorded separately at 1 min intervals increased steadily between 3 and 7 min, after which it progressively declined (Figure 4-7H). This is consistent with near exhaustion of a releasable pool of insulin-AF568 by 11 min following internalization, a time course consistent with that of the detection of continuous accumulation of pre-internalized native insulin in the HAMEC monolayer culture supernatant shown in Figure 1. Lastly, to determine whether uptake via fluid phase endocytosis might have resulted in TIRF-visualized exocytosis, we allowed HAMEC to internalize fluorophore-tagged dextran after a brief pulse (incubation). Under these conditions, HAMEC took up abundant dextran by endocytosis; however, we detected very few dextran exocytosis events by TIRF (Figure 4-9).

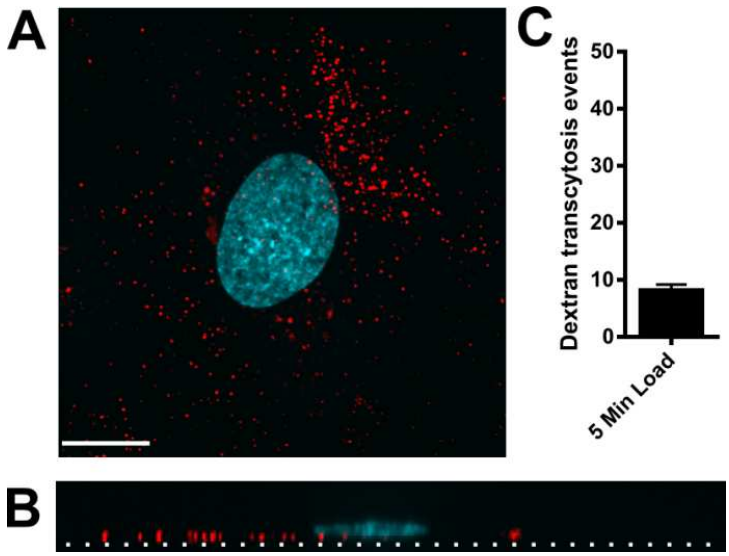

Figure 4-9 Minimal transcytosis of dextran occurs despite abundant uptake by HAMECs.

Cells were exposed to dextran tetramethylrhodamine during a 5 minute pulse. Fluorescent image (z-stack projection, panel A) demonstrates dextran-containing endosomes (cross-section shown in panel B); histogram (C) depicts average transcytosis events detected using TIRF microscopy. White scale represents 15µm.

4.4 Insulin transcytosis is dynamin-dependent and does not require cholesterol or caveolin-1

As shown above, a saturable step of insulin internalization defines the number of insulin transcytosis events. To further analyze the molecular route of this internalization, we explored the endocytic machineries that may be mediating this mechanism. Treatment of HAMEC with dyngo 4a, a specific inhibitor of the large GTPase dynamin [329] prior to and during insulin-AF568 pulsing, essentially abrogated its ensuing internalization and transcytosis (Figure 4-10). Dyngo 4a also prevented internalization of Alex Fluor 555-conjugated transferrin (transferrin-AF555, Figure 4-10), consistent with the well-known dependence of this phenomenon on dynamin [330].

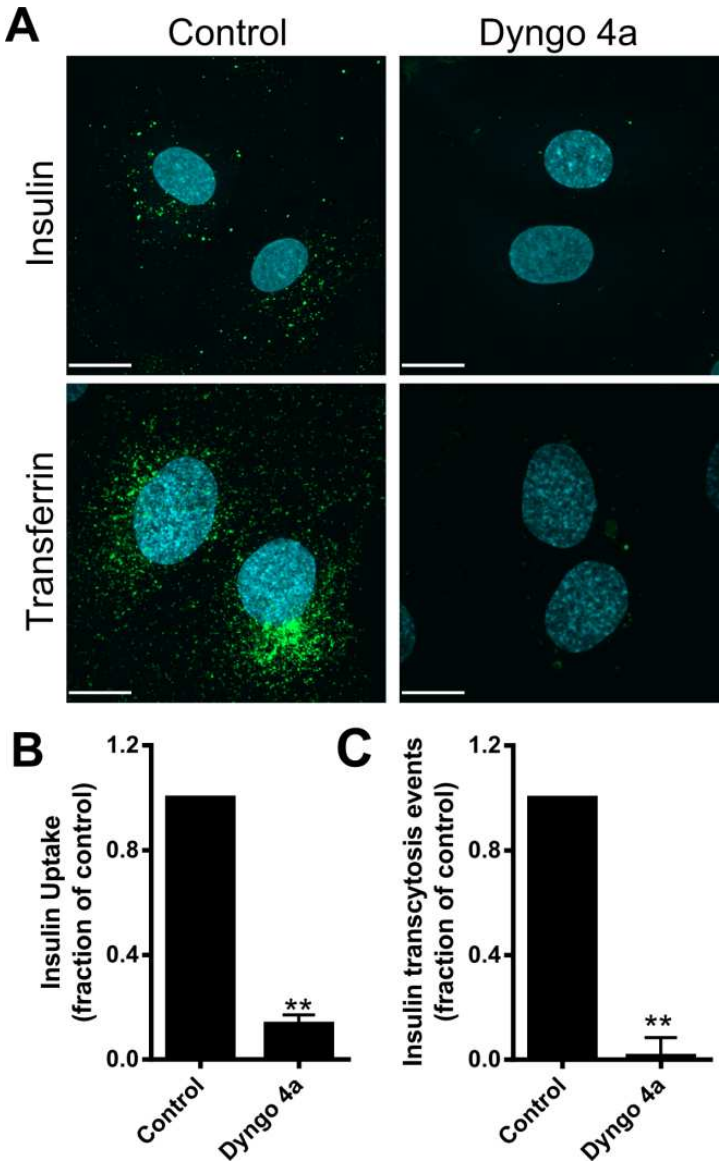

Figure 4-10 Insulin uptake and transcytosis are dynamin-dependent.

*(A) Cells were treated with insulin-FITC or transferrin-AF555 for 10 minutes after pre-treatment with 30uM dyngo 4a (right-hand panel) or vehicle (left) for 30 minutes to impair dynamin function. The white scale represents 15µm. (B) Quantification of insulin-FITC uptake in HAMECs after pre-treatment with dyngo 4a. **p < 0.01 by one sample t-test; data are normalized to control cells. (C) Average transcytosis events after pre-treatment with dyngo 4a. **p < 0.01 by one sample t-test; data are normalized to control cells.*

Dynamin is an essential component of both clathrin-dependent and caveolar-dependent endocytosis. Interestingly, Wang et al. observed that insulin uptake into macrovascular endothelial cells from bovine aortae is mediated by caveolae [265]. Caveolae are cholesterol-rich lipid microdomains and, accordingly, cholesterol depletion or sequestration causes caveolar disassembly. However, depletion of cholesterol using either methyl-β-cyclodextrin (MBCD) or nystatin did not inhibit insulin uptake or transcytosis in HAMEC, and instead tended to increase them (Figure 4-11, A, B, and C). Caveolin-1 is the major protein constituent of caveolae and is required for caveolae generation [331]. To our surprise, over-expression of dominant-negative caveolin-1 tended to promote insulin transcytosis events (Figure 4-11D), and knockdown of caveolin-1 by siRNA induced a similar trend (Figure 4-11, E and F). Consistent with these findings, insulin-FITC internalized for 1 minute exhibited little colocalization with caveolin-1 in HAMECs (Manders' coefficient of 0.196 ± 0.011; Figure 4-11G). Taken together, these data suggest that insulin transcytosis across adipose microvascular endothelial cells is dynamin-dependent, but does not occur via caveolae.

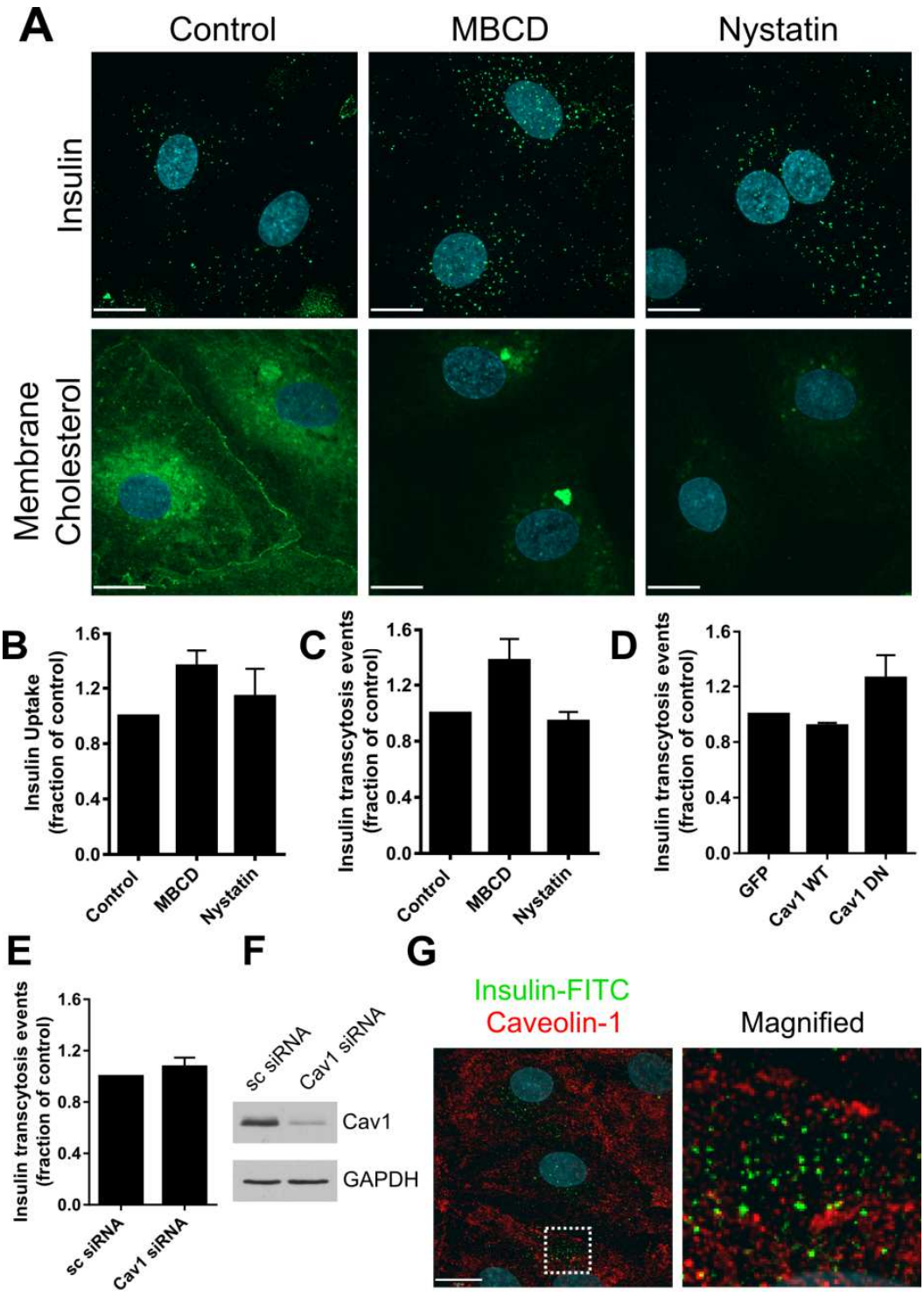

Figure 4-11 Insulin uptake and transcytosis do not require cholesterol or caveolin-1.

(A) Cells were treated with insulin-FITC or D4 membrane cholesterol probe for 10 minutes after pre-treatment with either 1uM methyl-β-cyclodextrin (MBCD) or 50ug/mL nystatin to deplete cells of cholesterol. The white scale represents 15µm. (B) Quantification of insulin-FITC uptake in HAMECs after pre-treatment with MBCD or nystatin; data are normalized to control cells. (C) Average transcytosis events after pre-treatment with MBCD or nystatin. (D) Average transcytosis events of insulin-AF568 after transfection with wild-type or dominant-negative (DN) caveolin-1 construct. (E) Average transcytosis events of insulin-AF568 after caveolin-1 was knocked down by siRNA. (F) Immunoblot of caveolin-1 protein after knockdown via siRNA. (G) Insulin-FITC colocalizes only modestly with caveolin-1 (red). Colocalization was quantified via Manders' coefficient which is 0.196 ± 0.011. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm.

4.5 Insulin uptake and consequent transcytosis by microvascular endothelial cells requires clathrin

The dependence of insulin uptake and transcytosis on dynamin but not on caveolae suggested an unsuspected role for clathrin in insulin transcytosis. Pitstop 2 is a cell-permeant small molecule that blocks the association of amphiphysin with the terminal domain of clathrin, thereby inhibiting clathrin-mediated endocytosis [332]. We first confirmed that Pitstop 2 effectively blocked the internalization of transferrin, a canonical clathrin-dependent process (Figure 4-12A). Under these conditions, uptake and transcytosis of fluorescent insulin were reduced by more than 50% (Figure 4-12, A, B, and C). As the specificity of Pitstop 2 has recently been questioned [333], we confirmed our findings by knocking down clathrin heavy chain via cognate siRNA. Depletion of clathrin significantly diminished insulin-AF568 transcytosis (Figure 4-12, D and E). Consistent with this finding and in contrast to what we observed with caveolin-1, we observed significant colocalization between clathrin and insulin-FITC internalized for 1 min (Manders' coefficient is 0.491 ± 0.020; Figure 4-12F). Hence, these results suggest that in HAMEC, insulin internalizes via a clathrin- and dynamin-dependent mechanism, which defines its subsequent availability for quantal exocytosis.

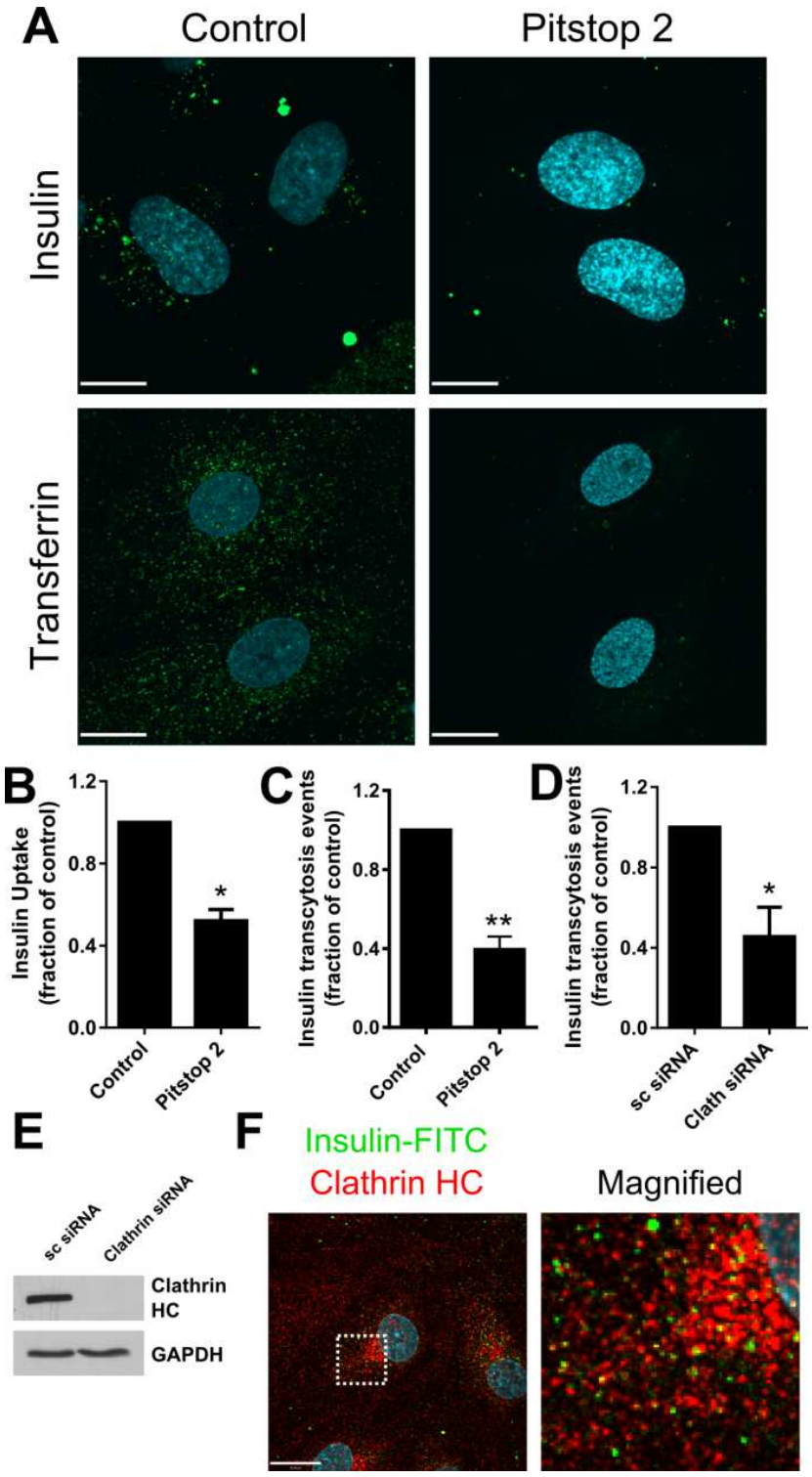

Figure 4-12 Insulin uptake and transcytosis are clathrin-dependent.

(A) Cells were treated with insulin-FITC or transferrin-AF555 for 10 minutes after pre-treatment with 10μM Pitstop 2 to impair clathrin-mediated uptake. The white scale represents 15μm. (B) Quantification of insulin-FITC uptake in HAMECs after pretreatment with Pitstop 2. (C) Average insulin-AF568 transcytosis events after pretreatment with Pitstop 2. (D) Average insulin-AF568 transcytosis events after clathrin heavy chain was knocked down by siRNA. (E) Immunoblot of clathrin heavy chain protein after knockdown by siRNA. (F) Insulin-FITC (green) colocalizes with clathrin heavy chain (red). Colocalization was quantified via Manders' coefficient which is 0.491 ± 0.020. Dashed box indicates area enlarged in right-hand panel and white scale represents 15μm.

In an attempt to reconcile these findings with the caveolar dependence of insulin uptake in bovine aortic endothelial cells [265], and cognizant of the heterogeneous nature of endothelial cells depending on the size and source of their vessels of provenance [84, 316], we explored the possible colocalization of internalized insulin with clathrin or caveolin in human aortic endothelial cells. As anticipated, in these large vessel-derived human endothelial cells, insulin colocalized significantly more with caveolin-1 than with clathrin (Manders' coefficient 0.411 ± 0.068 for caveolin-1; 0.196 ± 0.013 for clathrin; Figure 4-13), paralleling the observations reported for bovine aortic endothelial cells. These findings underscore the selective insulin uptake processes that take place in endothelial cells of different vascular beds, irrespective of whether they are of bovine or human origin.

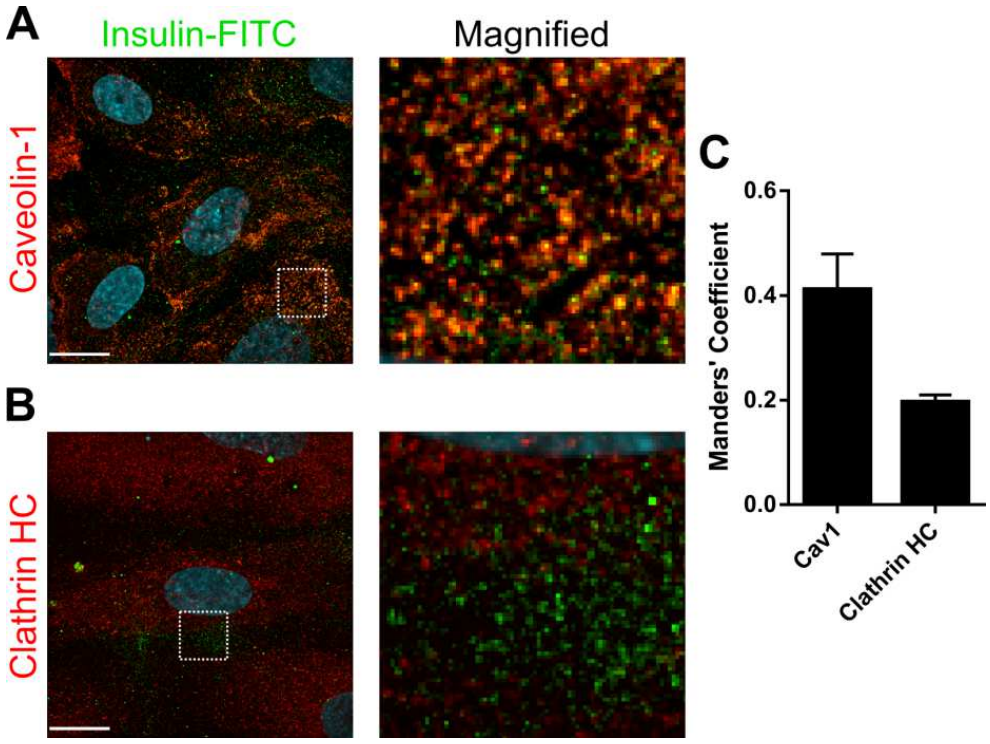

Figure 4-13 Insulin colocalizes with caveolin-1 in human aortic endothelial cells (HAEC).

(A) Insulin-FITC (green) colocalizes with caveolin-1 (red) in HAEC. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm. (B) Insulin-FITC (green) colocalizes with clathrin heavy chain (red) to a much lesser extent than caveolin-1 in HAEC. Dashed box indicates area enlarged in right-hand panel and white scale represents 15µm. (C) Quantification of colocalization of insulin-FITC with caveolin-1 (0.411 ± 0.068) or clathrin heavy chain (0.196 ± 0.013) via Manders' coefficient.

Chapter 5

Discussion & Future directions

The text presented in this chapter is adapted from Paymon M. Azizi, Roman E. Zyla, Sha Guan, Changsen Wang, Jun Liu, Steffen-Sebastian Bolz, Bryan Heit, Amira Klip, and Warren L. Lee (2014). Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. Mol Biol Cell.

5 DISCUSSION & FUTURE DIRECTIONS

The endothelium lining every blood vessel differs phenotypically and functionally depending on the size of the blood vessel (e.g. large versus small) and its location in the circulatory system (e.g. arterial versus venous) [334-336]. Importantly, the passage of nutrients and hormones such as insulin to cells occurs at the level of the *micro*vasculature, as opposed to the primary conduit function served by larger vessels like the aorta [84, 316]. While insulin delivery to critical tissues such as muscle and adipose is regulated by vasodilation or constriction that regulates capillary recruitment to perfuse tissue beds [337, 338], it is less well appreciated that the transendothelial movement of insulin out of the microvasculature is itself rate-limiting for insulin delivery and tissue action [307, 339, 340]. The vasoactive properties governing insulin delivery are relatively well studied; in contrast, surprisingly little is known about the molecular mechanism whereby insulin crosses the endothelium and exits the microvasculature. This process is particularly suitable for *in vitro* studies, since unlike experiments in whole animals, cell culture is not confounded by issues of blood flow and perfusion pressure.

The endothelium of microvessels supplying skeletal muscle and fat is continuous (i.e. without gaps, unlike the hepatic endothelium) and early work indicated that transendothelial insulin permeability *in vitro* is a saturable, temperature-sensitive, receptor-mediated process [312]. *In vivo*, however, it is debated whether the process of insulin transport across the endothelium is saturable [339, 341] or not [342]. The controversy may arise in part due to the influence of various parameters, including hemodynamic ones, on the measurements of trans-endothelial transport *in vivo*.

Recently, caveolin-1 was shown to be required for endothelial insulin uptake in the aorta and, by implication, for its transcytosis, analogous to its involvement in the transcytosis of albumin in lung endothelial cells [171, 265]. Thus, current evidence suggests that internalization of insulin by large vessel endothelia is likely mediated by caveolae. Whether the same is true for the microvascular endothelium, however, is unknown. Moreover, the physiological

significance of insulin uptake by endothelial cells of large vessels is unclear, although certainly insulin signalling in those vessels is of critical importance for the regulation of vascular tone.

Current assays for transcytosis have relied on cells seeded on transwells. Unfortunately, this approach is vulnerable to the induction of discontinuities in the endothelial monolayer causing potential confounding by the resulting paracellular leak. This, combined with the reported poor transfection efficiency of primary endothelial cells, has limited our knowledge of the cellular and molecular regulation of insulin transcytosis. In the present study, we report a novel single-cell assay for insulin transcytosis that obviates these issues. Using primary human adipose microvascular endothelial cells (HAMEC), this method is based on saturable insulin binding and intracellular delivery of the hormone in discrete vesicles that can be imaged at the TIRF zone and undergo exocytosis at the ventral membrane.

5.1 Distinctive characteristics of insulin uptake and transcytosis in HAMEC

Insulin uptake by HAMEC is distinct from the uptake of the hormone by muscle cells or by endothelial cells of larger vessels. First, insulin uptake in HAMEC is about 10-times higher than that in L6 myoblasts assayed under identical conditions. Second, while insulin internalized by myoblasts is routed to lysosomes and is readily degraded, insulin internalized by HAMEC is stable and a fraction of it undergoes recycling/exocytosis to the medium. This exocytosis is quantal and likely representative of insulin exocytosis at the basolateral side of endothelial cells *in vivo*. Of course, we cannot say with certainty whether insulin exocytosis at the ventral membrane of HAMEC cells adhered to a glass coverslip is directly equivalent to its exocytosis towards the interstitial space *in vivo*.

A large fraction of insulin internalized by HAMEC colocalizes with internalized transferrin, indicating its retention within elements of the endocytic pathway. Future studies should explore the nature of the insulin storage compartment in HAMEC, how it is sorted away from

delivery to lysosomes, and whether it can be further induced to undergo exocytosis through selective signals or cell growth conditions. In this regard, it is clearly acknowledged that HAMEC are only a tissue culture model and though being of primary origin they are expected to differ through cellular passages and *ex vivo* culturing conditions from the endothelial cells constituting the microvessels. Therefore, the results obtained with HAMEC are considered a guide for future exploration of the mechanism of insulin transcytosis across the actual microvasculature *in vivo*. Nonetheless, HAMEC represent a more faithful model of the endothelial cells enacting insulin transcytosis *in vivo* compared to endothelial cells derived from larger vessels. Indeed, our results evince that insulin internalization differs in HAMEC and aortic endothelial cells, being mediated by clathrin in the former and possibly by caveolae in the latter; it is also reasonable to speculate that if the *route* of uptake is different between macro- and microvascular endothelial cells, other important differences such as the *rate* of insulin uptake, may also exist. Intriguingly, although interference with clathrin endocytosis via Pitstop 2 and siRNA impaired insulin transcytosis, it did not completely block it, which we observed with dynamin inhibition. This suggests that there may be another mechanism that mediates insulin transcytosis that is dependent on dynamin, but independent of both clathrin and caveolae. The choice of human adipose tissue as the source of HAMEC was based on the importance of insulin delivery to this tissue, given the prominent role of insulin action in fat cells towards the control of lipolysis and adipokine secretion impacting on whole-body metabolism. It is possible that insulin transcytosis in HAMEC is representative of the equivalent phenomenon in endothelial microvascular cells of other metabolically relevant tissues such as muscle, but future studies could also explore for potential differences among microvascular cells of different tissue origins.

A further advance represented by the results reported herein lies in the recording of actual exocytosis of internalized insulin. To the best of our knowledge, our assay combining saturable insulin binding/internalization and subsequent analysis of its quantal delivery at the ventral membrane constitutes the first *in vitro* assay of cellular transcytosis of the hormone. Moreover, the system lends itself to molecular interventions that have allowed us to explore the mechanisms involved in insulin uptake.

At a broader level, our novel single-cell assay of transcytosis in HAMEC will greatly facilitate the study of endothelial transcytosis, a fundamental cellular process described decades ago for ligands including albumin, vitamin B12 and immunoglobulins [223]. Using this approach, we are continuing to elucidate the downstream signalling implicated in insulin transcytosis including an understanding of how insulin evades intracellular degradation. Although the insulin receptor would seem like the prime candidate to mediate transcytosis, a murine endothelial-specific knockout demonstrated little change in glucose homeostasis [343]. Thus, ongoing work in our labs will determine whether the insulin receptor is required for endothelial transcytosis or whether other receptors fulfill this role. Finally, it is possible that impaired insulin transcytosis contributes to the pathophysiology of insulin resistance or diabetes; elucidating its underlying molecular mechanisms may therefore be important for the identification of targeted therapeutic approaches to improve vascular insulin delivery to tissues.

With the development of the TIRF microscopy transcytosis assay, we can now interrogate various aspects of the transcytotic pathway for insulin. There are two major questions we have begun to address: (1) Which receptor mediates the transcytosis of insulin? (2) Is insulin transcytosis impaired during inflammatory conditions? In addition to beginning to answer these questions, we have begun developing a high throughput assay to identify compounds that may increase insulin transcytosis. Finally, in collaboration with the lab of Dr. Wolfgang Kuebler, we are developing the means to visualize insulin transcytosis by intravital microscopy so that we can study it *in vivo*.

5.2 **Receptor mediating insulin transcytosis**

An important question that has not been addressed is the identification of the receptor responsible for insulin transcytosis. Early work by King and Johnson [312] suggested that insulin transcytosis is receptor-mediated as transcytosis of labelled insulin was competed out by using unlabeled insulin. This has been confirmed by multiple labs including our own. Moreover, King and Johnson showed that treatment of endothelial cells with serum from insulin resistant

individuals, which was thought to contain blocking antibodies, was able to block transcytosis of labelled insulin.

An obvious candidate for the receptor responsible for insulin transcytosis is the insulin receptor (IR). However, endothelial-specific insulin receptor knock out mice (VENIRKO) do not exhibit any changes in glucose homeostasis under regular chow diets [343]. This suggests that insulin transcytosis does not require the insulin receptor. Another possible candidate is the insulin like growth factor 1 receptor (IGF1R) as it has been shown to bind insulin, although at higher concentrations. Moreover, endothelial cells have been shown to express much more IGF1R than IR that may offset their lower affinity for insulin. To study the role of IGF1R in insulin transcytosis, a group used blocking antibodies against IGF1R on endothelial cells grown on transwells and noted that insulin transcytosis was impaired [315].

A major caveat of these studies, however, is that they were studied in endothelial cells from macrovascular endothelial cells. The primary site of insulin transcytosis, though, is at the microvascular endothelium. As this thesis has shown, the route by which insulin is internalized differs in microvascular endothelial cells compared to macrovascular endothelial cells. Thus it is important to address the question regarding the receptor mediating insulin transcytosis in model that more closely reflects the cells responsible for doing so. As such, we have begun to identify the receptor that mediates insulin transcytosis in HAMECs.

Preliminary work in our lab has suggested that neither the IR nor the IGF1R are involved in insulin transcytosis. Insulin in endothelial cells does not colocalize substantially with either IR or IGF1R, at early or late time points (data not shown). Moreover, knock down of either IR or IGF1R does not influence insulin transcytosis measured by TIRF microscopy (Figure 5-1). However, as these are single knock down events, it may be possible that the other receptor may be compensating for the lack of the depleted receptor. We will use siRNA to knock down both receptors simultaneously and measure insulin transcytosis by TIRF microscopy. Furthermore, to confirm knockdown experiments, we will isolate endothelial cells from mice

lacking IR endothelial cells (VENIRKO) and from mice lacking both IR and IGF1R in endothelial cells and measure insulin transcytosis.

In accordance with our preliminary data, it is likely that neither receptor will be responsible for mediating insulin transcytosis. To identify the receptor necessary, we will first investigate other membrane receptors that have been previously shown to bind insulin. We currently have some candidates and we are investigating their role in insulin transcytosis. If however, none of these prove to be responsible for insulin transcytosis we will isolate membrane proteins that bind to insulin at the plasma membrane. This can be done by chemically ligating proteins together at the membrane. We will then use immunoprecipitation followed by mass spectrometry to identify what insulin is binding to on endothelial cells. Finally, using many of the assays described in this thesis, we will investigate the role of the potential candidates in their relation to insulin transcytosis. For example, we will use static imaging assays to assess whether or not insulin colocalizes with the candidate. This will be followed by functional assays (i.e. TIRF microscopy) while impeding the function of the candidate (via siRNA or inhibitor) to identify if it mediates insulin transcytosis. Ultimately, the contribution of any candidate receptor to insulin transcytosis will have to be assessed in vivo using a tissue-specific knockout mouse.

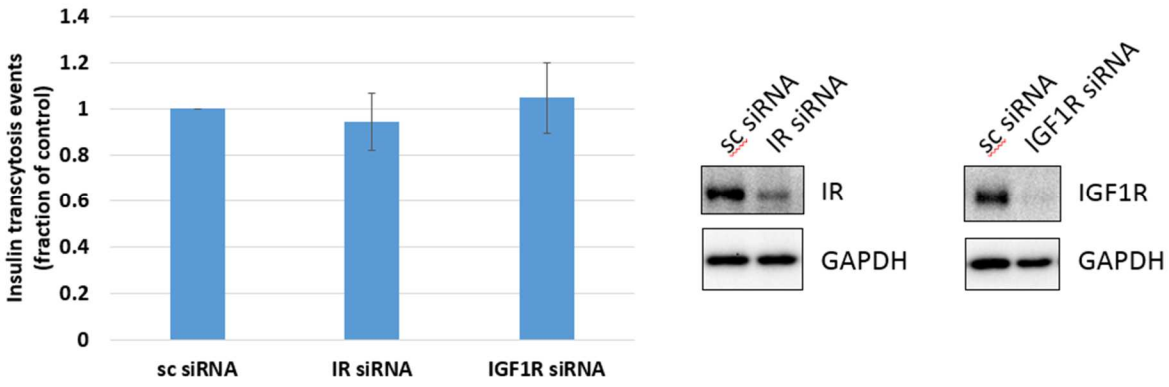

Figure 5-1 Knocking down the insulin receptor or insulin growth factor 1 receptor independently do not affect insulin transcytosis

Insulin receptor (IR) and insulin growth factor 1 receptor (IGF1R) were individually knocked down in HAMECs via siRNA using the protocol described in the methods. Insulin transcytosis was then assed via TIRF microscopy and knockdown was assessed by immunoblot. Despite reducing most of the receptor levels, insulin transcytosis was unaffected. N = 3.

5.3 **Endothelial dysfunction and insulin transcytosis**

The text presented in this section is adapted from Nicolas J Pillon, Paymon M Azizi* (*authors contributed equally), Yujin E Li, Jun Liu, Changsen Wang, Kenny L Chan, Kathryn E Hopperton, Richard P Bazinet, Bryan Heit, Philip J Bilan, Warren L Lee, Amira Klip (2015). Palmitate-induced inflammatory pathways in human adipose microvascular endothelial cells promotes monocyte adhesion and impairs insulin transcytosis. American Journal of Physiology-Endocrinology and Metabolism, ajpendo-00611. The figures represented here are produced by Paymon M. Azizi.*

As described previously, endothelial function is critical in the role of insulin delivery. The hallmark of endothelial dysfunction is reduced availability of nitric oxide (NO), which impedes the ability of the vasculature to vasodilate appropriately. This corresponds to impaired perfusion of the microvascular network, and in the case of insulin, impairs insulin delivery to the tissues.

Although endothelial dysfunction has been implicated in insulin delivery with respect to microvascular perfusion by a number of groups, it is currently unknown whether insulin transcytosis can be affected. A recent paper by Wang [314] investigated the role of insulin transcytosis with respect to NO and found that insulin transcytosis requires NO. Therefore, one can expect that impaired NO bioavailability as a result in impaired insulin transcytosis. However, as explained earlier, that used macrovascular endothelial cells that handle insulin differently compared to those of microvascular origin.

Thus we are interested in investigating the role of endothelial dysfunction with respect to insulin transcytosis. Elevated circulating free fatty acids have been implicated in the pathogenesis of insulin resistance and diabetes. Studies have looked at the role of fatty acids and insulin resistance in many tissues including the muscle [344], liver [345], pancreas [346], and vasculature [347]. Free fatty acids are also implicated in endothelial inflammation and dysfunction. Elevated circulating levels are associated with impaired endothelial-dependent vasodilation as a result of decreased eNOS activation [348, 349]. Because fatty acids have been

shown to cause endothelial inflammation and dysfunction, we hypothesized that they would impair insulin transcytosis as well.

For this study, we treated endothelial cells with palmitate (PA) or palmitoleate (PO). Both are fatty acids containing 16 carbons but PA is a saturated fatty acid (16:0), common in the western diet, while PO is a mono-unsaturated fatty acid (16:1). As PA and PO are insoluble in aqueous solutions, we conjugated the fatty acids to BSA. We treated HAMEC with either BSA, PA, or PO for 18 hours at a physiological concentration of 0.2mM after which insulin uptake and transcytosis were assessed.

PA treatment but not PO impaired insulin uptake by approximately 30% as assessed by confocal microscopy (Figure 5-2). Moreover, PA treatment impaired insulin transcytosis assessed by two methods: TIRF microscopy and transwells (Figure 5-3). PA induced endothelial inflammation has been shown to be mediated by toll-like receptor 4 (TLR4) signalling [350]. We therefore wondered if impairment of insulin transcytosis by PA was also mediated by TLR4. Blocking TLR4 function using the inhibitor TAK242 blocked the PA-induced impairment in insulin transcytosis (Figure 5-4). Moreover, knockdown of TLR4 also blocked PA's effect on insulin transcytosis, indicating that TLR4 was mediating impairments in insulin transcytosis.

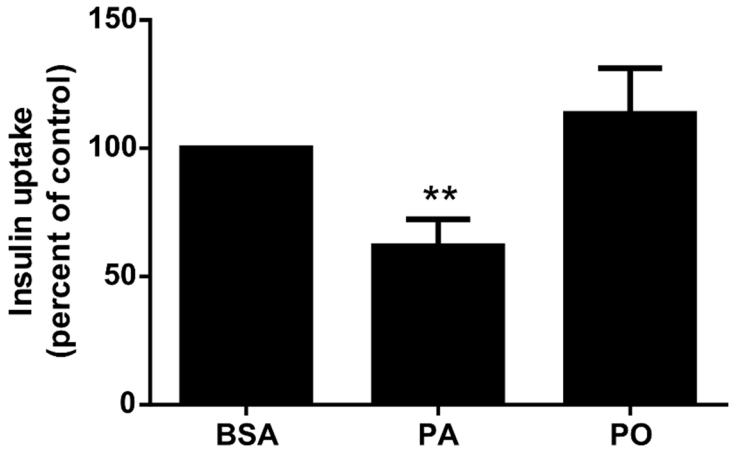

Figure 5-2 Palmitate impairs insulin uptake in HAMEC

Endothelial cells were treated with bovine serum albumin (BSA), palmitate (PA), or palmitoleate (PO) at 0.2mM for 18 hours. Cells were then treated with 50nM fluorescently tagged insulin (Insulin-FITC) for 15 minutes. Insulin uptake was assessed by confocal microscopy. PA treatment impaired the capacity of the cells to uptake insulin. N = 3.

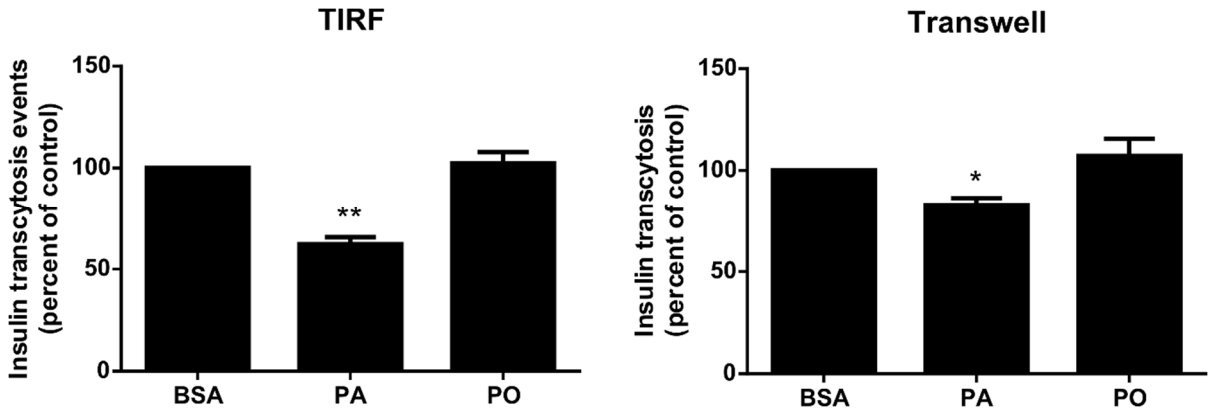

Figure 5-3 Palmitate impairs insulin transcytosis in HAMEC

HAMEC were treated with BSA, PA, or PO for 18 hours. PA treatment significantly impaired insulin transcytosis measured by TIRF microscopy and transwells. N = 3.

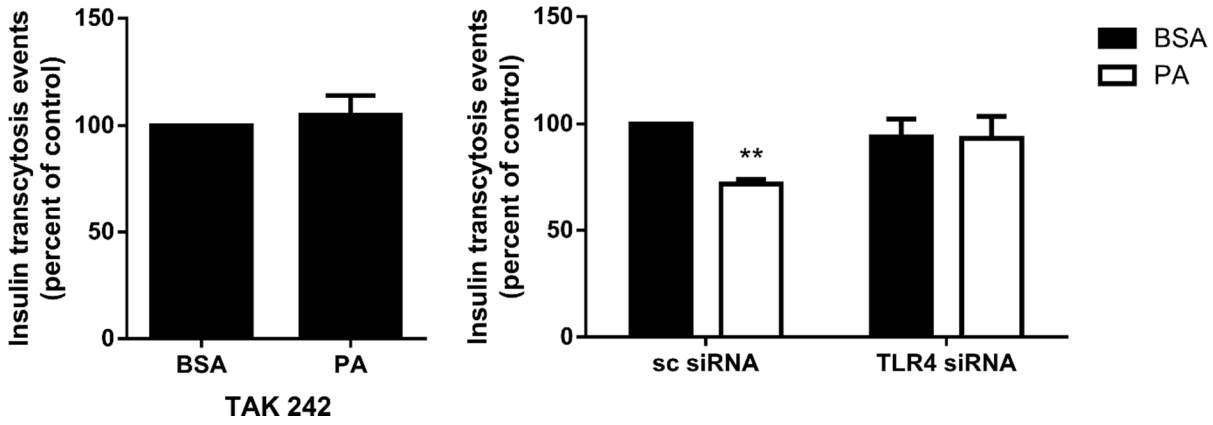

Figure 5-4 Impairing TLR4 function blocks PA mediated impairment in insulin transcytosis

HAMEC were treated with BSA or PA, with or without the TLR4 inhibitor TAK242 for 18 hours. Treatment with the TLR4 inhibitor blocked PA impaired insulin transcytosis. Knockdown of TLR4 restores insulin transcytosis in PA treated HAMEC. N = 3.

5.4 Effect of flow induced shear stress on insulin endocytosis and transcytosis

In vivo, endothelial cells are under constant flow. This flow imparts mechanical forces onto the cell that are able to modulate and alter many cell responses. In response to shear stress, the cytoskeleton changes (i.e. increased actin stress fibre formation), protein clustering at the membrane may be altered, and gene and protein expression may change. The effect shear stress has on endothelial transcytosis of insulin has not been studied. We are interested understanding if shear stress modulates insulin transcytosis and by what mechanism.

Our preliminary studies show that insulin uptake (Figure 5-5) and transcytosis (Figure 5-6) increases dramatically after a low level of shear stress (0.5 dyn/cm^2), typically experienced by the microvasculature. The mechanism by which this increase occurs is not known yet. One would expect that in part, this be mediated by NO, as endothelial cells increase NO production in response to flow. However, inhibition of eNOS by the chemical inhibitor L-NAME does not alter insulin transcytosis as detected by TIRF microscopy (data not shown). We will be investigating the mechanism by which endothelial cells are able to endocytose and transcytose insulin more after shear stress.

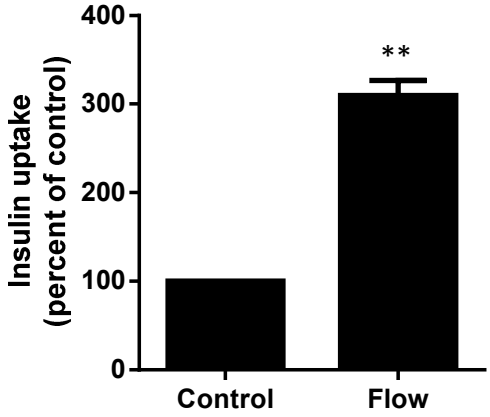

Figure 5-5 HAMEC endocytose more insulin after subject to flow induced shear stress

*HAMEC were either kept under static conditions or exposed to flow induced shear stress for 2 hours using the focht chamber system 2 at 0.5 dyn/cm^2. Cells were then treated with 500nM Insulin-FITC for 10 minutes under static conditions after which they were fixed and imaged. Cells exposed to shear stress significantly took up more insulin. ** P < 0.01. N = 2.*

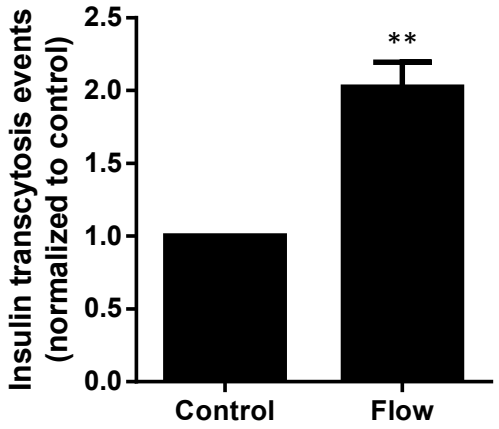

Figure 5-6 Transcytosis of insulin in HAMEC is increased after cells are subject to flow induced shear stress

*HAMEC were either kept under static conditions or exposed to flow induced shear stress for 2 hours using the focht chamber system 2 at 0.5 dyn/cm^2. Insulin-AF568 was then membrane bound for 10 minutes at 4°C. Unbound insulin was washed off using PBS prior to allowing insulin to internalize. Insulin transcytosis events were measured via TIRF microscopy. Cells exposed to shear stress had a higher rate of insulin transcytosis. ** P < 0.01. N = 3.*

5.5 High throughput assay to identify novel compounds to increase insulin transcytosis

As described previously, transport of insulin across the endothelium has been identified as rate-limiting in insulin mediated glucose uptake. In diabetic patients, the transport of insulin appears to be impaired, as interstitial insulin concentrations are lower. Identifying compounds that have the ability to increase insulin transcytosis may be able to improve insulin delivery to tissues and thus be a novel treatment for type 2 diabetes

To identify compounds that influence insulin transcytosis, we have been working to develop a high throughput screen that will potentially identify compounds that influence insulin transcytosis. The assay will utilize 96 well plates cultured with HAMECs. Upon reaching confluency, different wells of the plate will be treated with various pharmacological agents. Afterwards, fluorescently tagged insulin will be added to the wells and the cells will begin to endocytose the insulin. The cells will then be fixed, and the degree to which insulin is internalized will be measured by a plate reader.

At this stage, we are optimizing the conditions of the assay. We need to identify conditions that produce reliable measurements. Moreover, the kinetics of insulin endocytosis are important for the function of the assay. We would expect that insulin internalization is saturable in both concentration and time, and thus we need to ensure that the assay is measuring insulin endocytosis in the linear range.

Intriguingly, preliminary work with a time course of insulin endocytosis in HAMEC show that internalization plateaus early on at around 10 minutes, but then continues to increase again at around 30 minutes (Figure 5-7). The experiment needs to be repeated a number of times to confirm the phenotype, however, it opens up interesting questions. For instance, the plateau may occur because all of the receptors mediating insulin endocytosis on the surface may have been internalized initially. Insulin internalization can only then resume once the receptors are recycled back to the membrane. Another explanation for the delayed

internalization may lie with the fact that insulin signaling in HAMEC also occurs in a delayed fashion. Preliminary work in our lab has shown that Akt is phosphorylated at 30 minutes after insulin treatment. Insulin signaling in HAMEC could potentially increase the rate of macropinocytosis and thus increase insulin endocytosis.

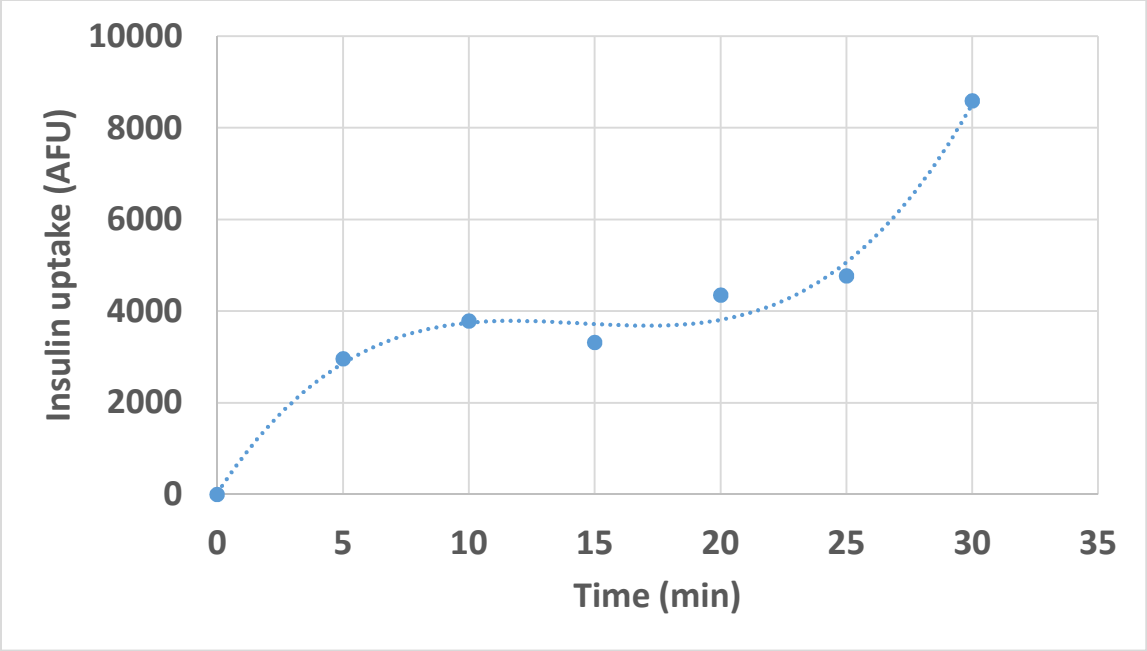

Figure 5-7 Time course of insulin endocytosis measured by a high throughput 96 well assay

HAMEC were seeded onto a 96 well plate and allowed to grow to confluence. HAMEC were treated with insulin-FITC for various time points before fixing and imaging. The data suggests that insulin uptake plateaus at the 10 minutes, but then increases again at 30 minutes. N = 1.

5.6 Development of an animal model to study insulin transcytosis using intravital microscopy

The work presented in this section was done in collaboration with the lab of Wolfgang Kuebler and his post-doctoral fellow Arata Tabuchi.

The method we developed for measuring transcytosis using TIRF microscopy is great to study transcytosis *in vitro*. We can measure insulin transcytosis on a single cell type and interrogate the molecular mechanism. However, due to the nature of the assay, it does not mimic a vascular bed *in vivo*. Vascular beds consist of many different cell types including the surrounding cells of the tissue (i.e. muscle cells or adipocytes), podocytes, and circulating cells. The interplay between these cells is capable of modulating and changing cell responses. The interaction between podocytes and endothelial cells, for instance, is critical to their proper function in the glomerulus of the kidney [351, 352]. As such, we were interested in developing an assay that would allow us to measure transcytosis in an animal. This would allow us to validate our findings in a live animal. Moreover, we would be able to measure insulin transcytosis in various mouse models (e.g. knock-out mice and mice on feeding studies). Finally, we will be able to test the compounds from our screen in section 5.5 in an animal model to see its effect *in vivo*.

In collaboration with Dr. Wolfgang Kuebler, we have developed an intravital imaging assay to measure the movement of fluorescent insulin in a live animal. We focus on a vascular bed, in this case the cremaster muscle, and image the microvasculature. Through the jugular vein we can inject fluorescently tagged wheat germ agglutinin to label the surface of the endothelium. We also inject fluorescently tagged insulin, and measure its location over time. We see that at early time after injection (3 minutes), insulin is localized to the endothelium (Figure 5-8). Over time, the insulin moves out of the blood and the vessel, and moves into the tissue.

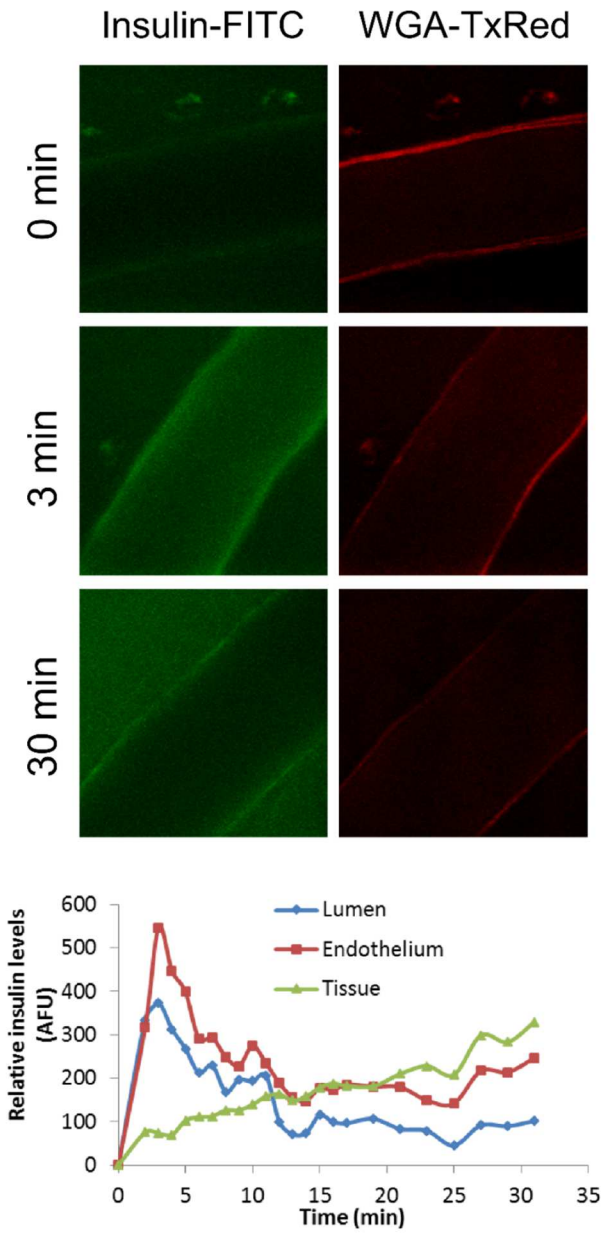

Figure 5-8 Live insulin-FITC detection in a mouse by intravital microscopy

Insulin-FITC (green) and Wheat Germ Agglutinin-Texas red (red) was injected i.v. into a mouse while imaging of the microvasculature of the cremaster. Initially, the Insulin signal localizes at the endothelium, but over time, it moves into the tissues. N = 1.

Chapter 6

Conclusion

6 CONCLUSION

In summary, we showed that endothelial cells handle insulin differently than most other insulin sensitive tissues. Unlike muscle cells that degrade the insulin they internalize, endothelial cells store and transport insulin, likely via transcytosis. To investigate insulin transcytosis, we developed an assay to measure transcytosis live, in single cells, using TIRF microscopy. Moreover, we showed that this assay is unaffected by paracellular leak, which is a confounding factor for all previous transcytosis assays.

Using the TIRF-based assessment of exocytic events we investigated the mechanism by which insulin is internalized in microvascular endothelial cells. Contrary to what was expected, we showed that insulin transcytosis in microvascular endothelial cells is not mediated by caveolae, but instead requires clathrin. Finally, we contrast endothelial cells from the microvasculature with the macrovascular endothelial cells and suggest that the mechanism of insulin transcytosis may differ depending on the endothelial origin.

Previously, the study of transcytosis was very difficult. Static assays such as electron microscopy were used in conjunction with transwell assays that are very susceptible to paracellular leak, making analyses tedious and difficult. Now, with the development of the TIRF microscopy assay to measure transcytosis, we can more easily investigate the mechanism of transcytosis. Understanding the mechanism by which insulin transcytosis occurs across microvascular endothelial cells may reveal novel therapeutic targets to combat type 2 diabetes.

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