Shear stress induces endothelial internalization and transcytosis of insulin independently of nitric oxide – potential role of the actin cytoskeleton

by

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Institute of Medical Science
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Abstract

Transport of circulating insulin out of the microvasculature to reach muscle is a potentially rate-limiting step to insulin action. Most evidence suggests that this occurs by transcytosis through endothelial cells. This study aimed to investigate the effect of shear stress on insulin transcytosis in endothelial cells. Exposing HAMEC to two hours of shear stress increased insulin transcytosis and uptake by approximately two-fold compared to static conditions. Exposure to the NO-donors sodium nitroprusside and spermine NONOate did not affect insulin uptake; furthermore, treatment with the nitric oxide synthase inhibitors L-NAME and L-NNA before and during exposure to shear stress did not ablate the shear-induced transcytosis. Treatment with cytochalasin D, which induces actin depolymerization, significantly enhanced shear-induced insulin uptake by HAMEC, while Jasplakinolide, which promotes actin polymerization, largely
abrogated the shear-induced insulin uptake. Together, these findings suggest a likely role for actin dynamics in regulating shear-induced insulin uptake by microvascular endothelial cells.
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Contributions

Unless otherwise indicated, all experiments were performed by Sha Guan.

Paymon Azizi performed some of the experiments in Figure 1.

Changsen Wang performed the experiments in Figure 3.

Michael Sugiyama performed the experiments in Figure 3BCD.

Javier Jaldin Fincati was responsible for conjugating Alexa Fluorophore 568 to insulin for use in the transcytosis assay.

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

Sha Guan, Amira Klip, and Warren Lee were responsible for designing the experiments and directing the project.
Table of contents

Abstract.................................................................................................................................................. ii
Acknowledgement.................................................................................................................................... iv
Contributions.......................................................................................................................................... v
List of abbreviations .............................................................................................................................. viii
List of Figures.......................................................................................................................................... x

1 Chapter 1 Literature Review ........................................................................................................... 1
  1.1 Overview- Type 2 Diabetes and Insulin Resistance ................................................................. 1
  1.2 Insulin ........................................................................................................................................... 3
    1.2.1 Synthesis and secretion ........................................................................................................ 3
    1.2.2 Physiological effects ............................................................................................................ 4
    1.2.3 Insulin Signaling ................................................................................................................ 6
  1.3 Overview of the mechanisms of insulin resistance .................................................................. 11
    1.3.1 Defective signaling in target tissues .................................................................................. 11
    1.3.2 Defective insulin delivery to target tissues ...................................................................... 12
    1.3.3 The vascular tree .............................................................................................................. 13
  1.4 Endothelium .................................................................................................................................. 20
    1.4.1 Endothelial cell heterogeneity ......................................................................................... 21
    1.4.2 Endothelial permeability .................................................................................................. 25
  1.5 Vascular Wall Shear stress ........................................................................................................ 36
    1.5.1 Effects of shear stress on endothelial cells ...................................................................... 37
    1.5.2 Mechano-sensors of shear stress ..................................................................................... 40

2 Chapter 2 Research Aims & Hypotheses ....................................................................................... 45

3 Chapter 3 Shear stress induces endothelial internalization and transcytosis of insulin independently of nitric oxide – potential role of the actin cytoskeleton ..................................................................................................................... 47
  3.1 Introduction ...................................................................................................................................... 47
  3.2 Methods ........................................................................................................................................ 50
    3.2.1 Cell culture ........................................................................................................................ 50
    3.2.2 Transcytosis assay ............................................................................................................. 50
    3.2.3 Shear stress experiments ................................................................................................... 52
    3.2.4 Insulin internalization assay ............................................................................................ 52
    3.2.5 Nitric Oxide Measurement ................................................................................................ 55
    3.2.6 Actin cytoskeleton modification and staining ................................................................. 56
    3.2.7 Statistical Analysis ............................................................................................................ 57
  3.3 Results ........................................................................................................................................... 58
    3.3.1 Shear stress induces insulin uptake and transcytosis ...................................................... 58
    3.3.2 Nitric oxide does not increase insulin uptake by adipose microvascular endothelial cells 60
    3.3.3 Nitric oxide induces insulin uptake by aortic endothelial cells ........................................ 63
    3.3.4 Shear stress induces remodelling of the actin cytoskeleton ........................................... 67
3.4 Discussion ................................................................................................................................. 70

4 Chapter 4 Discussion and Future Directions ........................................................................ 75
  4.1 Conclusions ............................................................................................................................ 91

5 References ................................................................................................................................. 93
List of abbreviations

A568- Alexa Fluor 555
ANG- Angiopoietin
ANOVA- Analysis of variance
Cav1- Caveolin-1
DAG- Diacylglyceride
DN- Dominant-negative
DTT- Dithiothreitol
ELISA- Enzyme-linked immunosorbent assay endothelial cell
eNOS- Endothelial nitric oxide synthase
ERK- Extracellular signal-regulated kinases
FBS- Fetal bovine serum
FFA- Free fatty acids
FITC- Fluorescein isothiocyanate
GLUT- Glucose transporter
HAEC- Human aortic endothelial cell
HAMEC- Human adipose microvascular
HUVEC- Human umbilical vein endothelial cell
ICAM-1- Intercellular adhesion molecule 1
ICAM1- Intercellular adhesion molecule 1
IGF1R- Insulin like growth factor 1 receptor
IKK- IκB kinase
IR- Insulin receptor
IRS- Insulin receptor substrate
JNK- c-Jun N-terminal kinase
MEK- Mitogen-activated protein kinase molecule 1
NO- Nitric oxide
PDK- Phosphoinositide-dependent kinase
PECAM1- Platelet endothelial-cell adhesion
PHLPP- PH domain and leucine rich repeat protein phosphatases
PI3K- Phosphoinositide 3-kinase
PIP2- Phosphatidylinositol 4,5-bisphosphate
PIP3- Phosphatidylinositol (3,4,5)-
PKC- Protein kinase C
PP2A- Protein phosphatase 2A
PTEN- Phosphatase and tensin homolog
PTP1B- Protein-tyrosine phosphatase 1B
ROS- Reactive oxygen species
siRNA- Small interfering ribonucleic acid
SOS- Son of Sevenless
TIRF- Total internal reflection fluorescence
TNF- Tumor necrosis factor
trisphosphate
VCAM- Vascular cell adhesion molecule
VEGF- Vascular endothelial growth factor
List of Figures

Figure 1 Insulin signaling pathways in endothelial cells .................................................. 10
Figure 2 Overview of blood vessels ................................................................................. 16
Figure 3 Exposure to 2 hours of shear stress (0.5 dynes/cm²) induced a two-fold increase in insulin uptake and transcytosis in HAMEC .............................................. 59
Figure 4 Nitric oxide does not affect insulin uptake and mediate the shear-induced increase in insulin transcytosis in HAMEC ...................................................... 61
Figure 5 Insulin uptake is increased by the NO-donor spermine NONOate in HAEC but not HAMEC ...................................................... 65
Figure 6 Remodeling of the actin cytoskeleton is induced by shear stress and may be involved in the shear-induced insulin uptake ...................................................... 69
Figure 7 Shear stress induces insulin release in HAMEC ................................................. 82
1 Chapter 1 Literature Review

1.1 Overview- Type 2 Diabetes and Insulin Resistance

Diabetes is one of the fastest growing diseases in the world. Every year, diabetes is responsible for 1.5 million deaths in the world and it is the 7th leading cause of death in Canada\textsuperscript{1,2}. While diabetes imposes a substantial financial burden on those living with the disease and their families, it is also a financial crisis for the healthcare system. It has been estimated by the Canadian Diabetes Association that in 2020, the cost of diabetes will reach almost $16 billion\textsuperscript{3}.

Diabetes is a metabolic disease in which the body fails to use the glucose that has been ingested, resulting in an increase in glucose levels in the blood. This state of high blood glucose, also known as hyperglycemia, if left untreated can over time have detrimental effects on organs in the body such as the cardiovascular system, kidney, eyes, and nerves\textsuperscript{4}. Of the people that have diabetes, 90\% have the form known as Type 2 Diabetes (T2D)\textsuperscript{5}.

T2D is characterized by the inability of the body to properly use the insulin that is being produced by the pancreas, also known as insulin resistance, which precedes the development of T2D\textsuperscript{6}. The key organs that are involved in the
pathophysiology of T2D are the liver, muscle, and fat or adipose tissue. In insulin resistant states, the liver is not able to properly control the release of glucose, and the adipose tissue and muscle are not able to uptake glucose for metabolism, resulting in increased blood glucose level.

About 90% of the people who have T2D are overweight or obese. Obesity has long been recognized to be associated with T2D, and this association is mainly due to the ability of obesity to cause insulin resistance. Obesity is a condition characterized by an excess amount of fat in the body to an extent that is harmful for one’s health. One is considered to be obese when his or her body mass index (BMI) is over 30kg/m², and overweight if his or her BMI is between 25 and 29.9. Obesity is a rising issue; in Canada, about 1 in 4 adults and 1 in 10 children are obese. Since 1980, the worldwide prevalence of obesity has almost doubled. As obesity is associated with T2D, this increase in prevalence of obesity means that the incidence of insulin resistance and T2D is also on the rise.

While there are several options of treatment available currently for diabetes, they are by no means cures and still pose a huge disturbance to one’s quality of life. Therefore, there is ongoing research to study insulin resistance and its molecular mechanisms in an effort to better understand the disease and to develop improved ways of treating T2D and insulin resistance.
1.2 Insulin

Insulin is a peptide hormone produced by the pancreas that regulates the metabolism of carbohydrates, proteins and fats. Insulin is made up of two peptide chains: the A- and B- chain, which are linked together by a disulfide bridge. It is comprised of a total of 51 amino acids and has a molecular weight of 5802 daltons.

1.2.1 Synthesis and secretion

Insulin is synthesized by the beta cells of the islets of Langerhans in the pancreas in response to a rise in blood glucose levels. It is first synthesized as a single polypeptide chain comprised of the B chain, the A chain, the connecting (C) peptide, and the signal peptide that together makes up the preproinsulin. The signal peptide directs the preproinsulin to the endoplasmic reticulum, where the signal peptide is cleaved and the preproinsulin becomes proinsulin. After folding into the correct configuration in the ER, the proinsulin then gets transported into the Golgi apparatus, where the C-peptide will be cleaved off and the insulin will mature. The mature insulin is stored in secretory granules as zinc-containing hexamers waiting to be released upon stimulation.
Upon sensing an increase in the level of glucose by the pancreatic beta cells, the insulin-storing granules are exocytosed releasing the insulin hexamers from the cell, which will enter the bloodstream through the fenestrated endothelium of the pancreatic microvasculature. It is in the bloodstream that the insulin hexamer will dissociate to become insulin monomers, which is the biologically active form of insulin that can exert its function on target tissues\textsuperscript{16}.

1.2.2 Physiological effects

After taking in a carbohydrate-rich meal, the glucose broken down from the carbohydrates will be absorbed by the intestines and be delivered into the bloodstream\textsuperscript{17}. This increase in blood glucose levels is sensed by pancreas, which will in response secrete insulin to regulate glucose disposal and metabolism\textsuperscript{15}. The two main physiological effects of insulin are to facilitate of the uptake of glucose by the adipose tissue and muscle, and to promote glucose storage in the liver as glycogen\textsuperscript{18}.

When insulin binds to its receptor on the muscle and adipose tissue, the signaling cascades lead to the translocation of the glucose receptor, GLUT4 that is stored in intracellular vesicles, to the cell membrane. Once the glucose transporters reach the cell surface, they are then able to uptake glucose into the cell decreasing the level of glucose in the blood\textsuperscript{19}. Once glucose is taken up by
the muscle and adipose tissue, it will be used towards processes such as glycolysis and glycogen synthesis, which will be induced by insulin signaling (which is explained in further detail below)⁰²⁰.

The liver, on the other hand, does not need insulin to stimulate glucose uptake, as the hepatocytes contain GLUT2, a glucose transporter that is always in the plasma membrane⁰²¹. However, it does need insulin to activate the pathways that will take the glucose to build the glycogen storage. Insulin signaling in the liver activates hexokinase enzyme, which phosphorylates the glucose keeping it confined in the cell, as well as inhibits the activity of glucose-6-phosphatase to enhance the efficiency of this process. Insulin also activates enzymes that are involved in glycogenesis such as glycogen synthase and phosphofructokinase, to convert the glucose within the cell into glycogen stores. At the same time, it inhibits the breakdown of glycogen stores and gluconeogenesis⁸.

In addition to its effects on glucose metabolism, insulin also regulates the metabolism of fat and proteins. When there is a high level of glucose, aside from building glycogen stores, it is also used towards the synthesis of fatty acids. In the adipose tissue, insulin receptor activation in the adipocytes of the adipose tissue leads to the activation of the pyruvate dehydrogenase (PDH) enzyme and acetyl-CoA carboxylase enzyme (ACC)⁰²²,⁰²³. PDH converts pyruvate into acetyl-CoA, which is in turn converted into malonyl-CoA, the substrate of fatty acid synthesis, by ACC⁰²²,⁰²⁴. Furthermore, insulin promotes the uptake of glucose by the
adipocytes, which are used to make glycerol and subsequently triglycerides, with the overall effect of increasing triglyceride storage in the adipose tissue\textsuperscript{25}. In the muscle, insulin receptor activation promotes the transport of amino acids into muscle promoting protein synthesis and inhibiting the breakdown of protein in muscle\textsuperscript{26}.

1.2.3 Insulin Signaling

To exert its effects on the target tissues, insulin must first bind to its receptors, the insulin receptor (IR), and the insulin-like growth factor-1 receptor (IGF-1R). IR and IGF1-R are composed of two extracellular alpha-subunits and two transmembrane beta-subunits that are all linked together by disulfide bonds to form a heterotetrameric complex\textsuperscript{27}. When insulin binds to the extracellular alpha-subunits, it induces a conformational change in the beta-subunits activating their kinase activity. This then leads to the autophosphorylation of tyrosine residues on the receptor, fully activating the receptor. Once activated, the IR and IGF-1R are now able to phosphorylate the tyrosine residues on downstream substrates such as those from the insulin receptor substrate family (IRS1, -2, -3, -4), Gab-1, Cbl, APS, Shc, and signal regulatory protein (SIRP) family members. The best-studied substrate out of these is the IRS, which is an important mediator of glucose homeostasis. Phosphorylated IRS will then bind to the p85 regulatory subunit of the phosphoinositide 3-kinase (PI3-K) activating the enzyme, which will go to the
plasma membrane and generate phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 will go on to activate the phosphoinositide-dependent protein kinase 1 (PDK), which activates a number of downstream kinases. One of which is protein kinase B (Akt), which mediates most of insulin’s metabolic effects, such as glucose transport, lipid synthesis, and glycogen synthesis. In addition, Akt also regulates cell proliferation and survival\textsuperscript{28}.

To mediate the uptake of glucose by target tissues such as muscle and adipose tissue, Akt phosphorylates the AS160 Rab GTPase-activating protein, which will associate with and activate Rab10 in adipocytes, and Rab8A and Rab13 in muscle cells to induce the translocation of vesicles containing the glucose transporter-4 (GLUT4) to the membrane. After reaching the membrane, GLUT4 is then able to facilitate the passive transport of insulin into the cell for metabolism and decrease blood glucose level. Akt also activates downstream targets to mediate glucose metabolism by indirectly activating the protein and enzymes that are associated with glycogen synthesis and storage, such as glycogen synthase, in tissues such as muscle and the liver. Other effectors of Akt mediate the inhibition of gluconeogenesis in the liver as well as the synthesis of fatty acids and proteins in the adipose tissue and muscle\textsuperscript{27}.

Another important branch of the insulin-signaling pathway is the mitogenic PI3K/Akt-independent MAPK signaling pathway, which mediates cell growth and
proliferation. The protein that initiates this branch of the signaling pathway is the Grb2 protein, which binds to phosphorylated IRS and recruit the protein Son of Sevenless (SOS). SOS is a guanine nucleotide exchange factor (GEF) for Ras; it activates Ras by converting it into the active GTP-bound form. Activated Ras then goes and activates the mitogen-activated protein kinase (MEK) which will activate ERK. ERK can phosphorylate a number of downstream effectors to influence cell proliferation and survival.\(^{27}\)

Another important site of insulin signaling is the endothelial cell. In endothelial cells, insulin signaling through the PI3K-Akt pathway leads to the activation of the endothelial nitric oxide synthase (eNOS), which generates nitric oxide (NO), a potent vasodilator that mediates capillary recruitment and increased blood flow. Contrarily, signaling through the mitogenic pathway leads to the expression of the vasoconstrictor endothelin-1. Therefore, insulin signaling in endothelial cells is mainly responsible for modulating vasomotor tone.\(^{27}\)

The insulin-signaling pathway is associated with various regulators that will activate or inhibit the pathway. Protein tyrosine phosphatase-1B (PTP1B) is known to reverse the autophosphorylation of IR\(^{27}\), while suppressor of cytokine signaling-1 (SOCS1) and SOCS3 inhibit the IR tyrosine kinase activity.\(^{29}\) Phosphatase and tensin homology (PTEN) protein converts PIP3 to PIP2 to downregulate PDK activity.\(^{30}\) Protein phosphatase 2A (PP2A) and PH domain and leucine rich repeat protein phosphatases (PHLPP) dephosphorylate Akt,
converting it back into its inactive form\textsuperscript{31}. These proteins act as controls of the insulin signaling pathway so over-signaling does not occur. As these proteins play such an important role in controlling the insulin-signaling pathway, any dysregulation in the activity of these proteins can lead to metabolic disorder.
Figure 1 Insulin signaling pathways in endothelial cells. Binding of insulin to the insulin receptor leads to the activation of two main signalling pathways, the PI3K/Akt signalling pathway, which mediates glucose uptake and metabolism, and MAPK signalling pathway, which mediates cell growth and proliferation.
1.3 Overview of the mechanisms of insulin resistance

1.3.1 Defective signaling in target tissues

The inability of the body to respond to insulin in insulin resistance is best known to be caused by defects in the insulin-signaling pathway within target tissues such as muscle and fat\(^{25}\). Defects have been documented at different points in the pathway, and have been shown to be caused by the conditions that lead to and are associated with insulin resistance, such as lipotoxicity and inflammation\(^{32}\).

A hallmark of insulin resistance is the excess accumulation of lipid intermediates in non-adipose tissue also known as lipotoxicity\(^{32}\). The increase in fatty acids has been shown to induce the activation of PKC, which inhibits IRS-1 by serine phosphorylation\(^{33}\). Unlike tyrosine phosphorylation, which activates proteins such as IR, IRS-1 and Akt, serine and threonine phosphorylation inhibits them\(^{34}\). The increase in sphingolipid ceramide that is seen in insulin-resistant subjects inhibits Akt activation by increasing the interaction between Akt and the protein phosphatase 2A (PP2A) protein, and increasing the threonine phosphorylation of Akt\(^{32}\). All of these proteins are upstream of the effectors that regulate GLUT4 translocation and glucose metabolism; thus a defect in their activation results in a failure to activate these processes\(^{32}\).
In addition, insulin resistance is also characterized by a low grade-inflammatory state. In this state, there is an increase in secretion of cytokines such as TNF-alpha, IL1B and IL6 by the immune cells and adipocytes, which have been shown to induce the activation of serine/threonine kinases, and decrease the expression of IRS-1 and GLUT4 expression.

In the skeletal muscle, insulin resistance is best known to be caused by the impairment in the tyrosine phosphorylation of IRS-1. This prevents the activation of IRS-1 and in turn the activation of PI3K, which is responsible for activating downstream effectors important for glucose metabolism.

Many proteins along the insulin-signaling pathway can be dysregulated in insulin resistance, in addition to the positive and negative modulators that act at different steps of the pathway. These dysregulations lead to the improper activation of downstream proteins, resulting in the metabolic syndrome we know as insulin resistance.

1.3.2 Defective insulin delivery to target tissues

While insulin resistance is widely known as a condition caused by defects in the insulin-signaling pathway within target tissues, the role of insulin delivery in
the pathogenesis of insulin resistance is less well-studied. Before insulin is able to exert its action, it must first reach the vascular bed of the target tissue and then exit out of the circulation to the target tissues by crossing the endothelial monolayer. Several studies have demonstrated that insulin must cross the vasculature first in order to exert its effects\cite{Gollan2012,Scott2013,Knowler2014}. Therefore, if there is impairment in the insulin delivery process to the target tissues, this is another way by which insulin resistance may arise. Indeed there have been several studies suggesting that an impairment in insulin delivery can contribute to the development of insulin resistance\cite{Dufour2007,Knowler2014}. They will be discussed below.

### 1.3.3 The vascular tree

In order to understand the notion of insulin delivery, it is important to understand the nature of the vascular tree. The vascular system is the organization of blood vessels in the body that constitutes a part of the circulatory system. It is important for the delivery of nutrients and oxygen to tissues and the removal of wastes. The vasculature is comprised of three main types of blood vessels: the artery, the veins, and the capillaries, which are located in different parts of the body serving different functions\cite{Rothwell2010}.

The largest blood vessels in the vascular system are the arteries and veins. The artery functions to carry blood out of the heart and distribute it to the body
through further branching into smaller blood vessels known as arterioles. The veins carry the blood back into the heart with the help of one-way valves built within the veins to prevent backward flow. The artery and veins are composed of three layers: the innermost layer called the tunica intima, which is made of endothelial cells supported by an internal elastic lamina; the middle layer called the tunica media, which is made of connective tissues and vascular smooth muscle; the outer layer called the tunica adventitia, which is made of connective tissues only. The artery and veins’ structure differ slightly in that the tunica media is much thicker in the artery than in the veins necessary for withstanding the high pressure generated from the heart.

The smallest blood vessel in the vascular system is the capillary. The capillaries mainly function to exchange nutrients and waste with the target organs and this is made possible with its unique yet simple structure. Unlike the artery and veins, the capillary is made up of only one single layer of endothelial cells; this allows small molecules to easily and quickly get to the target tissue without having to traverse between layers of smooth muscle and connective tissue. There are three types of capillaries: continuous, fenestrated or discontinuous. Continuous capillaries, also the most common type of capillaries, are composed of endothelial cells that are perfectly aligned forming a tight monolayer, that allows only the passage of very small molecules such as water and ions through the intercellular junctions. These capillaries are found in organs such as the muscle and adipose tissue. Fenestrated capillaries are composed of endothelial cells that
have pores allowing small molecules and some proteins to diffuse through. These capillaries are typically found in the intestines, endocrine glands, kidney and pancreas. Discontinuous or sinusoidal capillaries are composed of an endothelium in which the endothelial cells do not form a tight seal, but instead have gaps between endothelial cells that allow the free diffusion of blood cells and proteins. Discontinuous capillaries are typically found in the liver, endocrine organs, bone marrow, spleen and lymphoid tissues, where frequent exchange of materials takes place\textsuperscript{41}.
Figure 2 Overview of blood vessels. Artery and veins are comprised of three layers, the tunica intima (endothelium), tunica media which is composed of connective tissues and vascular smooth muscle, and tunica adventitia, which is made of connective tissues only. Capillaries are comprised of the tunica intima layer only. Figure was created using templates from Servier Medical Art.
1.3.3.1 Capillary recruitment and blood flow – role in insulin delivery

Once insulin is secreted into the bloodstream, it is able to enhance its own delivery to the target tissues by inducing capillary recruitment and increasing blood flow. Insulin signaling in endothelial cells leads to the production of nitric oxide (NO), a vasodilator, which acts on the smooth muscle layer of the blood vessel to cause the vessel to relax and increase in diameter. The dilation of terminal arterioles in turn leads to capillary recruitment or the increase in number of perfused capillaries. With more capillaries being perfused, more insulin can be delivered to the target tissues, as the surface area for insulin transport is increased. The dilation of resistance arterioles, on the other hand, leads to an increase in blood flow to the limbs, which increases the rate at which insulin is delivered to the target tissues. Consequently, any impairment in the insulin-induced capillary recruitment and increase in blood flow would lead to less insulin being delivered to the tissues, ultimately affecting glucose metabolism and leading to the development of insulin resistance. Indeed, there have been a number of studies showing a decrease in blood flow and capillary recruitment in obesity/insulin resistance. Increased levels of free fatty acid in the circulation, a condition that is typical in insulin-resistant obese subjects, have been shown to significantly decrease the recruitment of capillaries in the muscle and the uptake of glucose. This reduction in insulin-stimulated vasodilation has been shown to be associated with impairments in the pathways that activate the endothelial nitric oxide synthase (eNOS) in endothelial cells. It has been shown that
hyperlipidemia, a condition that contributes to the development of insulin resistance, causes a defect in the activation of eNOS, decreasing NO production\textsuperscript{46}. Inflammatory cytokines, which are increased in the circulation in insulin resistance, have also been shown to reduce the activation of eNOS in endothelial cells\textsuperscript{47}. In one study, when TNF-alpha is applied in the rat limb, the insulin-induced increase in capillary recruitment, blood flow, and glucose uptake are lost\textsuperscript{48}.

1.3.3.2 Transport of insulin out of the vasculature to the tissues – role of the endothelium

Once the insulin has reached the blood vessel of the capillary bed, another potentially rate-limiting step for insulin action is the transport of insulin across the endothelial barrier. Most evidence to date suggests that the major route by which insulin is transported across the endothelium is through individual endothelial cells, a process known as transcytosis\textsuperscript{35,49,50,51,52,53}; this is discussed in detail later on. The relative importance of this stage of insulin delivery is unclear. For example, it has been shown that the appearance of insulin in the interstitium is delayed in obese subjects compared to non-obese subjects during a hyperinsulinemic euglycemic clamp\textsuperscript{39,38}. Another study has shown that following an oral glucose tolerance test, the interstitial insulin levels from the adipose tissue and muscle of non-diabetic obese women were significantly lower than those of
the controls\textsuperscript{38}. While these findings indicate that there is an impairment in the delivery of insulin to the interstitium of the target tissue, it is not known whether this is contributed wholly by a defect in capillary recruitment and increase in blood flow or both an impairment in this process and the transcytosis of insulin. It is difficult to tease out the contribution of the two processes as a defect in either would result in lower interstitial insulin. As insulin transcytosis is technically challenging to study, it is not known whether or not this process also contributes to the defect in insulin delivery that has been observed in insulin resistance. In order to understand the context and potential role of endothelial transcytosis of insulin, the next sections will provide important background on endothelial cells and the determinants of endothelial barrier integrity.
1.4 Endothelium

The endothelium is the innermost layer of the blood vessel and it is made up of a single layer of endothelial cells\textsuperscript{40}. It serves as a selective-barrier between the lumen of the blood vessel and the tissue, regulating the transport of molecules between the blood and the tissues\textsuperscript{54}. The endothelial barrier is selective for both the size and charge of the molecule that pass through\textsuperscript{55}. It is permeable to small solutes and molecules, and impermeable to larger macromolecules. This size-selectivity of the endothelial barrier is determined by the interendothelial junctions, which link the endothelial cells together\textsuperscript{54}. These junctions restrict the passage of molecules greater than 3-5nm in molecular radius\textsuperscript{56}. The endothelial barrier’s charge selectivity is determined by the electrical charge on the vascular luminal surface, which is maintained by a layer of glycoproteins and proteoglycans on top of the endothelium known as the glycocalyx\textsuperscript{57}. The glycocalyx is between 0.1 to 1\textmu m thick, depending on the location along the vascular tree\textsuperscript{58}. It has a net negative charge due to the negatively charged glycosaminoglycan (GAG) side chains of the proteoglycans, and this negative charge is dependent on the GAG side chain sulphation pattern\textsuperscript{57}. Changes in sulphation result in changes in the charge of the glycocalyx and in turn the permeability of the endothelium. The glycocalyx repels negatively charged molecules as well as blood cells and platelets\textsuperscript{57}. 
Aside from its function as a barrier, the endothelium itself is able to produce chemicals and proteins to affect other processes, such as the vasomotor tone, immune and inflammatory responses, and hemostasis\textsuperscript{59}. Endothelial cells produce vasoactive substances such as nitric oxide and prostacyclin to cause vasodilation and inhibit platelet aggregation\textsuperscript{60}. The production of these substances by the endothelial cells is regulated by other chemical stimuli such as bradykinin, thrombin, as well as changes in blood flow\textsuperscript{37}. Under basal conditions, the endothelial cells are quiescent, non-thrombogenic, and do not express adhesion molecules on the cell surface for platelets and leukocytes to adhere\textsuperscript{61}. However, when the endothelial cells are activated, they enter into a proinflammatory and procoagulant state, in which the endothelium loses its integrity, starts to express adhesion molecules to prepare for leukocyte adhesion and extravasation, changes from an antithrombotic to prothrombotic stage, and produces cytokines to mediate the inflammatory response\textsuperscript{62}. Clearly, the endothelium is more than just a structure that makes up a blood vessel; it not only serves as a semi-permeable barrier to molecules, but also regulates many other processes such as those related to the inflammatory response.

1.4.1 Endothelial cell heterogeneity

As outlined above, the endothelium from different tissue beds and different parts of the vascular tree exhibit notable heterogeneity in structure and function.
As different tissues and locations of the vasculature have different functions, the endothelial cells need to be heterogeneous in structure and function in order to adapt to and accommodate for the differing needs of the tissue and vascular location\textsuperscript{41}.

In terms of structural heterogeneity, there is a variation in endothelial cell shape across the vascular tree. Endothelial cells are normally very flat and thin; however, in the venules, they are thicker and more cuboidal in shape\textsuperscript{63}. The thickness of endothelial cells also varies depending on which part of the vascular tree they are derived from; endothelial cells derived from the microvasculature usually, with some exceptions, have a thickness of less than 0.3 \(\mu\)M, while those from macrovasculature have a thickness of around 1\(\mu\)M\textsuperscript{64,65}. It is observed that endothelial cells derived from the arterioles of the mice cremaster muscle are much longer and larger in surface area than those from the veins. It has also been found that aortic endothelial cells from rat were more elongated and tapered, whereas those from the pulmonary veins were much rounder in shape\textsuperscript{63}.

One important function of the endothelium is the regulation of molecule transport across the vasculature. Molecules move across the endothelium either paracellularly between endothelial cells or transcellularly through each individual endothelial cells by a process called transcytosis\textsuperscript{41}. For the transcellular transport pathway, the internalization step can be mediated by clathrin-mediated endocytosis, caveolae, macropinocytosis, or phagocytosis\textsuperscript{66}. In general,
endothelial cells have more caveolae than clathrin-coated pits, with the exception of endothelial cells from the liver sinusoids, where more clathrin-mediated endocytosis occurs. The density of caveolae is the highest in continuous non-fenestrated endothelial cells and those derived from the capillaries; the only exception is the blood brain barrier, where the density of caveolae is particularly low. For the paracellular transport pathway, the movement of molecules is regulated by intercellular junctions such as the tight junction and adherens junction, which vary in composition and density across the vascular tree and beds. The tight junctions are dense and well developed in the arteries, as expected, due to their function of serving as the conduit for blood flow and withstanding the high pressure from the blood. The tightness of the junctions then decreases from arterioles to capillaries to venules, where a laxer paracellular junction is required for material transport and the modulation of leukocyte extravasation. As expected, the junctions are particularly tight in the endothelium of the blood brain barrier due to its high specificity in regulating molecule transport.

The expression of certain endothelial adhesion molecules also varies across the vascular tree, according to where leukocyte trafficking typically takes place. When there is tissue damage, leukocytes are recruited out of the bloodstream into the site of damage to fight off infections. The location in the vasculature that leukocytes typically exit at is the post-capillary venules. The initial step of leukocyte adhesion and rolling is mediated by interaction between carbohydrate ligands on the leukocyte and glycoproteins expressed by endothelial
cells known as selectins, specifically E- and P-selectin. The expression of E-selectin and P-selectin has been found to be the highest in the activated endothelial cells from the postcapillary venules.

The substances and mechanical forces that the endothelial cells are exposed to can all affect its phenotype, often induced as result of the activation of signaling pathways within the cell. Different tissues have a different environment to which the endothelial cells are exposed. For example, endothelial cells derived from the coronary artery are exposed to the mechanical forces of heart contractions as well as factors released by the cardiomyocytes, whereas endothelial cells derived from the brain are exposed to factors secreted by brain cells, which can affect their ability to form a tight blood-brain-barrier. In addition, the force that is applied against the endothelium by blood flow, also known as shear stress, have been shown to induce changes in endothelial cell phenotype such as the actin cytoskeleton and permeability to certain molecules; the specific effects of shear stress on endothelial cells is discussed in detail in a subsequent section.

Finally, while the location and surroundings of the endothelial cells is one way of generating and maintaining their heterogeneity, some evidences suggest that epigenetics may also have a role. Instead of depending on the environment to maintain its unique properties, the cells may also be epigenetically programmed to behave in certain ways. It has been shown by DNA microarray studies that when
endothelial cells from the microvasculature and microvasculature are taken into culture, they exhibit different expression profiles\textsuperscript{72}. Furthermore, when endothelial cells from the human coronary artery were treated with oxidized LDL, the changes in expression of genes involved in the development of atherosclerosis such as adhesion proteins and proliferation were much greater than endothelial cells derived from the leg vein\textsuperscript{73}. This suggests that the higher susceptibility of arteries to atherosclerosis than veins is likely due to the arterial endothelial cell itself being more sensitive to the development of this process than venous endothelial cells.

1.4.2 Endothelial permeability

The endothelium is a major site of material exchange between the blood and the tissues, serving to transport nutrients to and collecting metabolic waste from the tissues\textsuperscript{54}. The exchange of these materials can occur by movement between individual endothelial cells, known as paracellular transport, and through each individual endothelial cells, also known as transcellular transport or transcytosis\textsuperscript{54}. These processes are intricately regulated by the junctions between endothelial cells as well as intracellular vesicle trafficking to maintain a low and selective permeability across the vasculature\textsuperscript{54}. Below, the regulation of paracellular and transcellular transport will be discussed.
1.4.2.1 Paracellular transport

The paracellular transport pathway is regulated by interendothelial junctions. There are two types of cell-cell junctions in the endothelium that serve barrier functions and maintain endothelial permeability; they are the adherens junction and the tight junctions\textsuperscript{54}.

The adherens junction is comprised of the proteins cadherin, p120-catenin protein, β-catenin, plakoglobin, and α-catenin\textsuperscript{75}. Cadherins are transmembrane adhesion proteins that form homodimers in a calcium-dependent manner with the extracellular domain of other cadherins from adjacent cells\textsuperscript{75}. The p120 protein binds to the proximal end of the cytoplasmic tail of cadherin and its function is to regulate the trafficking and turnover of cadherin, as well as cell actin dynamics through modulating the activities of Rho family GTPases\textsuperscript{75}. β-catenin and plakoglobin bind to the distal end of the cadherin cytoplasmic tail, and are linked to the actin cytoskeleton via α-catenin\textsuperscript{75}. The catenin proteins are essential for the proper formation of the adherens junction as the disruption in interaction between cadherin and catenin was shown to significantly decrease the strength of the junction\textsuperscript{76}. Permeability of the paracellular pathway is regulated by changes in the adherens junction, such as the phosphorylation of the cadherins to induce cadherin internalization, as well as the promotion of stress fibres formation within the cell to break apart the adherens junction. Such processes usually occur when the junction needs to be temporarily disrupted to allow the movement of certain
molecules or cells across the endothelial barrier, for example during leukocyte extravasation\textsuperscript{64}.

The adherens junction adhesion protein that is exclusively expressed in endothelial cells is the VE-cadherin (vascular endothelial cadherin). VE-cadherins are critical in maintaining vascular integrity and permeability\textsuperscript{75}. It has been shown that disruptions in the interaction between VE-cadherins are seen in a lot of pathological conditions such as inflammation and acute lung injury\textsuperscript{77}. In addition, the administration of anti-VE-cadherin antibodies in mice has been shown to cause a significant increase in permeability, hemorrhages and vascular fragility\textsuperscript{77}.

The other interendothelial junction that regulates barrier integrity is the tight junction, which forms seals between endothelial cells\textsuperscript{64}. The tight junctions are formed after adherens junctions, which are built first to establish the architectural integrity of the endothelium for proper formation of the tight junction\textsuperscript{78,79}. The main proteins that make up the tight junctions are the claudin and occludin proteins\textsuperscript{80}. Claudins and occludins are transmembrane proteins that interact with claudin and occludin proteins from adjacent cells to form a tight seal arranged like a strand around the cell\textsuperscript{80}. Claudins are important for the assembly of the tight junctions and regulating the permeability of ions between cells\textsuperscript{81}. It has been shown that changing the expression of claudin proteins leads to changes in the conductivity of certain ions\textsuperscript{82}. The claudins that are responsible for forming channels that are selective to ions are claudins 2, 10, 15 and 17\textsuperscript{83}. There are 27 members in the
claudin family\textsuperscript{64}, however, only claudin-5 is exclusively expressed in endothelial cells\textsuperscript{64}. Claudin is associated with several intracellular adaptor proteins such as ZO-1, 2, 3, PATJ, and MUPP1, which connect the tight junction to the actin cytoskeleton\textsuperscript{80}. Occludins are responsible for the stabilization of the tight junction as well as serving barrier functions\textsuperscript{80}.

Adherens junctions are relatively ubiquitous along the vascular tree whereas tight junctions are more variable along different segments of the vascular tree\textsuperscript{85}. In large arteries where the main function is to serve as conduit for blood delivery, the tight junctions are well developed, strictly restricting the transport of molecules across the endothelium. However, in the capillaries, where constant exchange of materials occurs, the tight junctions are much less developed to facilitate this process. In the blood-brain, where the permeability to molecules is highly selective, the tight junctions are dense and highly developed\textsuperscript{64}.

1.4.2.2 Transcellular transport (transcytosis)

In addition to paracellular transport, another way by which molecules get across the endothelium is by moving through (rather than between) individual endothelial cells. This pathway is usually used for the transport of macromolecules and involves first vesicle-mediated endocytosis, then trafficking within the cell, followed by exocytosis at the basal side of the cell\textsuperscript{86}. 
The most studied pathway for transcytosis in endothelial cells is the caveolae-mediated transcytosis pathway. Caveolae are small 50-100nm invaginations in the plasma membrane that are rich in proteins and cholesterol. The caveolae-mediated transcytosis is first initiated by the binding of a ligand to its receptor in the lipid rich domains of the membrane. The caveolin proteins are then phosphorylated by Src kinase and recruited to the lipid rafts where they will aggregate and oligomerize to form caveolar endocytic vesicles. Once a bud is formed, the vesicle is excised from the membrane by the GTPase dynamin, pinching the vesicle off of the plasma membrane. The vesicles can then fuse with endosomes within the cell or move to the basolateral membrane, where they will dock and fuse with to release their contents into the interstitium. Caveolae are typically known to mediate the transcytosis of LDL in aortic and umbilical vein endothelial cells, albumin in rete mirabile microvascular endothelial cells, insulin in aortic and rete mirabile microvascular endothelial cells, and chemokines in venular endothelial cells. Albumin transcytosis is the most studied model for transcytosis in endothelial cells; it has been shown that caveolin-1-deficient endothelial cells are not able to transcytose albumin while wild-type endothelial cells can. In addition, the transcytosis of insulin in bovine aortic endothelial cells (bAECs) has been shown to be mediated by caveolae as well. When the expression of caveolin-1 was knocked down by siRNA in bAECs, the uptake of FITC-insulin was significantly reduced, whereas the overexpression of caveolin-1 increased insulin uptake. Similarly, aortic endothelial cells derived
from caveolin-1-null mouse did not take up insulin while the cells with caveolin-1 re-expressed did\textsuperscript{95}.

It has recently been found that clathrin also has a role in endothelial cell transcytosis, specifically the transport of insulin\textsuperscript{97}. Clathrin is a triskelion shaped protein that is composed of three clathrin heavy chains bound tightly to three light chains. They are recruited to the membrane by adaptor proteins, which bind to the cargo on the extracellular side of the cell and clathrin within the cell\textsuperscript{98}. As more and more clathrins get recruited to the membrane, they start to connect with each other eventually forming a curved lattice around the pit like a cage\textsuperscript{98}. The GTPase dynamin then comes in and pinches vesicle from the membrane, allowing the internalization of the cargo. Soon after scission, the clathrin coat will disassemble, before the vesicle goes on to fuse with other endosomes or be exocytosed\textsuperscript{98}. It was shown by Azizi and colleagues that when the clathrin heavy chain is depleted or inhibited in adipose microvascular endothelial cells, the internalization and transcytosis of insulin was reduced by 50%, suggesting that clathrin may also have a role in transendothelial transport\textsuperscript{97}.
1.4.2.3 Insulin transcytosis

While some older studies have suggested that insulin may cross the endothelial barrier by ways other than the transcellular pathway, most evidence to date supports that insulin crosses the endothelium by transcytosis\textsuperscript{35,49,50,53,52,53}. The process of insulin transcytosis is mediated by three main steps: the uptake of insulin into the endothelial cell, traffic within the cell, and exocytosis\textsuperscript{86}. There have been several studies showing that insulin transport across endothelium is a receptor-mediated process and that both the insulin receptor and IGF-1 receptor may be involved. It has been shown that when a blocking antibody to the insulin receptor (IR) was applied to a retinal endothelial monolayer along with insulin, the transport of insulin across the monolayer was significantly reduced\textsuperscript{53}. In addition, fluorescently labeled insulin taken up by aortic endothelial cells has been shown to colocalize partially with IR and the IGF-1 receptor\textsuperscript{99}.

Furthermore, there has also been in vitro evidence of vesicle-mediated insulin transport within the endothelial cells. The earliest study showing that insulin is carried in vesicles within endothelial cells was accomplished through electron microscopy imaging\textsuperscript{92}. More recently, this has been shown by insulin uptake experiments, where the uptake is significantly reduced in aortic endothelial cells with caveolin-1 is knocked down, and enhanced when caveolin-1 is overexpressed\textsuperscript{95}. Similar results were obtained from endothelial cells isolated from caveolin-1 knockout mice, where insulin uptake was abolished and only rescued
when caveolin-1 was re-expressed in these cells\textsuperscript{95}. Another study has shown in microvascular endothelial cells that insulin uptake and transcytosis is clathrin-mediated\textsuperscript{97}. In this study, it was shown that knockdown of clathrin expression attenuated insulin transcytosis. While there is a discrepancy between the transport mechanisms of insulin transcytosis, likely due to the different types of endothelial cells used, it is clear that insulin is transported across the endothelium at least in vitro by a receptor- and vesicle-mediated process.

The transport of insulin across the endothelium by transcytosis is further supported by findings from in vivo studies. It has been shown in dogs that during euglycemic clamp experiments, when they are infused with insulin and inulin, there are persistently higher plasma levels of insulin than in the lymphatics (a proxy for hormone’s concentration in the interstitial space), while inulin, a molecule with a similar molecular weight as insulin known to only cross the endothelium through the paracellular pathway, was equal in levels from the blood and the lymph\textsuperscript{100}. Another study by Ader and colleagues also found that when glucose tolerance test were performed in dogs, there was a persistently higher plasma level of insulin compared to the lymph, suggesting that the transport of insulin does not occur by simple diffusion\textsuperscript{35}. Other studies have looked directly at the saturability of this process in vivo. One study looked at the transport of radioactive insulin across coronary vessels with and without the presence of unlabeled insulin, and found that when radioactive insulin was perfused in the presence of unlabeled insulin, the presence of insulin in the capillary walls and
heart parenchyma was much lower than those without unlabeled insulin. When a modified version of the radioactive insulin that has lost its affinity to bind to the insulin receptor was applied, much less signal was observed in the capillaries and muscle; unlabeled insulin also did not result in a reduction of this signal\textsuperscript{101}. Another study by Herkner and colleagues observed that the interstitial insulin concentration was much less than that in the plasma during oral glucose tolerance test and that this ratio of plasma to interstitial levels of insulin is enhanced during a hyperinsulinemic clamp\textsuperscript{50}. All of these studies suggest that insulin transport across the microvasculature is a saturable process and also a potentially rate-limiting step to the initiation of insulin action as the insulin must reach the interstitial space before it is able to exert its actions.

1.4.2.3.1 Assays for insulin transcytosis

Insulin transcytosis is an important process to study as it has implications for the development of novel therapies for insulin resistance and T2D; however, knowledge in the regulation of this process is limited due to technical limitations in studying insulin transcytosis. Most studies on transcytosis in cell cultures have made use of the transwell assay, in which transcytosis is quantified by how much of the added insulin moves across the endothelial monolayer grown on a permeable membrane. This assay could be used to assess the saturability of insulin transport across the endothelium by measuring the amount of labeled
insulin that crossed the endothelium when in presence of unlabeled insulin. However, it is not ideal to investigate the specific regulators of this process as this would involve much harsher manipulations to the cells, such as siRNA, which can disrupt the endothelial monolayer and induce paracellular gaps. One of the major caveats of the transwell assay is that it is not able to distinguish between actual transcytosis and paracellular leak, which can be induced by pharmacological or molecular manipulation, and this can greatly cofound the measurement of actual transcytosis.\textsuperscript{102}

Previously, electron microscopy (EM) was also used to study transcytosis. However, it is mostly useful for studying the vesicular traffic of the molecule within the cell, such as its targeting to the lysosome and other endosome compartments. Only fixed cells can be analyzed using EM, which means the amount of molecule that is transported through the endothelial cells cannot be quantified.\textsuperscript{89} Most of all, electron microscope is very expensive for the amount of information it is able to provide to study transcytosis.

More recently, a novel assay for measuring insulin transcytosis by single cells in a confluent endothelial monolayer was developed by our lab that involves using total internal reflection fluorescence microscopy or TIRFM and fluorescently labeled insulin.\textsuperscript{97} TIRF microscopes allow the visualization of a very narrow range of the cell of approximately 100-150nm. We thus focus on the basolateral membrane of the cell after fluorescently-tagged insulin has been added. We are
then able to see vesicles bearing insulin appear and then disappear as they fuse with the basolateral membrane. In principle, this dynamic assay eliminates confounding from paracellular leak, avoids edge effects from transwell assays, and is not affected by low transfection efficiency of molecular constructs since single cells are selected\textsuperscript{102}. With this advancement in measuring insulin transcytosis, it is now easier to study insulin transcytosis and the regulation of this process. More details on the assay will be discussed in the Methods section.
Almost all of the in vitro studies described were performed under static conditions. Yet physiologically, blood vessels are constantly exposed to blood flow; this exerts a force on the endothelial surface known as shear stress. By definition, shear stress is the frictional force parallel to the vessel wall at the surface of the endothelium\(^{103}\). This force is determined by several factors as illustrated in the equation below:

\[
T = 4\eta Q/\pi r^3
\]

Where \(\eta\) is blood viscosity, \(Q\) is blood flow rate, and \(r\) is radius.

Shear stress is directly proportional to blood viscosity and flow rate, while it is inversely proportional to the cube of the radius of the blood vessel. As these factors vary along the vasculature, the shear stress that the endothelial cells are exposed to also varies across the vascular tree. The shear stress that is typically found in the arteries can range from 10 to 70 dynes/cm\(^2\), whereas that found in the capillaries and veins is much lower, ranging from 0.5 to 5 dynes/cm\(^2\)\(^{104,105}\). The application of shear stress to endothelial cells have been shown to induce various changes to the endothelial phenotype such as changes in gene expression, activation of signaling pathways within the cells, and changes in cell morphology, all of which will be discussed in more detail below\(^{71}\).
1.5.1 Effects of shear stress on endothelial cells

One of the many effects that shear stress has on the endothelial cells is the reduction in the rate of endothelial cell proliferation. The exposure to shear stress increases the number of cells arrested in the G0 and G1 phase of the cell cycle and has been shown to lower the DNA synthesis rate\textsuperscript{106,107}. The mechanism by which growth is suppressed in endothelial cells after being exposed to shear stress has been found to be mediated by the tumour suppressor p53\textsuperscript{106}. P53 is a transcription factor that induces the expression of a cyclin-dependent kinase (ckd) inhibitor called p21, which is a protein that inhibits the cdk proteins from promoting cell cycle and proliferation\textsuperscript{108}. It has been found that shear stress increases the association of the cdk proteins with the p21 inhibitor repressing their kinase activity that is required to phosphorylate downstream proteins involved in cell growth\textsuperscript{106}. Shear stress also induces the phosphorylation of the retinoblastoma protein, which when hyperphosphorylated, will bind to the transcription factors required for DNA synthesis, inhibiting DNA transcription and cell growth toward\textsuperscript{107,109}. In addition to the inhibition of endothelial cell growth and proliferation, shear stress also inhibits apoptosis. When endothelial cells that have been exposed to shear are treated with apoptosis inducers such as TNF-alpha and reactive oxygen species, apoptosis is suppressed\textsuperscript{110}. This is likely mediated by the increase in expression of inhibitors of apoptosis proteins 1 and 2 (IAP-1, -2) after exposure to shear stress, which inhibit active caspases and prevent them from inducing apoptosis\textsuperscript{111,112}. 

Another effect shear stress has on the endothelial cells is the induction of cell migration, as shown by studies that have looked at wound healing. These studies have shown that the exposure to shear stress leads to a higher rate of endothelial migration to the wound created in the endothelial monolayer compared to cells under static conditions, resulting in faster wound closure\textsuperscript{113,114}. This directional migration was shown to be mediated by the formation of protruding lamellipodia and focal adhesion remodeling, which are regulated by the Rho family of small GTPases\textsuperscript{115,116}. In addition, focal adhesion kinase (FAK), a protein that regulates the formation of focal adhesions required for providing traction during migration, has been shown to be involved in this shear-induced increase in wound healing, as inhibition of FAK lead to a slower wound healing\textsuperscript{117,118}.

Furthermore, the cytoskeleton is also another effector of shear stress. Shear stress has been shown to induce the elongation and alignment of endothelial cells towards the direction of shear stress\textsuperscript{71}. This alignment involves the remodeling of the cytoskeleton, which is also regulated by the Rho family of GTPases. The Rho family GTPases is comprised of the GTPases Rac, Rho, Cdc42, which switch between the inactive GDP-bound form and the active GTP-bound form\textsuperscript{119}. Rac is activated within 30 minutes of exposure to shear stress and it has been shown to be important for the alignment of the cytoskeleton towards the direction of shear stress, as cells transfected with dominant negative (DN) Rac1 do not become aligned in the direction of shear stress\textsuperscript{120,121}. The speed of
migration of endothelial cells has also been shown to be reduced in DN Rac1 cells\textsuperscript{122}. Unlike Rac, shear stress first induces a transient inactivation of Rho followed by its activation that peaks at 60 minutes and movement to the cell membrane\textsuperscript{121}. Rho has been shown to also be important for the alignment of the actin cytoskeleton, as exposing DN Rho-transfected cells to shear stress resulted in a failure of the cells to align in the direction of shear stress as well as a decrease in shear induced increase in stress fibres\textsuperscript{123}. Cdc42 is a GTPase known to be important for mediating the formation of filopodia, finger-like projections at the cell membrane\textsuperscript{124,125}. Like Rho, it is translocated to the membrane following exposure to shear stress and it has been shown to regulate the microtubule cytoskeleton rather than the actin cytoskeleton\textsuperscript{123,126}.

There has been some evidence suggesting that shear stress can also affect the transport of molecules across the endothelium. It has been shown that the transcytosis of albumin, the major protein in blood, is increased in response to shear stress in a dose-dependent manner\textsuperscript{127}. In addition, the internalization of LDL by aortic endothelial cells, the first step of the transcytosis pathway, has also been shown to be increased in response to high shear stress\textsuperscript{128}.

The shear stress that is experienced at the level of the macrovasculature and the level of microvasculature is different. In the aorta, for example, the blood flow is pulsatile, which results in fluctuations in shear magnitude, while in the capillaries, the blood flow is continuous, which results in a shear that is temporally
and spatially uniform\textsuperscript{129}. Pulsatile flow has been shown to induce significantly higher phosphorylation of ERK1/2 in bovine aortic endothelial cells as compared to those exposed to steady continuous flow\textsuperscript{130}. In another study, the change in endothelial cell shape is less rapid when exposed to pulsatile flow than steady flow; however, cells are more elongated in shape in the pulsatile flow condition\textsuperscript{131}. In addition to pulsatile flow, the blood flow in our circulatory system can be either laminar or turbulent. Laminar flow is when the fluid flows in parallel layers along the axis of the blood vessel; this occurs mostly at straight parts of the vascular tree. Turbulent flow is when the fluid flows in irregular patterns; this occurs usually at branching points of the vascular tree\textsuperscript{132}. Turbulent flow has been shown to affect the endothelial phenotype differently than laminar flow. Turbulent flow has been shown to upregulate the expression of genes that promote the development of atherosclerosis while laminar flow promotes the upregulation of expression of genes that are atheroprotective\textsuperscript{133}. As the shear stress resulting from different types of flow affects the endothelial cells differently, the phenotype of the endothelial cells derived from different locations along the vascular tree is also expected to be variable.

\subsection{1.5.2 Mechanosensors of shear stress}

The effect that shear stress exerts on endothelial cells is mediated by mechanosensors in the cell that sense the mechanical force and transduce it into
intracellular signaling. There are many molecules that have been suggested to have the role of being a mechanosensor in endothelial cells; the best-known mechanosensors are integrins, ion channels, G protein coupled receptors and G proteins, PECAM-1 and caveolin-1. They will be discussed below.

Integrins are transmembrane proteins that form a link between the cell's cytoskeleton and the extracellular matrix (ECM) by binding to specific molecules associated with the two structures. Integrins are heterodimers that are comprised of an alpha- and beta-chain. Tzima and colleagues have found that integrin αvβ3 is activated in just 5 minutes of exposure to 12 dynes/cm² shear stress in bovine aortic endothelial cells, as shown by an increase in WOW-1 (an antibody that binds to activated integrin αvβ3) staining after exposure to shear stress compared to static control. As integrins are activated, they are converted to a high affinity state, which increases their binding to extracellular matrix (ECM) proteins such as fibronectin and vitronectin. The increase in binding to ECM proteins can then induce various intracellular signaling cascades. Shear stress is known to activate Extracellular signal-Regulated Kinase-1 (ERK), c-Jun N-terminal kinases (JNK) and IkappaB (IKB); applying blocking-type anti- αvβ3 to aortic endothelial cells abolishes the activation of these proteins in response to shear stress. Similarly, blocking the activation of β1 integrins attenuated the previously found shear-induced phosphorylation of downstream proteins Akt, SFK, and eNOS. It is well known in the literature that shear stress increases nitric oxide production in aortic endothelial cells as a result of the activation of eNOS,
leading to the vasodilation of blood vessels\textsuperscript{138}. When a synthetic peptide that inhibits integrin binding to the ECM was applied intraluminally to isolated coronary arterioles during exposure to shear stress, shear-induced vasodilation is significantly reduced\textsuperscript{139}. In addition, the application of a blocking-antibody against the $\beta_3$ chain of integrin also caused a significant reduction in the shear-induced vasodilation of the coronary arterioles\textsuperscript{139}. These findings suggest that the shear-induced increase in nitric oxide and vasodilation is in part mediated by integrins.

Ion channels have also been reported to have a mechanosensory role in endothelial cells. Studies have shown that shear stress increases the permeability of the cell membrane to K\textsuperscript{+} inducing an increase in inward K\textsuperscript{+} current. In addition, shear stress has been shown to induce an inward flux of Ca\textsuperscript{2+} in endothelial cells as well, which can lead to various Ca\textsuperscript{2+} dependent cell responses and affect intracellular signaling and endothelial function\textsuperscript{71}. The ion channels that have been found to be shear-activated are the TRPV4 and TRPP1/2 channels\textsuperscript{140}.

Shear stress has been shown to activate G proteins on the endothelial cell membrane. G proteins are molecular switches within the cell that are activated by the G protein coupled receptor, with the active form being GTP bound and the inactive form being GDP bound\textsuperscript{141}. Once activated, the G protein can activate various signalling pathways to change the function of the cell. Ras is a GTPase that is responsible for regulating cell growth, differentiation and survival; it has been shown that within 5 seconds of exposing human umbilical vein endothelial
cells to flow, Ras activation increased by 10-fold as compared to the static control\textsuperscript{142}. However, transfecting the cells with antisense G\textalpha q inhibited this increase. Furthermore, transfecting the cells with constitutively active G\textalpha q did not enhance the shear-induced Ras activation, but transfecting the cells with the G\textbeta 1gamma2 subunit did, suggesting that Galphaq is required to initiate the mechanosensing of shear stress and that the shear-induced Ras activation is mediated by G\textbeta 1gamma2\textsuperscript{142}. As mentioned previously, shear stress activates the proteins ERK in endothelial cells. To test if G proteins are also important for mediating the activation of ERK1/2, cells were treated with the pertussis toxin (PTx), which inactivates Galphai and Galphao proteins, and then ERK activation was measured by the amount of phosphorylated ERK. Following treatment with PTx, the shear-induced phosphorylation of ERK1/2 was completely abolished. These findings provide some evidence for a role of the G proteins in mechanosensing shear stress in endothelial cells\textsuperscript{136}.

PECAM-1 is a surface adhesion protein located at the junctions between endothelial cells that is also found in platelets, monocytes, neutrophils, and some T cell. In endothelial cells, PECAM-1 mediates the migration of leukocytes across the endothelium, as well as processes such as angiogenesis, thrombosis, and mechanosensing of shear stress\textsuperscript{143}. PECAM-1 has been found to be phosphorylated 1 minute after exposure to shear stress\textsuperscript{144,145}. It is known that shear stress induces the translocation of the tyrosine phosphatase, SHP-2, to the endothelial junctions within 5 minutes of application of shear stress. However,
when PECAM-1 is downregulated in endothelial cells, the translocation of SHP-2 to the cell junctions is lost. In addition, the shear-induced ERK phosphorylation that is known to occur also decreases after exposure to shear stress when PECAM-1 expression is decreased\textsuperscript{146}.

Lastly, caveolae/caveolin-1 has also been suggested to have a role in mechanosensing in endothelial cells. Caveolae are small 50-100nm invaginations in the plasma membrane that are rich in proteins and cholesterol. Caveolae are formed with the protein component caveolin-1 and can mediate endocytosis\textsuperscript{87}. It has been shown that after exposing endothelial cells to shear stress for 24 hours, more cav-1 and caveolae were observed on the apical side of the cells\textsuperscript{147}. A more direct evidence for caveolae/caveolin having a role in mechanosensing was obtained from an \textit{in vivo} experiment, in which cav-1 knockout mice were examined\textsuperscript{148}. Shear stress is known to induce the dilation of the blood vessels; it was found that the shear-induced vasodilation was significantly reduced in the arteries of the cav-1 KO mice compared to that of the wild type control. However, this shear-induced vasodilation was restored to similar levels as that of wildtype when caveolin-1 was re-expressed in the endothelium\textsuperscript{148}. This effect was found to be due to the reduction in the level of phosphorylated eNOS, as phospho-eNOS levels were found to be much lower in the carotid arteries from the cav-1 KO mice as compared to wildtype (WT), and that the reconstitution of cav-1 in endothelial cells restored the level of p-eNOS to that in WT\textsuperscript{148}. These studies suggest that
caveolae/caveolin-1 is another important regulator of mechanotransduction in endothelial cells.

2 Chapter 2 Research Aims & Hypotheses

Type 2 diabetes is a disease in which the body is unable to metabolize glucose as a result of insulin resistance\textsuperscript{149}. While insulin resistance is widely known as a condition caused by defects in the insulin-signaling pathway within target tissues such as the muscle and fat, the role of the endothelium in the pathogenesis of insulin resistance is less well-studied\textsuperscript{25}. The transport of insulin across the microvasculature is a potentially rate-limiting step to the delivery of insulin and the initiation of its action. It has been shown that insulin appearance in the interstitial space is delayed in obese insulin-resistant subjects as compared to healthy subjects\textsuperscript{39}. This finding suggests that an impairment in the transport of insulin out of the vasculature could also be a contributing factor to insulin resistance in addition to the impaired insulin-signaling in target tissues. Understanding how insulin transport across the microvasculature is regulated is therefore important as it may lead to the identification of novel therapeutic targets for the treatment of insulin resistance. Most evidence to date suggests that insulin is transported across the endothelium through individual endothelial cells (transcytosis)\textsuperscript{35,49,50,51,52,53}, however, little is known about how this occurs.

Most \textit{in vitro} studies on endothelial cell biology have been done under
static conditions; however, *in vivo*, endothelial cells are constantly exposed to shear stress, a frictional force at the endothelial surface derived from blood flow\(^{103}\). This force has been shown to induce various phenotypic and molecular changes in the endothelial cell, such as the reorganization of the cell cytoskeleton as well as the change in expression of various genes\(^{71}\). In addition, exposure to shear stress has been shown to induce transcytosis of molecules such as albumin through endothelial cells\(^{127}\). However, whether shear stress affects the transcytosis of insulin in endothelial cells is unknown. Thus, we are interested in studying insulin transcytosis in endothelial cells under flow or exposed to shear stress, a condition more representative of the physiological milieu.

The purpose of this study was to determine whether physiological shear stress induced by affects insulin transcytosis by endothelial cells and if so, to determine the mechanism(s). As shear stress has been shown to induce transcytosis of certain molecules such as albumin in endothelial cells, we hypothesize that shear stress also induces the transcytosis of insulin in endothelial cells.
Chapter 3 Shear stress induces endothelial internalization and transcytosis of insulin independently of nitric oxide – potential role of the actin cytoskeleton

3.1 Introduction

While much research into the pathogenesis of insulin resistance has focused on signaling within target tissues such as skeletal muscle, there is growing appreciation of a potential role for the vascular endothelium\textsuperscript{150}. Insulin delivery to downstream tissues like muscle and fat is regulated by hemodynamic factors such as capillary blood flow and capillary recruitment. The dilation of upstream arterioles and precapillary sphincters increases blood flow and recruits non-perfused capillaries, augmenting the amount of insulin that can ultimately reach the downstream tissue bed\textsuperscript{151,152,153}. While precapillary sphincters have been described in the mesentery, it should be noted that it is still controversial whether it exist in the muscle\textsuperscript{206,207,208}. The significance of this regulation is suggested by the observation that insulin delivery is impaired in conditions associated with whole body insulin resistance such as obesity\textsuperscript{154}. Impairment of insulin delivery has been attributed to decreased endothelial nitric oxide (NO) production, as NO is a potent endogenous vasodilator\textsuperscript{153,150}. 
Notwithstanding the hemodynamic factors that regulate insulin delivery, it is important to note that in tissues like skeletal muscle and fat, the endothelial layer that lines capillaries is continuous\textsuperscript{63,155} – without intercellular gaps – thus potentially constituting a barrier to the diffusion of luminal insulin out of the capillary microvasculature. This is in contrast to the sinusoidal endothelium of the liver, which is fenestrated and allows for bidirectional movement of fluids and solutes between the interstitium and the vascular lumen. Thus in skeletal muscle and fat, endothelial permeability to insulin may occur via some combination of two potential routes: by diffusion between adjacent endothelial cells (i.e. paracellular diffusion) or by movement through the cytoplasm of individual cells in the microvascular endothelial monolayer, a process known as transcytosis\textsuperscript{156,157}. In the latter, luminal insulin is internalized at the apical endothelial surface by endocytosis, moves through the cell by vesicular traffic and is ultimately released by exocytosis to the interstitial space.

Despite being described over 30 years ago\textsuperscript{53}, the mechanisms and physiological importance of insulin transcytosis remain unclear. Numerous in vitro studies have supported the notion that endothelial cells may restrict the diffusion of insulin\textsuperscript{158,53,159,95}, but the cellular and molecular mechanisms remain elusive. Some of the uncertainty reflects technical difficulties in distinguishing paracellular diffusion from transcytosis in cultured cells\textsuperscript{102} and the underlying heterogeneity of the endothelium that was studied; for instance, while endothelial cells derived from
large vessels (e.g. the aorta) require caveolae for insulin uptake \(^95\), endothelial cells from the microvasculature appear to use clathrin-coated pits instead \(^{97,53}\).

A further limitation of existing *in vitro* studies is that they are almost uniformly performed under static conditions. *In vivo*, endothelial cells are constantly exposed to shear stress, the force exerted by blood flow which acts in the direction parallel to the cells. This physiological force can induce significant changes in cellular behavior, including cytoskeletal remodeling \(^{160}\) and altered gene expression. Furthermore, shear stress has been reported to enhance nitric oxide synthase activity \(^{161,162,163}\) leading to increased nitric oxide levels. NO, in turn, has been observed to enhance internalization of insulin by endothelial cells from the aorta \(^{164}\); whether this occurs in endothelial cells from the microvasculature was not determined.

To date, the effect of shear stress on endothelial internalization and transcytosis of insulin is unknown. In this study, we applied a defined shear stress to confluent adipose microvascular endothelial monolayers in culture, determined that endothelial internalization of insulin was enhanced, and elucidated the mechanism of this effect.
3.2 Methods

3.2.1 Cell culture

Primary human adipose microvascular endothelial cells (HAMEC) were purchased from ScienCell (Carlsbad, CA), and primary human aortic endothelial cells (HAEC) were purchased from Lonza (Allendale, NJ); both were grown in Endothelial Growth Media-MV (Lonza) at 37°C and 5% CO₂. HAMEC were expanded and used for experiments from passage 4 to 8, and the HAECs from passage 6 to 8. Cells were always seeded on gelatin-coated coverslips or plates and allowed to grow to confluency before experiments.

3.2.2 Transcytosis assay

To visualize and measure insulin transcytosis through individual endothelial cells, fluorescently labeled insulin and Total Internal Reflection Fluorescence Microscopy were used as previously reported. As FITC-insulin (used in the insulin internalization assays) photobleaches too quickly for visualization under TIRFM, Alexa Fluor 568-labeled insulin (A568-insulin) was used instead.

A568-insulin was generated by Dr. Javier Jaldin Fincati by conjugating human insulin (Sigma-Aldrich, St. Louis, MO) with Alexa Fluor 568-succinimidyl
ester using the protocol from the Molecular Probes manual (A20003; Life Technologies). Unreacted fluorophores were removed by extensive dialysis.

Cells were pulsed with 500nM Alexa Fluor 468-insulin for 10 minutes at 4°C to allow binding of the insulin to the membrane. After membrane-binding, the cells were washed twice with 4°C PBS (with Mg$^{2+}$ and Ca$^{2+}$) to remove any unbound insulin. The cells are then visualized under TIRFM on a heated stage in HEPES containing RPMI 1640 media.

Samples were imaged using an Olympus cell TIRF Motorized Multicolor TIRF module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany), at 150x magnification. Images were acquired using a laser with a 561nm excitation and a TIRF penetration depth value of 110nm, and acquisition was performed using Volocity software. Videos of each cell were taken at 10 frames per second for 15 seconds. Transcytosis events were quantified using a custom-written MATLAB scripts developed by Dr. Bryan Heit from the University of Western Ontario, as previously reported (7) and based on an approach described by Steyer et al.$^{165}$ Briefly, the algorithm identifies a vesicle containing A568-insulin by their expected size (9-36 pixels$^2$) and circularity (>0.2). Once the vesicles are identified, the algorithm then tracks the moving vesicles and only analyzes the ones that have stopped, representing cessation of vesicle transport as it docks at the plasma membrane preparing for cargo release. A transcytosis event is identified by a sudden loss in fluorescence of the vesicle (i.e. a decrease
in fluorescence intensity over the last 2 time points of their tracks equivalent to a drop of at least 2.5 standard deviations of the vesicular intensity over the entire period the vesicle has been tracked), as the vesicle fuses with the membrane and releases the fluorescent insulin.

### 3.2.3 Shear stress experiments

A parallel flow chamber connected to a pump (Bioptechs FCS2) was used to apply laminar flow to the HAMEC at 37°C and 5% CO2. The flow chamber contains a 0.1% gelatin-coated coverslip on which the HAMEC are seeded, and a 0.25mm thick gasket (14mm x 22mm) to separate the cells from the glass microaqueduct from which the media is delivered. The entrance to the chamber is attached to the pump and the exit is connected to the reservoir. Media for perfusion is taken up from the reservoir by the pump forming a closed circuit. The shear stress applied to the cells was 0.5 dynes/cm² based on published reports on the microvasculature. The amount of shear stress is calculated by \( \tau = 6 \mu Q/a^2b \), where \( u \) is the viscosity of the media, \( Q \) is the flow rate (ml/second), \( w \) is the width and \( h \) is the height of the flow region. Endothelial Growth Media-MV (Lonza, Allendale, NJ) was used as the perfusing media.
3.2.4 Insulin internalization assay

3.2.4.1 Insulin uptake in response to shear stress

Immediately following exposure to shear stress, HAMEC on a coverslip were taken out of the flow chamber and incubated with 500nM FITC-insulin (Sigma-Aldrich) at 37°C for 10 minutes to allow uptake. The cells were then washed with 4°C PBS (with Mg\(^{2+}\) and Ca\(^{2+}\)) and fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Following this, the cells were incubated with 0.15% glycine for 15 minutes, washed with PBS, and mounted using mounting media (Dako, Carpinteria, CA) supplemented with 1ug/ml DAPI. Insulin uptake after exposure to shear stress was imaged using an Olympus IX81 confocal spinning disk microscopy at 60x magnification, with the acquisition settings kept constant for all images captured. 30-50 cells were imaged per condition. Uptake was quantified using the ImageJ software (NIH, Bethesda, MD). Background correction was applied to all images.

For insulin transcytosis experiments, the cells were exposed to shear stress alone or shear stress supplemented with the nitric oxide synthase inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich) for 2 hours, followed by the measurement of insulin transcytosis using this assay. In separate experiments, a second inhibitor of nitric oxide synthase, L-N\(^\mathrm{G}\)-
Nitroarginine; $N^G$-nitro-L-Arginine (L-NNA) (Sigma-Aldrich) was tested; cells were exposed to shear stress alone or in the presence of 800uM of L-NNA followed by assessment of insulin uptake.

3.2.4.2 Insulin uptake in response to sodium nitroprusside (SNP) and spermine NONOate

HAMECs were serum-starved for 2 hours before being allowed to uptake FITC-insulin in the presence of 0, 0.01, 0.3, 0.5, 1uM SNP (Calbiochem) at 37°C for 30 minutes. In separate experiment, HAMECs were treated with 0, 0.1, 1 and 10uM spermine NONOate in serum-free media for 3 hours before being allowed to uptake 500nM FITC-insulin at 37°C for 10 minutes. In both cases, cells were then washed with 4°C PBS (with Mg$^{2+}$ and Ca$^{2+}$) and fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Following this, the cells were incubated with 0.15% glycine for 15 minutes, washed with PBS, and mounted using mounting media (Dako, Carpinteria, CA) supplemented with 1ug/ml DAPI.

Insulin uptake after exposure to shear stress was imaged using an Olympus IX81 confocal spinning disk microscopy at 60x magnification, with the acquisition settings kept constant for all images captured. 30-50 cells were imaged per condition. Insulin uptake was quantified by the number of punctas using ImageJ software. Background correction was applied to all images.
3.2.5 Nitric Oxide Measurement

To measure nitric oxide (NO) production in HAMEC and HAEC, we used DAF-FM diacetate (Thermo Fisher, Eugene, OR), a reagent that is non-fluorescent until it reacts with NO to form a fluorescent benzotriazole. DAF-FM diacetate is cell-permeant; once it reaches inside the cell, it is deacetylated by intracellular esterases to become DAF-FM, which can no longer leave the cell. Consequently, intracellular NO levels can be measured through the amount of fluorescence within a cell.

To validate that the assay is indeed able to detect a change in NO levels, spermine NONOate, an NO donor, was used. The cells were treated with 10uM spermine NONOate for 3 hours at 37°C. The spermine NONOate was then removed and the cells were loaded with 5uM DAF-FM in HBSS (with Mg^{2+} and Ca^{2+}) for 30mins at 37°C. Following DAF-FM loading, the cells were washed with HBSS (with Mg^{2+} and Ca^{2+}) and imaged immediately under the ImageXpress Micro XLS system. DAF signal was analyzed and quantified using the ImageXpress Software Molecular Devices.

To validate the effectiveness of L-N^G-Nitroarginine; N^G-nitro-L-Arginine(L-NNA) (Sigma-Aldrich) to inhibit nitric oxide production in HAMEC, the cells were treated with 800uM of L-NNA for 3 hours and then NO levels measured using the DAF-FM assay.
3.2.6 Actin cytoskeleton modification and staining

To modify the actin cytoskeleton, the actin cytoskeleton-modifying drugs, Cytochalasin D (CD) (Sigma-Aldrich) and Jasplakinolide (Jas) (Sigma-Aldrich), were used. Cytochalasin D binds to the barbed end of the F-actin, preventing polymerization, while Jas stimulates actin nucleation and promotes actin polymerization.

To stain for the actin cytoskeleton after exposure to shear stress, the cells were fixed immediately after exposure to shear stress with 4% PFA for 30 minutes at room temperature. Following this, the cells were incubated with 0.15% glycine for 15 minutes, washed with PBS (without Mg\(^{2+}\) and Ca\(^{2+}\)), and permeabilized with 0.1% Triton X-100 for 20 minutes. Following permeabilization, the fixed cells were incubated with 5% for 1 hour, followed by a wash and incubation with rhodamine-phalloidin for 30 minutes in the dark. Following staining, the cells were washed and mounted using mounting media (Dako, Carpinteria, CA) supplemented with 1ug/ml DAPI. The actin cytoskeleton staining was imaged using Olympus IX81 confocal spinning disk microscopy at 20x magnification, with the acquisition settings kept constant for all images captured.

For the insulin uptake experiments under static and shear stress conditions, HAMEC were treated with 20nM of CD and 5nM of Jas for 30 minutes immediately before uptake.
3.2.7 Statistical Analysis

All experiments were done at least 3 times, unless otherwise indicated. Data are presented as mean and standard error, unless otherwise indicated. One-sample test was performed for the normalized data. Comparison of multiple conditions was evaluated using one-way ANOVA with a post hoc test. All statistical analyses were performed using Prism software (Graphpad, La Jolla, USA).
3.3 Results

3.3.1 Shear stress induces insulin uptake and transcytosis

Our experimental system uses primary human adipose microvascular endothelial cells (HAMEC), which form continuous monolayers in culture that demonstrate circumferential tight and adherens junctions as well as polarized behaviour. Confluent monolayers of these cells were subjected to 2 hours of 0.5 dyn/cm$^2$ of continuous shear force, a level of shear stress similar to what has been reported to occur in the microvasculature. Afterwards, the cellular internalization of FITC-conjugated insulin over 10 minutes was then quantified. Cells that had been exposed to shear stress exhibited a two-fold increase in internalization of insulin (Figure 3AB); internalization of dextran, as a control for non-specific fluid phase uptake, was unaffected (not shown). To determine whether this increased uptake of insulin translated into an increase in insulin transcytosis, we used a recently reported assay based on total internal reflection fluorescence (TIRF) microscopy that measures the exocytosis of insulin-containing vesicles with the basal membrane of endothelial cells. Similar to the effect on uptake, shear stress induced a significant increase in insulin exocytosis events (Figure 3C).
Figure 3. Exposure to 2 hours of shear stress (0.5 dynes/cm2) induced a two-fold increase in insulin uptake and transcytosis in HAMEC. (AB) Cells treated with FITC-insulin for 10 minutes after exposure to 2 hours of shear stress. White scale, 10µM. **p<0.01 by one sample t test; data are normalized to control cells. N=4. (C) Average A568-insulin transcytosis events after exposure to 2 hours of shear stress. **p<0.01 by one sample t test; data are normalized to control cells. N=5. Inset shows the number of exocytosis events for each condition and the variability within one representative experiment.
3.3.2 Nitric oxide does not increase insulin uptake by adipose microvascular endothelial cells

Given that shear stress is known to induce nitric oxide (NO) production by endothelial cells, we hypothesized that the effect on insulin uptake was related to increased NO. We incubated HAMEC monolayers with increasing concentrations of sodium nitroprusside (Na2[Fe(CN)5NO], a well-established donor of nitric oxide\textsuperscript{164} that has been reported to induce insulin uptake by endothelial cells derived from the aorta. However to our surprise, sodium nitroprusside had no significant effect on insulin internalization across a broad range of concentrations (Figure 4AB). Furthermore, inhibition of nitric oxide synthases with the classical inhibitor L-NAME\textsuperscript{169} had no effect on shear-stress induced insulin transcytosis (Figure 4C).
Figure 4. Nitric oxide does not affect insulin uptake and mediate the shear-induced increase in insulin transcytosis in HAMEC. Insulin uptake is not affected by sodium nitroprusside (an NO-donor) in HAMEC and blocking nitric oxide synthase with L-NAME (a NOS inhibitor) during exposure to shear stress further enhances shear-induced transcytosis. (A) Images demonstrate cells which have been treated with insulin and increasing doses of SNP for 30 minutes. (B)
Quantification of insulin uptake in cells treated with FITC-insulin and SNP for 30 minutes. White scale, 35 µm. N=3 (C) Average A568-insulin transcytosis events after exposure to 2 hours of shear stress and shear stress supplemented with 100µM L-NAME. **p<0.01 by ANOVA and **p<0.01 compared to control group by post-hoc analysis. N=4.
3.3.3 Nitric oxide induces insulin uptake by aortic endothelial cells

The observation that sodium nitroprusside had no effect on insulin uptake by HAMEC monolayers was surprising given what has been reported using endothelial cells from the bovine aorta\textsuperscript{164}. Although classically sodium nitroprusside is thought to spontaneously release NO, some data have indicated that an active conversion of the drug into NO is required via an NADH oxidoreductase enzyme\textsuperscript{170}. Thus, to assess the role of NO on insulin internalization without this potential confounder, we incubated endothelial cells from both the aorta and adipose tissue with spermine NONOate, a NO donor that dissociates spontaneously into NO at physiological pH\textsuperscript{171}. We confirmed the release of NO by the drug in HAMEC monolayers using DAF-FM Diacetate (4-Amino-5-Methylamino-2',7'Difluorescein Diacetate), a highly sensitive and membrane-permeant fluorescent sensor for NO that is cleaved upon cellular internalization and then retained inside cells\textsuperscript{172}. As expected, incubation of cells with spermine NONOate induced a marked (almost 80-fold) increase in DAF-FM fluorescence (Figure 5A). However, while the drug increased insulin uptake in confluent human aortic endothelial cells (HAEC) in a dose-dependent manner, as well as a significant increase in uptake at 10µM spermine NONOate, it had no effect on HAMEC monolayers (Figure 5BC). Finally, to confirm the data we obtained with the eNOS inhibitor L-NAME (i.e. Figure 2C), we pre-incubated HAMEC monolayers with a second endothelial nitric oxide synthase (eNOS) inhibitor (L-NNA\textsuperscript{173}). After confirming that it reduced NO production (Figure 5D),
we determined its effect on shear stress-induced insulin internalization. Similar to the experiments with L-NAME, L-NNA had no effect on the increase in insulin uptake induced by shear stress (Figure 5E). Taken together, these data suggest that the effect of shear stress on the internalization and transcytosis of insulin by adipose microvascular endothelial cells is independent of nitric oxide.
Figure 5. Insulin uptake is increased by the NO-donor spermine NONOate in HAEC but not HAMEC, and blocking NO production during exposure to shear stress does not affect shear-induced insulin uptake. (A) Pre-treatment of HAMEC with spermine NONOate for 3 hours resulted in a 75-fold increase in NO production as measured by the DAF-FM assay. N=1. (B) Pre-treatment of human aortic endothelial cells (HAEC) with different doses of spermine NONOate
for 3 hours lead to a dose-dependent increase in FITC-insulin uptake. *p<0.05 by ANOVA and *p<0.05 compared to control group by post-hoc analysis. N=3 for control, 0.1 µM and 1 µM spermine NONOate. N=5 for 10µM spermine NONOate.

(C) Pretreatment of HAMEC with different doses of spermine NONOate for 3 hours did not affect insulin uptake. N=3. (D) Pre-treatment of HAMEC with the nitric oxide synthase-inhibitor L-NNA for 3 hours resulted in a dose-dependent decrease in NO production as measured by the DAF-FM assay. *p<0.05 by ANOVA and *p<0.05 compared to control group by post-hoc analysis. N=3 (E) Exposing HAMEC to 2 hours of shear stress supplemented with the endothelial nitric oxide synthase inhibitor, L-NNA, did not affect the shear-induced uptake. N=3.
3.3.4 Shear stress induces remodelling of the actin cytoskeleton

We then turned our attention to the actin cytoskeleton, a structure known to be affected by shear stress and which regulates endocytosis\textsuperscript{174}. We hypothesized that the effect of shear stress to induce insulin internalization required actin remodelling. As expected, shear stress induced re-alignment of cells in the HAMEC monolayer in the direction of shear stress (Figure 6A). To determine whether actin remodelling was required for shear-induced insulin internalization, we took advantage of well-characterized pharmacologic agents that act on the actin cytoskeleton in distinct and opposing ways. Specifically, we used cytochalasin D, which binds to the barbed ends of microfilaments and blocks actin polymerisation\textsuperscript{175}, and jasplakinolide, a cyclic peptide that stimulates actin polymerization\textsuperscript{176}. To focus on the role of shear stress, we first determined the dose and duration of exposure to cytochalasin D and jasplakinolide that had no effect on insulin uptake under static conditions (Figure 6BC). We then observed the effect of these agents under conditions of shear stress. Jasplakinolide completely prevented the shear-induced internalization of insulin by HAMEC monolayers, while cytochalasin D had the opposite effect, significantly potentiating shear-induced insulin internalization (Figure 6DE). Together, these data suggest that the effect of stress stress to induce insulin internalization by HAMEC monolayers involves actin remodeling.
A. Static Flow

B. Control 20nM CD

C. Control 5nM Jas

D. 20nM CD

E. 5nM Jas

Direction of flow

Insulin uptake (normalized to control)

Flow + CD

Flow + Jas
Figure 6. (see opposite page) Remodeling of the actin cytoskeleton is induced by shear stress and may be involved in the shear-induced insulin uptake. (A) Rhodamine-phalloidin staining of the actin cytoskeleton in HAMEC under static conditions and after exposure to 2 hours of shear stress. The actin cytoskeleton appears more organized and aligned in the direction of shear stress. N=1. (B, C) Treatment of HAMEC with 20nM CD and 5nM Jas for 30 minutes does not affect FITC-insulin uptake under static condition. N=3. (D, E) Exposing HAMEC to 2 hours of shear stress supplemented with 20nM CD in the last 30 minutes further enhanced the shear-induced increase in insulin uptake while 5nM Jas ablated the shear-induced effect. N=3. *p<0.05 and **p<0.01 by ANOVA, *p<0.05 and **p<0.01 compared to control group by post-hoc analysis.
3.4 Discussion

Although many mechanisms for insulin resistance have been described\textsuperscript{177}, most research has focused on the role of impaired signaling of insulin at the level of skeletal muscle. However for insulin to exert its effects on downstream organs, it first requires access to the capillaries that perfuse the tissues. The process by which circulating insulin reaches the tissues is known as insulin delivery. Pioneering work by Bergman’s group has demonstrated that plasma insulin levels exceed those in the interstitial fluid or the lymphatics of skeletal muscle by as much as a factor of 2 to $3^{35,51}$. In experiments in which insulin is injected into the vein, there is a delay before any increase in insulin concentration in the muscle interstitial fluid or muscle glucose uptake\textsuperscript{35}; this delay is obviated if insulin is instead injected directly into the muscle, bypassing the vasculature\textsuperscript{52}. These data suggest that insulin delivery is rate-limiting for insulin action.

While the hemodynamic component of insulin delivery – i.e. blood flow and capillary recruitment – has been well studied, the second half of insulin delivery – the transit of insulin across the endothelial monolayer – is much less well understood. Most \textit{in vitro} studies suggest that this process occurs by endothelial transcytosis\textsuperscript{178,53,159}, a facilitated process that integrates endocytosis of insulin at the luminal surface, vesicular traffic and finally exocytosis of insulin-containing vesicles at the basal membrane of the cells. As with insulin delivery in general, if insulin transcytosis is rate-limiting for insulin action, developing ways to accelerate
insulin transcytosis might constitute a novel and clinically useful approach for addressing insulin resistance.

Work by Wang and Barrett's group has implicated caveolae in the endothelial internalization and transcytosis of insulin. It has been proposed that pro-inflammatory cytokines such as IL-6 and TNF-α can inhibit caveolin-1 expression and thereby impair insulin transcytosis. In one study, TNF-α reduced insulin internalization by aortic endothelial cells and this was overcome through administration of the NO donor sodium nitroprusside. This conceptual framework is attractive given that systemic insulin resistance is associated with endothelial dysfunction, a broad term encompassing increased expression of adhesion molecules, cytokine production and diminished production of NO. However, endothelial cells are known to be heterogeneous depending on their tissue bed; furthermore, while large vessels such as the aorta possess a smooth muscle layer that can respond to NO, most of the microvasculature is lined only by endothelial cells. Thus, it is not apparent a priori that microvascular and macrovascular endothelial cells should produce or respond to NO in the same way.

In this report, we describe an effect of shear stress to enhance microvascular endothelial uptake and transcytosis of insulin that is independent of NO. We observed similar results using two different donors of nitric oxide and two distinct chemical inhibitors of endothelial nitric oxide synthase. Instead, our data
implicate a role for remodelling of the actin cytoskeleton in regulating the shear-induced endocytosis of insulin.

Actin remodelling is a feature of endocytosis in many cell types, with changes in actin filament dynamics being reported to both stimulate\textsuperscript{181} and inhibit\textsuperscript{182} vesicular internalization. These apparently contradictory roles of actin in endocytosis reflect a requirement for actin polymerization to generate force and to drive membrane invagination, but also the potential of a cortical actin microfilament network to obstruct vesicular internalization. Shear stress is a potent force for actin remodelling, inducing the depolymerisation of actin filaments within minutes and reducing cell motility\textsuperscript{160}. Interestingly, the actin polymerizing agent jasplakinolide has been reported to induce the arrest of clathrin-coated pits by epithelial cells\textsuperscript{182}, consistent with our data, in which we showed that jasplakinolide prevented the shear-induced insulin uptake (known to be mediated by clathrin in HAMEC\textsuperscript{97}).

Actin remodelling is a complex phenomenon regulated by members of the RhoGTPase family and numerous actin-binding and nucleation proteins, including formins, Arp2/3 and cofilin\textsuperscript{174}. Future work will be required to delineate the precise molecular mechanisms of the effect of shear-stress, which we postulate involves modulation of the activity and subcellular localization of Rac or RhoA. We speculate that this may involve mechanosensing of shear force by focal adhesions, which link the extracellular matrix with the cytoskeleton\textsuperscript{183,184}. 
Transcytosis can be conceptualized as consisting of three phases: ligand internalization, vectorial traffic of the vesicles, and exocytosis at the basal membrane. While we have focused on the first step, endocytosis, it is conceivable that shear stress affects the other stages in different ways. It is therefore reassuring that our data using total internal reflection fluorescence microscopy as an integrated measure of transcytosis also demonstrates induction by shear stress.

Our data are derived from adipose microvascular endothelial cells and should be confirmed in microvascular endothelial cells from skeletal muscle, since skeletal muscle is the major determinant of post-prandial glucose uptake. That said, our observations reaffirm the important differences between endothelial cells derived from the microvasculature and those originating from larger vessels. As nutrient exchange takes place at the level of capillaries, choosing the appropriate cellular system of study is clearly important. Finally, given that endothelial cells exhibit phenotypic drift in culture, it will be important to confirm our observations using ex vivo or in vivo approaches.

At a broader level, our data suggest the possibility of NO-independent strategies for enhancing insulin delivery to tissues like fat and skeletal muscle. Given that NO-mediated vasodilation is critical for insulin delivery by augmenting tissue perfusion, it is possible to envisage coupling NO-repletion strategies with
distinct downstream, microvascular endothelial-specific mechanisms for increasing endothelial transcytosis of insulin. Ongoing studies to elucidate the regulation of insulin transcytosis by the microvascular endothelium may therefore provide important insights for the management of insulin resistance.
Chapter 4 Discussion and Future Directions

Type 2 diabetes is a disease in which the body is unable to metabolize glucose as a result of insulin resistance\textsuperscript{149}. While insulin resistance is widely known as a condition caused by defects in the insulin-signaling pathway within target tissues such as the muscle and fat\textsuperscript{25}, the role of the endothelium in the pathogenesis of insulin resistance is less well-studied. The transport of insulin across the microvasculature is a potentially rate-limiting step to the delivery of insulin and the initiation of its action. It has been shown that insulin appearance in the interstitial space is delayed in obese insulin-resistant subjects as compared to healthy subjects\textsuperscript{39}. This finding suggests that an impairment in the transport of insulin out of the vasculature could also be a contributing factor to insulin resistance in addition to the impaired insulin-signaling in target tissues.

Understanding how insulin transcytosis in endothelial cells is regulated is thus important, as it will lend insights into whether or not this process also contributes to insulin resistance. In addition, identifying ways to induce insulin transcytosis in endothelial cells would have great implications for the development of novel therapeutics for the treatment of insulin resistance. We are therefore interested in studying insulin transcytosis and ways by which we can stimulate and enhance this process.
Most in vitro studies on insulin transcytosis have been done under static conditions. However, physiologically, the endothelial cells are constantly exposed to a force applied to the surface of the endothelium generated by blood flow called shear stress\textsuperscript{103}. This force has been shown to induce various changes to the endothelial phenotype, including the induction of albumin and LDL transcytosis\textsuperscript{127,128}. We are thus interested in studying insulin transcytosis under the influence of shear stress \textit{in vitro} to see if insulin transcytosis is also affected by this mechanical force, and if so, to determine the mechanism behind it.

Accordingly, we hypothesize that insulin transcytosis is increased after exposure to shear stress.

We have demonstrated that exposing HAMEC to two hours of flow with a shear stress of 0.5 dynes/cm\textsuperscript{2} induced a two-fold increase in transcytosis compared to the static control. To assess whether shear stress induces insulin transcytosis using another method and to also explore the mechanism behind the increase in transcytosis after exposure to shear stress, we looked at insulin uptake, the first step in transcytosis. From our results, we observed a two-fold increase in insulin uptake in HAMEC after exposure to shear stress compared to the static control, just like the increase we saw with insulin transcytosis. This provides further support that insulin transcytosis may be induced after exposure to shear stress and that this is in part due to the increase in internalization of insulin. However, this does not exclude other possible mechanisms for inducing transcytosis such as the induction of the exocytosis step. Further studies could be
done to test whether shear stress also induces exocytosis by allowing HAMEC to uptake insulin before exposing them to shear stress, and measuring the release of insulin during and after exposure to shear stress. An increase in release would indicate that shear stress also has an effect on the exocytosis step of insulin transit as the uptake was the same between both the shear stress and static conditions.

To investigate the mechanism behind the shear-induced increase in insulin uptake, a proxy for transcytosis, we turned to nitric oxide. Wang and colleagues have recently found that nitric oxide increases insulin uptake and flux in BAEC\textsuperscript{164}. When they applied SNP, an NO donor, or L-arginine, the substrate of the endothelial nitric oxide synthase, to BAEC, they observed a significant increase in insulin uptake\textsuperscript{164}. Similarly, when they applied SNP during a transendothelial transport assay using transwells, they also saw an increase in the transendothelial flux of insulin\textsuperscript{164}. This paper received much interest in the field, as this is the first study to identify a substance that is able to induce insulin uptake and transcytosis in endothelial cells. It is well known in the literature that shear stress induces the activation of the eNOS and production of NO\textsuperscript{137}. Since we found that shear stress induces insulin uptake, the role of nitric oxide in the shear-induced insulin uptake was investigated.

The most logical first step in approaching this investigation is to measure if eNOS activation indeed increases after exposure to shear stress in HAMEC.
However, due to technical limitations, such as the inability to extract enough protein from the small 14mm x 22mm flow region to probe for phospho-eNOS, we were not able measure this and prove that eNOS activation and nitric oxide production is increased after exposure to shear stress in HAMEC. Therefore, there is also a possibility that nitric oxide production is not stimulated in response to shear stress in HAMEC. Either way, we could still go ahead with the experiments in testing the hypothesis that NO is mediating the shear-induced increase in insulin uptake.

Following the insulin uptake protocol from Wang and colleagues’ study, we treated the HAMEC with different doses of SNP, and did not observe a significant change in insulin uptake at any of the doses compare to control. Similarly, treatment with spermine NONOate, another NO donor that we verified causes an increase in nitric oxide using the DAF-FM assay, also did not induce an increase in insulin uptake. However, like Wang et al.’s study\textsuperscript{164}, spermineNONOate did induce a dose-dependent increase in insulin uptake in aortic endothelial cells. This discrepancy in the effect of NO on insulin uptake in aortic and microvascular endothelial cells is likely due to the fact that these cells were derived from different tissue beds and locations in the vascular tree. Given that endothelial cells from different tissue beds display different functional characteristics\textsuperscript{63,155} and that the vessels involved in the direct delivery of insulin to the target tissues are not surrounded by smooth muscle for which sensitivity to nitric oxide would be
expected, microvascular endothelial cells might not respond the same way to NO as the macrovascular endothelial cells.

Taken together, our data suggest that NO is unlikely to account for shear-induced insulin uptake by microvascular adipose endothelial cells.

**Limitations**

**TIRF assay**

While the TIRF assay is a new and improved method of measuring insulin transcytosis, there are still limitations associated with this assay. It has been discovered that the transcytosis events that are measured and quantified in this assay may be confounded by photobleaching events. The algorithm for the TIRF assay differentiates a transcytosis event from a photobleaching event by looking for a sudden loss in fluorescent signal indicating the fusion of the vesicle bearing insulin with the membrane; the loss of fluorescence from fusion typically greatly exceeds the gradual loss due to photobleaching. However, we have recently found that when A568-insulin is applied to a glass coverslip with no cells, it produces signals that are similar to that of transcytosis events; this signal can also be detected by the algorithm. The fluorescence of a single fluorophore decays much faster than several fluorophores combined together; thus, the photobleaching of a single fluorophore may be detected as a transcytosis event.
Vesicles usually contain more than one internalized insulin, so the vesicles that get into the TIRF zone but are not transcytosed are unlikely have a sudden loss in fluorescence signal profile and be counted as transcytosis. However, A568-insulin applied to the cells during the membrane binding step could get under the cell, stick to the coverslip, and the photobleaching of a single A568-insulin molecule could be counted as a transcytosis event. In addition to the limitations of the algorithm to distinguish between transcytosis events and photobleaching events, the A568-insulin used in this experiment were found post-experiment to contain many free fluorophores, which could also contribute to the transcytosis signal and confound the true transcytosis events measured. Free fluorophores may be taken up by the cells by macropinocytosis and transcytosed contributing to a transcytosis event.

As the TIRF assay has this limitation, it would be wise to seek other means of confirming the shear-induced increase in insulin transcytosis. One way by which this can be done is to expose HAMEC to shear stress after they have taken up insulin, and then measure the amount of insulin released by the cells using an enzyme-linked immunosorbent assay (ELISA). This experiment will also provide information as to whether or not the shear-induced increase in transcytosis is a result of an increase in insulin internalization only or also an increase in the exocytosis of insulin. If shear stress also induces insulin exocytosis, then a higher release of insulin would be expected in the shear stress condition. However, if shear stress only increases the internalization of insulin and thereby the amount of
insulin available to be exocytosed, then an increase in insulin release may not necessarily be observed. We have performed this experiment wherein we measured the release of insulin under static conditions and in response to shear stress after uptaking the same amount of insulin, and have shown that after allowing the cells from the static and shear stress condition to uptake unlabelled insulin for 5 minutes and then exposing them to 15 minutes of shear stress, the amount of insulin released by the cells is much greater in the shear stress condition than the static condition (Figure 7). This provides further support that shear stress indeed induces insulin transcytosis and that this occurs by both an increase in internalization and the exocytosis of insulin.
Figure 7 Shear stress induces insulin release in HAMEC. Insulin release is increased upon exposure to shear stress over time. HAMEC were allowed to uptake insulin for 5 minutes, and then exposed to static condition or shear stress for 15 minutes. A sample of the supernatant at time points 0, 5 and 15 minutes after exposure to shear stress was taken.
Shear stress experiments

The shear stress that is applied in this study is 0.5 dynes/cm$^2$; however, physiologically, the shear stress that microvascular endothelial cells are exposed to ranges from 0.5 to 5 dynes/cm$^2$. It has been shown by several studies that exposure of endothelial cells to different shear stress actually results in different endothelial phenotype and behavior. Furthermore, the duration of exposure to shear stress has also been shown to affect endothelial cells differently. For example, after exposing bovine aortic endothelial cells to shear stress for 3 hours, the cells had an increase in stress fibers, thicker intercellular junctions, and more cortical actin. After 6 hours, the cells began to have their microtubule organizing centers and nucleus towards the upstream region of the cell. After 12 hours, the stress fibers became thicker and longer, and the height of the intercellular junctions was enhanced. In this study, we have only explored the effect of shear stress at one particular shear stress for a specific duration. Whether or not the exposure to higher shear stress and longer duration of shear stress would produce the same effect is unknown. Therefore, it would be worth exploring the effect of different amount of shear stress and duration of exposure on insulin uptake and transcytosis for the future.

Finally, one experiment that would have strengthened our hypothesis that the changes in the shear-induced phenotype produced by applying CD and Jas are indeed mediated by changes in the actin cytoskeleton would be to image the
actin cytoskeleton (e.g. phalloidin, GFP-tagged actin) under the different conditions. This will show more directly what changes occur in the actin cytoskeleton when the cells are exposed to shear stress and shear stress with CD/Jas and establish that the inhibitors are working in the way we propose.
Future Directions

*How does shear alter the cytoskeleton to favor insulin uptake by endothelial cells?*

Our data suggest (but do not prove) that remodeling of the actin cytoskeleton is behind the effect of shear stress to induce insulin uptake. While it is not known how changes in the actin cytoskeleton are able to affect insulin uptake, it is known what regulates actin cytoskeleton dynamics, and the regulators of this process have been shown to be affected by shear stress. Actin dynamics are regulated by the Rho family of GTPases, which includes Rac, Rho, and Cdc42. These molecules have all been shown to be activated in endothelial cells after exposure to shear stress. Rac is activated within 30 minutes of exposure to shear stress in bovine aortic endothelial cells, and has been shown to be important for the alignment of the actin cytoskeleton towards the direction of shear stress. Similarly, Rho GTPase is also activated following exposure to shear stress, although there is an initial transient inactivation at the start of exposure to shear stress, and it is translocated to the cell membrane. Like, Rac, it also mediates the alignment of the actin cytoskeleton as exposing DN Rho endothelial cells to shear stress resulted in a failure of the cells to align in the direction of shear stress. The role that Cdc42 plays in response to shear stress is mainly the regulation of the microtubule cytoskeleton rather than the actin cytoskeleton. As Rac and Rho have been shown to mediate actin
cytoskeleton, it is possible that they may be involved in the shear-induced increase in insulin uptake and transcytosis.

In addition to molecular regulators of the actin cytoskeleton, mechanosensors in endothelial cells can also be explored. One known mechanosensor is the integrins, which are proteins in the membrane that form a link between the extracellular matrix and the cell’s actin cytoskeleton\textsuperscript{134}. They are known to associate with regulators of the actin cytoskeleton such as parvin, filamin, alpha-actinin and tensin, and have been shown to recruit actin nucleating proteins such as arp2/3 complex and formin\textsuperscript{189,190}. Furthermore, they are required for the formation of stress fibres, a phenotype that is often observed in endothelial cells after exposure to shear stress\textsuperscript{191}. Integrins have been shown to be activated within 5 minutes of exposure to shear stress and are known to mediate various shear-induced effects in endothelial cells\textsuperscript{192}. For example, blocking the activation of b1 integrins attenuated the shear-induced phosphorylation of downstream proteins Akt, SFK, and eNOS\textsuperscript{137}. To investigate a possible role for integrins in the shear-induced insulin effect, a blocking-antibody against integrins may be applied during shear stress followed by insulin uptake/transcytosis. An ablation in the shear-induced effect would suggest a role of the integrins in mediating this effect.

Another well-known mechanosensor of shear stress is caveolin-1, a protein component of caveolae, which are cholesterol-rich invaginations in the plasma membrane\textsuperscript{87}. Caveolin-1 has been shown to mobilize to the apical side of the cell...
in response to shear stress and mediate the shear-induced activation of eNOS\textsuperscript{147}. However, whether or not it mediates the induction of insulin uptake/transcytosis in endothelial cells after exposure to shear stress is unknown. One can investigate this by inhibiting the formation of caveolae using cholesterol depletion drugs such as methyl-B-cyclodextrin (MBCD) and nystatin or knocking down caveolin-1 by siRNA to look at its effect on the shear-induced insulin uptake and transcytosis.

Transient receptor potential channels (TRP), such as the calcium channels TRPV4 and TRPP1/2, are also known mechanosensors of shear stress\textsuperscript{140}. The influx of Ca\textsuperscript{2+} has been shown to be significantly increased through these channels immediately following the application of shear stress on endothelial cells\textsuperscript{193}. Ca\textsuperscript{2+} is an important signalling molecule that is able to affect various signalling pathways and also the interaction between the myosin and the actin filaments\textsuperscript{194,195}. It would be interesting to test whether the increase in uptake/transcytosis after exposure to shear stress is a result of increased Ca\textsuperscript{2+} by applying inhibitors of the TRPV4 and TRPP1/2 channels or Ca\textsuperscript{2+} chelators during exposure to shear stress.

Is the effect specific to insulin?

In this study, we have shown that shear stress increases the uptake and transcytosis of insulin by microvascular endothelial cells; however, whether or not this effect is specific to insulin only is not known. In endothelial cells derived from
large vessels such as the aorta, shear stress has been shown to increase the uptake and transcytosis of albumin and LDL\textsuperscript{127,128}; thus, it is a possibility that shear stress induces a general enhancement in the uptake and transcytosis of molecules in endothelial cells. In fact, one study showed that exposing bovine aortic endothelial cells to increasing level of shear stress for 1 hour lead to a shear-dependent increase in pinocytosis\textsuperscript{196}. However, once again, this study was performed in aortic endothelial cells; whether this finding also applies in microvascular endothelial cells is unknown. Other molecules such as VLDL, IgG and transferrin are also recognized to traverse the endothelium by the transcytosis pathway\textsuperscript{197}. It would be interesting to apply the assays from this study on these other molecules known to transcytose in the endothelial cells and see if shear stress also affects their transport.

**Does the effect occur in vivo?**

As mentioned previously, most *in vitro* studies of endothelial cells have been done under static conditions, including those from our group reported above. However, *in vivo*, endothelial cells are constantly exposed to shear stress, a frictional force at the endothelial surface derived from blood flow\textsuperscript{198}. Due to this reason, we were interested in looking at insulin uptake and transcytosis under more physiological conditions by applying flow or shear stress to an endothelial monolayer. While we were able to study insulin transcytosis under a more physiological condition by applying shear stress to an endothelial monolayer, it is
not known if this effect actually occurs in vivo as endothelial cells taken into culture experience phenotypic drift. For example, the glycocalyx is completely lost when endothelial cells are taken into culture\textsuperscript{199}, and the number of caveolae decreases with increasing passages of the cells\textsuperscript{200}. In vivo, endothelial cells are covered apically by a layer of glycocalyx and surrounded by pericytes and connective tissues\textsuperscript{201,202}. It is known that the endothelial phenotype and function can be greatly affected by their environment\textsuperscript{41}. Due to these differences between the \textit{in vitro} and \textit{in vivo} environment, the endothelial cells may not respond the same way to shear \textit{in vivo}. A future direction for this study would be to validate our findings \textit{in vivo}; one way this can be approached is to perfuse an aorta \textit{ex vivo} with a set shear stress, and observe the uptake of fluorescent insulin in the aorta. Furthermore, it can be studied \textit{in vivo} where movement of fluorescent insulin across the microvasculature of the cremaster muscle can be imaged by intravital microscopy under controlled perfusion rates.

\textit{What are the physiological implications of our results?}

Our results show that applying shear stress to adipose microvascular endothelial cells induces the uptake and transcytosis of insulin. In addition, our preliminary data suggest that shear stress may also stimulate the exocytosis of insulin. Physiologically, after a carbohydrate-rich meal, insulin is released stimulating its own delivery to the tissue bed by increasing blood flow and capillary recruitment to the target tissues\textsuperscript{42}. Our data suggest that this increase in blood
Flow and capillary perfusion may further increase the delivery of insulin to the target tissue by enhancing the transcytosis of insulin in endothelial cells. It has been shown by Azizi and colleagues that after allowing HAMEC to uptake insulin for 5 minutes, 85% of the insulin is stored. It would be interesting to speculate whether the increase in blood flow and capillary perfusion could stimulate the mobilization of this insulin storage pool inducing its release to the basolateral side.

Furthermore, it is known that blood flow to skeletal muscle is greatly reduced in insulin resistant states. This has been attributed to the reduction in endothelial nitric oxide synthase activation by insulin as a result of an impairment in the insulin-signaling pathway. With a decrease in nitric oxide production, there is less vasodilation of the blood vessels and less increase in blood flow and capillary recruitment in response. This decrease in blood flow, in turn, further exacerbates insulin resistance as less insulin is delivered to the tissue bed on top of the tissue’s insensitivity to insulin. As our data shows that flow or shear stress induces insulin uptake/transcytosis in endothelial cells, the transport of insulin across the endothelial barrier may be another process that is affected in insulin resistance. The reduced blood flow in insulin resistance states may also cause a reduction in the amount of insulin transported across the endothelium, even further contributing to the development of insulin resistance.
4.1 Conclusions

Much of the existing literature on shear stress and nitric oxide originates from arteries and arterioles, large vessels that contain smooth muscle for which sensitivity to nitric oxide would be expected. However, flux of insulin out of the vasculature to its target organs occurs at the level of the microvasculature (e.g. capillaries and post-capillary venules), which lacks smooth muscle. While it is known that the transcytosis of macromolecules such as LDL and albumin in endothelial cells is able to be induced by shear stress, whether or not the transcytosis of insulin is affected by shear stress has never been studied. Our data indicate that shear stress is a potent stimulus for insulin transcytosis by microvascular endothelial cells, likely by increasing endothelial internalization of insulin, and that this effect is independent of nitric oxide. Instead, this effect is likely mediated by changes in the actin cytoskeleton, although the exact mechanism by which changes in the cytoskeleton can induce this effect is unknown.

Clearly, much work lies ahead for investigating the key regulators of this NO-independent pathway of inducing insulin uptake and transcytosis in microvascular endothelial cells. This work is significant as any regulator of this effect found may serve as a novel therapeutic target for insulin resistance and
Type 2 Diabetes to increase insulin delivery to the target tissues. The molecular mechanism for increasing insulin transport across the endothelial barrier could be harnessed as a complementary approach on top of NO-repletion strategies to further enhance the delivery of insulin in insulin resistance and type 2 diabetes.
5 References

19. Regulation of glucose transport by insulin: traffic control of GLUT4: Article: Nature Reviews Molecular Cell Biology. Available at:


76. Catenin-dependent and -independent functions of vascular endothelial cadherin. - Google Search. Available at: https://www.google.ca/search?q=Catenin-dependent+and+independent+functions+of+vascular+endothelial+cadherin.&ie=utf-8&oe=utf-8&gws_rd=cr&ei=d8RRVjaMuOUjwStjp1w. (Accessed: 3rd June 2016)


169. Schubert, W. et al. Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-NAME, restores


202. Endothelial/Pericyte Interactions. Available at: http://circres.ahajournals.org/content/97/6/512.full. (Accessed: 5th June 2016)


