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Physiology 31:336-345, 2016. ;
doi: 10.1152/physiol.00010.2016

Physiology (formerly published as News in Physiological Science) publishes brief review articles on major physiological developments. It is published bimonthly in February, April, June, August, October, and December by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. ©2016 Int. Union Physiol. Sci./Am. Physiol. Soc.. ESSN: 1548-9221. Visit our website at http://www.the-aps.org/.

PHYSIOLOGY 31: 336–345, 2016. Published August 10, 2016; doi:10.1152/physiol.00010.2016

Endothelial Transcytosis of Insulin: Does It Contribute to Insulin Resistance?

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Most research on insulin resistance has focused on impaired signaling at the level of target tissues like skeletal muscle. Insulin delivery is also important and includes recruitment and perfusion of capillaries bearing insulin, but also the transit of insulin across the capillary endothelium. The mechanisms of this second stage (insulin transcytosis) and whether it contributes to insulin resistance remain uncertain.

Insulin Resistance: Beyond Impaired Metabolic Action in the Tissue Parenchyma

Insulin resistance refers to a decreased metabolic action of insulin, particularly to an impaired ability to promote glucose uptake in muscle and adipose tissues and to inhibit hepatic glucose production. It is a predisposing factor and a defining feature of Type 2 diabetes, a disease afflicting almost 10% of Americans and projected to affect as many as 33% by the year 2050 (15); globally, the number of adults living with diabetes has increased almost fourfold since 1980, and the disease now affects 422 million adults (98). Furthermore, about one in three American adults have "pre-diabetes," a pre-morbid condition also characterized by insulin resistance that, without intervention, may progress to frank diabetes within 5 years (15).

Although numerous mechanisms of insulin resistance have been described (as reviewed in Ref. 69), much research has focused on the impairment of insulin signaling in skeletal muscle since this is the major site for postprandial glucose disposal (1). Insulin resistance in skeletal muscle is the dominant contributor to total body insulin resistance (23) and is one of the earliest signs of Type 2 diabetes. Moreover, it can even be observed in the normoglycemic offspring of patients with Type 2 diabetes (65).

In skeletal muscle, a hallmark of insulin resistance is impaired phosphorylation of insulin receptor substrate 1 (IRS-1), a major docking protein immediately downstream of the insulin receptor (IR) (26); this defect, in turn, impairs the ability of IRS-1 to activate phosphatidylinositol 3-kinase (PI3-kinase) (21), a critical enzyme necessary for most of the downstream metabolic actions of insulin, including glycogen synthesis and glucose uptake (71). In contrast, signaling of IRS-1 to the mitogen-activated protein kinase (MAPK) pathway remains functional during insulin resistance (21), resulting in persistent stimulation of cell proliferation (56).

However, for insulin to exert its metabolic effects, in addition to needing a competent signaling pathway in its target tissues, blood carrying the hormone must first perfuse the relevant tissue bed, and insulin itself must then leave the blood stream; this integrated process is known as insulin delivery (FIGURE 1). Although the first step, i.e., perfusion of tissues and its status during insulin resistance, has been studied at length (reviewed in Ref. 56), the physiology of the second component (i.e., the egress of insulin from the blood stream) has been technically difficult to study, particularly because it has not been possible to study this step in isolation of changes in blood flow. As a result, it is far less understood. Importantly, as we discuss below, there are documented changes in the speed and extent of insulin delivery to muscle in obesity and diabetes. However, it is not known whether insulin transit across the microvascular endothelium per se is altered. If the passage of insulin out of the circulation contributes to whole body insulin resistance, enhancement of this process might constitute a novel therapeutic approach. Thus the purpose of this concise review is to discuss the evidence supporting and discounting a role for this step in insulin delivery in the pathogenesis of insulin resistance. We build on earlier comprehensive reviews by Barrett and colleagues (9, 10) by updating the literature to date. We begin by reviewing the relevant physiology underlying insulin delivery as a whole and then focus on experimental data from the literature.

Blood Flow, Capillary Recruitment, and Endothelial Permeability

The flux of nutrients to the tissue parenchyma and the removal of metabolic wastes take place at the level of the microvasculature, in particular at the capillary beds. These vessels [~5–8 μm in diameter in humans, slightly smaller in rodents (63, 96)] are lined by a single layer of endothelial

cells, unlike the upstream tissue arterioles that possess an additional layer of smooth muscle (the arterial media) and extra connective tissue (the vascular adventitia). It has also been proposed that the perfusion of capillaries is regulated by precapillary sphincters located immediately upstream and composed of a single muscle fiber (34), in addition to control by the more abundant smooth muscle in the wall of upstream arterioles and arteries; however, the existence of precapillary sphincters in muscle is controversial, unlike in the mesentery, where they were originally described (reviewed in Refs. 33, 38, 66). In any case, vascular smooth muscle permits the control of blood flow in response to local and systemic metabolic signals [as reviewed recently by Kusters and Barrett (47)]. Importantly, insulin itself increases blood flow to the capillaries by dilating upstream vascular smooth muscle (75, 102); the net effect is both an increase in total blood flow and the recruitment of capillaries. As described earlier with skeletal muscle, insulin signaling in the endothelium leads to activation of phosphatidylinositol 3-kinase. However, in endothelial cells, this induces activation of endothelial nitric oxide synthase (eNOS) and the production of nitric oxide (NO) (72, 85, 101). NO then inhibits calcium influx into smooth muscle cells of arteries/arterioles and blocks their contraction (20), increasing blood flow. Interestingly, during insulin resistance, this pathway is impaired, leading to decreased NO, whereas persistent signaling of insulin to the MAPK pathway results in the production of endothelin-1, a potent vasoconstrictor (56).

In principle, increasing blood flow through filled capillaries or by perfusion of previously collapsed capillaries (capillary recruitment) will increase both the total amount of insulin delivered and (in the case of capillary recruitment) the endothelial surface area exposed to insulin. These changes will potentially increase insulin

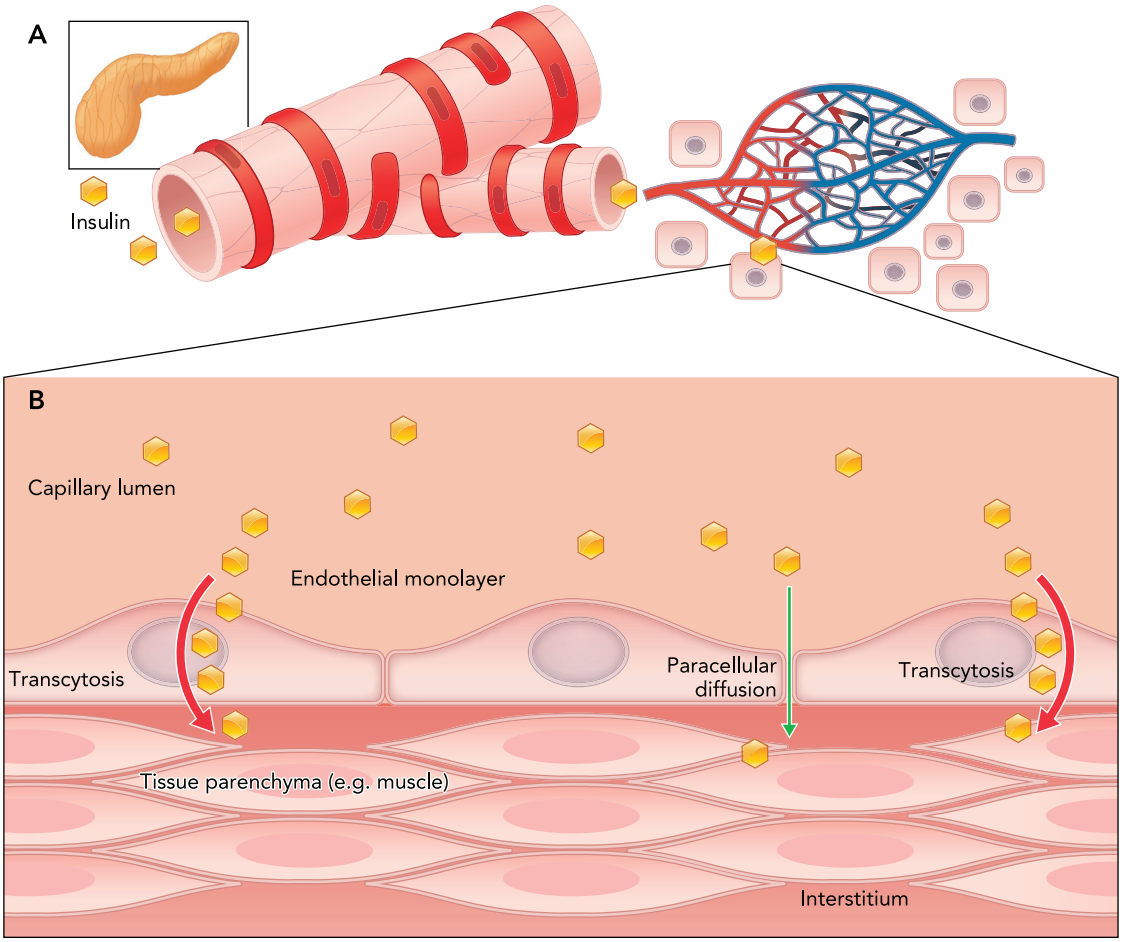

FIGURE 1. **The two stages of insulin delivery to skeletal muscle**
Insulin delivery may be thought of as being comprised of two stages. *A*: in the first stage, insulin (yellow hexagons) is secreted into the circulation by the pancreas and reaches downstream tissues (e.g., skeletal muscle) through capillaries. The recruitment of collapsed capillaries or an increase in blood flow through already perfused capillaries may therefore increase the supply of insulin to tissues. *B*: the second component of insulin delivery reflects the fact that insulin must exit capillaries, which (in tissues like skeletal muscle and fat) are lined by a layer of continuous endothelium. Insulin must therefore pass between endothelial cells (paracellular diffusion, green arrow) or be transported across individual cells by transcytosis (red arrow) to access the interstitium.

delivery across capillaries to the interstitium by its simple diffusion across the endothelial layer (FIGURE 1A). However, in muscle and adipose tissue, the capillaries across which nutrient exchange occurs are delimited by a layer of continuous endothelial cells. These cells are held together by cell-cell junctions, of which adherens junctions and tight junctions are the major constituents (53). These junctions confer a degree of barrier integrity against the free movement of cells, fluids, and large molecules across the endothelial monolayer, and restrict the passage of molecules between adjacent endothelial cells to those smaller than 3–5 nm in effective radius (54). This is in contrast to the liver, where the sinusoidal endothelium is discontinuous, allowing free mixing of luminal and interstitial contents (3), and to the brain, in which the endothelium is characterized by much tighter intercellular junctions (particularly the tight junctions) and very low rates of transcytosis (59). Little is known about how the microvascular endothelium differs structurally and functionally between skeletal muscle and adipose tissue, although some data suggest that the hemodynamic response of these tissues to insulin is similar (49, 55).

Thus the delivery of insulin from the circulation to tissues like skeletal muscle and fat may be regulated by both upstream hemodynamic factors, such as blood flow and capillary recruitment, and also by a distinctly local factor: the permeability of the capillary endothelium to insulin. In capillary beds with low permeability (i.e., "tight" endothelia), the flux of insulin across the endothelium has been postulated to be a facilitative, regulated, and rate-limiting process (42). Monomeric insulin, the form which is biologically active, has a molecular radius of 1.34 nm (77), whereas dimeric and hexameric forms, which are generated as a result of aggregation, are far larger (79). Such aggregates have been detected in the circulation of diabetic patients receiving exogenous insulin (52). Based on size alone, monomeric insulin might be expected to diffuse between adjacent endothelial cells and to freely access the interstitium. However, as discussed in detail below, experimental data on the permeability of the endothelium in vivo to insulin are not definitive on this issue. This may reflect the tendency of insulin to aggregate in aqueous solution and suggests that additional restrictions on paracellular diffusion exist beyond size alone. For instance, endothelial permeability may be affected by a molecule's net charge (89) (which, in the case of insulin, is slightly negative at neutral pH) and by noncellular structures, such as the endothelial glycocalyx (30).

Role of the Microvascular Endothelium as a Barrier to Insulin

In Vitro Evidence

Surprisingly, notwithstanding theoretical considerations based on molecular radius, data from cell culture studies suggest that the endothelial cell monolayer can restrict permeability to insulin. This has been demonstrated using microvascular endothelial cells freshly isolated from tissue (80) derived from both animals and humans (7, 42). Those findings indicate that endothelial cells bind and internalize insulin and notably that the internalized hormone is not significantly degraded (24, 25, 40, 80), in contrast to its degradation by other cell types like myocytes, adipocytes, and hepatocytes (7, 27). The lack of degradation by endothelial cells is particularly notable in cells derived from the microvasculature (24), which in vivo would be the ones across which insulin would pass to supply the tissues. Based on these studies, it has been hypothesized that the endothelium plays a role in regulating the transport of insulin out of the circulation and even potentially as a storage depot for circulating insulin (35).

In principle, insulin could cross the endothelial monolayer through gaps between adjacent cells (paracellularly) or alternatively across the cytoplasm of individual cells [transcytosis (60), and as reviewed in Ref. 43; FIGURE 1B]. There is precedent for the latter: transcytosis is important in the delivery of other hormones such as human chorionic gonadotropin (36) and follicle-stimulating hormone (92). Transcytosis is essentially an integrated process involving endothelial endocytosis of the hormone (e.g., insulin) at the vascular lumen, traffic of the insulin-bearing vesicles across the cell, and exocytosis of the insulin-bearing vesicles at the basal membrane to release the hormone into the interstitium. With regard to the first step, binding and transport of radioactive insulin across a retinal endothelial monolayer in vitro was inhibited by an antibody to the insulin receptor (IR) (42) and was significantly attenuated by the addition of an excess of unlabeled insulin. Similar data were obtained using FITC-conjugated (i.e., fluorescent) insulin and bovine aortic endothelial cells grown in culture (94). Here, fluorescent insulin colocalized partially with both IR and the receptor for insulin-like growth factor (IGF). Flux of fluorescent insulin across the endothelial cell monolayer was attenuated by both unlabeled insulin and the addition of IGF-I. Together, these data suggest that insulin transit across these endothelial monolayers in vitro is likely receptor-mediated rather than occurring

by simple diffusion and that both the IR and receptor for IGF (IGF-1R) may participate. This last point is discussed in further detail later in the context of in vivo studies.

Some post-receptor events involved in insulin transport through endothelial cells have also been studied in vitro. An early report observed insulin in intracellular vesicles in endothelial cells analyzed by electron microscopy (11) (with the caveat that a very high concentration of insulin, 1 mM, was used); more recent studies using immunofluorescence have suggested that the vesicles bearing insulin may be caveolae (95). Caveolae are 50- to 70-nm diameter invaginations of the plasma membrane that can internalize to become intracellular vesicles (4), a proportion of which can move through the cells (61); their major protein constituent is caveolin-1, and the caveolar membrane is enriched in cholesterol and sphingolipids. Caveolin-1 knockout mice lack caveolae (74), and endothelial cells from the aortas of these *cav1*$^{-/-}$ mice demonstrate reduced insulin internalization. This phenotype is recapitulated by siRNA-mediated knockdown of caveolin-1 in endothelial cells from the bovine aorta (95). Interestingly, loss of caveolin-1 also attenuated expression of the insulin receptor itself in adipose tissue, although levels in the endothelium of the tissue were not examined (19). Early work also noted that treatment of endothelial cells with filipin (which disrupts caveolae) prevented insulin transport across cultured aortic endothelial cells (73), a finding reproduced more recently by others (95). However, these data implicating caveolin-1 in insulin internalization and transcytosis are in apparent contradiction with recent observations that insulin colocalizes partially with clathrin (and not caveolin-1) in primary human microvascular adipose endothelial cells (7). In this study, we observed that knockdown of clathrin expression (but not caveolin-1) partially attenuated insulin transcytosis. Another study using electron microscopy of retinal vascular endothelial cells also observed insulin in clathrin-coated pits and never in caveolae or at intercellular junctions (86). The discrepancy between these studies may be due to well documented heterogeneity between endothelia from distinct tissues and different-sized vessels [e.g., macrovascular vs. microvascular (2, 3)], but further investigation is needed to resolve this issue.

Together, most but not all (68) of the in vitro data strongly suggest that insulin crosses endothelial cells by transcytosis. Despite being postulated decades ago (42), deciphering the molecular mechanisms of insulin transcytosis has been relatively elusive due to difficulties in distinguishing the transcellular route from paracellular leak (5). Most studies have used endothelial cells seeded on transwells and compared the permeability of endothelial cells to labeled-insulin in the absence or presence of an excess of unlabeled hormone; the ability of the unlabeled insulin to inhibit flux of the labeled ligand across the transwell is used to indicate transcytosis. Although informative, this approach is cumbersome due to imperfections in cell seeding and the high transfection efficiency required to manipulate the endothelial monolayer for mechanistic studies (e.g., siRNA or overexpression studies). We recently reported a single-cell assay to study insulin transcytosis using total internal reflection fluorescence microscopy (TIRF) that will hopefully permit more facile delineation of the regulation of insulin transcytosis (7) and the transcytosis of other ligands (6).

In Vivo Evidence

While the in vitro studies are useful for determining the potential molecular mechanisms of insulin transcytosis, their physiological relevance must be confirmed in vivo, since even primary endothelial cells display rapid and abundant phenotypic drift in culture (48); for example, the number of caveolae in endothelial cells decreases with repeated passages in culture (83). In vivo experiments to determine the physiological significance of insulin transcytosis generally support the notion that transendothelial permeability is rate-limiting for insulin action, although this is not universal (84). These studies are briefly reviewed below.

Seminal studies by Bergman and coworkers in the late 1980s and early 1990s measured insulin concentrations in lymphatics after systemic injection as a proxy for the hormone's concentration in the interstitial fluid. For instance, Yang and colleagues simultaneously measured plasma and lymphatic (thoracic duct) insulin concentrations during a euglycemic clamp in dogs; they also infused labeled inulin as a control for passive transendothelial transport (i.e., paracellular leak). They observed that plasma insulin was persistently higher than lymphatic insulin levels, even though the levels of the control tracer inulin became equal between the two compartments. Importantly, the change in lymphatic insulin levels closely paralleled whole body glucose disposal, suggesting that the movement of insulin from the systemic circulation to the interstitial fluid was rate-limiting (100). In a follow-up study, Ader and colleagues performed intravenous glucose tolerance tests on dogs and measured both lymph and plasma insulin concentrations. Again, they observed a delay in the appearance of insulin in lymph and a persistently lower concentration in lymph relative to plasma (99).

Using an ex vivo approach, Bar and colleagues perfused rat hearts with radioactive insulin in the

presence or absence of an excess of unlabeled insulin (8). The radioactive insulin was observed within capillary walls and heart parenchyma, and its presence was significantly reduced in the presence of unlabeled insulin. In contrast, in hearts perfused with a radioactive, modified desoctapeptide insulin with no affinity for the insulin receptor, there was noticeably less signal in capillaries and muscle, and unlabeled insulin did not influence the signal. Lastly, treatment of capillaries with low concentrations of the protease trypsin significantly reduced the appearance of insulin in muscle, suggesting that insulin binding to receptor proteins on the capillary surface was being attenuated.

Similar studies have been performed in humans. Jansson and colleagues (39) used microdialysis to measure insulin concentrations in subcutaneous interstitial fluid during a euglycemic hyperinsulinemic clamp (or controls perfused with inulin). Their data revealed a persistent plasma-interstitial gradient for insulin with no difference for inulin. Herkner and colleagues used a similar approach with microdialysis probes inserted into the thigh muscles of human volunteers, measuring plasma and interstitial fluid insulin concentrations during an oral glucose tolerance test (OGTT) and during a hyperinsulinemic euglyemic clamp (37). They observed that interstitial insulin concentrations were less than half those in plasma during the OGTT and that the ratio of the two fell even further during the hyperinsulinemic clamp, consistent with insulin delivery to the tissue involving a saturable process.

More recently, Eggleston and colleagues used contrast-enhanced ultrasound to measure microvascular blood volume in the forearms in 14 healthy volunteers during a hyperinsulinemic euglycemic clamp. They calculated insulin uptake as the difference between arterial and venous insulin in the forearm. Using insulin concentrations within the physiological range, the study documented an acute increase in microvascular blood volume after insulin infusion that preceded an increase in total arm blood flow. Despite this, the fraction of insulin extracted from the arterial blood fell during hyperinsulinemia, which the authors ascribed to a saturable mechanism for muscle insulin uptake (31).

In contrast, Steil and colleagues performed euglycemic clamps in dogs, this time using both physiological and supraphysiological doses of insulin (84). Of note, in this study, lymphatic vessels in the limbs were cannulated as a proxy for skeletal muscle interstitial fluid, since this method reflects the interstitium of insulin-sensitive tissues (e.g., skeletal muscle) more accurately than measurements from the thoracic duct (64). Whether using physiological or supraphysiological doses of insulin, plasma insulin levels exceeded those measured in the lymph of the hindlimbs. By this approach, the authors noted that the gradient between plasma and lymph was actually lower at the higher dose of insulin, the opposite of what was expected if transendothelial transport were saturable. Clearance of insulin from the muscle interstitial fluid was not affected, implying that the reduced plasma/lymph gradient at the higher insulin dose was not a result of impaired removal from the interstitial fluid. However, it is also possible that the supraphysiological doses of insulin caused capillary recruitment and enhanced insulin delivery, which might confound any assessment of insulin transendothelial permeability.

Indeed, in a follow-up study, the same investigators injected insulin directly into the hindlimb muscle of dogs to bypass the capillary endothelium. Using this approach, interstitial insulin concentrations rose rapidly, and the delay in the onset of muscle insulin action normally observed after intravenous injection was eliminated (17), consistent with the capillary endothelium in skeletal muscle constituting a bona fide barrier to insulin delivery.

In summary, findings from healthy animals and humans demonstrate a significant gradient between circulating insulin and interstitial insulin levels, suggesting that transendothelial transport of insulin is rate-limiting. However, many of the studies did not control for potentially confounding input by microvascular blood flow, the first and essential component of insulin delivery that is known to regulate skeletal muscle glucose uptake (91). Thus a delay in the arrival of insulin to the interstitial space may have reflected an impairment in capillary recruitment or delayed blood flow rather than an effect on transendothelial permeability of insulin per se. Beyond these considerations, the in vivo studies measuring the transendothelial insulin gradient, even when using physiological insulin doses, are limited in the ability to discern the molecular mechanisms of insulin transendothelial delivery. In particular, if insulin transendothelial transport is saturable and therefore receptor-mediated, the identity of the receptor(s) involved cannot be determined simply from this approach.

Role of the Insulin Receptor in Transendothelial Permeability: In Vivo Studies

A powerful strategy to study insulin transcytosis in vivo is to generate mice deficient in specific molecules thought to be essential for the process. A logical starting point would be the insulin receptor, which in vitro studies suggested was essential for

insulin transcytosis (as discussed earlier). In contrast, the in vivo data are less clear-cut.

Vicent and colleagues generated mice genetically depleted of IR selectively in cells expressing the Tie2 promoter (90). Tie2 is largely confined to endothelial cells (97) but is also expressed in a subset of monocytes (50). Strikingly, these mice (endothelial cell-specific insulin receptor-knockout, VENIRKO) exhibited normal glucose homeostasis and no evidence of skeletal muscle insulin resistance, even though endothelial nitric oxide synthase (eNOS) and endothelin-1 levels were reduced. The only circumstance under which these mice displayed insulin resistance was when placed on a low-salt diet. Regrettably, this study did not analyze effects on insulin-induced capillary recruitment or insulin delivery to muscle interstitial fluid.

Similarly, Duncan and colleagues generated mice with endothelial-targeted overexpression of a dominant-negative human IR (ESMIRO mice) (29). As expected, the mouse exhibited endothelial dysfunction (e.g., blunted vasodilation) and blunted eNOS activation in response to insulin. However, there was no change in glycemic control or fasting insulin levels. In a separate study, in hearts from ESMIRO mice perfused with insulin ex vivo, myocardial Akt phosphorylation (a proximal readout for IR signaling) was not different from that in wild-type mice, suggesting that transendothelial transport of insulin was unaffected by expression of the dominant-negative IR (76).

In contrast, targeting insulin-signaling in endothelia suggested participation of the pathway in whole-body insulin sensitivity. Specifically, Kubota and colleagues generated mice with endothelial-specific knockout of IRS2 (also using the Tie2 promoter, and dubbed ETIrs2KO mice) (45). IRS2 is an adaptor protein immediately downstream of both the IR and the IGF-1R, although not exclusively engaged by these receptors. These mice displayed reductions in eNOS activation but unchanged levels of endothelin-1, suggesting that loss of IRS2 conferred a selective impairment of the insulin-signaling pathway. Importantly, insulin-induced capillary recruitment and insulin delivery were directly measured in ETIrs2KO mice and found to be diminished. Moreover, unlike the case of VENIRKO mice, ETIrs2KO mice had markedly reduced skeletal muscle glucose uptake. These impairments could be reversed by pharmacological induction of eNOS phosphorylation. In contrast, the delay in insulin action was not observed in the livers of the knockout mice, even though the endothelium of hepatic blood vessels was also depleted of IRS2. The authors attributed this distinction to structural differences in the microcirculation of skeletal muscle compared with the liver, since, as mentioned earlier, although the liver endothelium is fenestrated and allows free paracellular diffusion of molecules (including insulin), the microvascular endothelium of skeletal muscle is continuous and may preclude paracellular diffusion of insulin.

Collectively, these studies suggest that the IR is not required for transendothelial insulin transport, but IRS2 (and potentially IR) can still contribute to overall insulin delivery. However, it is important to note that none of the in vivo studies can distinguish between bona fide transendothelial permeability to insulin vs. an effect on total insulin delivery (e.g., via increased blood flow or capillary recruitment). In other words, insulin transendothelial transport per se was not actually measured. Rather, although ETIrs2KO mice displayed insulin resistance, this phenotype is consistent with the observed loss in hemodynamic effects of insulin and does not necessarily imply an impediment to its transendothelial permeability. Indeed, the restoration of capillary blood volume and normalization of skeletal muscle glucose uptake achieved by pharmacological induction of eNOS phosphorylation (activation) in the KO mice is consistent with this notion.

If the insulin receptor is not required for transendothelial insulin transport, how can one explain the extensive in vitro data discussed earlier, demonstrating that excess unlabeled insulin decreases the transendothelial transport of labeled insulin? One possibility is that the in vitro studies do not accurately reflect normal physiology (e.g., by using supraphysiological concentrations of insulin; potential confounding by insulin aggregation). Alternatively, if the in vitro data are a faithful representation of what occurs in vivo, a potential explanation is that another (as of yet uncharacterized) receptor or receptors mediate insulin transcytosis across the endothelium. Even if that proves to be the case, however, it is important to note that it would not automatically mean that insulin transcytosis is rate-limiting for its action on tissues.

Impaired Insulin Delivery to Tissues During Disease

Finally, if insulin transendothelial permeability is rate-limiting during normal physiology, how is it affected during pathological states?

A number of studies have described defects in insulin delivery in association with obesity. For instance, microdialysis sampling of forearm muscle interstitial fluid of obese and insulin-resistant subjects revealed the delayed appearance of insulin in the interstitium (relative to non-obese individuals) during a hyperinsulinemic euglycemic clamp (78). Blood flow rate in the forearm in response to insulin was also reduced. Interestingly,

similar kinetics were observed for the appearance of the tracer inulin in the interstitial fluid, leading to the conclusion that both insulin and inulin are transported by a non-receptor-mediated mechanism, likely diffusion.

In contrast to the above findings, interstitial insulin concentrations in adipose tissue and forearm muscle from non-diabetic obese women measured after an oral glucose tolerance test were significantly lower compared with controls (70). However, as with other in vivo studies, neither of these reports specifically examined whether transendothelial insulin transport was affected. This is important since a number of studies have established that insulin's ability to increase blood flow and recruit capillaries is reduced in obesity (41, 93). As alluded to earlier, in animal studies, this specific defect is ascribed to defective endothelial signaling and reduced nitric oxide production. Thus dogs fed a high-caloric high-fat diet developed insulin resistance and a diminished volume of distribution to the tracer inulin that could not be enhanced by insulin administration (32). These data imply that insulin delivery can be compromised without the need to invoke impaired insulin transendothelial permeability.

At this point, it has not been possible to determine whether delayed transendothelial transport contributes to insulin resistance in obesity. An elegant study by Castillo and colleagues (14) in obese and non-obese subjects without diabetes measured circulating and peripheral lymphatic insulin concentrations during a euglycemic-hyperinsulinemic clamp. Although a gradient between circulating and lymphatic insulin concentrations was observed in both groups, it was not greater in obese than in non-obese subjects. Furthermore, the most obese subjects had the smallest gradients, revealing lesser coupling between lymphatic insulin levels and glucose uptake in this group than in lean controls. Thus, at least in this study, obesity did not further impair transendothelial flux of insulin. One caveat is that insulin measurements were taken from the subcutaneous lymphatics draining the foot, meaning that the systemic-peripheral gradient may have included contributions from tissues other than skeletal muscle.

Elevated circulating free fatty acids are a feature of obesity (12) and are known to inhibit intracellular insulin signaling in skeletal muscle (13). Nonetheless, their effect on endothelial insulin transcytosis is uncertain (62). Although acutely elevated free fatty acids impair insulin-induced vasodilation (18), Kolka and colleagues found that lipid infusion in dogs had no effect on the ability of insulin to access the interstitial fluid surrounding skeletal muscle, as assessed from the lymph of the hindlimb (44). Similar findings have been reported by Szendroedi and colleagues (87) using microdialysis in the inner thigh skeletal muscle of human volunteers. Hence, at least in this paradigm of acutely induced insulin resistance, there is appears to be normal insulin delivery to muscle. In all instances, insulin availability in lymph or cannulated interstitia was measured, providing a surrogate measure for the molecular transfer of insulin across the endothelium.

Interestingly, both obesity (81) and diabetes (57) have been associated with increased endothelial permeability, with the important caveat that most of the research has not been performed in skeletal muscle or adipose tissue. The increase in permeability has been attributed to a variety of mechanisms, including direct disruption of cell-cell junctions by numerous paracrine mediators [e.g., visfatin (16), VEGF (28)], alterations to the endothelial glycocalyx (67), and increased endothelial activation leading to leukocyte recruitment (58). If the microvascular endothelium does constitute a rate-limiting barrier to insulin leaving the circulation, in conditions like diabetes or obesity, the increase in endothelial permeability could represent an attempt at compensation for impaired insulin delivery to capillaries due to loss of nitric oxide (81).

The effect of diabetes and obesity on endothelial transcytosis per se is largely unknown. Although the cytokines TNF-α and IL-6 [which are elevated in insulin resistance (22)] have been reported to decrease insulin internalization by aortic endothelial cells, the transcytosis of albumin by microvascular endothelial cells is known to be stimulated by other inflammatory mediators such as endotoxin (88) and thrombin (46).

Conclusions and Future Directions

It has been more than 30 years since a role for endothelial transcytosis in the regulation of insulin delivery was postulated (42), yet controversy remains as to its physiological importance. In particular, it remains unclear whether insulin transcytosis constitutes a rate-limiting step to insulin action. Some of the uncertainty is a result of tissue heterogeneity: the fact that certain endothelial capillary beds are continuous (e.g., muscle, adipose) whereas others are not (e.g., liver) is certain to affect the relevance of transcytosis. Another likely confounder when interpreting experiments is the effect of insulin concentration, which may alter insulin's tendency to self-aggregate (and thus its effective molecular radius) and its binding to potential receptors (e.g., IR, IGF-1R) (51). Fundamentally, however, the basic dilemma in the field is how to dissect out the contributions of capillary recruitment and blood flow from those of an intra-

cellular traffic process. Animal studies are confounded by the inability to control perfusion and capillary recruitment, whereas in vitro studies with endothelial cells, although useful for delineating molecular mechanism, will never establish in vivo relevance. In vitro studies are also complicated by the clear heterogeneity between endothelial cells derived from different tissues [e.g., large vessels vs. the microvasculature (2, 3)], the phenotypic drift of cultured cells over time, and, until recently, the lack of facile quantitative methods for studying transcytosis (6, 7).

Thus, although it is fairly clear that insulin's effect to increase blood flow and recruit capillaries is important to its overall metabolic action (82), the role (if any) of insulin transcytosis remains uncertain. The solution to this experimental conundrum may lie in the artful exploitation of both ex vivo and intravital approaches, since the issues of perfusion and capillary recruitment are easier to control in these settings compared with standard live animal experiments. The ex vivo approach may be particularly valuable for validating the findings from cell culture, in particular, to prove that specific receptors and downstream signaling molecules are required in the vasculature without incurring the risk and expense of generating tissue-specific knockout animals.

Although not simple, proving that endothelial insulin transcytosis is rate-limiting is not simply an academic exercise. If this can be established, identifying methods to induce insulin transcytosis by the microvasculature might then constitute a useful and novel therapeutic approach for the treatment of insulin resistance. However, it is clear that such an ambitious goal would require a deep understanding of its molecular mechanisms. ■

This work was supported by Canadian Institutes of Health Research Grant MOP-130493 to A. Klip and W. Lee.

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author contributions: W.L.L. prepared figures; W.L.L. drafted manuscript; W.L.L. and A.K. edited and revised manuscript; W.L.L. and A.K. approved final version of manuscript.

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