Published in final edited form as:

Microcirculation. 2013 July ; 20(5): 434–439. doi:10.1111/micc.12044.

Endothelial cells actively concentrate insulin during its transendothelial transport

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Abstract

We examined insulin's uptake and transendothelial transport (TET) by cultured bovine aortic endothelial cells (BAECs) in order to: a) ascertain whether insulin accumulates within the cells to concentrations greater than in the media; b) compare the TET of insulin to that of inulin (using the latter as a tracer for passive transport or leak); and c) determine whether insulin's TET depended on insulin action. Using ^{125}I -insulin at physiologic concentrations, we found that BAECs accumulate insulin >5-fold above media concentrations and that the TET of insulin, but not inulin, is saturable and requires intact PI-3-kinase and MEK-kinase signaling. We conclude that the insulin receptor and downstream signaling from the receptor regulate endothelial insulin transport. Based on comparison of the kinetics of BAEC insulin uptake with insulin TET, we suggest that insulin uptake is rate limiting for insulin TET.

Introduction

In skeletal muscle, insulin, at physiologic concentrations, can increase total blood flow(1) and recruit microvasculature(19). The latter action increases the endothelial surface available for nutrient exchange. As insulin's movement from plasma to muscle interstitium is rate limiting for insulin's metabolic action(28), regulation of blood flow and its distribution within muscle links insulin's metabolic and vascular actions(2). Studies using compartmental modeling(22), lymphatic sampling(28) and microdialysis(23) for assessing muscle interstitial insulin concentrations each support an important role for insulin's trans-endothelial transport (TET) in the regulation of muscle metabolism.

Early studies of cultured endothelial cells (ECs) implicated the insulin receptor in insulin's TET(6, 13). This was confirmed more recently and extended by data suggesting that insulin acts on the EC to regulate its own trans-endothelial movement(26). In vivo in rat skeletal muscle(16) and human forearm muscle (8) the clearance of insulin declines with physiological hyperinsulinemia, a finding consistent with a saturable process mediating insulin uptake by muscle in vivo.

Thus, in muscle insulin's TET regulates the kinetics and magnitude of insulin action. To address whether insulin TET in vivo involves a trans-cellular or paracellular pathway, serial muscle biopsies were taken from rats given FITC-labeled insulin during a 10 mU/min/kg euglycemic hyperinsulinemic clamp. The FITC-insulin appeared to quickly concentrate within the endothelium(24). However, it was not possible to quantitate that process in vivo. We undertook the current studies to assess whether, when insulin is present at physiologic concentrations, the EC accumulates insulin against a concentration gradient. We also addressed whether inhibiting insulin action interfered with insulin TET when insulin was

present at physiologic concentrations. Finally, we addressed whether insulin had non-specific effects on endothelial barrier permeability.

Methods

Culture of bovine aortic endothelial cells (BAECs)

BAECs were obtained from Lonza (Walkersville, MD) and cultured in EGM-MV complete media at 37°C, 5% CO₂. Cells from passages 3–8 were used for experiments.

¹²⁵I-insulin uptake by BAECs

BAECs were grown to approximately 90% confluence then serum starved overnight prior to the experiment. Before adding labeled insulin the cells were washed once with phosphate buffered saline (PBS) pH 7.4. For each experiment, ¹²⁵I-insulin, at a final concentration of 80 pM (2200.0 Ci/mmol, Perkin Elmer, Shelton, CT) with or without adding 1 μM unlabeled insulin to the cells in Hepes binding buffer (0.1M Hepes, 0.12M NaCl, 5mM KCl, 1.2mM MgSO₄, 8mM glucose, 1% BSA pH 7.8) for the times indicated in results. To stop insulin uptake cells were washed with ice-cold Hepes buffer and surface bound insulin was removed with a 6 min incubation on ice with a solution containing 0.5M NaCl and 0.2M acetic acid, pH 2.5 (12). Cells were lysed with a buffer containing 50mM Tris pH 7.4, 1% triton X-100 and 0.5% SDS. ¹²⁵I-insulin uptake was assessed by gamma counting and protein content was determined using a Bradford protein assay.

Measurement of EC intracellular volume

The BAEC intracellular volume was measured as 3-O-methyl-glucose (3-MG) accessible space. 3-O-methyl-glucose is not concentrated or metabolized by the EC and enters the EC rapidly via the Glut 1 transporter. Cells were serum-starved overnight then washed with PBS supplemented with 5mM glucose and 1 μCi ³H-3MG was added to each well for 0, 5, 15 or 30min. To stop the reaction, the labeled media was removed and cells were rapidly rinsed four times with ice-cold PBS. Cells were then lysed and radioactivity was assayed using a liquid scintillation counter and protein quantified using Bradford reagent. The 3-MG space was calculated using the following formula: $V_{3-MG} = A_i/[3-MG]_O$, Where A_i equals the intracellular 3-MG (dpm/μg protein) when [3-MG] reaches steady-state and [3-MG]_O equals the dpm/μL of 3-MG in the incubation buffer. Results are expressed as μL/μg protein. This method was adapted from Latzkovits et al (14).

Measurement of ¹²⁵I-insulin trans-endothelial transport (TET)

ECs were grown in 24-well Transwell plates (Corning, Pittston, PA) and trans-endothelial electrical resistance (TEER) was measured each day using an EndOhm device (World Precision Instruments, Sarasota, FL). Cells were used when a peak TEER was reached. The TEER of the endothelial monolayer averaged (119 ± 2 ohms/cm²) at the time of the experiments. Prior to addition of ¹²⁵I-insulin, cells were washed with serum-free media. Trans-endothelial transport (TET) was assessed using 80pM and 400pM ¹²⁵I-insulin with or without addition of 1 μM unlabeled insulin. In some experiments wortmannin (100nM) or PD98059 (25 μM) or IGF-1 receptor neutralizing antibody (Ab-3, 1 μg/ml, Calbiochem, San Diego, CA) was incubated with the cells for 30 min prior to adding labeled insulin to inhibit PI3-kinase and MAPK-kinase, respectively.

As insulin might traverse the EC monolayer grown on Transwell plates by a trans-cellular path, a para-cellular path or some combination, we also measured the movement of ³H-inulin (80nM) to assess passive, presumably para-cellular, movement of this ~ 5kD polymer. We also measured the TET of ³H-inulin (80nM) in monolayers exposed to wortmannin, PD98089 or insulin to assess any non-specific effects on the endothelial monolayer integrity.

While inulin is comparable to insulin in size it does not have the same charge as insulin. Therefore, we also measured the TET of heat-denatured ^{125}I -insulin. To denature the ^{125}I -insulin it was heated at 70°C for 90min. This did not remove the ^{125}I tag, however this denatured insulin is unable to bind to the insulin receptor. Thus, the rate of TET movement of heat-denatured insulin, like that of inulin, was considered “passive”. In Results (see figures 3 and 4) we have subtracted this passive ^{125}I -insulin transport from the measured TET of native ^{125}I -insulin, thus giving transport rates solely due to an insulin-receptor mediated transport process.

Statistical analysis

One way Anova and T-tests were used as indicated in results with p values less than 0.05 considered statistically significant.

Results

BAEC insulin uptake

Serum starved BAECs rapidly accumulated ^{125}I -insulin and the intracellular radioactivity reached a plateau by 10min (Fig 1A). Labeled insulin uptake was inhibited by the presence of $1\mu\text{M}$ unlabeled insulin (Fig 2A). In previous studies from our laboratory that used 50 nM FITC-insulin both insulin and IGF-I receptors contributed to insulin uptake (12). To assess whether IGF-I receptors mediate insulin uptake at the physiologic insulin concentrations used here we pre-incubated the cells with an antibody against the exofacial domain of the IGF-I receptor (Ab-3) that we previously observed inhibits BAEC uptake of 50 nM FITC-insulin. ^{125}I -insulin uptake was not affected at either 80pM (Fig 2B) or 400pM (data not shown) indicating that the IGF-I receptor is not involved in insulin trans-endothelial transport at these physiological insulin concentrations. We have previously shown that the Ab3 antibody inhibits uptake of 10 or 50 nM insulin by BAECs(24). Our current findings are also consistent with our previous observation that insulin at concentrations $< 500\text{pM}$ does not phosphorylate the IGF-1 receptor in BAECs (15).

To estimate the intracellular insulin concentration, we measured the intracellular volume of the BAECs using ^3H -3-MG (Fig. 1B). Uptake of ^3H -3-MG was rapid and reached a steady-state within 5 min. Based on the equilibrium distribution of ^3H -3-MG we estimated the volume of our BAECs as $\sim 1.0\mu\text{l}/\text{mg}$ protein ($n=3$). Using this volume, we estimated that when the extracellular insulin concentration was 80 pM, the cellular insulin concentration was 5–6 times higher.

Trans-endothelial insulin transport studies

The TET of heat-denatured ^{125}I -insulin was not affected by adding a high concentration of native unlabeled insulin (Fig 3A). Likewise, ^3H -inulin crossed the EC monolayer barrier and this was not affected by the addition of high concentrations of unlabeled inulin (Fig 3 B). This suggests that inulin TET is a non-saturable process. Addition of insulin at the concentrations used here did not affect inulin TET (data not shown). We noted that the TEER of the BAEC monolayer declined significantly with time during incubation in serum-free media, indicating a greater ionic conductance. This however was not affected by the presence or absence of insulin in the media (Fig 3C).

To measure TET of native insulin we added 80 or 400pM native or heat-denatured ^{125}I -insulin to the upper chamber of Transwell plates containing a confluent layer of BAECs. At both 80 and 400pM ^{125}I -insulin, the TET of native insulin exceeded that of heat-denatured insulin ($p < 0.001$). The latter was subtracted from the measured TET of native ^{125}I -insulin.

Addition of excess (1 μ M) unlabeled insulin significantly inhibited this specific transport of labeled insulin (Fig 3D).

To examine the role insulin signaling in regulating the TET of ^{125} I-insulin we used wortmannin (100nM) and PD98059 (25 μ M) to inhibit of the PI3-kinase and MAPK signaling pathways, respectively. We had previously shown that insulin at concentrations as low as 100 pM phosphorylated Akt and MEK(15) and this effect was blocked by wortmannin and PD 98059, respectively(26). Neither wortmannin nor PD98059 affected non-specific para-cellular transport as assessed by ^{3} H-inulin movement (Fig 4A) or TEER (Fig 4B). At 80pM native ^{125} I-insulin, wortmannin significantly inhibited insulin transport ($p<0.05$) and PD98059 showed a trend to decrease the transport (data not shown). At 400 pM native ^{125} I-insulin both wortmannin and PD98089 each significantly decreased labeled insulin TET (Fig 4C).

Discussion

Previous studies from our lab (24) and others (13) have strongly indicated that insulin is transported in a saturable insulin-receptor dependent manner across the endothelial cell. The studies presented here confirm that finding and furthermore show that the endothelial cell accumulates insulin to concentrations well above those in the surrounding media. Thus, insulin uptake by the EC cannot be ascribed to simple pinocytosis, which would lead to cellular concentrations below or at best near equivalent to those in the extracellular media depending upon the volume of pinocytotic vesicles within the EC. The finding that both wortmannin and PD 98059 inhibit insulin TET is consistent with an active role for insulin in regulating its own transport. Schnitzer et al (21) first suggested that insulin and other proteins traversed the EC in association with caveolae. Indeed, immunohistochemical data clearly demonstrate a relationship between insulin, insulin receptor and caveolae(24). In adipocytes insulin receptors have been shown to preferentially localize to the neck region of caveolae(9). Whether this is the case in endothelial cells where insulin uptake appears to be part of a trans-endothelial transport process is not known.

Previous studies of insulin uptake and transport by cultured adipocytes and endothelial cells have also shown that there is a rapid internalization of ^{125} I-insulin(11, 12, 17). In adipocytes internalized insulin traverses an endocytic degradative pathway where the majority of internalized insulin is released as small molecular weight insulin degradation products within minutes of exposure (17). However, studies by several groups indicate that ECs degrade insulin very slowly (5, 7).

Dernovsek and Bar have also examined the release of ^{125} I-insulin from ECs pre-loaded with labeled insulin(6). They found that the majority of total cell-associated radioactivity is released within the first 10min using either macrovascular or microvascular ECs. Analysis of the released ^{125} I-insulin found the majority to be intact and not degraded. This was particularly true for microvascular ECs. Pretreatment of endothelial cells with a high concentration of unlabeled insulin prior to the addition of ^{125} I-insulin decreased subsequent entry of ^{125} I-insulin and release by 50–90%. This suggests that initial binding to insulin (or at high insulin concentrations IGF-I receptors limits ^{125} I-insulin EC uptake and subsequent release(5). Other studies confirmed that approximately 70–75% of internalized ^{125} I-insulin was released from the EC by 60min and that this largely consisted of intact insulin(11, 12). Likewise, in a study of trans-endothelial insulin transport, King and Johnson concluded that insulin can be transported across the endothelial cells with only a small amount of degradation(13). Those authors also showed that an antibody to the exofacial domain of the IR blocked insulin transport. The rapid release of ^{125} I-insulin seen in these studies compares well with our findings that suggest that the uptake of insulin is the limiting factor in the

trans-endothelial transport of insulin rather than the actual transport of insulin through the endothelial cell.

Using adipocytes, Goldberg et al. showed that gold-labeled insulin uptake involves uncoated membrane invaginations (10). In recent years abundant data has indicated that serum proteins such as albumin are transported through endothelial cells via a vesicular transport pathway involving caveolae (18). By contrast, inulin or sucrose traverse the endothelium by paracellular diffusion(3). Caveolae are believed to play a major role in the regulation of endothelial vesicular trafficking and signal transduction including insulin signaling(20). Cohen et al examined insulin receptor signaling in caveolin-1 - deficient (Cav-1 null) mice and found that young Cav-1 null mice are insulin resistant, compared to their wild-type counterparts (4). We have reported aortic ECs derived from caveolin-1 null mice do not take up insulin, however, transfection of these cells with FLAG-tagged caveolin-1 DNA rescued insulin uptake(25).

As noted above, by estimating the volume of our endothelial cells using 3-MG we were able to estimate that insulin is approximately 5–6 fold more concentrated in the BAECs in comparison to the surrounding media. Findings from extensive immunocytochemical localization studies as well as co-immunoprecipitation studies are consistent with insulin residing within caveolae in BAECs(24). Consistent with this, siRNA knockdown of caveolin-1 as well as germ-line knockout of caveolin-1 strongly inhibits BAEC insulin uptake(25). If insulin is indeed concentrated within caveolae then its concentration within these vesicles is actually much higher. It is estimated that in endothelium, where caveolae are abundant, they may occupy up to 10–15% of the cytosol volume. Thus, if the insulin within the EC is all or largely within caveolae, then insulin's concentration in the vesicle would be 30–50 fold higher than in the surrounding media.

In the current study we found that wortmannin and PD98059 each significantly decreased trans-endothelial insulin transport. We had previously reported that these inhibitors blunted the EC uptake of FITC-labeled insulin (9). As much higher insulin concentrations were used in that study (typically 50 nM), insulin transport was occurring via binding with both the insulin and the IGF-1 receptors. In the current study using radiolabeled insulin, we observed that these inhibitors specifically inhibit insulin TET at physiologic insulin concentrations. Moreover, this ^{125}I -insulin TET is not blocked by an antibody to the IGF-1 receptor. This suggests that the insulin receptor specifically is involved in insulin TET and that this requires insulin signaling through each of its two major downstream signaling pathways. Interestingly, caveolae are enriched with insulin receptors, PI 3-kinase and MAPK (ERK-2) (4) and over-expression of caveolin augments insulin signaling(27). Inhibition of PI 3-kinase and MAPK may interfere with caveolae trafficking although the mechanism remains to be elucidated.

The Transwell system used in this study to examine insulin TET, though useful, has significant limitations. As it is a cultured system it is unlikely to exactly mimic the behavior of an endothelial barrier in vivo. In addition, the polyester membrane on which the cells grew contributes a barrier to insulin movement. After 2hr only 40% of the ^{125}I -insulin added to the Transwell's upper chamber had moved to the bottom chamber when there were no cells on the membrane. When fibronectin was present this decreased to 36%. This compares well with earlier studies where within 2hrs 44% of the ^{125}I -insulin diffused from the upper to the lower chamber in the absence of cells(13).

It is of interest to compare the rate of BAEC insulin uptake with its rate of TET. At a media insulin concentration of 80pM these measured $40 \pm 9 \times 10^{-6}$ and $50 \pm 5 \times 10^{-6}$ pmols/min/

mg protein, respectively. This may suggest that the uptake rather than transport through the endothelial cell is the rate-limiting factor for insulin trans-endothelial transport.

In conclusion, at physiological concentrations, insulin is actively concentrated within the endothelial cell by a process that requires intact insulin action. For ECs in culture, insulin TET at physiologic insulin concentrations is a saturable process and may be limited by the rate of EC insulin uptake. Whether this is also the case in the intact vasculature is yet to be defined.

Acknowledgments

This work was supported by research grants from the NIH (DK057878 and DK073059) and ADA 11-BS6 to EJB.

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Figure 1.

Panel A. 80pM ^{125}I -insulin cellular uptake, n = 3. Panel B. ^3H -3-MG cellular uptake was used as a measure of intracellular volume in order to calculate the intracellular concentration of ^{125}I -insulin and thus the rate of uptake, n = 3.

Figure 2. Panel A. 80pM ^{125}I -insulin uptake into BAECs (black bars) +/- 1 μM cold unlabeled insulin (white bars) at 30min, * $p < 0.05$, $n = 9$. Panel B. 80pM ^{125}I -insulin uptake into BAECs (black bars) +/- IGF-IR antibody (grey bars) at 30min.

Figure 3.

Panel A. Transport of heat-denatured 400pM ^{125}I -insulin (black bar) with or without 1 μM cold unlabeled insulin (white bar). Panel B. Movement of 80nM ^3H -inulin (black bar)(+ 80μM unlabeled inulin) (white bar) which is expected to travel by a non-receptor mediated pathway. Panel C. Decrease in TEER with media only (black bar) incubation for one hour and one hour incubation with 1 μM insulin (white bar). Panel D. 400pM ^{125}I -insulin trans-endothelial transport is inhibited by addition of excess (1μM) cold unlabeled insulin at 30min. n = 5–8. * p = <0.05.

Figure 4. Panel A. ^3H -inulin trans-endothelial transport. Panel B. TEER change and Panel C. 400pM ^{125}I -insulin transport. Black bars are in the absence of inhibitors, wortmannin indicated by grey bars, and PD98059 by white bars in each panel. n = 6 * p < 0.05