Insulin Signaling Stimulates Insulin Transport by Bovine Aortic Endothelial Cells

Hong Wang, Aileen X. Wang, Zhenqi Liu, and Eugene J. Barrett

OBJECTIVE—In vivo evidence suggests that insulin entry into skeletal muscle is rate limiting for its overall metabolic action. Although there has been controversy regarding whether insulin crosses the endothelium by a passive (transcellular or paracellular) or mediated process, accumulating data favor the latter. Here, we addressed whether insulin signaling within the endothelial cell is required for the first step of transendothelial insulin transport: its uptake by the endothelial cell.

RESEARCH DESIGN AND METHODS—Bovine aortic endothelial cells (bAECs) were incubated in serum-free medium for 6 h before addition of 50 nmol/l fluoroisothiocyanate (FITC)-labeled insulin for 30 min, and uptake of FITC insulin was quantified by confocal immunocytochemistry.

RESULTS—Cellular insulin uptake was temperature dependent, being greater at 37 vs. 4°C ($P < 0.05$). Inhibiting phosphatidylinositol 3-kinase (PI 3-kinase) (wortmannin), mitogen-activated protein kinase kinase (MEK) (PD98059), the cSrc-family tyrosine kinase (PP1), or the insulin receptor tyrosine kinase (genistein) markedly diminished FITC insulin uptake ($P < 0.05$ for each). In contrast, inhibiting the phosphotyrosine phosphatase protein tyrosine phosphatase 1B further stimulated insulin uptake ($P < 0.05$). Addition of the inflammatory cytokine 5 ng/ml tumor necrosis factor-α (TNF-α) for 6 h before adding 50 nmol/l FITC insulin diminished insulin uptake significantly ($P < 0.05$). This inhibitory effect of TNF-α could be partially reversed by a specific p38 MAPK inhibitor (SB203580).

CONCLUSIONS—Insulin uptake by bAECs requires intact insulin signaling via both the PI 3-kinase and MEK signaling cascades and the cSrc-family tyrosine kinases, and endothelial cell insulin uptake is sensitive to cytokine-induced insulin resistance. ***Diabetes* 57:540–547, 2008**

Muscle is a key target for insulin action and accounts for >75% of insulin-mediated glucose disposal under euglycemic insulin clamp conditions (1). Nearly 20 years ago, Yang et al. (2) showed that interstitial insulin concentrations (measured in lymphatic fluid) correlated more closely than plasma insulin with muscle glucose uptake. Olefsky and colleagues (3,4) later measured the half-time for increases in interstitial muscle insulin concentration, muscle glucose transport, and insulin receptor tyrosine phosphorylation during an insulin clamp. They concluded that insulin entry into the muscle interstitium from plasma is rate limiting for overall insulin-mediated glucose uptake. Other studies suggest that this transendothelial transport step contributes to the diminished insulin-mediated skeletal muscle glucose uptake in states of insulin resistance (5,6), although this is not a uniform finding (7).

Unresolved is whether transendothelial insulin movement is a passive (8,9) or receptor-mediated (10) process. We have recently reported that in vivo insulin enters skeletal muscle via a transcellular pathway (11) and that during its transport, insulin could associate with either the insulin or IGF-I receptor (11,12) in a concentration-dependent fashion. Given that the endothelial cell is involved in mediating transendothelial insulin transport, the question arises as to whether insulin exerts a biological action on the cell to facilitate the transport process. As a result of these and other observations, we initiated the studies reported here addressing whether insulin action on the endothelial cell is required for the process of insulin uptake and transport. To our knowledge, there are currently no reports relating to whether insulin action on the endothelial cell is required for insulin uptake or transport.

Using cultured bovine aortic endothelial cells (bAECs) that have been extensively characterized in our laboratory, we examined fluoroisothiocyanate (FITC) insulin uptake using confocal fluorescence microscopy with quantitative measurement of fluorescence intensity of individual cells. We observed that FITC insulin uptake was temperature dependent; inhibited by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin, the cSrc-kinase inhibitor PP1, the general tyrosine kinase inhibitor genistein, and the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor PD 98059; and stimulated by protein tyrosine phosphatase 1B (PTP1B) inhibition, indicating that endothelial cell insulin uptake required insulin action on the endothelial cells and indicating involvement of both the PI 3-kinase and MEK pathways in this process. We also observed that the inflammatory cytokine tumor necrosis factor-α (TNF-α), which interferes with insulin signaling in endothelial cells, inhibits endothelial insulin uptake.

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bAEC, bovine aortic endothelial cell; FITC, fluoroisothiocyanate; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PTP1B, protein tyrosine phosphatase 1B; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α.

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RESEARCH DESIGN AND METHODS

Cell culture. bAECs (BioWhittaker, Walkersville, MD) (passage numbers 2–8) were grown in EGMMV medium supplemented with human epidermal growth factor, hydrocortisone, gentamicin, amphotericin, bovine brain extract, and 5% fetal bovine serum in eight-well slide chambers. The bAECs were incubated in serum-free basal medium for 6 h and then treated with either 50 nmol/l FITC insulin (Molecular Probes, Eugene, OR) or 10 nmol/l regular insulin (Humulin R; Eli Lilly, Indianapolis, IN) for 30 min at 37°C. Cells were then fixed with cold methanol for 10 min at −20°C before processing for immunocytochemical analysis (see below). For incubations treated with 100 nmol/l wortmannin, 50 mmol/l PD98059, 50 mmol/l genistein (all from Sigma-

Aldrich), 10 μmol/l PP1 (Biomol International), or 250 μmol/l PTP1B inhibitor (Calbiochem), these reagents were added to cell incubations 30 min before insulin addition. TNF-α (5 ng/ml) (Sigma-Aldrich) with or without 10 μmol/l SB203580 (a specific inhibitor of p38 MAPK) (Calbiochem) as indicated in figures was added to cell incubations 6 h before addition of insulin.

Immunocytochemistry. After the fixation, cells were washed three times in Tris-buffered saline (TBS), permeabilized in TBS containing Triton X-100 (0.05%) and 1% horse serum for 30 min at room temperature, and incubated with two different primary antibodies against two different target proteins (double labeling) overnight at 4°C. The following primary antibodies were used: rabbit polyclonal antibody against phospho–caveolin-1 (1:200; Cell Signaling Technology, Beverly, MA); mouse monoclonal anti–caveolin-1 (1:25; BD Transduction Laboratories); rabbit polyclonal anti-fluorescein (FITC) (1:100; Molecular Probes); rabbit polyclonal anti–phospho-insulin receptor (Tyr^{972}) (1:50; Stressgen Bioreagents, Ann Arbor, MI). The cells were washed three times in TBS and then incubated with species-specific secondary antibodies conjugated with a fluorophore at 1:200 dilutions for 45 min at room temperature. The following secondary antibodies were used: donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA) and donkey anti-mouse IgG conjugated to Cy2. The cells were washed three times in TBS and then coverslipped with the antifade mounting medium.

Imaging. The immunocytochemical labeling was examined using a two-color Olympus BX50 WI confocal microscope equipped with Krypton and Argon laser as described previously (11). An *x-y-z*-axis scanning method was used. The images were scanned through up to ×60 objectives, acquired at a resolution of 1,024 × 768 pixels, and stored in 24-bit TIFF format. Fluorescence intensity of individual cells reflecting FITC insulin uptake was quantified using Image J software (National Institutes of Health). Forty-five cells were measured for each treatment group. Because the fluorescence intensity of cells incubated in the cold or with several inhibitors was low, the intensity output was adjusted to such a level that the cell profiles could be clearly identified but the maximum fluorescence intensity would not be saturated in some treatment groups (e.g., PTP1B inhibitor treatment group) in Photoshop and then exported for subsequent quantitation by NIH Image J software. All images from each experimental group were processed identically. During image acquisition, the individual microscopic field was selected to include at least 15 cells but was otherwise random. Individual cells were outlined by polygonal method, and the integrated fluorescence intensities were measured.

Statistical analysis. Data are presented as means ± SE. Statistical comparisons among different groups were made using one-way ANOVA with post hoc testing as indicted in results. Statistical significance is defined as $P \leq 0.05$.

RESULTS

We first examined the temperature dependence of insulin uptake by bAECs incubated with 50 nmol/l FITC insulin. As shown in Fig. 1*C*, cells exposed to FITC insulin for 30 min at 37°C and then incubated with an anti-FITC antibody before fluorophore labeled anti-IgG stain intensely for insulin. Omitting FITC insulin greatly diminished the fluorescence intensity (Fig. 1*A*). Because active uptake of insulin by endothelial cells is reported to be temperature sensitive (13), we examined the temperature dependence of FITC insulin uptake to further exclude a nonspecific surface binding phenomena and to be certain that cell-associated staining was not attributable to cells being relatively freely permeable to insulin. bAECs incubated with FITC insulin at 4°C had much diminished staining with anti-FITC antibody (Fig. 1*B* and *D*). Quantitating these responses confirmed the temperature dependence of FITC insulin uptake (46.9 ± 1.3 at 37°C vs. 21.3 ± 1.2 at 4°C; $P < 0.05$). Additionally, because these images were obtained using confocal microscopy, we were able to assure that the staining was not limited to the outer surfaces of the plasma membrane but was present within the cytosol of the bAECs as well.

We next examined the effect of inhibiting insulin action using the PI 3-kinase inhibitor wortmannin, the MEK inhibitor PD98059, cSrc-family tyrosine kinase inhibitor PP1, or the tyrosine kinase inhibitor genistein. In each case (see Fig. 2, *top*), there was a substantial reduction in

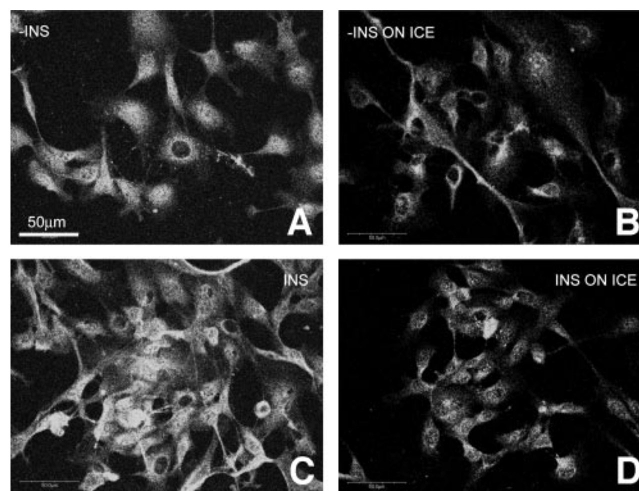


FIG. 1. Temperature dependence of insulin uptake. *A*: Cells incubated with the basal medium at 37°C without FITC insulin before processing for immunocytochemical staining for FITC. *C*: Cells were incubated with FITC insulin for 30 min at 37°C before fixation. Cells in *B* (−INS ON ICE) and *D* (INS ON ICE) were treated identically to those in *A* and *C* except the incubation was at 4°C. INS, FITC insulin; −INS, incubated in basal medium without FITC insulin. Representative images of three independent experiments.

FITC insulin fluorescence associated with the endothelial cells. Interestingly, incubation of these cells with the PTP1B inhibitor appeared to enhance FITC insulin staining of the bAECs. The pattern of cellular staining in the presence of the PTP1B inhibitor differed from each of the other incubations in that both the cytosol and the nuclear areas of the cell appeared to be stained. We also observed that 5 ng/ml TNF-α inhibited FITC insulin uptake as effectively as wortmannin, PD 98059, or genistein.

Figure 2, *bottom panel*, provides quantitative assessment of FITC insulin uptake under each of the experimental conditions. Signal intensity was compared with that observed with cells incubated at 37°C with both primary and secondary antibody but omitting FITC insulin in the incubation medium. Results are the mean of three separate stainings with the fluorescence intensity in 15 cells quantified with each incubation (a total 45 cells under each experimental condition). The decreases in fluorescence intensity in response to wortmannin, genistein, PP1, PD 98059, and TNF-α and the increase seen with PTP1B inhibitor are all highly statistically significant (one-way ANOVA on ranks with post hoc Dunnett's test, $P < 0.05$ for each).

These findings indicate that the process by which insulin is accumulated within endothelial cells requires the biological action of insulin on the endothelial cell acting through the insulin receptor and intact downstream signaling through PI 3-kinase and MEK as well as cSrc-family tyrosine kinase signaling pathways. We attempted to use these inhibitors to examine whether intact insulin signaling was required for movement of FITC insulin across a confluent endothelial monolayer using cells cultured on Transwell plates. However, we observed that each agent caused leakiness of confluent endothelial monolayers as indicated by fall of transendothelial electrical resistance by ~75% as measured using an epithelial voltohmmeter (WPI EVOM) (11).

Considering the inhibitory effect of TNF-α (Fig. 2), cells were pretreated with the cytokine for 6 h before the addition of FITC-labeled insulin. We have previously

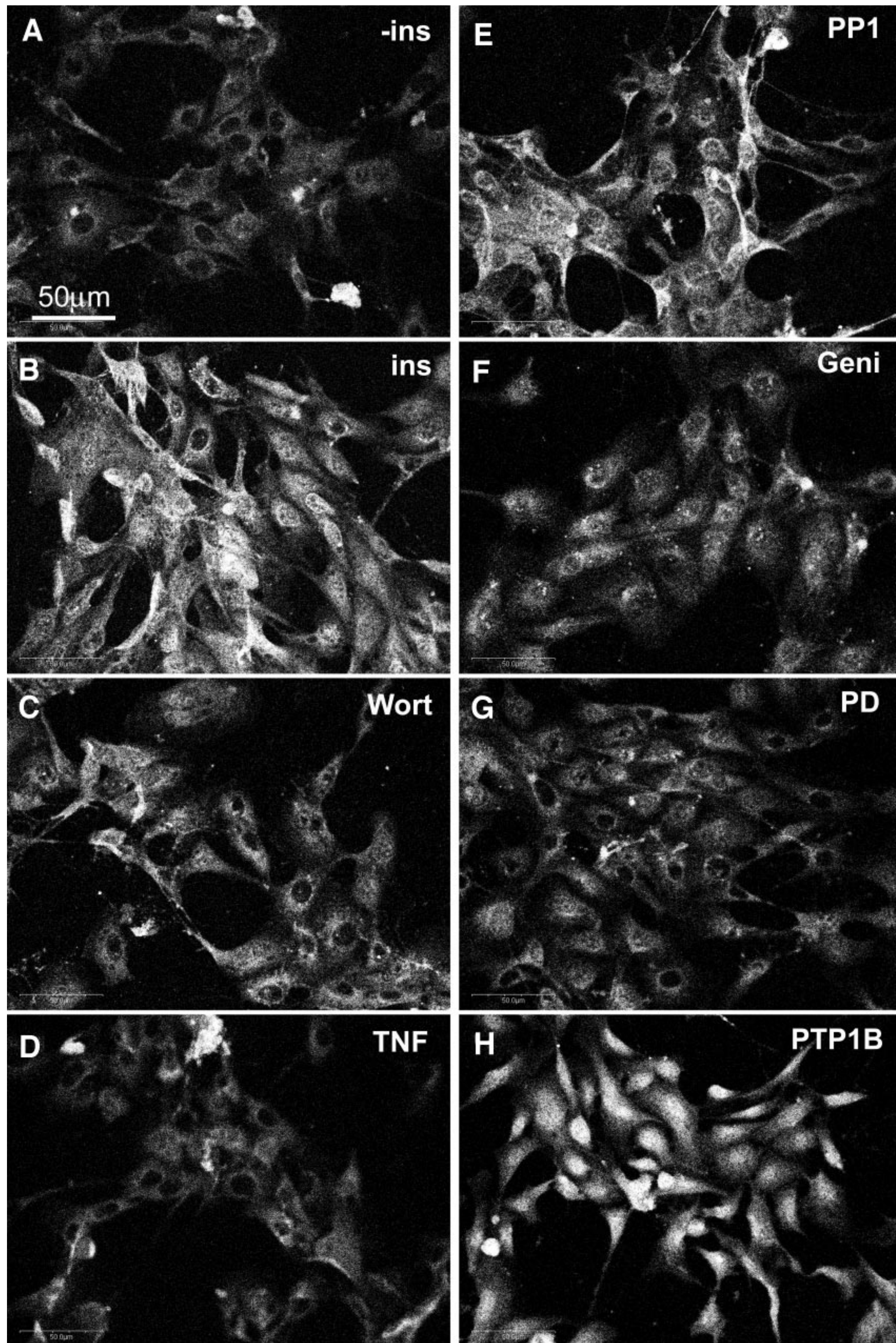


FIG. 2. Insulin uptake is controlled by its own signaling. ***Top panel*****: Confocal images of bAECs.** ***A*****: Cells maintained in the basal medium without FITC insulin for 30 min before being stained with anti-FITC.** ***B*****: Cells were treated identically to those in** ***A*** **except FITC insulin (INS) was present during the 30 min incubation.** ***C–H*****: Cells were likewise incubated with FITC insulin for 30 min before fixation and subsequent immunocytochemical staining with anti-FITC antibody. Wortmannin (WORT) (*C*), TNF-α (*D*), PP1 (*E*), genistein (GENI) (*F*), PD98059 (PD) (*G*), or PTP1B inhibitor (PTP1BI) (*H*) was added (at concentrations indicated in RESEARCH DESIGN AND METHODS) to cells 30 min before addition of FITC insulin.** ***I*****: Quantification of the fluorescence intensity from three experiments for each experimental condition. Highly significant inhibition of insulin uptake was observed in response to inhibition of insulin signaling while inhibition of tyrosine phosphatase activity significantly enhanced cell fluorescence intensity. *P < 0.05, one-way ANOVA on ranks with post hoc Dunnett's test.**

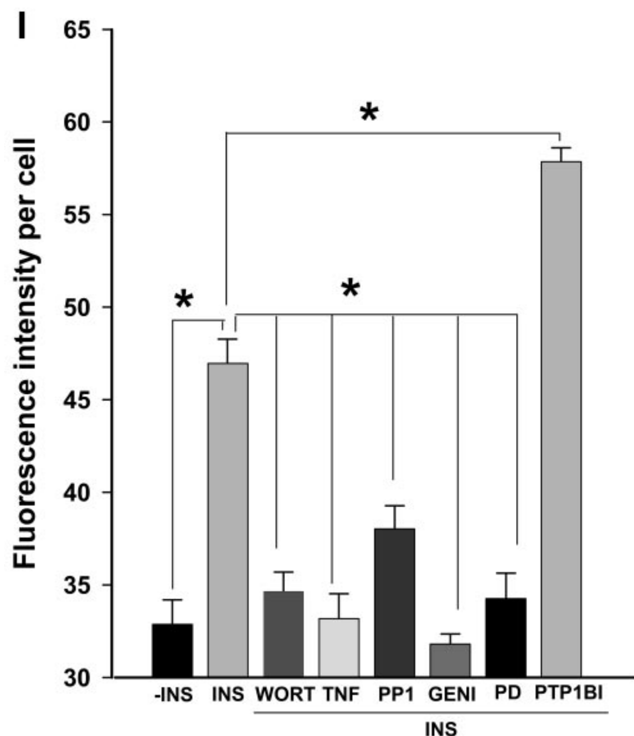


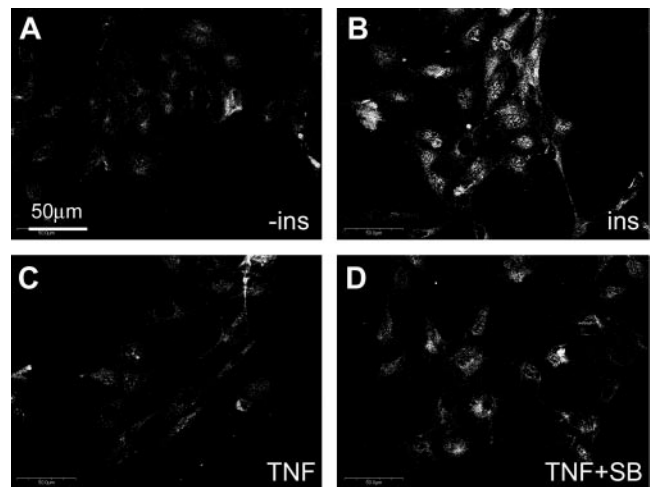
FIG. 2—Continued.

shown that in bAECs incubated under similar conditions, TNF-α inhibits downstream insulin signaling pathways and that this appears to be due to activation of P38 MAP kinase by TNF-α with subsequent serine phosphorylation of insulin receptor substrate-1 (IRS-1) proteins (14). Although the P38 MAP kinase inhibitor SB203580 appears to block the action of TNF-α on IRS-1 serine phosphorylation (14), this only partly restored the ability of bAECs to take up insulin (Fig. 3, *top*). This suggests that TNF-α may exert additional actions to impede insulin uptake beyond the activation of P38 MAPK.

Next, we measured insulin receptor phosphorylation immunocytochemically using an anti–phospho-insulin receptor (Tyr^{972}) antibody. We observed that genistein did in fact inhibit insulin receptor phosphorylation by quantifying insulin receptor phosphorylation immunocytochemically in cells incubated in the presence and absence of genistein (Fig. 4*A*). Conversely, PTP1B inhibition amplified the signal intensity for the phospho–insulin receptor staining. These changes are quantified in Fig. 4, *bottom*. We note that staining for phospho-insulin receptor displayed a preferential nuclear localization after cells were treated with the PTB1B inhibitor (Fig. 4*A*, *d*), whereas treatment with FITC insulin, genistein, TNF-α, or wortmannin did not show the same cellular distribution (Fig. 4*A* [*b* and *c*] and *B* [*c* and *d*]), and we do not have an explanation for this phenomenon.

As shown in Fig. 4*B*, we observed that whereas insulin enhanced the phosphorylation of the insulin receptor, TNF-α and wortmannin had no effect on insulin-induced insulin receptor phosphorylation. Therefore, the inhibitory effect of TNF-α on insulin uptake does not appear to originate with effects on insulin receptor phosphorylation

We have previously reported that in bAECs, insulin receptors appear to associate with caveolae (11). Phosphorylation of caveolin-1 is thought to be involved in



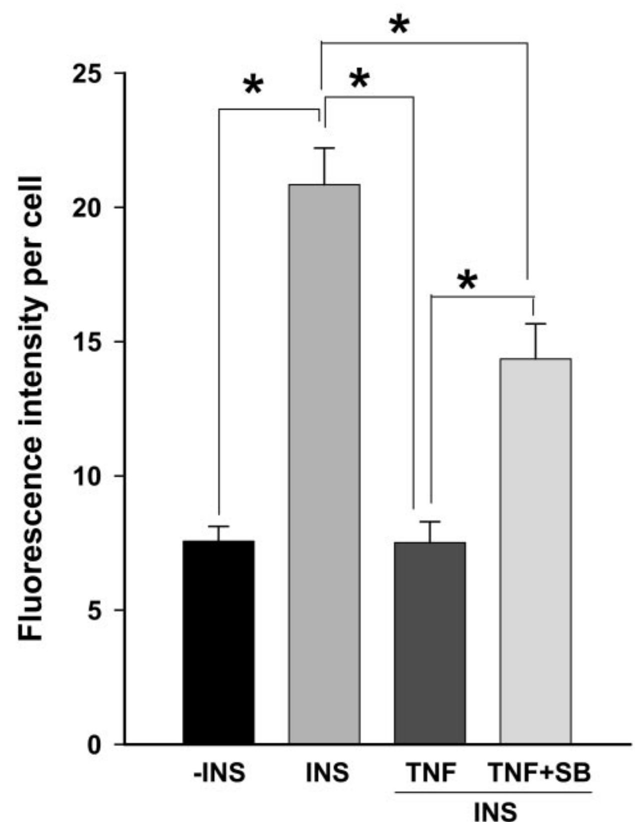


FIG. 3. Effect of TNF-α on cellular insulin uptake. Six hours of preincubation of bAECs with 5 ng/ml TNF-α reduced cellular insulin uptake essentially to background levels. Addition of SB203580, a specific P38 MAPK inhibitor, restored insulin uptake to ~50% of that seen in control cells. Representative images are shown above the quantitative analysis of cellular insulin uptake. *A*: Basal medium only. *B*: FITC insulin. *C*: FITC insulin plus TNF-α. *D*: FITC insulin plus TNF-α and SB203580 (SB). *P < 0.05, one-way ANOVA.

modulating endocytosis by caveolae in endothelial cells. We therefore examined whether 10 nmol/l insulin increased caveolin-1 phosphorylation and whether TNF-α interfered with this process. This is shown in Fig. 5*A* and *B*. Stimulation of bAECs with 10 nmol/l insulin for 30 min did not increase Tyr^{14} caveolin-1 phosphorylation. However, TNF-α appeared to modestly inhibit the phosphorylation at Tyr^{14} ($P = 0.06$). Wortmannin had no such effect, and neither agent affected total caveolin-1 content of the bAECs (Fig. 5*A* and *C*).

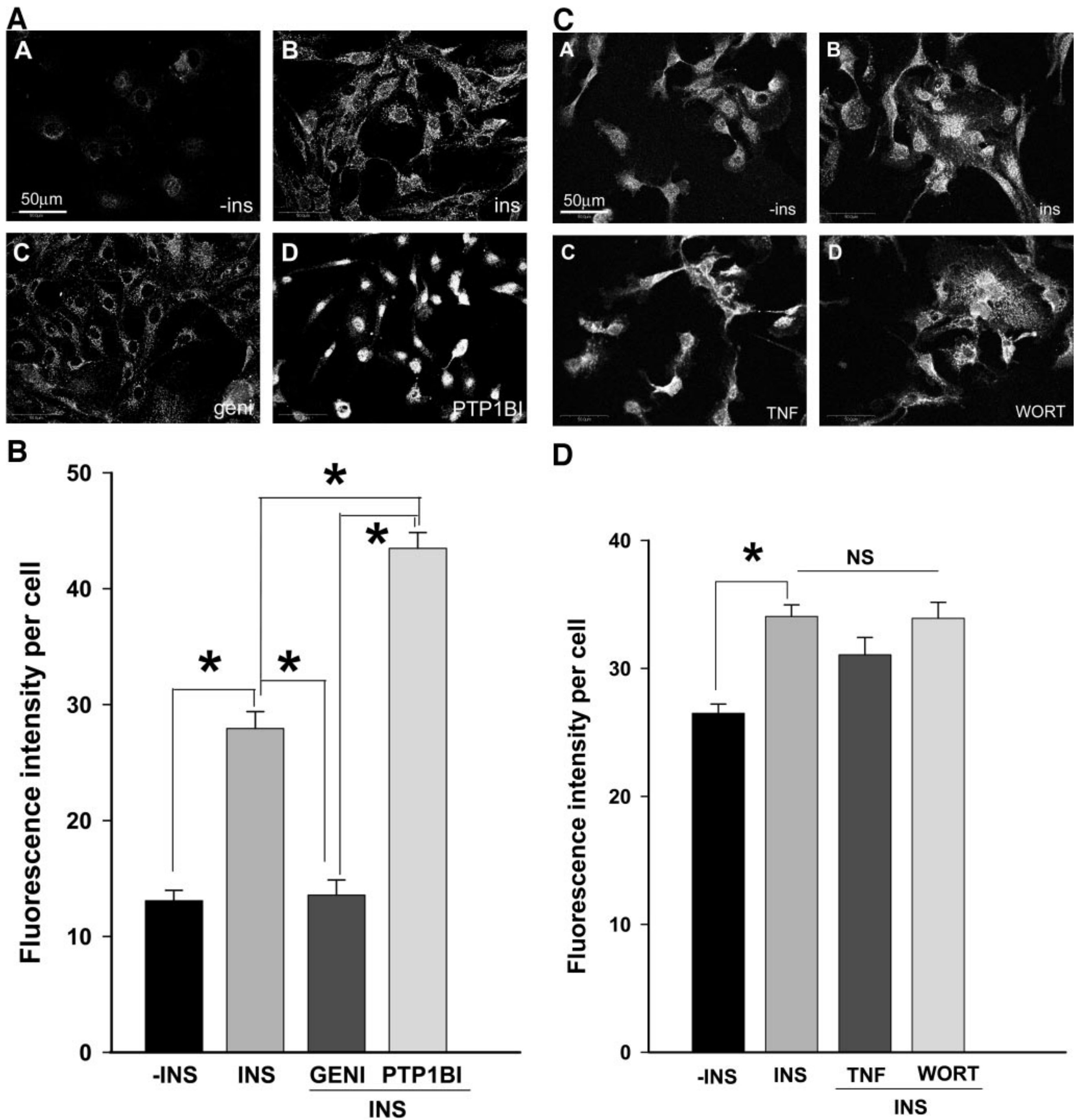


FIG. 4. Insulin-induced insulin receptor phosphorylation in bAECs. In the *top panels*, representative confocal images illustrate the effect of 10 nmol/l insulin to increase the phosphorylation of insulin receptor as detected by an anti–phospho-insulin receptor Tyr^{972} antibody. Whereas insulin clearly stimulated insulin receptor phosphorylation (*B*), this effect was blocked by genistein (*C*) and enhanced by PTP1B inhibition (*D*). Cells incubated in the basal medium without exposure to insulin show little staining for phosphorylated insulin receptor (*A*). The *right panels* illustrate that neither TNF-α (*C*) nor wortmannin (*D*) affected insulin action on the insulin receptor. The *bottom panels, B* and *D*, illustrate quantification of images. **$P < 0.05$; NS,** $P \geq 0.05$**.**

DISCUSSION

We are pursuing the hypothesis that there are at least three discrete steps at which insulin may act on the vasculature to enhance its own delivery to muscle: *1*) at resistance vessels, which can be relaxed by insulin to increase total blood flow (15); *2*) at terminal arterioles, which, when relaxed by insulin, increase the microvascular bed (capillaries and probably postcapillary venules) perfused within skeletal muscle (16–19); and *3*) at the transporting endothelial cell where insulin actually moves across the endothelium (11). There is no consensus currently extant as to whether transendothelial insulin movement is a passive (8,9) or a mediated (10,11) process. We previously (11) showed that transendothelial insulin transport is a transcellular process that may involve caveolae and insulin and/or IGF-I receptors. The current experiments particularly addressed whether insulin action on the endothelial cell through its classical signaling pathways regulates endothelial cell insulin uptake; i.e., must insulin exert a biological action on the cell for uptake to occur? The data

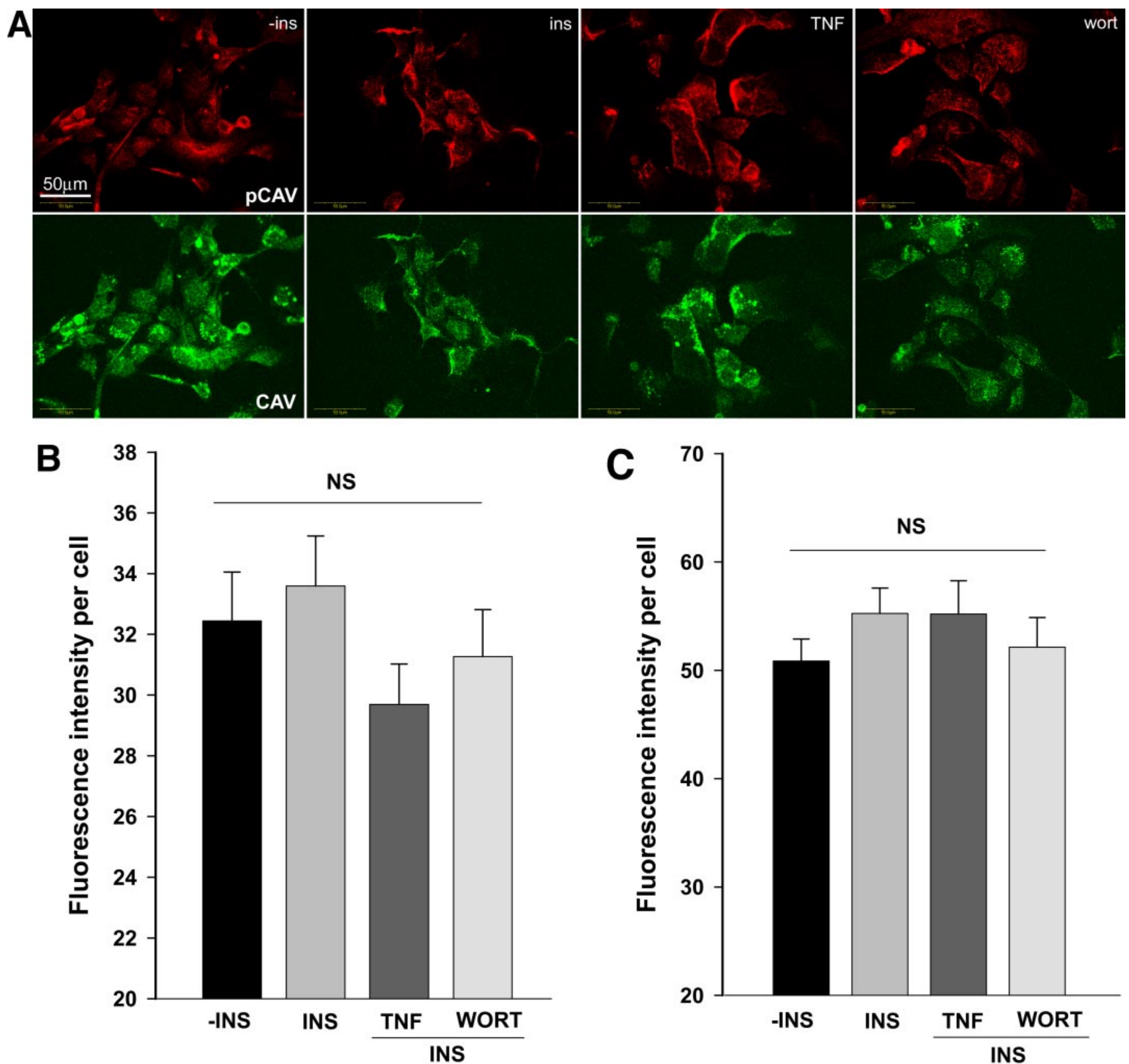


FIG. 5. Effect of insulin signaling on caveolin-1 phosphorylation. *A*: Confocal images obtained using anti–phospho-CAV1 at Tyr^{14} (*top row*, revealed by Cy3, red) and anti-CAV1 (*bottom row*, revealed by Cy2, green). *B*: The fluorescence intensity attributable to phospho-caveolin-1 at Tyr^{14} staining in endothelial cells incubated with either insulin alone or with TNF-α or wortmannin added. *C*: Total fluorescence intensity attributable to caveolin-1 content in these cells. NS, $P \geq 0.05$. (Please see http://dx.doi.org/db07-0967 for a high-quality digital representation of this figure.).

presented here provide, to our knowledge, the first evidence that this first step in transendothelial transport of insulin by the endothelial cell requires multiple intact pathways for insulin signaling. Inhibition of either the PI 3-kinase or MEK signaling pathways markedly diminished insulin uptake while inhibiting cSrc-kinase signaling had a lesser, yet still significant, effect. In contrast, inhibiting PTP1B, a phosphatase that regulates insulin receptor and IRS-1 signaling (20), significantly enhances insulin uptake. Beyond demonstrating that insulin action on the endothelium is required for the first step of transendothelial insulin transport, i.e., insulin uptake, we find that this process is altered by exposure of bAECs to TNF-α. This would suggest that inflammatory cytokines that provoke insulin resistance at the endothelium might affect the normal uptake of insulin by endothelial cells. In muscle and fat, which have relatively tight endothelial junctions, inhibiting insulin transport could materially contribute to tissue insulin resistance (5,6). We have previously reported that TNF-α infusion in rats effectively inhibits insulin's vascular action to recruit microvasculature and diminishes insulin-mediated glucose disposal (21). The current findings suggest that an effect of TNF-α on endothelial insulin uptake may have contributed to this in vivo insulin resistance. Of note, aortic endothelial cells in culture may behave differently than microvascular endothelial cells within skeletal

muscle. The latter cannot be accessed for in vitro studies of the type described here. The full physiological significance of the findings reported here will require follow-up in vivo studies. We also recognize that the transit pathway by which insulin, once taken up by the endothelial cell, crosses the cell and is then released is not known. It does appear, at least based on studies in cultured endothelial cells, that insulin is only slowly degraded by endothelial cells (<15% after 1 h for ^{125}I-labeled insulin (22) and ~14% in our hands for FITC insulin in the course of 1 h). It is possible that insulin taken up by the cell (likely in association with caveolae) remains within a vesicular compartment, and these vesicles may subsequently bud off from the luminal plasma membrane of the endothelial cell and migrate to the abluminal side to subsequently fuse and release their content (rev. in 23). Accumulating evidence indicates that albumen transport across the endothelium occurs in this manner (24). Net transendothelial insulin transport would occur only if the insulin concentration in the interstitium is lower than in plasma. Clearly clarifying the multiple steps involved in transcellular movement and abluminal membrane release of insulin will be important for understanding the overall kinetics of transendothelial insulin transport.

We recognize that it is unusual to observe similar inhibition of insulin-dependent processes when either the PI 3-kinase or MAPK pathways are inhibited. Several considerations suggest that these are not nonspecific toxic effects of the inhibitors. First, the concentrations of each of these inhibitors used are quite typical of those used in numerous studies examining diverse actions of insulin (25). Second, we have recently demonstrated that treatment of endothelial cells with 25 μmol/l PD98059 (same dose and incubation duration as used in the present study) does not affect insulin-induced Ser^{473} Akt phosphorylation, whereas treatment with 100 nmol/l wortmannin (same dose and incubation duration as used in the present study) does not affect insulin-stimulated MEK 1/2 phosphorylation. In both cases, insulin-induced IRS-1 tyrosine phosphorylation remains intact (14). These data suggest that these inhibitors are pathway specific.

The current studies were carried out using insulin concentrations above the physiological range. We have previously shown that with these concentrations, both insulin and IGF-I receptors are tyrosine phosphorylated and that downstream insulin/IGF-I signaling pathways are activated in bAECs (12). In addition, we have shown that IGF-I receptors are capable of mediating transendothelial insulin transport in cultured bAECs (11). As a result, we cannot be certain of the proportional contributions of the insulin, IGF-I, or insulin/IGF-I hybrid receptors (which are also present in bAECs [12]) to the uptake of FITC insulin observed in the current studies. It would seem likely that the IGF-I receptors are making a significant contribution, given the more abundant expression of these receptors on bAECs (26).

The observation that PTP1B inhibition actually augments the uptake of labeled insulin further underscores the importance of insulin (and IGF-I) action to facilitate the transport process. It also suggests that even at 50 nmol/l insulin, the activation of the transport mechanism is not maximal. Again, whereas insulin action via insulin receptor would be expected to be saturated at this concentration, insulin action via IGF-I or hybrid receptors would not be (12).

The role of the cSrc-kinase in insulin action is not clear. However, in vascular tissue, cSrc-kinase has been implicated in IGF-I action to stimulate cell proliferation via the MEK-MAPK signaling cascade (27). Possibly, this accounts for the effect seen here. Alternatively, it is interesting that albumin, which is transported across the endothelium by a caveolae-mediated pathway (28,29), relies on the activation of cSrc-kinase (30) via a G-protein–linked albumin binding protein (GP60) on the endothelial cell plasma membrane. The finding that inhibition of cSrc-kinase diminished insulin uptake might either relate to a role of cSrc-kinase in regulating the trafficking of caveolae (31) or in modulating insulin or IGF-I signaling.

We (11) and others (32) have previously reported that disruption of lipid rafts that are enriched with caveolae using either filipin or methyl-β-cyclodextrin decreases endothelial cell insulin uptake. We examined whether TNF-α might be acting to inhibit caveolin-1 phosphorylation on Tyr^{14}. Phosphorylation is thought to facilitate caveolae trafficking (33), and there is evidence that insulin stimulates caveolin-1 phosphorylation at Tyr^{14} (33). Several authors have previously reported that insulin transiently (maximum by 10 min) enhances caveolin-1 phosphorylation and that this persists for at least 30 min using Western blotting methods (33,34). However, we did not observe in bAECs under our experimental conditions either a consistent increase or decrease in tyrosine phosphorylation of caveolin-1 by insulin. Possibly either the methods used here or the 30-min incubation insulin limited assessment of caveolin-1 phosphorylation in the current studies. Also, with the concentration of TNF-α and the time period of treatment we used in this study (5 ng/ml, 6 h), we did not observe a significant inhibition of caveolin-1 phosphorylation at Tyr^{14}, although an inhibitory tendency existed (P = 0.06). Further study with higher doses and longer treatment times seems to be warranted to clarify this issue.

We have been interested in the vascular actions of insulin to increase skeletal muscle blood flow and to recruit microvasculature (capillaries) within muscle. Each of these processes has the potential to increase insulin delivery to muscle interstitium, and each appears to involve an action of insulin to increase endothelial cell production of nitric oxide and relax vascular smooth muscle. In studies comparing the dose response (18) to insulin and the time course (35) for insulin action, we have found that microvascular recruitment occurs more quickly and at a lower insulin concentration compared with changes in total blood flow. Beyond that, we have found that microvascular recruitment is impaired in states of insulin resistance (36,37), and this could, by diminishing insulin delivery to the microvascular endothelium where transendothelial transport occurs, contribute to impaired insulin-mediated glucose disposal.

The current studies suggest that endothelial cell uptake of insulin is yet another locus at which insulin resistance might impact insulin action in skeletal muscle and perhaps adipose tissue. Clarifying the significance of the findings reported here will require careful physiological studies of insulin transendothelial transport in vivo in normal and insulin-resistant animals and humans.

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REFERENCES

1. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP: The effect of insulin on the disposal of intravenous glucose: results from indirect calorimetry and hepatic and femoral venous catherization. *Diabetes* 30:100–107, 1981
2. Yang YJ, Hope ID, Ader M, Bergman RN: Insulin transport across capillaries is rate limiting for insulin action in dogs. *J Clin Invest* 84:1620–1628, 1989
3. Freidenberg GR, Suter S, Henry RR, Nolan J, Reichart D, Olefsky JM: Delayed onset of insulin activation of the insulin receptor kinase in vivo in human skeletal muscle. *Diabetes* 43:118–126, 1994
4. Miles PD, Levisetti M, Reichart D, Khoursheed M, Moossa AR, Olefsky JM: Kinetics of insulin action in vivo: identification of rate-limiting steps. *Diabetes* 44:947–953, 1995
5. Miles PD, Li S, Hart M, Romeo O, Cheng J, Cohen A, Raafat K, Moossa AR, Olefsky JM: Mechanisms of insulin resistance in experimental hyperinsulinemic dogs. *J Clin Invest* 101:202–211, 1998
6. Sjostrand M, Gudbjornsdottir S, Holmang A, Lonn L, Strindberg L, Lonnroth P: Delayed transcapillary transport of insulin to muscle interstitial fluid in obese subjects. *Diabetes* 51:2742–2748, 2002
7. Castillo C, Bogardus C, Bergman R, Thuillez P, Lillioja S: Interstitial insulin concentrations determine glucose uptake rates but not insulin resistance in lean and obese men. *J Clin Invest* 93:10–16, 1994
8. Steil GM, Ader M, Moore DM, Rebrin K, Bergman RN: Transendothelial insulin transport is not saturable in vivo: no evidence for a receptor-mediated process. *J Clin Invest* 97:1497–1503, 1996
9. Hamilton-Wessler M, Ader M, Dea MK, Moore D, Loftager M, Markussen J, Bergman RN: Mode of transcapillary transport of insulin and insulin analog NN304 in dog hindlimb: evidence for passive diffusion. *Diabetes* 51:574–582, 2002
10. King GL, Johnson SM: Receptor-mediated transport of insulin across endothelial cells. *Science* 227:1583–1586, 1985
11. Wang H, Liu Z, Li G, Barrett EJ: The vascular endothelial cell mediates insulin transport into skeletal muscle. *Am J Physiol Endocrinol Metab* 291:E323–E332, 2006
12. Li G, Barrett EJ, Wang H, Chai W, Liu Z: Insulin at physiological concentrations selectively activates insulin but not insulin-like growth factor I (IGF-I) or insulin/IGF-I hybrid receptors in endothelial cells. *Endocrinology* 146:4690–4696, 2005
13. Bertelsen M, Anggard EE, Carrier MJ: Oxidative stress impairs insulin internalization in endothelial cells in vitro. *Diabetologia* 44:605–613, 2001
14. Li G, Barrett EJ, Barrett MO, Cao W, Liu Z: Tumor necrosis factor-{alpha} induces insulin resistance in endothelial cells via a p38 mitogen-activated protein kinase-dependent pathway. *Endocrinology* 148:3356–3363, 2007
15. Baron AD, Steinberg H, Brechtel G, Johnson A: Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. *Am J Physiol* 266:E248–E253, 1994
16. Vincent MA, Clerk LH, Barrett E: Skeletal muscle microvascular recruitment is more insulin sensitive than femoral blood flow or glucose disposal. *Diabetes* 52:A48, 2003
17. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S: Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol Endocrinol Metab* 285:E123–E129, 2003
18. Zhang L, Vincent MA, Richards SM, Clerk LH, Rattigan S, Clark MG, Barrett EJ: Insulin sensitivity of muscle capillary recruitment in vivo. *Diabetes* 53:447–453, 2004
19. Clerk LH, Vincent MA, Lindner JR, Clark MG, Rattigan S, Barrett EJ: The vasodilatory actions of insulin on resistance and terminal arterioles and their impact on muscle glucose uptake. *Diabete Metab Res Rev* 20:3–12, 2004
20. Taniguchi CM, Emanuelli B, Kahn CR: Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85–96, 2006
21. Zhang L, Wheatley CM, Richards SM, Barrett EJ, Clark MG, Rattigan S: TNF-alpha acutely inhibits vascular effects of physiological but not high insulin or contraction. *Am J Physiol Endocrinol Metab* 285:E654–E660, 2003
22. Dernovsek KD, Bar RS: Processing of cell-bound insulin by capillary and macrovascular endothelial cells in culture. *Am J Physiol* 248:E244–E251, 1985
23. Tuma PL, Hubbard AL: Transcytosis: crossing cellular barriers. *Physiol Rev* 83:871–932, 2003
24. Mehta D, Malik AB: Signaling mechanisms regulating endothelial permeability. *Physiol Rev* 86:279–367, 2006
25. Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105, 2000
26. Chisalita SI, Arnqvist HJ: Insulin-like growth factor I receptors are more abundant than insulin receptors in human micro- and macrovascular endothelial cells. *Am J Physiol Endocrinol Metab* 286:E896–E901, 2004
27. Lieskovska J, Ling Y, Badley-Clarke J, Clemmons DR: The role of Src kinase in insulin-like growth factor-dependent mitogenic signaling in vascular smooth muscle cells. *J Biol Chem* 281:25041–25053, 2006
28. Schubert W, Frank PG, Razani B, Park DS, Chow CW, Lisanti MP: Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo. *J Biol Chem* 276:48619–48622, 2001
29. Minshall RD, Sessa WC, Stan RV, Anderson RG, Malik AB: Caveolin regulation of endothelial function. *Am J Physiol Lung Cell Mol Physiol* 285:L1179–L1183, 2003
30. Tiruppathi C, Song W, Bergenfeldt M, Sass P, Malik AB: Gp60 activation mediates albumin transcytosis in endothelial cells by tyrosine kinase-dependent pathway. *J Biol Chem* 272:25968–25975, 1997
31. Shajahan AN, Timblin BK, Sandoval R, Tiruppathi C, Malik AB, Minshall RD: Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. *J Biol Chem* 279:20392–20400, 2004
32. Schnitzer JE, Oh P, Pinney E, Allard J: Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol* 127:1217–1232, 1994
33. Lee H, Volonte D, Galbiati F, Iyengar P, Lublin DM, Bregman DB, Wilson MT, Campos-Gonzalez R, Bouzahzah B, Pestell RG, Scherer PE, Lisanti MP: Constitutive and growth factor-regulated phosphorylation of caveolin-1 occurs at the same site (Tyr-14) in vivo: identification of a c-Src/Cav-1/Grb7 signaling cassette. *Mol Endocrinol* 14:1750–1775, 2000
34. Kimura A, Mora S, Shigematsu S, Pessin JE, Saltiel AR: The insulin receptor catalyzes the tyrosine phosphorylation of caveolin-1. *J Biol Chem* 277:30153–30158, 2002
35. Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S, Barrett EJ: Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* 53:1418–1423, 2004
36. Clerk LH, Vincent MA, Jahn LA, Liu Z, Lindner JR, Barrett EJ: Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes* 55:1436–1442, 2006
37. Rattigan S, Clark MG, Barrett EJ: Acute insulin resistance in rat skeletal muscle in vivo induced by vasoconstriction. *Diabetes* 48:564–569, 1999