Mode of Transcapillary Transport of Insulin and Insulin Analog NN304 in Dog Hindlimb
Evidence for Passive Diffusion

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A defect in transcapillary transport of insulin in skeletal muscle and adipose tissue has been proposed to play a role in the insulin resistance that leads to type 2 diabetes, yet the mechanism of insulin transfer across the capillary endothelium from plasma to interstitium continues to be debated. This study examined in vivo the interstitial appearance of insulin in hindlimb using the fatty acid acylated insulin analog Lys329-tetradecanoyl des-(B30) human insulin, or NN304, as a marker for insulin transport. If the insulin transport were a saturable process, then “swamping” the capillary endothelial insulin receptors with native insulin would suppress the subsequent appearance in interstitial fluid of the insulin analog NN304. This analog binds to insulin receptors with an affinity of ~50% of native insulin. Experimental conditions established a physiologic NN304 dose in the absence or presence of pharmacologic and saturating concentrations of regular human insulin. Euglycemic clamps were performed in dogs under inhalant anesthesia with deep hindlimb lymphatic sampling, representative of skeletal muscle interstitial fluid (ISF). In group 1 (n = 8), NN304 alone was infused (3.6 pmol · min⁻¹ · kg⁻¹) from 60 to 360 min. In group 2 (n = 6), starting at time 0, human insulin was infused at a pharmacologic dose (60 pmol · min⁻¹ · kg⁻¹) with the addition of NN304 infusion (3.6 pmol · min⁻¹ · kg⁻¹) from 60 to 360 min. In group 3 (n = 4), the human insulin infusion was increased to a saturating dose (120 pmol · min⁻¹ · kg⁻¹). Pharmacologic insulin infusion (group 2) established steady-state human insulin concentrations of 6,300 ± 510 pmol/l in plasma and 5,300 ± 540 pmol/l in ISF. Saturating insulin infusion (group 3) achieved steady-state human insulin concentrations of 22,000 ± 1,800 pmol/l in plasma and 19,000 ± 1,500 pmol/l in ISF. Total (bound and unbound) NN304 plasma concentrations rose from a steady state of 1,900 ± 110 (group 1) to 2,400 ± 200 pmol/l (group 2) and 3,100 ± 580 pmol/l (group 3), consistent with a competition-driven decline in NN304 clearance from plasma as the human insulin level increased (P < 0.05 by ANOVA). Steady-state interstitial NN304 concentrations also rose with increasing human insulin levels but did not achieve significance in comparison with analog alone (162 ± 15 vs. 196 ± 22 and 241 ± 53 pmol/l for group 1 versus groups 2 and 3, respectively; P = 0.20), yet the steady-state plasma:ISF ratio for NN304 remained essentially unchanged in the absence and presence of elevated human insulin levels (12.6 ± 1.2 vs. 12.4 ± 0.5 and 13.1 ± 1.5 for group 1 versus groups 2 and 3, respectively; P = 0.93). Last, NN304 rate of appearance in interstitial fluid (i.e., half-time to steady state) was similar between groups; mean half-time of 92 ± 4 min (NS between groups). In conclusion, appearance of the insulin analog NN304 in skeletal muscle interstitial fluid was constant whether in the absence or presence of human insulin concentrations sufficient to saturate the endothelial insulin receptors. These findings support the hypothesis, provided that the mechanism of insulin and NN304 transcapillary transport is similar, that transcapillary transport of insulin in skeletal muscle occurs primarily via a nonsaturable process such as passive diffusion via a paracellular or transcellular route. Diabetes 51:574–582, 2002

Insulin resistance is a primary defect that leads to type 2 diabetes. Skeletal muscle and adipose tissue are primary targets for insulin action, and a defect in any of the steps that lead to insulin action could contribute, in whole or in part, to insulin resistance. Whereas the intracellular insulin signaling cascade has received much of the attention regarding possible defects in insulin action, the slowness of insulin action in vivo seems to be due to the sluggish transport of the hormone across the tight capillary endothelium of skeletal muscle (1–3). Because transendothelial insulin transport has been suggested to play a role in insulin resistance (4,5), it is critical to understand the mechanism of transcapillary insulin transport.

Two distinct hypotheses have emerged regarding the mode of transport of insulin across the capillary endothelium from plasma to interstitium: passive diffusion versus receptor-mediated transcytosis. Skeletal muscle capillary endothelium has been described as a nonfenestrated continuous endothelium across which hydrophilic solutes with a radius of <30 Å can diffuse through small pores or paracellular pathways (6). Early studies (7–10) suggested...
passive diffusion as the primary mechanism of transport of molecules in the size range of insulin (Stokes-Einstein radius of 21 Å). More recently, Vincent et al. (11) demonstrated no change in glucose tolerance or insulin action in vascular endothelial insulin receptor knockout mice, a finding consistent with non–receptor-mediated transcapillary insulin transport. In contrast to skeletal muscle and adipose tissue, insulin transport across the brain microvascular endothelium in vivo was demonstrated by Schwartz and colleagues (12,13) to be a saturable, receptor-mediated process. Thus, it seems that heterogeneity exists with respect to transcapillary insulin transport in various tissue beds. Evidence for receptor-mediated transendothelial insulin transport emerged from studies of bovine aortic endothelial cells; King and Johnson (14) reported saturability of the transendothelial insulin transport across a cell monolayer consistent with receptor-mediated transport. In contrast, Milton and Knutson (15,16) reported that delivery of insulin across a cell monolayer, based on permeability and diffusion coefficients, was consistent with passive diffusion and not receptor-mediated endocytosis. The reason for the discrepancy between in vitro studies remains unclear. Therefore, we have pursued studies of this transport process in vivo models (2,3,17,18).

One approach to studying whether a transport process is mediated by specific carrier molecules is to observe the movement of labeled molecules in competition with saturating amounts of the unlabeled moiety. High-affinity binding sites for insulin have been identified in endothelial cell cultures (19). Negative cooperativity has also been demonstrated in vitro in endothelial cells with displacement of iodinated insulin when in the presence of unlabeled insulin (19,20). However, with respect to iodinated insulin, use of radioactive tracer to assess transport processes has been limited by nonspecific binding and by altered binding properties of the labeled compared to native ligand (21,22). The recent availability of analogs to insulin with a long-chain fatty acid bound to the position 29 on the B-chain has provided a novel alternative approach to studying transcapillary insulin transport. Because of the specificity of enzyme-linked immunospecific assay (ELISA) methods, native insulin and Lys**B**29-tetradecanoyl des-(B30) human insulin, or NN304, can be measured separately and virtually independently from native insulin in the same plasma (or lymph) sample. NN304 resembles native insulin with the exception that the ε-amino group of lysine**B**29 is acylated with myristic acid after removal of threonine**B**30 (23). This acylated insulin reversibly binds to albumin at the long-chain fatty acid binding sites, and thus the onset of its effect on glucose turnover is protracted in comparison to native insulin. However, the receptor-binding domains of the insulin molecule remain unchanged in this analog, as evidenced in vivo and in vitro (23,24). Also, the molecular size is similar to the native molecule (Stokes-Einstein radius 21 Å for native insulin and 22Å for NN304) (15,25).

Given the availability of the insulin analog NN304, we tested the hypothesis that transcapillary transport of insulin in skeletal muscle is a nonsaturable, non–receptor-mediated process such as transcytosis and/or passive diffusion. We measured the appearance of a physiologic dose of NN304 in deep hindlimb lymph (representative of skeletal muscle interstitial fluid) in the absence and presence of pharmacologic and saturating human insulin concentrations sufficient to “swamp” the capillary endothelial insulin receptors. If the hypothesis were to be accepted that transcapillary insulin transport in skeletal muscle is via a nonspecific process such as passive diffusion that is non–receptor mediated and not saturable, then high plasma levels of native insulin would be expected to have little, if any, effect on the subsequent appearance of the insulin analog in interstitial fluid.

### RESEARCH DESIGN AND METHODS

**Animals.** A large animal model was used to allow for successful sampling of hindlimb lymphatic fluid. Experiments were conducted on healthy male mongrel dogs that weighed 23 ± 0.8 kg (range 19.1–30). Dogs were housed under controlled kennel conditions (12-h light, 12-h dark) in the University of Southern California School of Medicine Vivarium. Animals had free access to water and standard diet (24% protein, 9% fat, 40% carbohydrate, 17% fiber; Wayne Dog Chow, Alfred Mills, Chicago, IL). Food was withdrawn 18 h before experiments. Dogs were used for experiments only if judged to be in good health. The experimental protocols were approved by the University Institutional Animal Care and Use Committee.

**Surgical preparation.** Surgery was performed at ~0700 h. All experiments were done on anesthetized animals after catheter implantation. Dogs were preanesthetized with acepromazine maleate (0.1 mg/lb) (Prom-Ace; Aueco, Fort Dodge, IA) and atropine sulfate (0.05 cc/lb) (Western Medical, Arcadia, CA). Anesthesia was induced with sodium pentobarbital (Nembutal, 9.2 cc/lb; Abbott Laboratories, North Chicago, IL) and maintained with halothane and nitrous oxide. Indwelling silastic catheters were implanted in the right jugular vein (saline drip) and left carotid artery (sampling). Right and left cephalic vein intracatheters were placed for insulin, somatostatin, and exogenous glucose infusions as detailed below. A perivascular ultrasonic flowprobe (2 mm diameter; Transonic, Ithaca, NT) was placed around the right femoral artery for measurement of blood flow. Hindlimb lymphatic fluid, representative of skeletal muscle interstitial fluid (26,27), was sampled via a polyethylene catheter (usually PE50) inserted into a deep lymph vessel. To expose the hindlimb lymphatic vessels, a longitudinal incision was made distal to the femoral triangle, and the lymphatic vessels lying between the femoral artery and vein were carefully freed of fascia within a window of ~0.5–1.0 cm. The sampling catheter was then threaded through a pinhole, advanced 1–2 cm beyond the incision point (to avoid any lymphatic valves), and secured with silk suture. Lymph flow was initiated by gentle massage of the hindlimb muscle. Skin incisions were closed with silk suture and kept moist with saline-soaked gauze. Body temperature was maintained with warming pads. Blood pressure, heart rate, and respiratory CO₂ were monitored continuously. Dogs received a saline drip throughout both the surgery and the experiment (10 cc/lb administered during the first 60 min of surgery and a slow drip thereafter). Urine was collected throughout the experiment. After experiments, animals were killed by an overdose of sodium pentobarbital (Eutha-6; Western Medical, Arcadia, CA).

**Experimental protocols.** A total of 18 experiments were performed. In all experiments, a 90-min stabilization period followed the catheter placement. After the stabilization period, a continuous infusion of somatostatin (0.8 μg · min⁻¹ · kg⁻¹) (Bachem, Torrance, CA) was initiated and continued to 300 min. Basal insulin was replaced (1.2 pmol · min⁻¹ · kg⁻¹) (Novolin-R; Novo Nordisk A/S, Bagsvaerd, Denmark) via continuous peripheral infusion. Arterial Euglycemia was maintained by exogenous glucose infusion into the left cephalic vein at rates calculated according to online measurements of plasma glucose.

**Group 1: Analog alone (n = 8).** At time 0, an intravenous infusion of the fatty acid acylated insulin analog Lys**B**29-tetradecanoyl des-(B30), or NN304 (3.6 pmol · min⁻¹ · kg⁻¹), was initiated and continued to 300 min. This protocol was absent of any additional human insulin infusion above basal replacement (1.2 pmol · min⁻¹ · kg⁻¹).

**Group 2. Analog + pharmacologic human insulin (n = 6).** At time 0, a continuous intravenous infusion of human insulin (60 pmol · min⁻¹ · kg⁻¹; Novolin-R) was initiated and continued throughout the experimental period. The analog NN304 infusion (3.6 pmol · min⁻¹ · kg⁻¹) was then initiated at 60 min and continued to 360 min.

**Group 3. Analog + saturating human insulin (n = 4).** At time 0, a continuous intravenous infusion of human insulin (120 pmol · min⁻¹ · kg⁻¹; Novolin-R) was initiated and continued throughout. Again, the analog NN304
RESULTS

Glucose. Basal glucose values (6.6 ± 0.5 mmol/l or 120 ± 9 mg/dl) and clamp glucose values were not different between groups (6.7 ± 0.1 mmol/l or 121 ± 2 mg/dl; coefficient of variation, 8%). The exogenous glucose infusion rates to maintain euglycemia differed between groups as a result of differences in the hormone doses (doses: NN304 at 3.6 pmol/min−1·kg−1 for group 1 versus NN304 at 3.6 pmol/min−1·kg−1 + human insulin at 60 and 120 pmol/min−1·kg−1 for groups 2 and 3, respectively). Steady-state glucose infusion rates were 7.2 ± 0.6 mg·min−1·kg−1 for analog alone (group 1) versus 13.9 ± 1.3 and 18.2 ± 1.0 mg·min−1·kg−1 for groups 2 and 3, respectively (P < 0.05; Fig. 2).

Blood flow and mean arterial pressure. Average basal femoral artery blood flow was similar between groups (Table 1). During analog and insulin infusions, femoral

FIG. 1. Plasma (A) and lymph (B) samples measured for cross-reactivity of human insulin with ELISA specific to NN304. Samples were spiked with increasing concentrations of human insulin (10–7,500 pmol/l in plasma and 10–2,800 pmol/l in lymph); n = 8 assays.
artery blood flow showed a modest increase of ~11% (range, 8–26%) above basal in all groups. Mean arterial pressure also remained constant from basal throughout the experimental protocol and did not differ between groups.

**Insulin.** Basal endogenous insulin levels were similar between groups for plasma (44 ± 7 pmol/l) and for lymph (24 ± 3 pmol/l; P = 0.02 versus plasma). In group 2, pharmacologic human insulin infusion caused plasma insulin levels to rise rapidly to a steady state of 6,300 ± 510 pmol/l (Fig. 3A), whereas interstitial insulin rose more slowly to a steady state of 5,300 ± 540 pmol/l. At the saturating insulin dose (Fig. 3B), plasma insulin rose to 22,000 ± 1,800 pmol/l with an interstitial insulin level of 19,000 ± 1,500 pmol/l. A true plateau was not achieved with the saturating insulin dose, consistent with saturation of receptor-mediated insulin clearance. A steady-state plasma:ISF insulin ratio of 1.9 ± 0.3 at basal insulin (group 1) decreased to 1.2 ± 0.1 with pharmacologic insulin in group 2 and slightly further to 1.1 ± 0.1 with saturating insulin in group 3 (P < 0.05 versus basal for both; Fig. 4). The observed decrement in the plasma:ISF insulin ratio from basal to pharmacologic and saturating insulin levels is consistent with previous reports of a reduction in the ratio at supraphysiologic in comparison to physiologic insulin concentrations (18). The theoretical minimum ratio would be 1.0, in which case there would no gradient or barrier between the plasma and interstitial fluid compartments.

**NN304.** Plasma and interstitial NN304 concentrations reflect the total, i.e., albumin-bound and unbound, concentration. The majority of analog is reversibly bound to albumin (~98% (23,24)) at all concentrations. During infusion of analog alone (group 1), plasma NN304 rose to a steady-state level of 1,900 ± 110 pmol/l (Fig. 5A), consistent with previous studies that used a similar NN304 dose (30,31). For groups 2 and 3, plasma NN304 rose to steady states of 2,400 ± 200 and 3,100 ± 580 pmol/l, respectively (P < 0.05 versus group 1). Thus, it is apparent that the added saturating dose of human insulin caused a significant decrement in the metabolic clearance rate for NN304 (P = 0.03, group 1 versus group 3).

As expected, interstitial steady-state NN304 concentrations were much lower than those in plasma (30). Steady-

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**TABLE 1**

<table>
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<tr>
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<th>Group 1 (analog)</th>
<th>Group 2 (human insulin + analog)</th>
<th>Group 3 (human insulin + analog)</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>Basal</td>
<td>76 ± 3</td>
<td>73 ± 3</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>Steady state</td>
<td>74 ± 2</td>
<td>74 ± 2</td>
<td>72 ± 5</td>
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<tr>
<td>Femoral flow (ml/min)</td>
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<tr>
<td>Basal</td>
<td>179 ± 28</td>
<td>200 ± 27</td>
<td>205 ± 10</td>
</tr>
<tr>
<td>Steady state</td>
<td>212 ± 14</td>
<td>210 ± 38</td>
<td>224 ± 29</td>
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Data are means ± SD. MAP, mean arterial pressure
state NN304 in ISF was 160 ± 15 pmol/l for group 1 (Fig. 5B). The rise in plasma NN304 with pharmacologic and saturating insulin induced a concomitant rise in interstitial NN304 levels to 196 ± 22 and 241 ± 53 pmol/l for groups 2 and 3, respectively (P = 0.06, group 1 versus group 3). The mean steady-state plasma:ISF ratio for NN304 was not significantly different between groups at 12.6 ± 1.2 for group 1 vs. 12.4 ± 0.5 and 13.1 ± 1.5 for groups 2 and 3, respectively (NS; Fig. 6). Similar to the steady-state results, the dynamics (half-times to steady state) of NN304 in plasma and ISF were virtually identical in the presence and absence of supraphysiological plasma insulin concentrations (Table 2). Thus, neither the steady-state ratio of plasma to ISF NN304 nor the dynamics of NN304 were significantly influenced by modifying plasma insulin concentrations. Plasma and ISF levels for NN304 rose concurrently during progressive elevation in the ambient insulin concentration, consistent with the hypothesis of passive diffusional transcapillary insulin transport.

**DISCUSSION**

Transcapillary transport of insulin has been identified as a rate-limiting step in insulin action in vivo (1–3,32). The rate of appearance of insulin in ISF is a determinant of glucose uptake by insulin-sensitive tissues. Recently, insulin transport was implicated in insulin resistance (4,5). However, the mode by which insulin traverses the capillary endothelium in vivo continues to be an area of inquiry. The present study used the fatty acid acylated human insulin analog...
The appearance of NN304 in deep hindlimb ISF was examined in the absence and in the presence of plasma insulin concentrations sufficient to saturate the insulin receptors on the capillary endothelium (16,33). We hypothesized that transcapillary transport of insulin is a nonsaturable and non-receptor-mediated process such that high plasma levels of native insulin would be expected to have little, if any, effect on the subsequent appearance of the insulin analog in interstitial fluid. We found the rate of appearance of NN304 in hindlimb ISF (i.e., half-time to steady state) remained constant in the absence and presence of saturating plasma insulin concentrations. These findings support the hypothesis that transcapillary transport of insulin occurs primarily via a nonsaturable process, such as transcytosis or passive diffusion.

The use of the fatty acid acylated insulin analog NN304 as a marker for insulin transcapillary transport seems to be justified. First, examination of the crystal structure of the NN304 molecule (25) revealed only a small change of the molecular radius as compared with that of unmodified insulin. The Stokes-Einstein radius for native insulin is 21 Å and for NN304 is 22 Å (15,25). The diffusion capacity of NN304 with respect to endothelial pore size thus would be equivalent to native insulin. Second, only the COOH-terminal of the insulin molecule has been modified in NN304. This terminal portion of the B-chain is not involved in insulin receptor binding and is distal to the bioactive region of the insulin molecule (34). Thus, it is likely that the mode of transport of NN304 across the skeletal muscle capillary endothelium is similar to that of native insulin.

It is notable that the appearance of NN304 in ISF is considerably delayed in comparison with native insulin. This is a result of retention of NN304 by binding to plasma albumin rather than a slowing of the transcapillary transport rate (35). NN304 dissociates from plasma albumin at a constant rate and is free to traverse the capillary endothelium. The reversible binding of NN304 to albumin via the fatty acid ester thus explains the large pool of biologically inactive plasma NN304 and high steady-state plasma:ISF ratio in comparison with native insulin. Supraphysiologic plasma insulin concentrations did not influence the interaction between plasma albumin and NN304 in this study as evidenced by the similar plasma half-times of the analog between groups (Table 2). This result was not surprising because native insulin does not bind to albumin and would not be expected to impede the NN304-albumin binding. Neither the steady-state ratio of plasma to ISF NN304 nor the dynamics of NN304 were significantly influenced by changing plasma insulin concentrations. Thus, it is apparent that the sequestration of NN304 by plasma albumin remains the rate-determining factor for its appearance in interstitial fluid and is not influenced by elevating plasma insulin concentrations. Upon dissociation from albumin, NN304 freely diffused across the capillary endothelium at a constant rate irrespective of the circulating insulin concentration, providing evidence for passive diffusion of insulin across the capillary endothelium. The insulin analog demonstrates full efficacy in ISF, such that the ability of NN304 to bind to ISF albumin has little influence on its peripheral action (35).

The plasma and interstitial NN304 levels rose in parallel as the plasma insulin concentration was increased consistently with decreased clearance from plasma (Fig. 5). The likelihood of a decrease in hepatic extraction of NN304 seems plausible as an explanation for the increased plasma and interstitial concentrations of NN304 when in the presence of supraphysiologic human insulin concentrations compared with analog alone. Partial occupancy of hepatic insulin receptors may have occurred. Across several studies that included various species (36–38), fractional hepatic insulin extraction has been estimated to decrease from 50% to ~35% at plasma insulin concentrations of 6,000 pmol/l. Indeed, in the present study, with

![Graph showing plasma:ISF ratio for insulin analog NN304.](image)

**TABLE 2**

<table>
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<th>Group 1 (analog)</th>
<th>Group 2 (human insulin + analog)</th>
<th>Group 3 (human insulin + analog)</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
<td>4</td>
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<tr>
<td>NN304 half-times (t 1/2)</td>
<td></td>
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<tr>
<td>Plasma t1/2 (min)</td>
<td>19 ± 3</td>
<td>27 ± 3</td>
<td>28 ± 8</td>
</tr>
<tr>
<td>ISF t1/2 (min)</td>
<td>91 ± 6</td>
<td>95 ± 9</td>
<td>91 ± 8</td>
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Data are means ± SD.

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concentrations to supraphysiologic levels. Several factors increases rather than decreases with elevating insulin. This is consistent with a “nonsaturation theory.” The reduced ratio for insulin suggests that the rate of transcapillary transport increases rather than decreases with elevating insulin concentrations to supraphysiologic levels. Several factors may have contributed to the increase in transcapillary insulin transport, including an increase in nonspecific binding. An increase in transcapillary insulin transport has been attributed to an increase in capillary recruitment, diversion of capillary flow, and/or dilation of capillary endothelium. In this study, femoral artery blood flow increased modestly during insulin infusions, indicating that a decrease in the plasma:ISF insulin ratio may have been attributed, in part, to an increase in blood flow as well as to changes in capillary recruitment or local capillary flow. An additional component that influences the plasma:ISF insulin ratio was explained previously as possible changes in the clearance of insulin from the interstitial compartment, i.e., a relative decrease in irreversible binding and degradation when at saturating insulin concentrations for insulin action.

Unlike native insulin, that there was no change in the plasma:ISF ratio for NN304 with increasing plasma insulin concentrations (Fig. 6) provides additional confirmation of the sequestration of NN304 by plasma albumin as the rate-determining factor for its appearance in interstitial fluid. As well, the unchanged plasma:ISF ratio for NN304 provides evidence for passive diffusional transcapillary transport of the moiety. High concentrations of native insulin would have been expected to displace, via competition, the binding of NN304 to the endothelial insulin receptor and as such block receptor-mediated transport of the insulin analog. Therefore, we conclude that, in vivo, NN304 and insulin traverse the skeletal muscle capillary endothelium via a nonsaturable and non–receptor-mediated process. This finding is consistent with previous studies in which the capillary permeability of different tissue beds was assessed. Liver, muscle, intestine, and cervical region capillary permeability was demonstrated by Mayerson et al. (39) to vary according to endothelial pore size distribution. They reported that in dogs, small pores were predominant in muscle capillary endothelium and permeable to molecules up to ∼30–45 Å. Guyton (40) reported that the intercellular cleft that lies between adjacent endothelial cells has a width of up to 60–70 Å. Finally, Haraldsson (6) found that small anionic solutes (<30 Å) were transported across the rat skeletal muscle capillary endothelium predominately by diffusion through small pores. Endothelial pores of these sizes would allow for passive diffusion of insulin and NN304 across the capillary endothelium but would restrict diffusion of albumin. Thus, it is possible that a small fraction of NN304 could traverse the capillary endothelium bound to albumin, via albumin receptor-mediated transport. However, the relative small transfer of albumin from plasma to ISF would very likely remain constant under the conditions of this study and therefore not influence the outcome with respect to NN304 transport.

Factors in addition to pore and molecular size may significantly influence the passage of molecules across the capillary endothelium. Temperature, capillary hemodynamics, net molecular charge on the molecule of interest, electrical resistance of the capillary endothelium, and Starling forces have been shown to be important determinants of capillary permeability and transcapillary transport (6). These factors are important when comparing results from in vitro and in vivo studies of transendothelial transport. Previous in vitro studies (43) that used cultured endothelial cells, epithelial cells, and fibroblasts have yielded conflicting results with respect to transendothelial transport of insulin (44). From a monolayer preparation, Hachiya et al. (44) concluded that in aortic and adipose capillary endothelial cells, insulin traverses the endothelium via a receptor-mediated process. Rabkin et al. (45) demonstrated similar findings in cultured glomerular endothelial cells. However, subsequent in vitro studies have yielded results that support the passive diffusion hypothesis (15). Differences between studies might be explained by methodological considerations, including indicators of endothelial permeability or transport, i.e., radioactive tracer versus permeability coefficient.

Therefore, to avoid potential complexities of cell culture preparations, we sought to perform experiments in vivo by exploiting the technology of an insulin analog. One assumption was required in these experiments: that the fatty acid acylated insulin analog NN304 is transported via the same mode as native insulin. Because this molecule is bioactive in vitro and in vivo, it is more than likely that its behavior is similar to insulin, yet we cannot fully exclude the possibility that the mechanism by which NN304 traverses the capillary endothelium may be different from native insulin. The presence of the fatty acyl moiety might enhance the lipophilic nature of the molecule (46). However, NN304 shows no affinity for cell membranes using a variety of cell types (23). Because the solubility of NN304 is similar to native insulin, we consider it likely that this moiety is transported by a mechanism similar to insulin itself.

An additional caveat to the present results is our inability to detect a saturable receptor-mediated transport process that may be of small magnitude. Clearly, if most of insulin transport is diffusional and a small amount is via specific transport molecules, then it would be possible that we could be blocking the receptor-mediated component and not detect this with our methodology. However, if such a mechanism exists, it seems to account for only a small fraction of transcapillary insulin transport under conditions of physiological increments in glucose utilization. The previous work of Steil et al. (18), demonstrating that transendothelial insulin transport was likely nonsaturable, supports the supposition of a minor receptor-mediated component. In this study, we have advanced the notion that transcapillary transport of the insulin analog NN304 and, very likely, also insulin is neither a saturable nor a receptor-mediated process.

The present studies have important clinical implica-
tions. Saturable insulin transport at the level of the endothelium supports the concept that insulin resistance could reside at the level of insulin movement into the interstitium. In fact, Miles et al. (5) suggested that such resistance can exist in the hyperinsulinemic dog model in which slowed activation of peripheral glucose disposal was in part due to slowed transcapillary insulin transport. In a pair of studies, obesity-induced insulin resistance in humans (47) and in rats (48) was associated with markedly increased plasma and interstitial insulin levels compared with lean controls. However, Castillo et al. (47) demonstrated no apparent defect in transcapillary insulin transport and maintenance of the “normal” steady-state plasma:ISF insulin ratio in obese compared with lean men. In contrast, Holmang et al. (48) demonstrated an apparent loss of the plasma:ISF insulin gradient in obese Zucker rats, suggestive of either a loss of the barrier function of the capillary endothelium or reduced clearance of insulin from the interstitial space. It is interesting that in a study of nonobese individuals with type 2 diabetes, Sjostrand et al. (49) showed near-normal insulin levels and no alteration in the steady-state plasma:ISF ratio compared with matched controls. However, kinetic analysis of transcapillary transport was not assessed in that study. The present study results suggest that if there is resistance at the transcapillary transport step, then the resistance may be more related to changes in local blood flow (50), capillary recruitment, or capillary permeability (51) rather than to changes in the ability of specific receptors to shuttle insulin across the capillary boundary. In skeletal muscle, changes in transcapillary transport of insulin as a result of alterations in capillary hemodynamics or permeability could contribute at least in part to the insulin resistance that leads to type 2 diabetes (T2T defect). Finally, it should be remembered that absence of a receptor-mediated transport mechanism in skeletal muscle by no means rules out specific transport mechanisms in other tissue beds, such as in brain, as reported by Schwartz and colleagues (12,52).

In summary, the present study results demonstrate that, in this in vivo preparation, the appearance of insulin in hindlimb lymph is not saturable and does not seem to be receptor-mediated. Thus, skeletal muscle transcapillary transport of this insulin analog and, most likely, native insulin occurs via passive diffusion by transcellular or paracellular routes.

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