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

Marianthe Hamilton-Wessler,1 Marilyn Ader,1 Melvin K. Dea,1 Donna Moore,1 Mette Loftager,2 Jan Markussen,2 and Richard N. Bergman1

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 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 = 0.98). 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

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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 in vitro 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 LysB29-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-aminogroup of lysineB29 is acylated with myristic acid after removal of threonineB30 (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 22 Å 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 22 ± 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; Aveco, 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, NY) 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 distal to the incision point (beyond 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) to suppress endogenous insulin release was begun at ~30 min and maintained throughout the experiment. Basal insulin was replaced (1.2 pmol · min⁻¹ · kg⁻¹) (Novolin-R; Novo Nordisk A/S, Bagsvaerd, Denmark) via continuous peripheral infusion. Arterial eu glycemia 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 min, an intravenous infusion of the fatty acid acylated insulin analog LysB29-tetradecanoyl des-(B30) human insulin, or NN304 (3.6 pmol · min⁻¹ · kg⁻¹) (Bachem, Torrance, CA) to suppress endogenous insulin release was begun at ~30 min and maintained throughout the experiment. Basal insulin was replaced (1.2 pmol · min⁻¹ · kg⁻¹) (Novolin-R; Novo Nordisk A/S, Bagsvaerd, Denmark) via continuous peripheral infusion. Arterial eu glycemia was maintained by exogenous glucose infusion into the left cephalic vein at rates calculated according to online measurements of plasma glucose.

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
infusion (3.6 pmol · min⁻¹ · kg⁻¹) was then begun at 60 min and continued to 360 min. For groups 2 and 3, human insulin only was infused for the first hour in an effort to “swamp” the insulin receptors on the capillary endothelium before commencing the NN304 infusion. Both pharmacologic and saturating doses of human insulin were used to test fully the hypothesis of non–receptor-mediated transcapillary transport.

Arterial sampling (~3 ml blood) was coupled with hindlimb lymphatic sampling (continuously from ~1.5 min before to ~1.5 min after arterial sample time; 300–700 μl lymphatic fluid). Basal samples were taken at ~20, ~10, and ~1 min. Arterial and hindlimb lymph samples for human insulin, NN304, and glucose were collected every 5 min from 5 to 40 min, at 50 and 60 min, then every 5 min from 65 to 120 min. Remaining samples were collected every 10 to 360 min.

**Assays.** Arterial blood samples were collected in ice-chilled Vacutainer tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Hindlimb lymph was collected in tubes precoated with sodium fluoride and lithium heparin (Brinkman Instruments, Westbury, NY). Blood and lymph samples were kept on ice until centrifugation. Plasma and lymph samples were stored at −20°C until assayed. Online plasma glucose was assayed by the glucose oxidase method on an automated analyzer (Yellow Springs Instrument, Yellow Springs, OH). Novo-Nordisk A/S provided materials for the insulin and NN304 assays. Human and canine insulins were measured in arterial and hindlimb lymph with an ELISA for the appropriate insulin (28). NN304 total concentrations (bound and unbound) were measured in arterial and hindlimb lymph using a specific ELISA method developed by Novo Nordisk A/S and adapted in our laboratory. This method uses monoclonal antibodies specific to NN304, which exhibit no measurable cross-reactivity with either human or dog insulin. Eight assays of human or dog insulin with concentrations up to 7,500 pmol/l in plasma and 2,800 pmol/l in lymph did not read above the detectable limit of 10 pmol/l by the NN304 assay (Fig. 1). Intra- and interassay coefficients of variation were 3 and 5%, respectively.

**Data analysis.** Steady-state values were calculated by averaging data from the last 30 min of the experiment (i.e., 530–360 min). Plasma:interstitial fluid (ISF) ratio, or gradient, was calculated by dividing the steady-state increment above basal for plasma insulin or NN304 by the respective increment in interstitial level.

To compare the kinetics of the physiologic dose of NN304 in the absence and presence of pharmacologic or saturating insulin, we expressed data from individual experiments as a percentage of steady-state levels. Half-times were then estimated as the time (minutes) required to reach 50% of the increment between basal and steady state. Standard statistics (independent and paired Student’s t tests) and one-way ANOVA (29) with Tukey’s procedure were conducted with Minitab (State College, PA) statistical software on a personal computer. P < 0.05 was considered statistically significant. Data are presented as means ± SE.

**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

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**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.

**FIG. 2.** Exogenous glucose infusion rate during infusion of insulin analog NN304 (3.6 pmol · min⁻¹ · kg⁻¹, group 1, n = 8; bars), human insulin + analog (60 + 3.6 pmol · min⁻¹ · kg⁻¹, group 2, n = 6; bold line) or human insulin + analog (120 + 3.6 pmol · min⁻¹ · kg⁻¹, group 3, n = 4; thin line).
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>
<thead>
<tr>
<th></th>
<th>Group 1 (analog)</th>
<th>Group 2 (human insulin + analog)</th>
<th>Group 3 (human insulin + analog)</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
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<td>72 ± 5</td>
</tr>
<tr>
<td>Femoral flow (ml/min)</td>
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<td></td>
<td></td>
</tr>
<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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</tbody>
</table>

Data are means ± SD. MAP, mean arterial pressure

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**FIG. 3.** Human insulin profile in plasma (●) and hindlimb lymph (or ISF; □) concentrations. A: Infusion of human insulin + analog at 60 + 3.6 pmol · min⁻¹ · kg⁻¹, group 2. B: Infusion of human insulin + analog at 120 + 3.6 pmol · min⁻¹ · kg⁻¹, 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 supraphysiologic 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
Lys\textsuperscript{B29}-tetradecanoyl des-(B30), or NN304, as an indicator or marker for insulin movement across skeletal muscle capillary endothelium. 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. The reversible binding of NN304 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

![FIG. 6. Steady-state plasma:ISF ratio for insulin analog NN304. Group 1, basal insulin replacement (1.2 pmol·min\(^{-1}·kg\(^{-1}\)) during analog infusion (3.6 pmol·min\(^{-1}·kg\(^{-1}\)); Group 2, human insulin + analog (60 + 3.6 pmol·min\(^{-1}·kg\(^{-1}\)); Group 3, human insulin + analog (120 + 3.6 pmol·min\(^{-1}·kg\(^{-1}\)).](image)

### TABLE 2

<table>
<thead>
<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>NN304 half-times (t 1/2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
</tr>
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Data are means ± SD.
elevating plasma insulin to supraphysiologic concentrations, plasma clearance was reduced for both native insulin and insulin analog NN304.

Pharmacologic and saturating human insulin concentrations were also associated with a decrease in the plasma: ISF ratio for insulin at steady state in comparison with basal insulin (Fig. 4) and to previously reported physiologic steady states (17,18). This observation 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 (22). An increase in transcapillary insulin transport also 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 predominantly 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 (6,15,40–42). 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 (15,16,44,45). 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 (15,16). 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 (23), 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 (T2D 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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