

Albumin Binding of Acylated Insulin (NN304) Does Not Deter Action to Stimulate Glucose Uptake

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NN304 [Lys^{B29}-tetradecanoyl des(B30) human insulin] is a potentially therapeutic insulin analog designed to exhibit protracted glucose-lowering action. In dogs with infusion rates similar to insulin itself, NN304 exhibits similar glucose uptake (R_d) stimulation with delayed onset of action. This compartmental modeling study was to determine if NN304 action could be accounted for by the ~2% unbound NN304 concentration. NN304 (or human insulin) ($n = 6$ each) was infused at $10.2 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ under euglycemic clamp conditions in anesthetized dogs. NN304 appearance in lymph, representing interstitial fluid (ISF), was slow compared with insulin ($t_{1/2} = 70 \pm 7$ vs. 14 ± 1 min, $P < 0.001$). R_d was highly correlated with the ISF concentration for insulin and NN304 ($r = 0.86$ and 0.93 , respectively), suggesting that slow transendothelial transport (TET) is responsible for sluggish NN304 action. Insulin and NN304 concentration data were fit to a two-compartment (plasma and ISF) model. NN304 plasma elimination and TET were reduced to 10 and 7% of insulin, respectively. Thus, there was reduction of NN304 transport, but not to the degree expected. In ISF, there was no reduction in NN304 elimination. Thus, this acylated insulin analog demonstrates blunted kinetics in plasma, and full efficacy in the compartment of action, ISF. *Diabetes* 51: 762–769, 2002

Tight glycemic control has been shown to forestall the microvascular complications in both type 1 and type 2 diabetic patients (1,2). An important component of glycemic control is the delivery of constant basal replacement of insulin. Currently available therapy intended for basal replacement of insulin can be improved, as the longest acting insulin formulations do not provide unchanging plasma insulin concentrations throughout the overnight fast (3). Recently, new insulin analogs with the goal of prolonged action have been designed by ϵ -amino group acylation of human insulin with a saturated fatty acid. The saturated fatty acid imparts low-affinity binding of the molecule to the fatty acid binding sites on albumin (4). Indeed, these

analogues exhibit protracted onset of action (5–7). Although it has been hypothesized that albumin binding retards the analogue's action, the specific mechanism(s) of slowed action has not been clearly defined.

Normal human insulin acts slowly in vivo to stimulate glucose uptake. The capillary endothelium provides a barrier for insulin to reach skeletal muscle cells, a primary site of insulin action. The transport of insulin across the capillary endothelium of muscle (transendothelial transport [TET]) has been shown to determine the time course of insulin action in the intact organism. The effect of TET to determine insulin's rate of action has been demonstrated by the slow increase in interstitial insulin relative to plasma insulin and the high correlation between interstitial fluid (ISF) insulin concentrations and the rate of glucose disposal (8). A similar close relationship between interstitial hormone levels and time course of action was demonstrated for very slowly acting insulin analog acylated Lys^{B29}-tetradecanoyl des-(B30) human insulin, or NN304 (9). NN304 is bound by plasma albumin due to the free fatty acid moiety bound to the insulin molecule. However, it has not been clarified from previous studies whether the slowed appearance of NN304 in ISF is due to reduced TET per se or to sequestration by plasma albumin.

For albumin-bound ligands such as NN304, it is generally assumed that only unbound hormone binds hormone receptors. Based on in vitro binding affinity to albumin and the albumin concentration, it may be predicted that 1.7 and 3.3% of NN304 is not bound to albumin at steady state in plasma versus ISF, respectively. The difference between relative binding in the two pools is due to the reduced albumin concentration in ISF. Previous studies of NN304 have revealed an enigma regarding its mode of action: the fraction of unbound hormone in ISF estimated from in vitro kinetics is too low to account for the compound's potency to enhance glucose uptake (7). Other hormones, metabolites, and drugs extensively bound to albumin and other plasma proteins have also exhibited this apparent "enhanced action" (10–16). Therefore, the behavior of NN304 with respect to the unbound hormone hypothesis merits examination at the levels of hepatic clearance, TET, and at the site of insulin action itself in the interstitium.

One approach to examine the cause of NN304 retardation of action compared with insulin and the unexpected potency of ISF NN304 is to study the distribution kinetics of the compound. For this purpose, we have applied compartmental modeling to NN304 plasma and lymph concentration data obtained during constant infusion (ac-

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Received for publication 25 August 2001 and accepted in revised form 27 November 2001.

ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; ISF, interstitial fluid; TET, transendothelial transport.

tivation) and deactivation periods. The NN304 data has been compared with the kinetics of human insulin controls. Thus, the modeling is based upon NN304 and insulin measurements in plasma and ISF as previously reported (9).

RESEARCH DESIGN AND METHODS

Experimental protocol. Experiments were conducted on healthy male mongrel dogs under anesthesia (24.1 ± 0.9 kg, range 12.5–31.4, $n = 12$). Protocol was approved by the USC institutional Animal Care and Use Committee. Surgery was as follows: indwelling catheters were implanted in the carotid artery (sampling) and jugular vein (saline drip). Left and right cephalic vein intracatheters were inserted for infusions as detailed below. Hindlimb muscle lymphatic fluid was sampled via a polyethylene catheter (PE10 to PE90, predominantly PE50) inserted into a deep lymph vessel as previously described (17). Lymphatic flow was initiated by gentle massage of the limb muscle.

In all experiments, a primed tracer infusion of HPLC-purified [$3\text{-}^3\text{H}$]-D-glucose (25 μCi bolus followed by 0.25 $\mu\text{Ci}/\text{min}$; Dupont-NEN, Boston, MA) was used during euglycemic clamps to assess glucose turnover. 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 of lymphatic fluid).

The experimental protocol consisted of four temporal phases: a basal phase (from -40 to -30 min), an insulin replacement phase (from -20 to 0 min), an “activation” phase (from 0 to 180 min for human insulin [$n = 6$], from 0 to 320 min for NN304 [$n = 6$]), and a “deactivation” phase (from 180 to 360 min for human insulin, from 320 to 660 min for NN304). Somatostatin (0.8 $\mu\text{g} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$; Bachem California, Torrance, CA) was infused to suppress endogenous insulin release, and basal insulin secretion was replaced with a systemic infusion of regular human insulin for the human insulin protocol and regular porcine insulin for the NN304 protocol (1.2 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$; Novo Nordisk A/S, Bagsvaerd, Denmark). During activation, either human insulin or NN304 was infused at a rate of 10.2 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$. During the deactivation phase, suprabasal human insulin or NN304 infusion was discontinued, with continued basal insulin replacement. Porcine insulin, identical in amino acid sequence to canine insulin, was utilized for basal replacement in the NN304 protocol. Porcine insulin could not be utilized in the human insulin protocol, since it crossreacts with the human-insulin assay; therefore, human insulin was utilized for basal replacement. Human and porcine insulin should be equally efficacious for basal replacement in these experiments.

During the experiments, glucose was clamped at 121 ± 1 mg/dl for human insulin experiments and 117 ± 2 mg/dl for NN304 experiments ($P < 0.03$) with an exogenous infusion of glucose labeled with [$3\text{-}^3\text{H}$]glucose (Dupont-NEN) to a specific activity of 2.7 $\mu\text{Ci}/\text{g}$. Glucose was clamped with an overall coefficient of variation of $3.6 \pm 0.3\%$ for human insulin experiments and $5.0 \pm 0.6\%$ for NN304 experiments.

Assays. Total NN304 concentrations (bound and unbound) were measured in plasma and hindlimb lymph using a specific enzyme-linked immunoabsorbent assay (ELISA) method developed by Novo Nordisk A/S and adapted in our laboratory. The assay identifies a moiety on NN304, which is not blocked by albumin when bound. Therefore, total NN304 concentrations are measured, but relative bound versus unbound concentrations cannot be determined in vivo. The ELISA for NN304 utilizes monoclonal antibodies specific to NN304 and exhibits no crossreactivity with either human or dog insulin. The NN304 assay did not read above the detectable limit (10 pmol/l) in samples with increasing human or dog native insulin concentrations to 7,500 pmol/l in plasma and 2,800 pmol/l in lymph.

Human insulin was measured in plasma and hindlimb lymph with an ELISA method developed for human plasma by Novo-Nordisk (18) and adapted for dog plasma in our laboratory.

Pharmacological model. Because of crossreactivity of endogenous dog insulin in the human ELISA assay, basal insulin in plasma and lymph was subtracted by linear interpolation between the beginning and the end of each experiment. Human insulin and NN304 data were each fit to a two-compartment model (Fig. 1), with compartment 1 representing plasma and compartment 2 representing ISF. The compounds are eliminated from both compartments; mass balance equations were as follows:

$$\frac{dM_1}{dt} = \text{infusion} + k_{12}M_2 - (k_{21} + k_{01})M_1 \quad (1)$$

$$\frac{dM_2}{dt} = k_{21}M_1 - (k_{12} + k_{02})M_2 \quad (2)$$

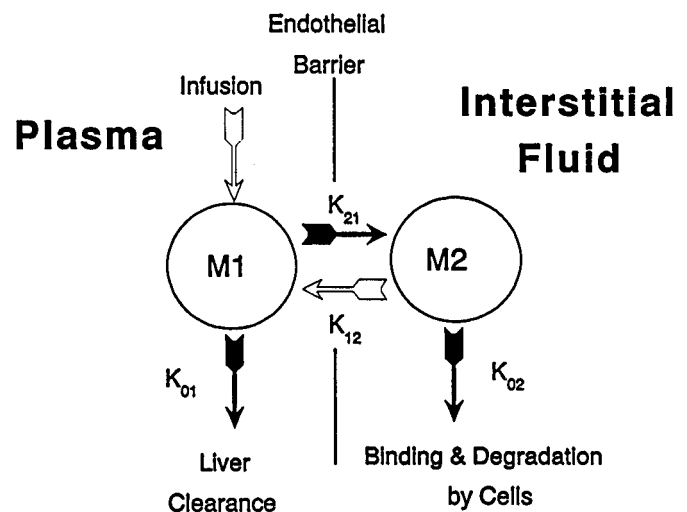


FIG. 1. A compartmental model portraying transport of insulin and NN304 in vivo. An endothelial barrier separates plasma from ISF. The compounds are cleared from both compartments.

where M_1 and M_2 are the masses of the compounds in the plasma and ISF compartments, respectively. Parameter k_{21} is the rate constant from plasma to ISF, parameter k_{12} is the rate constant from ISF to plasma, parameter k_{01} is the rate constant for elimination from plasma, and parameter k_{02} is the rate constant for elimination from ISF. All rate constants are expressed as min^{-1} . Movement across the endothelium was assumed to be driven by the concentration gradient (19,20), as in diffusion:

$$\frac{k_{12}}{V_1} = \frac{k_{21}}{V_2} \quad (3)$$

where V_1 and V_2 are the distribution volumes of plasma and ISF, respectively. To allow for identifiability, V_2 was assumed to be 17% of body weight (21). It was not necessary to assume V_1 , as the protein was infused directly into this accessible pool. Therefore, for both NN304 and human insulin, the parameters identified by iterative curve fitting were: k_{21} , k_{01} , k_{02} , and V_1 .

Flux rates were calculated by multiplying the compartment mass by the appropriate rate constant as described in the differential equation.

Numerical methods. Steady state was taken as the average of the last 30 min of the activation period. Modeling analysis was performed using MLAB (Civilized Software, Bethesda, MD) implemented on an IBM-compatible computer. Parameter identifications were obtained by nonlinear least-squares using a Marquardt-Levenberg algorithm with inverse-variance weights. Data are reported as means \pm SE. Statistical analysis was performed by ANOVA between insulin and NN304 using Mini-Tab, and P values are reported with values < 0.05 considered significant. Confidence intervals were calculated with a significance of 5%.

RESULTS

Insulin and NN304 concentrations (Fig. 2). Human insulin achieved a steady-state concentration of 570 ± 50 pmol/l in plasma, and a steady-state concentration of 300 ± 30 pmol/l in ISF (plasma-to-ISF ratio $\sim 2:1$). Total NN304 achieved a steady-state plasma concentration of $5,000 \pm 300$ pmol/l ($P < 0.001$ vs. human insulin) and 420 ± 30 pmol/l in ISF ($P < 0.03$ vs. human insulin; plasma-to-ISF ratio $\sim 12:1$). NN304 dynamics were slowed in both the plasma and ISF compartments compared with insulin. The half time to steady state in plasma was 4.5 ± 0.8 min for insulin and 19 ± 0.8 min for NN304 ($P < 0.001$). In ISF, the half time to steady state was 14 ± 1 min for insulin and 70 ± 7 min for NN304 ($P < 0.001$).

Glucose turnover (Fig. 3). The mean amount of exogenous glucose required to maintain euglycemia at steady state (Fig. 3A and B) was slightly higher for NN304 than for

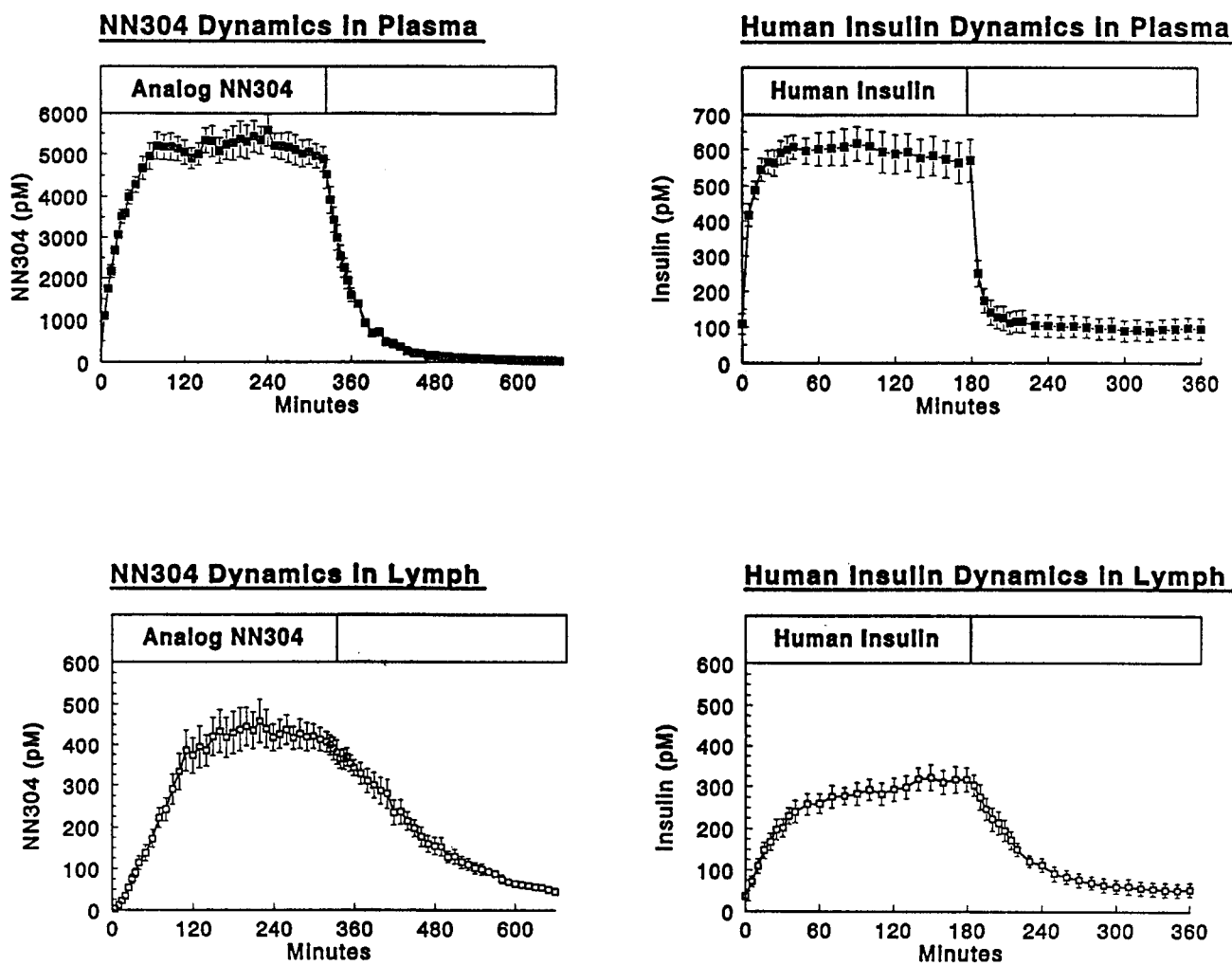


FIG. 2. Average NN304 and insulin concentrations throughout the experiment. Note that the time scales are different between NN304 and insulin, and that the *y*-axis is on a different scale for plasma concentrations. In plasma, NN304 reached higher concentrations and reached steady state more slowly. In ISF, similar total NN304 concentrations were achieved as equimolar insulin infusions but were attained more slowly.

insulin (11.1 ± 1.1 vs. 9.0 ± 0.6 , NS.), although this difference did not reach statistical significance.

Glucose uptake with the two compounds (Fig. 3C and D) was similar, achieving a steady-state level of 9 ± 1 mg \cdot kg⁻¹ \cdot min⁻¹ for human insulin and 11 ± 2 mg \cdot kg⁻¹ \cdot min⁻¹ for NN304 (NS). However, the half time to steady-state glucose uptake was 45 ± 22 min for insulin but 97 ± 7 min for NN304 ($P < 0.001$). Glucose uptake rates measured during the clamps were linearly correlated with ISF concentrations (Fig. 4) for both human insulin ($r =$

0.9) and NN304 ($r = 0.8$).

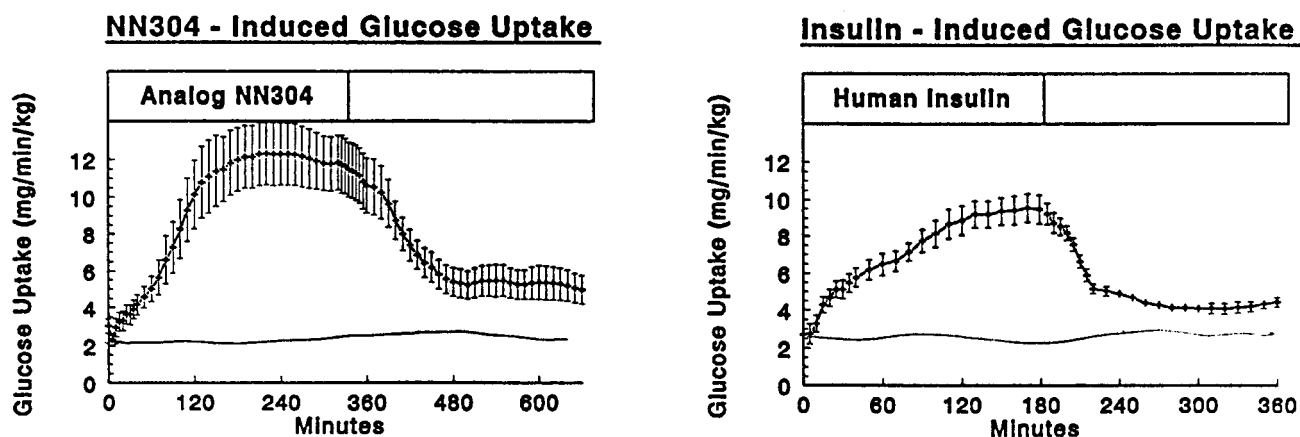


FIG. 3. Average time course of glucose infusion rates, glucose uptake, and glucose production for NN304 and insulin activation and deactivation. For all three measures, steady-state values were similar (NS), but the time course to achieve steady state differed between NN304 and insulin ($P < 0.05$). Please note the difference in scale for the time axis for NN304 and insulin studies.

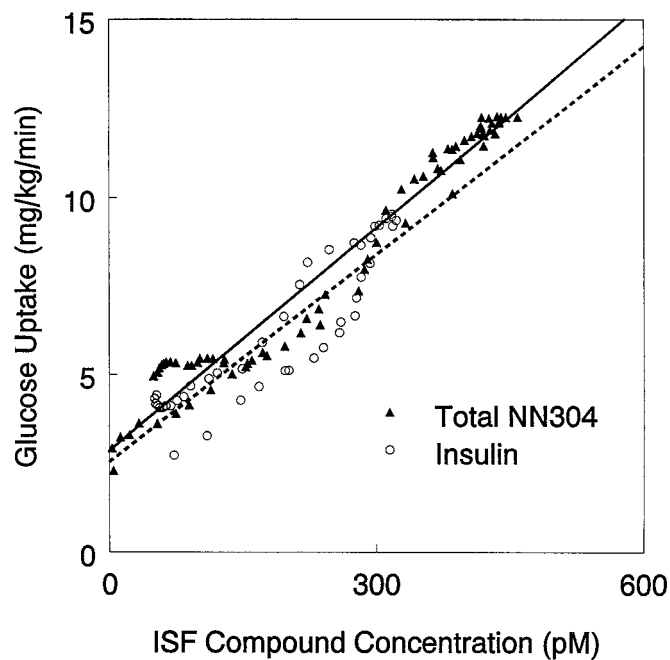


FIG. 4. The time course of glucose uptake as a function of hormone concentration demonstrates high linearity. The linear regressions (solid for NN304 and dashed for insulin) were similar in slope, suggesting that all NN304 in the ISF compartment may stimulate insulin receptors to increase glucose uptake. Error bars removed for clarity.

0.86 ± 0.03 , $R_d = 0.019 * [\text{insulin}] + 2.4$) and for total NN304 ($r = 0.93 \pm 0.01$, $R_d = 0.020 * [\text{NN304}] + 3.2$). Mild hysteresis in the curves suggests that there is a short time delay of intracellular signaling after insulin receptor activation. Importantly, equivalence of the relation between ligand and glucose uptake (NS) was surprising. If one assumes most of the NN304 in ISF to be bound to albumin, one might expect a substantial reduction in efficacy of the molecule in the interstitial space. However, total measured NN304 was equivalent to soluble native insulin in efficacy, measured as glucose uptake per total ligand molarity.

Glucose uptake at the end of the experiment did not return to basal in either the NN304 or the insulin experiments. This may have been due to over-replacement of basal insulin. Replacement commenced at -20 min, and if over-replaced would result in a slow rise in glucose uptake. This would be superimposed on the increased glucose uptake during the activation phase and would finally be observed as persistent elevated rates of glucose uptake at the end of the experiment, as seen in these studies. Furthermore, the effects of prolonged fast, as well

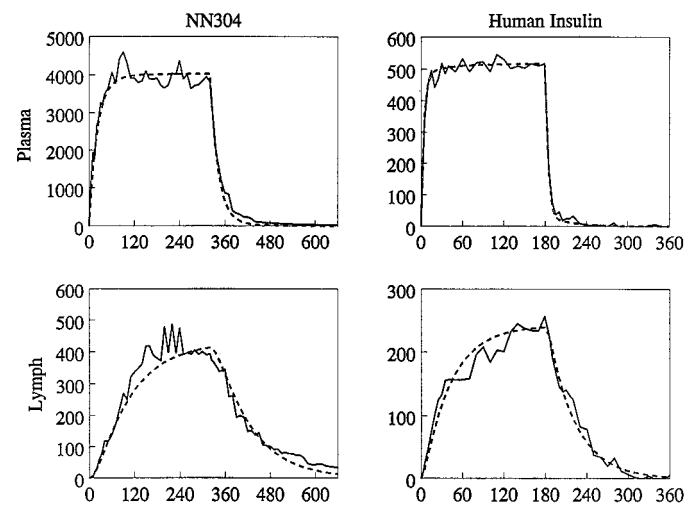


FIG. 5. Representative fits for the two-compartment models for NN304 and human insulin.

as decreased glucagon and growth hormone in the face of elevated somatostatin, cannot be ruled out.

Glucose production (Fig. 3E and F) was similar at steady state between the two compounds, achieving steady-state levels of $0.4 \pm 0.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for human insulin and $0.9 \pm 0.6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for NN304 (NS). Suppression of glucose production was not observed in one NN304 experiment, leading to the high variability observed in the averaged glucose production time course. However, this experiment did not meet outlier criteria. Therefore, analysis of glucose production rates plotted against interstitial peptide concentration has not been included.

Curve fitting. A representative set of insulin and NN304 curve fits are shown in Fig. 5. The median percent residual was 0.6% for plasma insulin, 4.7% for interstitial insulin, 8.4% for plasma NN304, and 5.7% for interstitial NN304. Adding more compartments to the model did not yield improved fits as tested by Akaike Information Criterion (22). Therefore, the representation presented here represents a parsimonious model for the available data.

Parameter identification (Table 1). Parameter values and associated coefficient of variation (CV) for the estimates are shown in Table 1 for NN304 and human insulin. The CV for the estimates appeared reasonable, confirming the adequacy of the distribution model ($<24\%$).

Plasma volume. Plasma volume of distribution was not different between the NN304 group and the insulin group

TABLE 1

Comparison of parameters for NN304 and human insulin identified by iterative curve fitting (and associated CV) or calculated from identified parameters

	NN304		Human insulin	
	Value	CV (%)	Value	CV (%)
k_{01} (min^{-1})	0.027 ± 0.002	3.4 ± 0.4	0.28 ± 0.02	23 ± 3
k_{02} (min^{-1})	0.011 ± 0.002	7.6 ± 1.7	0.014 ± 0.002	7.3 ± 0.6
k_{21} (min^{-1})	0.003 ± 0.001	8.4 ± 1.6	0.043 ± 0.005	21 ± 3
V_1 (l)	1.6 ± 0.1	3.1 ± 0.4	1.6 ± 0.2	24 ± 3
k_{12} (min^{-1})	0.0011 ± 0.0002		0.018 ± 0.002	

Data are means \pm SD.

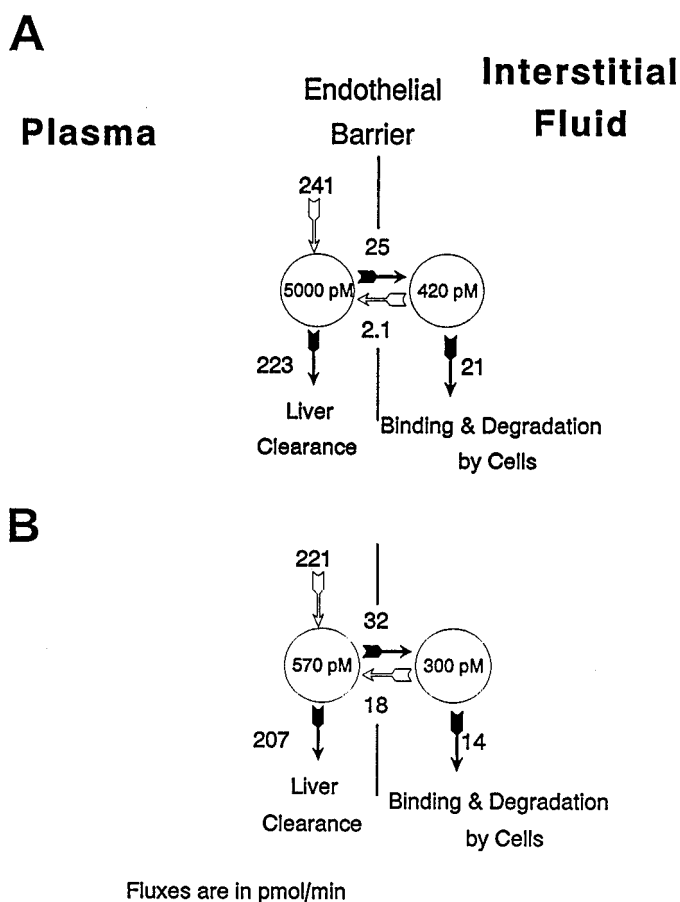


FIG. 6. Mean mass flux at steady state for NN304 (A) and human insulin (B). Liver clearance, peripheral clearance, and appearance in ISF were similar between NN304 and insulin at steady state.

(1.6 ± 0.11 vs. 1.6 ± 0.21 , respectively; NS), suggesting that there was no nonspecific binding in the plasma compartment of NN304 compared with insulin. Despite the fact that NN304 is highly bound to albumin, similar plasma distribution volumes were expected for the two molecules because both bound and unbound NN304 were assayed in toto.

Rate constants. The fractional rate constant for elimination of NN304 from the plasma pool (k_{01}) was only 9.6% of that for human insulin (0.027 ± 0.002 vs. $0.28 \pm 0.02 \text{ min}^{-1}$, NN304 vs. human insulin; $P < 0.001$). Presumably, this low fractional rate constant reflects protection of NN304 from hepatic degradation, the main fate for native insulin. The fractional rate constant for transendothelial transport of NN304 (k_{21}) was only 7.0% of that for human insulin (0.003 ± 0.001 vs. $0.043 \pm 0.005 \text{ min}^{-1}$, $P < 0.001$). Peripheral elimination (k_{02}), presumably nonhepatic, was similar between NN304 and human insulin (0.011 ± 0.002 vs. $0.014 \pm 0.002 \text{ min}^{-1}$, NS). Thus, rate constants describing unidirectional movement of NN304 out of plasma and into the liver or periphery were reduced. In contrast, no difference between insulin and NN304 was observed in the rate constant describing clearance from the interstitial compartment. The similarity in clearance of NN304 and insulin from the ISF is consistent with equivalent efficacy of total interstitial NN304 versus insulin.

Mass flux rates. Mass flux rates (Fig. 6) equal the product of the mass in the compartment and the fractional disap-

pearance rate. As NN304 had higher concentrations but lower fractional disappearance rates, NN304 mass flux rates through k_{01} , k_{02} , and k_{21} were similar to native insulin (k_{01} : 223 ± 8 vs. $207 \pm 15 \text{ pmol/min}$, NS; k_{02} : 21 ± 6 vs. $14 \pm 9 \text{ pmol/min}$, NS; k_{21} : 25 ± 6 vs. $32 \pm 2 \text{ pmol/min}$, NS). This comparability suggests that, despite the difference in the plasma-to-ISF ratio between insulin and NN304, at steady state a similar proportional amount of each ligand is eliminated in the liver versus the periphery. Additionally, the steady-state mass transfer from plasma to ISF is the same for insulin and NN304; however, because the mass of NN304 at steady state is much greater than insulin, the fractional clearance of NN304 is much less. As the total plasma NN304 concentrations were greatly elevated compared with human insulin, but resulted in similar flux rates away from the plasma compartment, acylation of NN304 results in reduction of clearance relative to the total concentration. Sequestration by albumin does reduce transport from the plasma pool. In contrast to this, the similar ISF concentrations of total NN304 and insulin result in similar k_{02} flux rates, suggesting that the presumed ability of NN304 to bind albumin in the interstitium does not deter its mass clearance from the interstitial compartment or its action on skeletal muscle to increase glucose uptake.

Mass flux through k_{12} , the return from ISF to plasma, appeared lower for NN304 compared with insulin at steady state (2.1 ± 0.5 vs. $18 \pm 3 \text{ pmol/min}$, $P < 0.001$). Thus, while binding to albumin in the interstitial compartment did not appear to restrict action of NN304 on cells, return of ISF NN304 to plasma was retarded compared with native insulin itself.

DISCUSSION

In the present study, companion two-compartment models were used to compare human insulin and NN304, an insulin analog designed to complex with the fatty acid binding sites of albumin. It was hypothesized that, based on the in vitro binding affinity to albumin, 98.3 and 96.7% of total NN304 would be bound to albumin in plasma or ISF, respectively, and that the bound component would be inactive. This hypothesis was tested by compartmental modeling of the plasma and lymph data for human insulin and NN304 to determine if the rate constants for NN304 were reduced to the degree expected based on the binding affinity to albumin.

A major mechanism of slowed NN304 action in the clinical setting is the absorption of the compound from the subcutis after injection. Stabilization of NN304 hexameric subunits and subcutaneous binding to albumin have been proposed as mechanisms for this delayed absorption (5). In the current study, NN304 was infused intravenously, circumventing delay from subcutaneous absorption.

NN304 dynamics were slower in the plasma and ISF compartments in comparison with human insulin (Fig. 2). The slowed NN304 plasma dynamics were due primarily to reduced liver clearance. The near linearity of the ISF concentration data versus glucose uptake for both insulin and NN304 suggests that the rate-limiting step for the action of these two molecules is the appearance rate in the active compartment, ISF. A modest hysteresis was noted in both the NN304 and human insulin curves, consistent

with an additional delay after insulin appears in ISF. This delay is most likely due to intracellular insulin signaling and recruitment of GLUT4 transporters to the plasma membrane. However, the slowed appearance rate of NN304 into ISF compared with insulin, together with the elevated total plasma concentrations, suggests that albumin binding retards TET of NN304. Also, the similarity of slope between NN304 and insulin when R_d is plotted against ISF concentration data suggests that the expected ability of the molecule to bind to albumin in ISF does little to retard NN304 action in the periphery. These observations were further examined by mathematical modeling of concentration data.

Current NN304 assay technology limits the measurement to total NN304 (bound and unbound) and is unable to differentiate between the two species. In the current study, we estimated the "unbound" portion by determining the transport parameters for the molecule in a two-compartment model. It might be suggested that we could separate the bound and unbound species by immunoprecipitation or size exclusion techniques, but it would not be possible to separate the species in an environment identical to the *in vivo* situation and could lead to errors.

The rate constants describing TET elimination from plasma were lower in comparison with human insulin. The degree of reduction was similar (9.6% of the native insulin value for k_{01} and 7.0% for k_{21}), suggesting that similar mechanisms may be responsible for the low values of both parameters. It is reasonable to presume that albumin binding of NN304 would account for its lower hepatic clearance and TET. However, it was anticipated that NN304 transport parameters would be only 1.7% of human insulin values, as *in vitro* kinetics predict that 98.3% of NN304 should be bound to albumin in plasma. Thus, somewhat more NN304 is transported from plasma than expected. This suggests that either the albumin-bound compound may still be transported or that there is more unbound hormone than predicted based on the *in vitro*-derived equilibrium constant for NN304.

It is interesting to note that the higher-than-expected transport rates from plasma are consistent with other albumin-bound substrates. For example, free fatty acids (FFAs) exhibit a remarkably high single-pass extraction of 40% through the heart. Very few FFAs are not bound to albumin (<1%), and the unbinding rate of FFAs from albumin is slower than the time for passage through the heart (27). The mechanism for the high extraction is not known and may also apply to the current study.

Analysis of plasma elimination can yield an estimate of the hepatic single-pass extraction of NN304. Assuming that plasma clearance is primarily hepatic, assumed hepatic plasma flow of 574 ml/min (23) yields an estimated single-pass extraction of 8%. The 8% single-pass extraction of NN304 is much lower than that of regular insulin in dogs, which is 57% (24).

The rate constant describing elimination from the peripheral compartment was similar for NN304 and human insulin (0.011 ± 0.002 vs. $0.014 \pm 0.002 \text{ min}^{-1}$). Therefore, it appears that once NN304 is in the peripheral compartment, predicted albumin binding does not dissuade NN304 from binding to the insulin receptor, despite the predicted 96.7% bound to albumin.

Several assumptions, which merit discussion in terms of impact on the accuracy of parameter identification, were made in the development of these compartmental models. The ISF volume of distribution was assumed to be the same percentage of body weight in both the insulin and NN304 compartmental models. However, because of the lipid moiety, it could be suggested that there would be a larger degree of nonspecific lipophilic binding to cell membranes for NN304, amplifying the apparent volume of distribution (V_2) larger than the assumed volume would likely result in an inappropriately large k_{02} rate constant. It would be expected that similar steady-state k_{02} flux should result in similar rates of glucose uptake, and the model-determined k_{02} mass flux was indeed similar between NN304 and insulin. Therefore, it does not appear likely that there is more nonspecific binding (to cell membranes) for NN304, and the equal volume of distribution assumption is likely to be valid in this analysis.

It was assumed that insulin and NN304 move across the endothelium in a manner reflective of the concentration gradient across the endothelium, rather than via a saturable mechanism, such as receptor-mediated transport. The assumption that insulin transport is not saturable has been tested before in our laboratory (19,20); however, a receptor-mediated component of insulin transport has not been totally excluded, as has been suggested by some *in vitro* preparations (25). Also, in preliminary results we have not seen a diminution in transport of NN304 even with pharmacologically high plasma insulin values (20). Finally, recent evidence from Kahn et al. (26) showing that tissue specific endothelial insulin receptor knockouts have little effect on glucose tolerance in mice supports nonreceptor-mediated TET. Thus, the assumption that TET of insulin (and, by inference, NN304) is not saturable appears to be supported by the weight of the available evidence.

The diffusion of NN304 or insulin from ISF back into plasma is likely not responsible for the high plasma concentrations. However, it may be important for calculation of elimination from ISF, as k_{12} and k_{02} are highly dependent on one another. For example, an underestimated k_{12} may result in an overestimated k_{02} . It is not possible from this set of data to precisely calculate k_{12} without the diffusion assumption while ensuring identifiability. However, the accuracy of the k_{02} estimate is justified by the similarity in steady-state glucose uptake and k_{02} mass flux for insulin and NN304. Total clearance from ISF ($k_{02} + k_{12}$) was accurate, as the model accounts for the ISF mass of NN304 dynamically and at steady state. Thus, it is logical to presume that the calculation of k_{12} is accurate.

It was found that mass flux from the interstitium back into plasma was greater for insulin than for NN304. This was because the ISF concentrations were not drastically different between insulin and NN304 (300 vs. 420 pmol/l), while k_{12} for NN304 was reduced to 6% of human insulin. This suggests that all transport processes for NN304 were deterred by albumin binding to a similar degree except the binding to insulin receptors of nonhepatic insulin-sensitive cells. Additionally, transport away from the peripheral pool is dissimilar for endothelial transport compared with peripheral clearance.

The simplest explanation of the lack of deterrence of albumin-bound NN304 in the interstitium is that NN304, when bound to albumin, can still bind to the insulin receptor. In ISF, NN304 appears to act as effectively to stimulate glucose uptake as if it were not bound. However, the concept of concurrent binding of NN304 to albumin and an insulin receptor has been excluded *in vitro* (5). Additionally, TET, which is faster than expected (7% rather than 1.7% of human insulin), is not explained by this reasoning, since it is likely a diffusional mechanism. A possible explanation for the relatively high TET of NN304 may lie in the hydrophobicity of the lipid moiety. Hydrophobic substances are known to traverse the endothelial barrier more rapidly, and the myristylation of insulin may allow NN304 to cross the capillary endothelium more readily. Additional studies would be required to confirm this hypothesis.

Several mechanisms have been proposed to explain the paradoxical high potency of other protein-bound ligands. One hypothesis that has been put forth to explain this behavior involves the binding of albumin to its receptors. It has been hypothesized that cargo-bearing albumin may bind cell surface albumin receptors, causing a conformational change in albumin and expelling the cargo (10–12,27–31). Thus, when measuring the action or clearance of an albumin-bound hormone, it appears that the effective binding affinity to albumin is reduced. Effectively, there may be more active NN304 than unbound NN304 as calculated with *in vitro*-derived k_a . However, this albumin-receptor hypothesis is controversial, and there is some evidence against the hypothesis (32,33). Other hypotheses involve nonspecific charge interactions between the carrier protein and the cell surface and local inhibitors of albumin binding (13).

A possible explanation for the difference in activity in plasma and ISF is the effect of flow rate. In the unstirred ISF, albumin binding and unbinding may be so fast that effectively all NN304 is active, while in plasma, the limited interaction with the receptors due to blood flow may limit the apparent activity of NN304. It has been suggested that, under certain conditions, the activity of protein-bound ligands in plasma for transport processes may be highly dependent on the permeability-capillary surface area product and the transit time through the capillary (34). This stipulation may be further extended to the case of zero flow, as in the unstirred layer of the peripheral compartment. According to this model, as the transit time approaches infinity, the activity approaches 100%, consistent with the observed full activity in ISF. Because albumin binding kinetics were not explicitly included in this model of NN304, albumin binding and transport characteristics are lumped together. Thus, in the rapidly flowing plasma compartment, transport characteristics may be perceived to be slower because the apparent albumin binding is affected by flow, while in the slowly moving interstitium, the effect of albumin binding is minimal and the perceived peripheral clearance is unaffected. Studies on cell culture have not been performed to address the issue of flow rate in the apparent activity of NN304 with albumin.

In this study, it was hypothesized that the unbound NN304, as calculated by *in vitro* binding affinity to albumin, is the only active compartment for transport processes.

This hypothesis was found to be incorrect. Although the plasma transport processes were not slowed to the degree expected, they were reduced. These slow transport processes explain the elevated plasma NN304 concentration compared with equimolar human insulin infusion, as well as the prolonged time to steady state in plasma. The reduced TET elucidates the slow appearance in ISF, and rate of appearance in ISF remains the rate-limiting step for NN304 action, as human insulin. This study indicates that there is no reduction in NN304 action or clearance once it is in ISF, despite the high albumin concentration in ISF. By acylating insulin, an analog having blunted kinetics in plasma and full efficacy in the compartment of action, ISF, was created.

ACKNOWLEDGMENTS

This work was supported by grants to R.N.B. from Novo Nordisk A/S and the National Institutes of Health (DK-29867 and DK-27619). M.K.D. was supported by a predoctoral National Institutes of Aging Training Grant Fellowship (AG00093-15). M.H.-W. was supported by a postdoctoral National Institute of Aging Training Grant Fellowship (AG00093-15). M.A. was supported by a National Institutes of Health grant (AG00544).

The authors thank Dr. Jang Youn for helpful discussions throughout the preparation of this article.

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