Transendothelial Insulin Transport Is Not Saturable In Vivo

No Evidence for a Receptor-mediated Process

Garry M. Steil, Marilyn Ader, Donna M. Moore, Kerstin Rebrin, and Richard N. Bergman
Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles, California 90033

Abstract

In vitro, insulin transport across endothelial cells has been reported to be saturable, suggesting that the transport process is receptor mediated. In the present study, the transport of insulin across capillary endothelial cells was investigated in vivo. Euglycemic glucose clamps were performed in anesthetized dogs ($n = 16$) in which insulin was infused to achieve concentrations in the physiological range (1.0 mU/kg per min + 5 mU/kg priming bolus; $n = 8$) or pharmacologic range (18 mU/kg per min + 325 mU/kg priming bolus; $n = 8$). Insulin concentrations were measured in plasma and hindlimb lymph derived from interstitial fluid (ISF) surrounding muscle. Basal plasma insulin concentrations were twice the basal ISF insulin concentrations and were not different between the physiologic and pharmacologic infusion groups (plasma/ISF ratio 2.05 ± 0.22 vs 2.05 ± 0.23; $P = 0.0003$). The plasma/ISF gradient was, however, significantly reduced at steady-state pharmacologic insulin concentrations (1.37 ± 0.25 vs 1.98 ± 0.21; $P = 0.0003$). The reduced gradient is opposite to that expected if transendothelial insulin transport were saturable. Insulin transport into muscle ISF tended to increase with pharmacologic compared with physiologic changes in insulin concentration (41% increase; $1.37 \pm 0.18\ 10^{-2}$ to $1.93 \pm 0.24\ 10^{-2}$ min^{-1}; $P = 0.088$), while at the same time insulin clearance out of the muscle ISF compartment was unaltered ($2.53 \pm 0.26\ 10^{-2}$ vs $2.34 \pm 0.28\ 10^{-2}$ min^{-1}; $P = 0.62$). Thus, the reduced plasma/ISF gradient at pharmacologic insulin was due to enhanced transendothelial insulin transport rather than changes in ISF insulin clearance. We conclude that insulin transport is not saturable in vivo and thus not receptor mediated. The increase in transport efficiency with saturating insulin is likely due to an increase in diffusionary capacity resulting from capillary dilation or recruitment. (*J. Clin. Invest.* 1996. 97:1497–1503.) Key words: insulin transport • insulin action • glucose uptake • blood flow • endothelial cells

Introduction

For plasma insulin to increase peripheral glucose uptake, two separate processes must occur: (*a*) Insulin must first migrate from plasma to interstitial fluid bathing insulin-sensitive tissues, and (*b*) insulin must bind to its receptor and initiate a sequence of intracellular events. While details regarding the intracellular events after receptor binding are rapidly emerging (1), less is known about how insulin traverses the capillary endothelial wall. That the endothelial wall provides a barrier to insulin was originally demonstrated by Rasio and colleagues (2–4), who reported a slow rise in lymph insulin after changes in plasma concentrations. Later, Yang et al. demonstrated that the transendothelial transport step was actually rate limiting for insulin action during euglycemic glucose clamps (5); that is, Yang et al. demonstrated that thoracic duct lymph insulin dynamics were not only slower than plasma insulin dynamics, but also that it was the lymph concentration of insulin, rather than plasma insulin, that was correlated with peripheral glucose uptake. These results suggested both that the transport step was rate limiting and that thoracic duct lymph insulin concentrations were indicative of insulin concentrations in the interstitial fluid bathing insulin-sensitive cells (predominantly muscle and adipose tissue). However, since thoracic duct lymph is derived from ISF bathing both splanchnic (insulin insensitive) and muscle tissue beds (insulin sensitive) (6), a later study was conducted in which the muscle lymphatics were directly cannulated (7). In this later study, the appearance of insulin in the interstitial fluid compartment was not only rate limiting for insulin action on glucose uptake, but also for insulin action on hepatic glucose output (i.e., suppression of hepatic glucose output was also linearly correlated with interstitial insulin levels). Based on such data, our group put forth the hypothesis that transendothelial insulin transport (TET_{ins})[1] was rate limiting for both peripheral glucose uptake and hepatic glucose output—the so-called single-gateway hypothesis (8, 9).

Previous lymph studies did not address the mechanism by which insulin crosses the endothelial barrier. Large macromolecules are generally thought to traverse the capillary barrier by passive diffusion (10), via movement in vesicles (11), or by receptor-mediated transcytosis (12). Insulin has been suggested by several groups to be transported by the latter "receptor-mediated process" (13–16). In particular, King and Johnson (13) reported that the transport of insulin across cultured endothelial monolayers was both unidirectional and saturable. This, together with the existence of insulin receptors on the endothelial wall (17), strongly suggested that transendothelial insulin transport was receptor mediated. Nevertheless, despite such in vitro evidence, there are few in vivo data to support such a hypothesis. The present study was therefore undertaken to measure the in vivo transport of insulin from plasma to interstitial fluid (ISF) surrounding muscle cells. Concentration of insulin in the ISF surrounding dog hindlimb muscles was ascertained by sampling hindlimb lymphatic fluid during anesthesia. Dynamics associated with insulin transport into, and clearance from, the ISF compartment were analyzed during ei-

Address correspondence to Richard N. Bergman, Department of Physiology and Biophysics, University of Southern California School of Medicine, 1333 San Pablo Street, MMR 626, Los Angeles, CA 90033. Phone: 213-342-1920; FAX: 213-342-1918.

Received for publication 3 August 1995 and accepted in revised form 3 January 1996.

J. Clin. Invest.

0021-9738/96/03/1497/07 $2.00
Volume 97, Number 6, March 1996, 1497–1503

1. *Abbreviations used in this paper:* ISF, interstitial fluid; TET_{ins}, transendothelial insulin transport; TET_{inu}, transendothelial inulin transport.

ther physiologic or pharmacologic (saturating) changes in plasma insulin. Transport of inulin, a diffusionary marker with molecular weight similar to that of insulin, was also determined.

Methods

Animals. Experiments were conducted on 16 male mongrel dogs (12.5–30.0 kg). Dogs were housed under controlled kennel conditions (12 h light, 12 h dark) in the University of Southern California (USC) Medical School Vivarium. Animals had free access to standard chow (25% protein, 9% fat, 49% carbohydrate, 17% fiber; Wayne Dog Chow, Alfred Mills, Chicago, IL) and tap water. Food was withdrawn 15 h before experiments. Dogs were used for experiments only if judged to be in good health as determined by visual observation, weight stability, body temperature, and hematocrit. The experimental protocol was approved by the USC Institutional Animal Care and Use Committee.

Surgical preparation. Surgery was performed in the morning of each experiment. Dogs were preanesthetized with acepromazine maleate (Prom-Ace; Aueco, Fort Dodge, IA; 0.1 mg/lb) and atropine sulfate (0.05 cc/lb; H. Schein, Port Washington, NY). Anesthesia was induced with sodium pentobarbital (0.1 cc/lb; Western Medical, Arcadia, CA) and maintained with halothane and nitrous oxide. In-dwelling catheters were implanted in the carotid artery (sampling), jugular vein (saline drip), and left femoral vein (sampling). Left and right cephalic vein intracatheters were inserted for various infusions as detailed below. Hindlimb muscle lymphatic fluid was sampled via a small polyethylene catheter (typically PE50 but as small as PE10 and as large as PE90) inserted into a lymph vessel. To insert the lymph catheter a longitudinal incision was made in the left hindlimb, distal to the femoral triangle, and the hindlimb lymphatic vessels were carefully exposed. The catheter was threaded through a pinhole and advanced 1–2 cm beyond the insertion point (i.e., beyond any lymphatic valves) and secured with a silk suture. Flow was started by gently massaging the limb muscle. A perivascular ultrasonic flow probe (2-mm diameter; Transonic, Ithaca, NY) was placed around the right femoral artery (contralateral to the femoral vein and hindlimb catheters) for measurement of blood flow. Incisions were kept moist with saline-soaked gauze, and body temperature was maintained with heating pads. Blood pressure, heart rate, and respiratory CO_2 were continuously monitored. Dogs received a saline drip throughout both the surgery and the experiment to improve stability ($\sim$ 1 liter was administered during the first 90 min of surgery and a slow drip thereafter). After experiments, animals were given an overdose of sodium pentobarbital (65 mg/kg).

Experimental protocol. Dogs were maintained under anesthesia throughout the experimental protocol. After the surgical preparation, arterial, venous, and hindlimb lymph sampling was begun. Arterial and venous samples were taken simultaneously ($\sim$ 3 ml blood), whereas hindlimb lymphatic samples were taken over 3 min ($\sim$ 1.5 min before sample time to 1.5 min after sample time). Hindlimb lymph sampling generally resulted in 300–600 μl of lymphatic fluid. The experimental protocol consisted of four experimental phases: a basal phase (−90 to −60 min), an insulin replacement phase (−60–0 min), an insulin activation phase (0–180 min), and an insulin deactivation phase (180–300 min). During the insulin replacement phase, somatostatin was infused (0.8 μg/min per kg, right cephalic vein; Bachem, Torrance, CA) to suppress endogenous insulin release, and the endogenous insulin secretion was replaced with a systemic insulin infusion (0.2 mU/min per kg; Novo-Nordisk (Copenhagen, Denmark), also into the right cephalic vein). During the insulin activation phase, a primed physiological (1.0 mU/min per kg, 5 mU/kg prime; n = 8) or pharmacological (18 mU/min per kg, 325 mU/kg prime; n = 8) insulin infusion was begun. The priming bolus was chosen to achieve approximate stepwise increments in plasma insulin concentration. The higher ratio of priming bolus/infusion rate for the pharmacologic vs the physiologic insulin infusions (i.e., 18.1 min vs 5 min) was necessary to compensate for the saturation of plasma insulin clearance during pharmacologic infusions. [^{14}C]Inulin was infused (0.09 μCi/min; ICN Pharmaceuticals, Irvine, CA) concomitantly with insulin to assess diffusional transport. During the insulin deactivation phase, the insulin/inulin infusion was terminated (somatostatin and basal insulin replacement were continued throughout the experiment). The exogenous glucose infusion rate necessary to maintain arterial glucose at the basal replacement concentration was calculated according to on-line measurements of plasma glucose. Arterial and venous blood samples were taken at the following times (in minutes): −90, −80, −70 (basal); −30, −20, −10 and −2 (replacement insulin phase); 1, 2, 3, 4, 5, 8, 10, 12, 14, 16, 20, 25, 30, 35, 40, 50, 60, 70, 80, 100, 120, 140, 160, 178 (activation phase); 181, 182, 183, 184, 185, 188, 190, 195, 200, 205, 210, 220, 230, 240, 250, 260, 280, and 300 (deactivation phase). All samples were collected into tubes containing sodium fluoride and heparin and stored on ice until centrifugation; once separated, plasma was stored at −20°C. Hindlimb lymph samples were collected at the following times (in minutes): −90 −80, −70, −30, −20, −10, −2, 3, every 3 min until 30 min, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 178.5, 183, 186, 189, 192, 195, 198, 201, 204, 207, 210, 215, 220, 225, 230, 240, 250, 260, 280, and 300.

Assays. Plasma glucose was assayed using glucose oxidase on an automated analyzer (model 23A; Yellow Springs Instrument Co., Yellow Springs, OH) with 25 μl of sample. Arterial and hindlimb lymph insulin concentrations were measured with an ELISA using 15 μl of sample. The assay was originally developed for human plasma by Novo-Nordisk (18) and adapted for dog plasma in our laboratory. The method is based on two murine mAbs that bind to different epitopes on the insulin molecule. Proinsulin is not bound by the antibodies. The insulin assay was established in our laboratory with the kind assistance of B. Dinesin of Novo-Nordisk. [^{14}C]Inulin was assayed using 100 μl of sample mixed with 1.0 ml tissue solubilizer (BTS-450; Beckman Instruments, Inc., Fullerton, CA). These samples were then incubated at room temperature for 30 min and counted in 10 ml of organic scintillation fluid (Ready Organic, Beckman Instruments, Inc.) on a liquid scintillation counter with automatic quench correction.

Insulin dynamics. If insulin traverses the capillary endothelial barrier in both the forward (plasma to ISF) and reverse (ISF to plasma) directions, both fluxes will affect the ISF insulin dynamics. These fluxes are schematically shown in Fig. 1 (K_{21} and K_{12}, respectively; units of min^{-1}), as is the binding of insulin to its receptor and its subsequent degradation (K_{02}; units min^{-1}). This representation may be described by the whole-body compartmental model shown; however, such a model is a priori unidentifiable from plasma data alone. Thus, rather than identifying the whole-body model, which describes both plasma and ISF kinetics, we focused on a reduced formulation describing only the ISF compartment. The mass balance equation for this compartment is:

$$\frac{dV_2C_2}{dt} = k_{21}V_1C_1 - (K_{12} + K_{02})\,V_2C_2 \tag{1}$$

where V_1 and V_2 represent the volumes of plasma and ISF respectively, and C_1 and C_2 are the respective concentrations. Eq. 1 can be expressed in terms of the concentration profiles as:

$$\frac{dC_2}{dt} = TET_{\text{ins}}C_1 - K_2C_2 \tag{2a}$$

$$TET_{\text{ins}} = \frac{k_{21}V_1}{V_2} \text{ and } K_2 = (k_{12} + k_{02}) \tag{2b}$$

For this formulation, TET_{ins} and K_2 (total fractional insulin clearance from the interstitial compartment) can be estimated from the dynamics in plasma and ISF (see below). Note that the transendothelial insulin transport parameter (TET_{ins}) combines the fractional transport rate of insulin from plasma to ISF (K_{12}; min^{-1}) with the ratio of

plasma (V_1) to ISF volume (V_2). The insulin ISF clearance parameter (K_2) measures the total clearance from the ISF compartment (i.e., the sum of the reverse flux K_{12} and the fractional degradation K_{02}). If the transport of insulin is unidirectional, as suggested in reference 13, then $K_{12} = 0$; alternatively, if the transport is by diffusion alone, then $K_{21}V_1 = K_{12}V_2 = D$ (the diffusion coefficient; ml/min). Further, if the transport process is receptor mediated, then TET_{ins} should decrease (saturate) as plasma insulin is increased, whereas, if the process is by simple diffusion, then TET_{ins} should be unchanged with changes in plasma insulin.

The representation shown in Fig. 1, with minor modifications, may also be used to describe inulin movement. Since inulin is not cleared by the muscle tissue bed, K_{02} can be set to zero; also, since inulin is transported into the ISF by diffusion alone, $K_{12}V_2$ can be set equal to $K_{21}V_1$. Thus, for inulin, Eq. 1 reduces to:

$$\frac{dC_2}{dt} = TET_{inu}C_1 - TET_{inu}C_2 \quad (3a)$$

$$TET_{inu} = \frac{K_{21(inu)}V_1}{V_2} = K_{12(inu)} \quad (3b)$$

Note that if both plasma and ISF concentrations are measured, then the whole-body compartmental structure of inulin need not be specified to identify the parameters in Eqs. 2a or 3a. Eqs. 2a and 3a do not attempt to describe the plasma dynamics; rather, plasma dynamics are used as an input (C_1), and the parameters (TET_{ins}, K_2, TET_{inu}) are identified by fitting the output (C_2) to the lymph measurements.

Numerical methods. Modeling analysis and statistics were performed using MLAB (Civilized Software, Bethesda, MD) implemented on an IBM-compatible computer. Parameter identifications were obtained by weighted nonlinear least-squares using a Marquardt-Levenburg algorithm with inverse variance weights. When plasma data were used as an input to Eqs. 2a and 3a, the plasma concentration between sample times was determined by linear interpolation. Data are reported as mean±SEM with *t* tests (paired and unpaired as appropriate) used to evaluate differences; *P* values are generally reported with values < 0.05 considered significant.

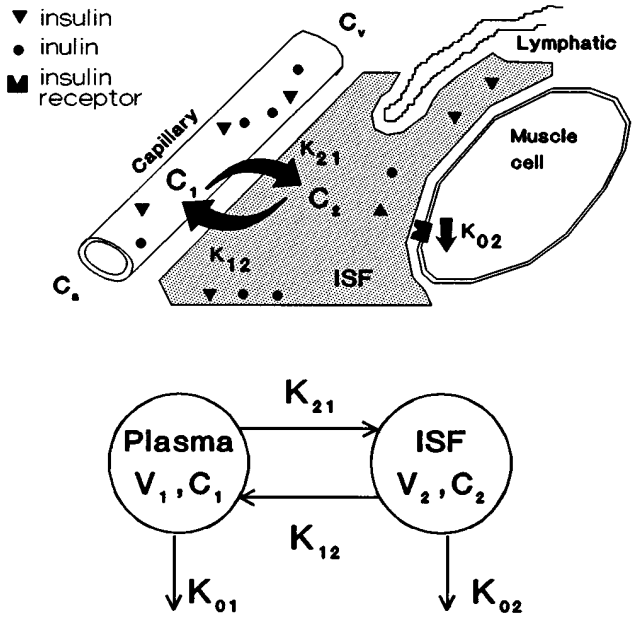

Figure 1. Schematic model used to identify transendothelial insulin transport and fraction insulin clearance from the remote interstitial insulin compartment (see text for details).

Results

Glucose. Basal plasma glucose levels were not different between the physiologic and pharmacologic insulin experiments (117±4 vs 119±5; $P = 0.8$); however, plasma glucose was slightly lower during the insulin replacement phase in the pharmacologic group (119±5 to 104±4 mg/dl; $P = 0.01$) but not in the physiologic group (117±4 to 110±6 mg/dl; $P = 0.22$; Fig. 2 *A*). Nonetheless, glucose was well clamped during the insulin activation and deactivation periods for both groups (mean coefficient of variation = 7±1 and 8±0.5%; physiologic and pharmacologic insulin infusions, respectively). As expected, the steady-state exogenous glucose infusion rate was higher during the pharmacologic insulin infusion than during the physiologic insulin infusion (14.3±1.7 vs 6.1±0.9 mg/min per kg), and the glucose infusion was slower to decrease after the termination of the pharmacologic insulin infusion (Fig. 2 *B*; insulin deactivation period, 180–300 min). The slower decline in glucose infusion rate can be related to the saturation of whole-body insulin clearance.

Blood flow. Basal femoral artery blood flow (Fig. 3) was not different between the physiologic and pharmacologic insulin infusion groups (8.59±0.59 vs 7.71±1.39 ml/min per kg; $P = 0.57$, unpaired *t* test), and no steady-state increase from the basal flow rate was observed during insulin infusion for either physiologic (8.59±0.59 vs 8.68±0.49 ml/min per kg; $P = 0.49$)

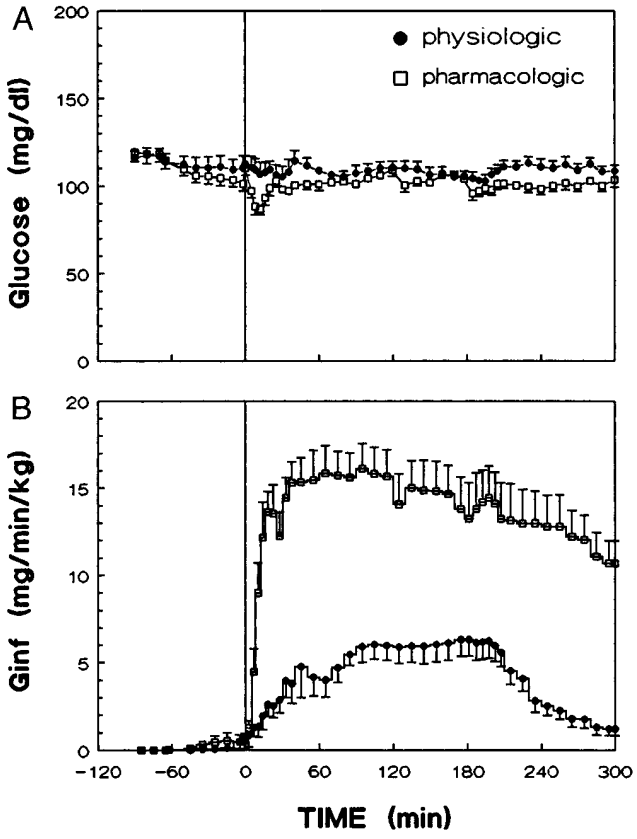

Figure 2. Plasma glucose concentrations (*A*) and exogenous glucose infusion rates (*B*) during physiologic (●) and pharmacologic infusions (□) of insulin. Time −90 to −60 min represents basal; −60–0 replacement insulin; 0–180 activation phase; and 180–300 deactivation. Data are the mean±SEM for eight experiments each.

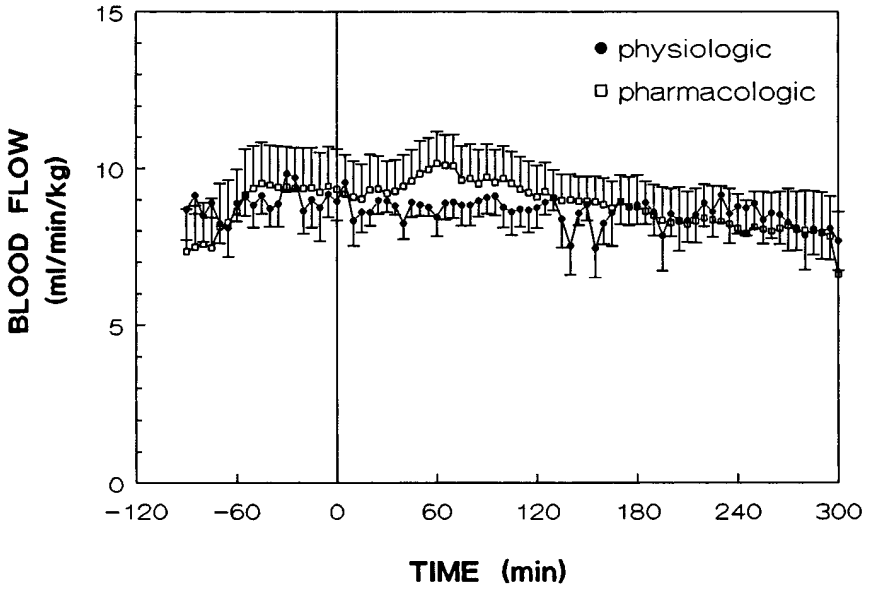

Figure 3. Femoral artery blood flow during physiologic (●) and pharmacologic insulin infusion (□) experiments. Data are the mean±SEM for eight experiments in each group.

or pharmacologic insulin infusions (7.71±1.39 vs 9.08±0.91 ml/min per kg; $P = 0.14$, paired t tests for both).

Insulin. Basal plasma insulin concentrations were not different between the two infusion groups (71±0.8 vs 77±14 pmol/liter; physiologic vs pharmacologic, $P = 0.79$); however, during the insulin replacement phase, plasma insulin concentration significantly increased in the physiologic group (Fig. 4; 71±0.8 to 101.4±9.6 pmol/liter, $P = 0.007$). This unexpected increase in mean insulin concentration (insulin was infused at the anticipated basal replacement rate) was also observed in the pharmacologic group (77.1±14 to 94.8±13.7 pmol/liter) but did not reach statistical significance ($P = 0.085$). Nevertheless, insulin concentrations during the replacement phase were within the normal range for dogs. Steady-state lymph insulin concentrations were lower than plasma insulin concentrations for both groups during all three phases (basal: 71±0.8 vs 36.5±6.2 pmol/liter and 77.1±14.0 vs 40.4±7.0, $P < 0.05$ for both; replacement phase: 101.4±9.6 vs 40.3±5.3 and 94.8±13.7 vs 48.8±3.8 pmol/liter, not tested (see below); steady-state activation: 617.6±48.4 vs 314.7±20.2 and 32,899±1760 vs 24,143±1,397, $P < 0.05$ for both). The difference in plasma vs lymph insulin concentration during the replacement insulin phase was not tested because the unexpected rise in the plasma insulin concentrations during the replacement phase prevented lymph insulin from achieving steady state.

The lower lymph insulin concentrations (compared with plasma) during the basal and steady-state activation phases resulted in steady-state plasma/lymph insulin gradients > 1.0 (Fig. 5). The basal gradients were not different between the physiologic and pharmacologic insulin groups (2.05±0.22 vs 2.05±0.23; $P = 0.84$); however, the plasma/lymph insulin gradient during the steady-state activation period was significantly reduced with pharmacologic insulin compared with the corresponding physiologic infusion period (1.37±0.25 vs 1.98±0.21; $P = 0.0003$). This observed reduction in the plasma/lymph insulin gradient contrasts with that expected for a saturable insulin transport process; that is, saturation of the transport process would result in an increase, rather than a decrease, in the plasma/lymph gradient.

A change in the steady-state plasma/lymph insulin gradient cannot, in and of itself, be used to infer a change in transendothelial insulin transport since the gradient depends on both transport into and clearance from the ISF space. These two processes (transport into and clearance from) were simulta-

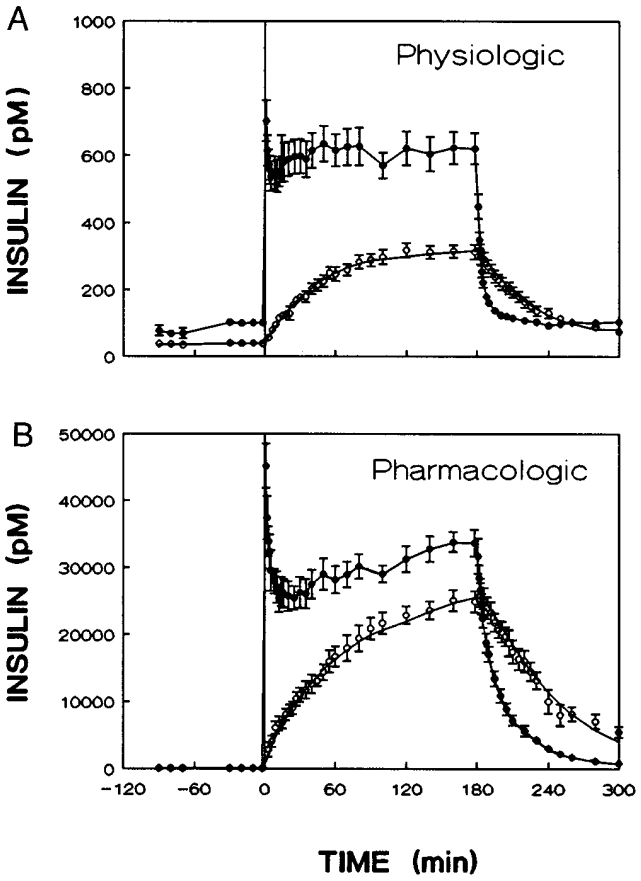

Figure 4. Arterial (●) and hindlimb lymph (○) insulin concentrations during physiologic (*A*) and pharmacologic (*B*) insulin infusions. Solid line through hindlimb lymph data represents the average fit obtained with Eq. 2 (see text).

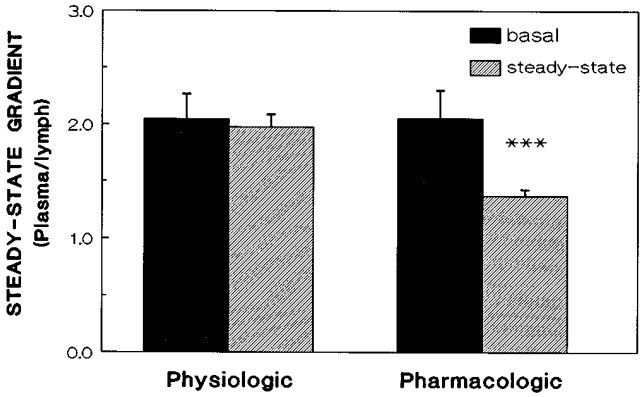

Figure 5. Basal and steady-state plasma/lymph insulin gradients observed during physiologic and pharmacologic infusions of insulin. *** The gradient is significantly lower than the corresponding gradient during physiologic insulin infusion.

neously estimated from the insulin dynamics (Fig. 4) using the transport model of Fig. 1 (cf Eq. 3 and Methods). The lymph insulin dynamics were well described by the model during both the physiologic and pharmacologic increments in plasma insulin (cf Fig. 4). Transendothelial insulin transport (Fig. 6), which should have decreased if the process had been receptor mediated, exhibited a paradoxical tendency to increase (Fig. 6, 41% increase; $1.37\pm0.18 \times 10^{-2}$ to $1.93\pm0.24 \times 10^{-2}$ min^{-1}; $P = 0.09$). At the same time, ISF insulin clearance (K_2) was not significantly altered ($2.53\pm0.0026 \times 10^{-2}$ vs $2.34\pm0.0028 \times 10^{-2}$ min^{-1}; $P = 0.62$). Thus, the decreased gradient (Figs. 4 and 5) was a result of an increase in transendothelial insulin transport rather then a change in ISF insulin clearance.

Inulin. Consistent with the fact that inulin is not cleared from muscle ISF, the steady-state plasma and lymph inulin concentrations (Fig. 7) were not different for either the physiologic (2,086±271 vs 1,907±239 dpm/ml; $P = 0.22$) or pharmacologic insulin infusion groups (2,095±291 vs 2,110±254 dpm/ml; $P = 0.84$, paired t tests for both). The lack of a steady-state concentration gradient for inulin argues against any concentration or dilution of the ISF within the lymphatics. As was the case with insulin, transendothelial inulin transport also tended to be greater during pharmacologic vs physiologic insulin infusions (3.73 ± 0.6 10^{-2} vs 3.03 ± 0.5 10^{-2} min^{-1}); this difference was not, however, statistically significant ($P = 0.25$). However, the transport model for this solute fit the rise in lymph inulin during the physiologic insulin infusion very well, but was less able to follow the rise in lymph inulin during pharmacologic insulin infusion. During the pharmacologic insulin infusion period, the measured lymph inulin concentrations between 30 and 180 min were higher than the model prediction (residual run), consistent with the concept that TET_{inu} was increasing with time.

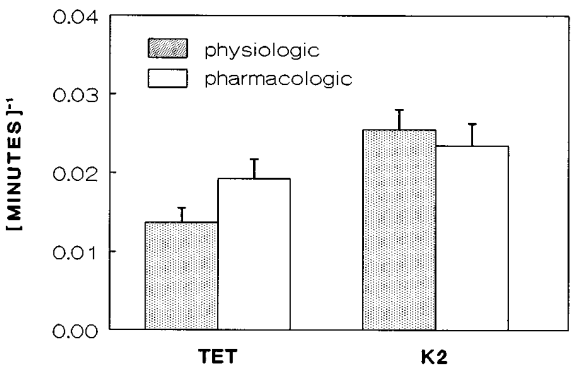

Figure 6. Comparison of transendothelial insulin transport (TET_{ins}) to ISF, and fractional clearance (K_2) from ISF, during physiologic and pharmacologic insulin infusions. Transendothelial transport and clearance (TET, K_2) were identified from Eq. 2 using data shown in Fig. 3.

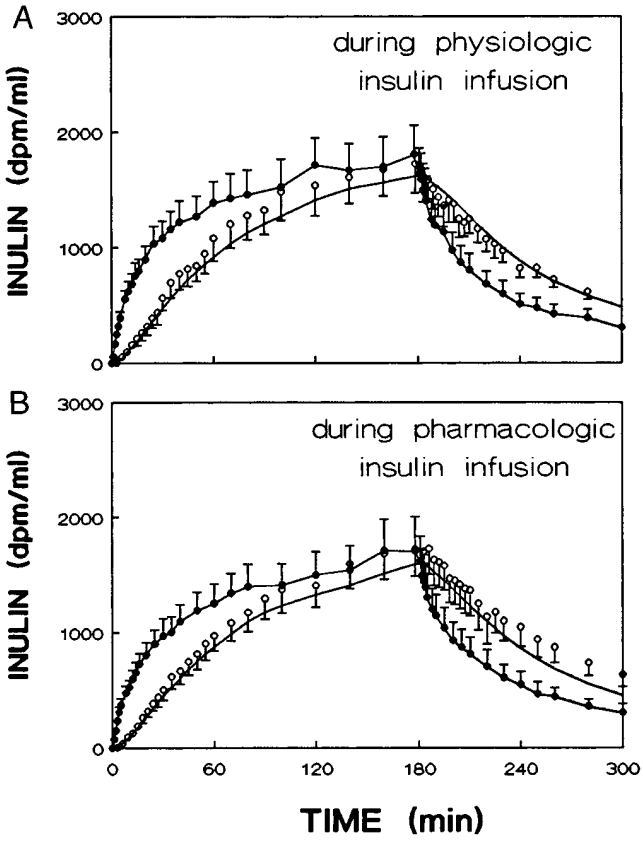

Figure 7. Arterial (●) and hindlimb lymph (○) inulin dynamics obtained during the infusion of inulin with a simultaneous physiologic (*A*) or pharmacologic insulin (*B*) infusion. Solid line passing through hindlimb lymph data represent the average fit obtained with Eq. 3 (see text).

Discussion

The current study demonstrated that insulin transport across capillary endothelial cells is not saturable in vivo. Results indicate that the steady-state plasma/ISF gradient is reduced, rather than increased, at pharmacologic insulin levels (Figs. 4 and 5), and that the reduced gradient is due to an increase in fractional transport of insulin rather than a decrease in ISF insulin clearance (Fig. 6). Thus, rather than saturating the transport mechanism, transendothelial insulin transport appeared to be 41% higher at the pharmacologic plasma insulin concentrations (Fig. 6). Possible explanations for this paradoxical increase in transport with pharmacologic plasma insulin are an increase in diffusion area due to dilation of capillaries or an increase in the capillary number exposed to insulin (capillary re-

cruitment). Capillary recruitment during hyperinsulinemia has previously been suggested by Baron and co-workers (19, 20) as a mechanism to explain enhanced blood flow to muscle. Although we did not observe a statistically significant increase in hindlimb blood flow with hyperinsulinemia, it is possible that the flow was shifted from tissues such as skin to the muscle bed itself. Such a shift would result in increased capillary perfusion of the muscle and could account for the increase in TET. Alternatively, diverting blood to tissues with more permeable capillaries, or increasing lymph formation from such tissues, could also account for the increase in TET. Finally, capillary dilation could increase TET without either an increase or a shift in blood flow. During capillary dilation, the cross-sectional surface area of the capillaries would increase, and, if the corresponding flow velocity (cm/s) decreased, there would be no change in flow volume (flow volume in ml/min equals cross-sectional surface area [cm^2] times flow velocity [cm/min]). Dilation of the vessels would increase the overall surface area and pore size associated with the capillary bed, and this, along with the decreased flow velocity, would increase the diffusion capacity (TET).

The lymphatic system itself may also play a role in determining the dynamics and steady-state insulin gradients. There is some evidence that solute concentrations in lymph are altered by osmotic forces during transit through lymph nodes (21). However, the steady-state plasma and lymph inulin concentrations observed in the present study were not different, indicating that the concentration of this solute (and insulin, which has a similar molecular weight) was not altered by the lymphatics (cf Fig. 7). It is also conceivable that the concentration of insulin in lymph could be lowered by insulin degradation within the lymph system; however, the steady-state plasma/HL-lymph gradient observed in the present study was similar to that previously observed between plasma and thoracic duct lymph (5, 22). This suggests that lymph insulin concentrations are not significantly altered during transit to the thoracic duct.

While we did not observe a saturation of transendothelial insulin transport, it might be expected that the irreversible loss of insulin from muscle ISF is a process that should have saturated. This loss of insulin is due to internalization and degradation of insulin by muscle cells—events that must be preceded by insulin binding to its receptor. The loss of insulin from the ISF compartment is necessary for there to be a significant plasma/lymph insulin gradient (this gradient was statistically significant during all steady-state periods, cf Results). Nonetheless, the change in ISF insulin clearance (K_2) observed with pharmacologic insulin was not statistically significant. However, the estimated ISF clearance (K_2) includes both the irreversible loss (K_{02}), which should saturate, and the movement of insulin back to plasma (K_{12}). It is therefore possible that the receptor-mediated clearance described by K_{02} was decreased (i.e., saturated), while at the same time the flux of insulin from ISF back to plasma described by K_{12} was increased, giving the appearance of little or no change in K_2 (K_2 is the sum of K_{02} and K_{12}). That K_{12} was increased in the present study is supported by the fact that TET_{ins} was increased (i.e., $TET_{ins} = K_{21}V_1/V_2$; if the increase in TET_{ins} was due to a diffusional increase in K_{21} then K_{12} would increase equally). Further, if insulin moves by diffusion alone ($K_{21}V_1 = K_{12}V_2$), then K_{02} and K_{12} need to be approximately equal to achieve the 2:1 plasma/ISF gradient observed in the present study (Eq. 1, steady-state $C_1/C_2 = 2$). This suggests that an increase in diffusion could effectively mask an equal decrease in muscle insulin clearance.

Results obtained from analyzing the inulin dynamics support the idea that pharmacologic insulin increased the diffusionary capacity of the capillary bed. Transendothelial transport of inulin increased by an estimated 23% with pharmacologic compared with physiologic insulin infusion. Whereas this increase did not achieve statistical significance ($P = 0.25$), two important observations must be made. First, there was insufficient power to detect such an increase ($1 - \beta = 13\%$); and second, the model was able to follow accurately the rise in lymph inulin during physiologic increments in plasma insulin, but not during pharmacologic increments (cf Fig. 7). This "residual run," in which the measured inulin concentrations are higher than the model prediction (Fig. 7 *B*; $30 < t < 180$) suggests that transendothelial inulin transport (TET_{inu}) was slowly increasing. As with insulin, such an increase could be due to an increase in perfusion of the muscle bed or an alteration in the site of lymph formation. However, unlike the insulin data, the inulin residuals suggest that the increase in diffusion capacity occurred after $\sim$ 30 min (that insulin did not exhibit a residual run suggests the increase in insulin diffusion capacity was virtually instant). In any case, the inulin residual run would suggest an underestimation of the true TET_{inu} rate. Thus, if TET_{inu} is estimated from the data during the insulin activation period alone (0–180 min rather than 0–300 min for both the physiologic and pharmacologic groups), the increase in TET_{inu} is estimated to be 35% rather than 23%, and the corresponding P value is reduced to 0.18 (data not shown). A 35% increase in transendothelial inulin transport is close to the value observed for the increase in transendothelial insulin transport (41%), suggesting that both increases can be accounted for by an increase in diffusion capacity alone.

Conclusions in the present study conflict with some other in vivo studies (15, 16). A study by Sonksen and colleagues (15), using arteriovenous differences in the dog hindlimb, indicated that the extraction of labeled insulin was reduced as the concentration of unlabeled insulin was increased. However, the ^{125}I-labeled insulin used in that study has different binding kinetics than native insulin (23, 24) making it difficult to draw definitive conclusions. A study by Jansson et al. (16), using microdialysis to assess ISF insulin concentration, indicated that the plasma/subcutaneous fluid insulin gradient was increased as the plasma insulin concentration was increased. Also, a second study by this group, in which the gradient was assessed in muscle ISF per se, was consistent with this earlier finding (25). However, rapid measurements of ISF concentration are not possible with microdialysis, making it difficult to assess clearance. Since changes in clearance affect the gradient, it is critical to assess this parameter if an increase in the gradient is to be interpreted as a decrease in transport. Finally, there is evidence for receptor-mediated transport of insulin across the blood–brain barrier. M. Schwartz et al., in collaboration with our group, were able to simultaneously measure insulin dynamics in plasma and cerebrospinal fluid (anesthetized dog). From these dynamics, the transport rate of insulin into brain ISF was obtained from a compartmental model (26). Pharmacologic elevation of the plasma insulin concentration was then shown to decrease (i.e., saturate) the plasma-to-brain ISF transport rate (27). However, transport across the blood–brain barrier is highly specialized; thus, it is possible that insulin

transport across the blood–brain barrier is saturable, whereas insulin transport across the muscle capillary endothelial barrier is not.

In vitro, insulin transport across cultured endothelial monolayers was shown to be both unidirectional and saturable, while at the same time inulin was shown to be free to move in either direction (13). However, there are many differences between in vivo and in vitro endothelial barriers. Most notably, endothelial cells grown in vitro do not actually form capillary-like structures but rather are confluent monolayers. The permeability characteristics of such monolayers depend on many factors (28); how the layer was originally seeded (monolayers seeded at supraconfluent densities do not form as tight cell–cell junctions as those seeded at lower densities and allowed to grow to confluence), the type of culture media (the presence of albumin or histamine may alter permeability), and how long the cell culture is incubated. It is possible that, in vitro, cells form such tight junctions as to eliminate the diffusion pathway, leaving all transport to be via receptors; whereas, in vivo, insulin transport may be dominated by diffusion with only a small component transported by the receptor-mediated pathway. In the current study, a small receptor-mediated component may not have been observable because of the paradoxical increase in diffusion capacity at pharmacologic insulin. That is, since TET_{ins} (Eq. 2) is the sum of a diffusionary component and a receptor-mediated component, the possibility remains that the receptor-mediated component decreased with saturating insulin while at the same time the diffusionary component increased. The sum of these two processes would then increase, masking any decrease in receptor-mediated transport. However, if this scenario were true, we would have expected to see a larger increase in the transport of inulin (the diffusionary marker) than for insulin. The observed increase in TET_{inu} was actually less than or similar to (see above) the increase in TET_{ins}. Thus, our data would suggest that, if a receptor-mediated component does exist, it is not quantitatively significant for the transport of insulin in vivo.

In conclusion, our data indicate that the insulin diffusionary pathway is enhanced during pharmacologic increases in plasma insulin. This enhanced diffusionary transport is likely due to dilation of existing capillaries or new capillary recruitment. While the present study cannot rule out the existence of a small insulin receptor-mediated transport pathway, it is clear that diffusion is the dominant process in vivo and that this process cannot be saturated.

Acknowledgments

The authors thank Dr. Jang H. Youn for insightful comments in the preparation of the manuscript, Dr. Joyce M. Richey for help with experiments, and Lena Roumian Minassian for technical assistance.

This work was supported by National Institutes of Health grants DK-27619 and DK-29867. R.N. Bergman was supported by the Salerni Collegium of the University of Southern California. M. Ader was supported by the NIH (AG005402). This study was performed as part of the doctoral studies of Garry M. Steil.

References

1. White, M.F., and C.R. Kahn. 1994. The insulin signaling system. *J. Biol. Chem.* 269:1–4.

2. Rasio, E.A., E. Mack, R.H. Egdahl, and M.G. Herrera. 1968. Passage of insulin and inulin across vascular membranes in the dog. *Diabetes.* 17:668–672.

3. Rasio, E.A., C.L. Hampers, J.S. Soeldner, and G.F.J. Cahill. 1967. Diffusion of glucose, insulin, and Evans blue protein into the thoracic duct of man. *J. Clin. Invest.* 46:903–910.

4. Rasio, E.A. 1982. The capillary barrier to circulating insulin. *Diabetes Care.* 5:158–161.

5. Yang, Y.J., I.D. Hope, M. Ader, and R.N. Bergman. 1989. Insulin transport across capillaries is rate limiting for insulin action in dogs. *J. Clin. Invest.* 84:1620–1628.

6. Steil, G.M., M.A. Meador, and R.N. Bergman. 1993. Thoracic duct lymph: relative contribution from splanchnic and muscle tissue. *Diabetes.* 42: 720–731.

7. Poulin, R.A., G.M. Steil, D.M. Moore, M. Ader, and R.N. Bergman. 1994. Dynamics of glucose production and uptake are more closely related to insulin in hindlimb lymph than in thoracic duct lymph. *Diabetes.* 43:180–190.

8. Bradley, D.C., R.A. Poulin, and R.N. Bergman. 1993. Dynamics of hepatic and peripheral insulin effects suggest common rate-limiting step in vivo. *Diabetes.* 42:296–306.

9. Bergman, R.N., D.C. Bradley, and M. Ader. 1995. On insulin action in vivo: the single gateway hypothesis. *In* New Concepts in the Pathogenesis of NIDDM. S. Efendic, C.G. Ostenson, and M. Vranic, editors. Plenum Press, New York.

10. Landis, E.M., and J.R. Pappenheimer. 1963. Exchange of substances through capillary walls. *In* Handbook of Physiology, Circulation. Section 2, Vol. 2. Am. Physiol. Soc., Washington, D.C. 961–1034.

11. Bundgaard, M. 1983. Vesicular transport in capillary endothelium: does it occur? *Fed. Proc.* 42:2425–2430.

12. Ghitescu, L., A. Fixman, M. Simionescu, and N. Simionescu. 1986. Specific binding sites for albumin restricted to the plasmalemmal vesicles of continuous capillary endothelium: Receptor mediated transcytosis. *J. Cell Biol.* 102: 1304–1311.

13. King, G.L., and S.M. Johnson. 1985. Receptor-mediated transport of insulin across endothelial cells. *Science (Wash. DC).* 227:1583–1585.

14. Bar, R.S., M. Boes, and A. Sandra. 1988. Vascular transport of insulin to rat cardiac muscle. Central role of the capillary endothelium. *J. Clin. Invest.* 81: 1225–1233.

15. Sonksen, P.H., J.R. McCormick, R.H. Egdahl, and J.S. Soeldner. 1971. Distribution and binding of insulin in the dog hindlimb. *Am. J. Physiol.* 221: 1672–1680.

16. Jansson, P.E., J.P. Fowelin, H.P. von Schenck, U.P. Smith, and P.N. Lonnroth. 1993. Measurement by microdialysis of the insulin concentration in subcutaneous interstitial fluid. *Diabetes.* 42:1469–1473.

17. Bar, R.S., J.C. Hoak, and M.L. Peacock. 1978. Insulin receptors in human endothelial cells: identification and characterization. *J. Clin. Endocrinol. Metab.* 47:699–702.

18. Andersen, L., B. Dinesen, P.N. Jorgensen, F. Poulsen, and M.E. Roder. 1993. Enzyme immunoassay for intact human insulin in serum or plasma. *Clin. Chem.* 39:578–582.

19. Laakso, M., S.V. Edelman, G. Brechtel, and A.D. Baron. 1990. Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J. Clin. Invest.* 85:1844–1852.

20. Baron, A.D. 1994. Hemodynamic actions of insulin. *Am. J. Physiol.* 267: E187–E202.

21. Adair, T.H., D.S. Moffatt, A.W. Paulsen, and A.C. Guyton. 1982. Quantitation of changes in lymph protein concentration during lymph node transit. *Am. J. Physiol.* 12:H351–H359.

22. Ader, M., R.A. Poulin, Y.J. Yang, and R.N. Bergman. 1992. Dose-response relationship between lymph insulin and glucose uptake reveals enhanced insulin sensitivity of peripheral tissues. *Diabetes.* 41:241–253.

23. Genuth, S.M. 1972. Metabolic clearance of insulin in man. *Diabetes.* 21: 1003–1012.

24. Iszzo, J.L., A. Roncone, M.J. Izzo, and W.F. Bale. 1964. Relationship between degree of iodination of insulin and its biological electrophoretic and immunochemical properties. *J. Biol. Chem.* 239:3749–3754.

25. Holmang, A., K. Mimura, P. Bjorntorp, and P.N. Lonnroth. 1995. Measurement by microdialysis of muscle insulin concentration in the rat. *Diabetes.* 44:339*a.* (Abstr.)

26. Schwartz, M.W., R.N. Bergman, S.E. Kahn, G.J. Taborsky, L.D. Fisher, A.J. Sipols, S.C. Woods, G.M. Steil, and D.J. Porte, Jr. 1991. Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. *J. Clin. Invest.* 88:1272–1281.

27. Baura, G.D., D.M. Foster, D.J. Porte, Jr., S.E. Kahn, R.N. Bergman, C. Cobelli, and M.W. Schwartz. 1993. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J. Clin. Invest.* 92:1824–1830.

28. Schaeffer, R.C.J., F. Gong, and M.S.J. Bitrick. 1992. Restricted diffusion of macromolecules by endothelial monolayers and small-pore filters. *Am. J. Physiol.* 263:L27–L36.