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Lymphatic Absorption Is a Significant Contributor to the Subcutaneous Bioavailability of Insulin in a Sheep Model

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Purpose: This study was conducted to explore the role of the peripheral lymphatics in insulin absorption following subcutaneous (SC) administration using a sheep model that allows continuous collection of peripheral lymph and simultaneous assessment of systemic bioavailability.

Methods: In a parallel group design, soluble human insulin (0.5 IU/kg) was administered by bolus SC injection into the interdigital space of the hind leg of non-cannulated control sheep, and sheep in which the efferent popliteal lymph duct was cannulated. A separate group received a bolus IV injection (0.15 IU/kg). Blood was sampled from all animals, and lymph was collected continuously over 12 h post-dosing. Samples were assayed for insulin by ELISA.

Results: The SC bioavailability of insulin in control sheep was 31.5 ± 3.2%, which was significantly higher than when the peripheral lymph was continuously collected (18.4 ± 1.7%). In the lymph-cannulated animals, 17.3 ± 1.0% of the dose was collected in peripheral lymph.

Conclusions: Based on the direct measurement of insulin in regional lymph and on the decrease in the systemic bioavailability when regional lymph was continuously collected, the results demonstrate that lymphatic absorption contributed significantly to the overall insulin bioavailability following SC administration to sheep.

KEY WORDS: insulin; subcutaneous; absorption; lymph; protein; bioavailability.

INTRODUCTION

Since its commercial introduction in the 1920's, insulin has been extensively utilized in the treatment of diabetes mellitus and arguably represents the best characterized of all therapeutic proteins. Considerable research has been directed toward the development of insulin formulations and delivery systems to mimic physiological insulin secretion and provide normalized glycemic control throughout a 24-h period. The results of The Diabetes Control and Complication Trial (DCCT) reported in 1993 emphasized the importance of strict glycemic control for the prevention of degenerative diabetic complications thus providing further incentive for the continued search for improved therapies (1).

A number of commercially available insulin preparations provide short, intermediate, and long durations of action. Regular human insulin is a neutral, soluble form containing zinc that exhibits a delayed absorption rate with an onset effect of approximately 0.5 h and maximal effect between 2.5 and 5 h. The delayed absorption rate is thought to be due to the requirement for dissociation of insulin hexamers and dimers within the interstitium prior to absorption (2–4).

Using radiolabelled tracer techniques, a series of elegant studies characterized the absorption of insulin after SC injection in pigs (3,5,6) and humans (4,6,7). A strong correlation was demonstrated between the average state of insulin association and the rate of disappearance of radiolabel from the injection site. Based on these trends, insulin analogues with a reduced tendency for self-association, such as Lispro (8) and Aspart insulin (3,6), have been designed to provide a more rapid onset of action and more closely mimic endogenous postprandial insulin secretion.

Insulin absorption is influenced by numerous factors including the formulation concentration (9,10) and injection volume (9), subcutaneous blood flow (7,11), site of injection (12,13), skin-fold thickness, and injection depth (12,13). Application of external factors such as heat (6), exercise (14), and massage (15) result in an increased rate of absorption.

After SC administration, protein drugs may be transported to the systemic circulation directly via blood capillaries or indirectly via the lymphatics, both of which contribute to the absorption of materials from the subcutaneous interstitial region. Studies by Supersaxo *et al.* (16,17) and Charman *et al.* (18) have shown that greater than 50% of a SC dose may be taken up by the peripheral lymphatics for macromolecules exceeding approximately 20 kDa. Presently, molecular weight, or size, is thought to be the primary determinant for the absorption of macromolecules via either the blood or the lymph with the lymphatics becoming increasingly important for larger molecules (17,19).

The lymphatic route is generally not considered a significant contributor to the SC absorption of insulin based primarily on the early work of Binder (9). In these studies, insulin was administered subcutaneously into the forearm and blood was sampled via a catheter in a vein distal to the cubital fossa that directly drained the injection site. Blood was also collected via a catheter in a contralateral arm vein. Insulin concentrations in blood from the injection arm were significantly higher than those from the contralateral arm verifying that at least a portion of the dose was absorbed directly via the blood. However, given that insulin concentrations in the contralateral arm would be subject to dilution effects due to distribution (whereas concentrations in the injection arm would not), these results do not exclude a potential contribution from lymphatic absorption. Given the evidence for lymphatic absorption of macromolecules and the dependence of insulin absorption on various factors that affect lymph flow (i.e. heat, massage, exercise), it seems likely that insulin could be at least partially absorbed via the lymphatics.

The objective of this study was to explore the role of the peripheral lymphatics in the absorption of insulin following SC administration using a sheep model that allows continuous and complete collection of the regional lymph draining the injection site and simultaneous assessment of the systemic bioavailability.

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MATERIALS AND METHODS

Study Design

Using a parallel group design, soluble human insulin was administered by bolus SC injection to non-lymph cannulated control sheep and to sheep containing a peripheral lymph duct cannula. Blood was collected in each group, and lymph was continuously collected in the lymph-cannulation group. Insulin was also administered by bolus IV injection to a separate group of animals.

Surgical Procedures

Surgery was performed by a veterinary surgeon under aseptic conditions, and all procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee. Adult male merino sheep weighing 33–56 kg were used. Jugular vein catheters were inserted in all animals for blood sampling, and IV doses were administered into the opposite jugular vein. In the lymph-cannulation group, the efferent duct of the popliteal lymph node was cannulated as previously described (20). During the recovery period, the lymph cannula was kept patent by immersion in a polypropylene collection bottle containing heparin, which was held in a pouch at the side of the animal. Animals were housed in metabolism cages and allowed to recover for approximately 36 h following surgery with free access to food up to approximately 12 h prior to dosing. Water was available *ad libitum* during both the recovery and sampling periods, and animals were fasted from 12 h prior to dosing until the end of the sampling period.

Insulin Administration and Sample Collection

Neutral, soluble human insulin (Atrapid®, Novo Nordisk, 100 IU/ml) was administered by bolus IV injection at doses of 0.15 and 0.39 IU/kg. The 0.39 IU/kg dose was administered without dilution by venipuncture. For the 0.15 IU/kg dose, the formulation was diluted immediately prior to administration by adding to a mixture of saline and each animal's own blood (to minimize adsorption with low concentration) and administered via a jugular vein catheter that was removed after dosing. Blood samples were collected into dipotassium EDTA tubes (Sarstedt, Australia) prior to dosing and over 2 h post-dosing. Tubes were centrifuged at 3,000 rpm for 10 min, and plasma was separated and frozen at −20°C until analysis.

Soluble human insulin was administered by bolus SC injection (0.5 IU/kg) into the interdigital space of the hind leg. For the lymph-cannulation group, SC injections were given into the same leg as the lymph cannulation. Blood was sampled prior to dosing and over 12 h post-dosing and was transferred to polypropylene tubes containing EDTA and treated as described above.

In the lymph-cannulation group, peripheral lymph (approximately 4–8 ml/h) was continuously collected into preweighed dipotassium EDTA tubes, which were changed at hourly intervals for the duration of the experiment. At the conclusion of each interval, the volume of lymph was determined gravimetrically, samples were centrifuged at 3,000 rpm for 10 min and the supernatant separated and frozen at −20°C until analysis.

Assay Methodology

Insulin concentrations in plasma and lymph were determined using a commercially available one-step sandwich ELISA (Active™ Insulin ELISA, DSL, Texas). Reference standards were reconstituted with blank fasted sheep plasma to provide an assay range of 0.112 to 12.40 ng/ml. Where necessary, samples were diluted with pooled blank fasted sheep plasma to ensure detection within the validated range. Samples were assayed in duplicate and concentrations were calculated by comparison to a log-log calibration curve for absorbance vs. concentration. The same batch of blank sheep plasma was used for sample dilution and standard preparation.

Short term sample stability to support sample handling and processing was conducted by spiking insulin into fresh whole blood and centrifuging immediately or following 60 min at ambient temperature after which plasma was collected and frozen at −20°C until assay. Quality control samples prepared in blank sheep plasma and stored along with the study samples were assayed in duplicate within each analytical run.

Given the homology between sheep and human insulin (92%), the cross-reactivity of endogenous sheep insulin with the human insulin ELISA was examined over a representative post-dosing period. Two fasted, lymph-cannulated sheep were dosed with normal saline by bolus SC injection into the interdigital space and blood and lymph samples were collected, processed and assayed as previously described.

Data Analysis

Pharmacokinetic parameters for insulin after IV administration were determined by fitting the data to a biexponential equation using non-linear curve fitting. The area under the plasma concentration versus time profile (AUC) following IV dosing was calculated by:

$$AUC_{IV} = \frac{A}{\alpha} + \frac{B}{\beta}$$

where α and β represent the hybrid first-order rate constants for the distribution and elimination phases, respectively, and A and B represent the corresponding extrapolated intercepts. Plasma clearance was calculated by dose/AUC_{IV} and the volume of distribution during the elimination phase ($V_{d,\beta}$) was calculated by dose/(AUC_{IV} *β).

For the SC groups, maximum plasma concentrations (C_{max}) and the time to reach the maximum (T_{max}) were determined from the concentration vs. time data, and terminal rate constants were obtained by exponential regression of the terminal portion of the profiles. The linear trapezoidal method was used to calculate the area up to the last time point ($AUC^{0\text{-}t}$). The area to infinity ($AUC^{0\text{-}\infty}$) was determined by adding the extrapolated area (the last measurable concentration/terminal rate constant) to $AUC^{0\text{-}t}$. Bioavailability calculations for the SC groups were conducted using:

$$\text{Bioavailability} = \frac{AUC_{SC} * Dose_{IV}}{AUC_{IV} * Dose_{SC}} * 100$$

where AUC_{SC} represents the area to infinity after SC administration in each animal and AUC_{IV} and $Dose_{IV}$ represent the mean values for the low-dose IV group. Statistical compari-

sons were performed using a student's t test testing for significance at $\alpha = 0.05$.

The mass of insulin collected in each lymph sample was calculated as the product of the measured concentration and the volume of lymph. The cumulative amount collected for each animal was expressed as a percentage of the administered dose. The fraction absorbed was calculated for individual animals in the lymph-cannulated group as the sum of the percentage of the dose collected in peripheral lymph and the systemic availability.

RESULTS

Assay Validation

Endogenous sheep insulin concentrations in blank fasted sheep plasma used to prepare standards and provide for sample dilution were below the limit of quantitation of the assay (0.112 ng/ml). Comparable assay responses were obtained when insulin standards were prepared in either blank sheep plasma or lymph verifying that lymph samples could be accurately assayed by dilution with blank plasma and comparison to plasma calibration curves. Plasma quality control samples prepared at 1 and 10 ng/ml exhibited a within-assay precision (% CV) of <10% at each concentration. The accuracy of quality control samples across the study period was within ±25% of the nominal concentrations consistent with recent recommendations for immunoassays (21).

Pre-dose lymph samples exhibited assay responses that were similar to that for the zero-concentration kit standard. Pre-dose plasma concentrations were below the limit of quantitation (LOQ, 0.112 ng/m) in eight out of thirteen sheep and just above the LOQ (0.14–0.3 ng/ml) in the remaining sheep. In the lymph-cannulated sheep dosed with a SC injection of normal saline, endogenous sheep insulin concentrations averaged approximately 0.3 ng/ml across the sampling period. The endogenous sheep insulin AUC for the saline treated group was estimated to be approximately 10% of the AUC measured for the SC insulin treatment group. Given the low predose insulin concentrations in relation to the concentrations measured in the saline treated sheep, the possible 10% AUC contribution was assumed to be the maximum likely contribution. No attempt was made to correct for endogenous insulin concentrations given the likely variability between animals. Sheep were maintained in a fasted state during the sampling period to minimise potential contributions due to endogenous insulin secretion.

Pharmacokinetics of Insulin after IV Injection in Sheep

Figure 1 illustrates plasma concentration vs. time profiles for insulin after IV injection in sheep at 0.15 and 0.39 IU/kg and pharmacokinetic parameters are shown in Table I. There was no evidence of hypoglycemia following insulin administration at either dose. Concentrations for the two doses declined in parallel in a biexponential manner with a terminal half-life of approximately 13–14 min. This half-life is comparable to that for regular human insulin in other animal species (22) but slightly shorter than that recently reported for humans (23). There were no significant differences in the half-life, clearance, and volume of distribution values for the two doses indicating that insulin IV pharmacokinetics in sheep

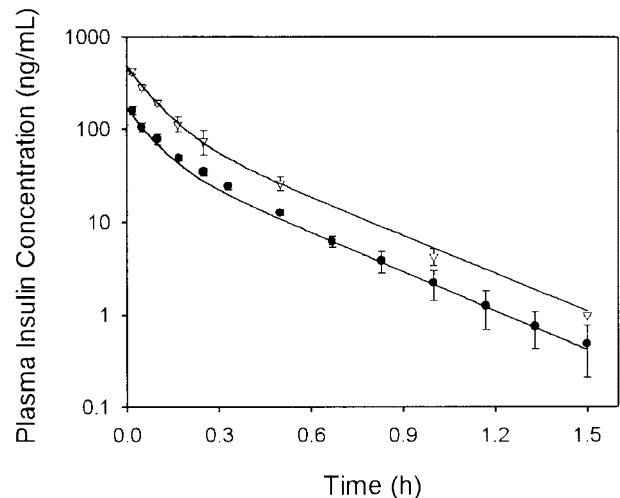

Fig. 1. Plasma insulin concentration vs. time profiles following IV bolus administration of 0.15 (●) or 0.39 (△) IU/kg neutral, soluble human insulin to sheep (mean ± SEM)

were linear within this dose (0.15–0.39 IU/kg) and concentration range.

Absorption of Insulin after SC Administration to Sheep

Mean plasma concentration vs. time profiles for soluble insulin after SC administration are shown in Fig. 2 and pharmacokinetic parameters are listed in Table I. Peak plasma concentrations were detected at approximately 1–2 h post-dosing and concentrations declined with a half-life of approximately 2 h. Significant double peaks in the individual plasma profiles were observed in two out of five animals in both the lymph-cannulated and the non-cannulated control groups. In the lymph-cannulated group, the two sheep with double peaks in the plasma profiles also exhibited delayed absorption into lymph (Table II).

Insulin bioavailability in the SC control group was 31.5 ± 3.2% (mean ± SEM, n = 5) in comparison to a value of 59% reported for regular human insulin after SC injection in humans (24). The bioavailability was significantly lower ($p<0.05$) in the lymph-cannulated group (18.4 ± 1.7%) than in the SC control group consistent with the lymphatics being a significant contributor to the overall SC bioavailability of insulin (Table I). The reduced bioavailability in the lymph-cannulated group could be readily accounted for by the collection of 17.3 ± 1.0% of the dose in the peripheral lymph draining the SC injection site (Fig. 3). The total fraction absorbed after SC administration was estimated for each animal in the lymph-cannulation group from the sum of the systemic availability and the cumulative proportion of the dose collected in lymph to give a value of 35.7 ± 1.6% which was not significantly different ($\alpha = 0.05$) from the bioavailability measured in the SC control group (31.5 ± 3.2%).

DISCUSSION

The lymphatic route represents a potentially major contributor to the absorption of macromolecules after SC injection because of their inherently low membrane permeability and their large size, both of which limit transport across the vascular endothelium. The lymphatic capillaries differ from

Table I. Pharmacokinetic Parameters for Neutral, Soluble Human Insulin following IV and SC Administration to Sheep

	IV bolus		SC bolus to non-cannulated control sheep	SC bolus to lymph-cannulated sheep
	0.15 IU/kg	0.39 IU/kg	0.5 IU/kg	0.5 IU/kg
C_{max} (ng/ml)	—	—	11.0 ± 3.4	4.8 ± 0.9
T_{max} (h)[a]	—	—	1.2 ± 0.1	2.1 ± 0.8
Terminal half-life (h)	0.22 ± 0.03	0.23 ± 0.02	2.0 ± 0.2	2.1 ± 0.5
AUC (ng.h/ml)	28.0 ± 3.2	65.9 ± 8.0	29.3 ± 2.9	16.8 ± 1.6
CL (ml/min/kg)	3.66 ± 0.36	4.12 ± 0.53	—	—
$V_{d,\beta}$ (mL/kg)	69.5 ± 7.4	82.2 ± 18.3	—	—
Systemic availability (%)	—	—	31.5 ± 3.2	18.4 ± 1.7
Lymph recovery (% of dose)	—	—	—	17.3 ± 1.0
Fraction absorbed (% of dose)[a]	—	—	—	35.7 ± 1.6

[a] Fraction absorbed = systemic availability + lymph recovery in each lymph-cannulated animal.
Values represent the mean ± SEM for n = 3 (IV) or n = 5 (SC) sheep.

subcutaneous blood capillaries in that they consist of overlapping endothelial cells and lack a well-defined basal lamina and inter-endothelial tight junctions. The structure of the lymphatic capillary provides a "flap" or valve mechanism that responds to changes in interstitial osmotic and oncotic pressure and facilitates the uptake and transport of plasma exudate to the systemic circulation. The peripheral lymphatic absorption of a few proteins including human growth hormone (hGH, 22 kDa) (18), interferon-α-2a (IFN, 19 kDa) (17), cytochrome C (12.3 kDa) (17), and the low molecular weight compounds, inulin and fluorodeoxyuridine (17), after SC injection has been reported. Even with this relatively limited database, a direct relationship between the extent of lymphatic absorption (expressed as a percentage of the dose) and the molecular weight has been observed (Fig. 4).

A sheep model was used in the current study to enable cannulation of the efferent popliteal lymph duct and the quantitative collection of lymph draining the SC injection site. Sheep have been extensively utilized for studying the peripheral lymphatics and the lymphatic capillary microstructure as a result of their relatively large size and the feasibility of cannulating various lymphatic vessels. The site of injection was based on practical considerations, and it is recognized that differences in regional blood and lymph flow may influence the proportion of the dose being absorbed by each pathway. The rationale behind using sheep, and the comparison of sheep to other animal models, is discussed elsewhere (20).

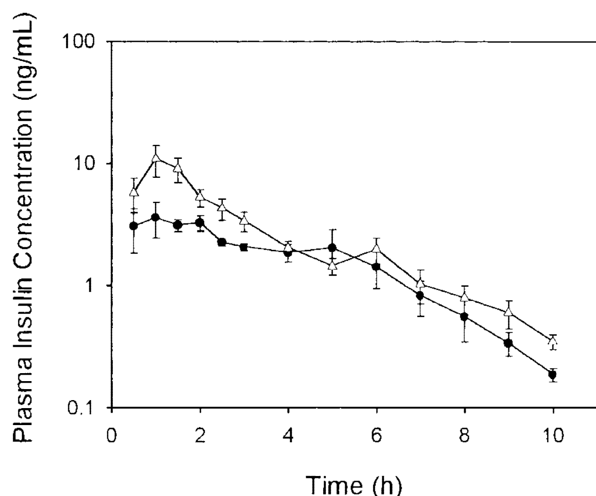

Fig. 2. Plasma insulin concentration vs. time profiles following SC bolus administration of 0.5 IU/kg neutral, soluble human insulin to non-lymph cannulated control (△), or peripheral lymph-cannulated (●) sheep (mean ± SEM).

To our knowledge, these results represent the first report of lymphatic insulin absorption after SC injection. In the non-cannulated control sheep, insulin was absorbed both via the blood and the lymph and each route contributed to the overall bioavailability of 31.5%. In comparison, the bioavailability was reduced to 18.4% in the lymph-cannulated group due to the continuous and complete collection of lymph draining the SC injection site. The conclusions, therefore, are based not only upon the direct measurement of significant amounts of insulin in regional lymph (17.3%), but also on the significant decrease in the systemic bioavailability of insulin in the lymph-cannulated group (i.e. when the lymphatic contribution was removed) compared with the non-cannulated control group.

The total fraction of insulin absorbed in the lymph-cannulated animals (obtained by adding together the cumulative percent of the dose in lymph and the systemic bioavailability in the cannulated sheep) was approximately 35% suggesting that a significant proportion of the dose was lost at the injection site. There are conflicting reports in the literature regarding the subcutaneous degradation of insulin in humans with some accounts indicating that degradation may be significant (25,26) while others consider it to be of only minor

Table II. Individual Times for Maximum Plasma Concentrations (T_{max}) and Times for 50% Maximal Lymphatic Absorption ($T_{50\%}$) following SC Administration of 0.5 IU/kg Soluble Human Insulin to Lymph Duct-Cannulated Sheep

Sheep number	Plasma T_{max} (h)	Lymph $T_{50\%}$ (h)	Lymph recovery (% of dose)
1	3.1 and 5.0	4.8	19.7
5	0.5	2.0	14.6
6	1.5	2.0	18.4
9	2	1.6	18.3
11	1.5 and 5.0	4.7	15.4

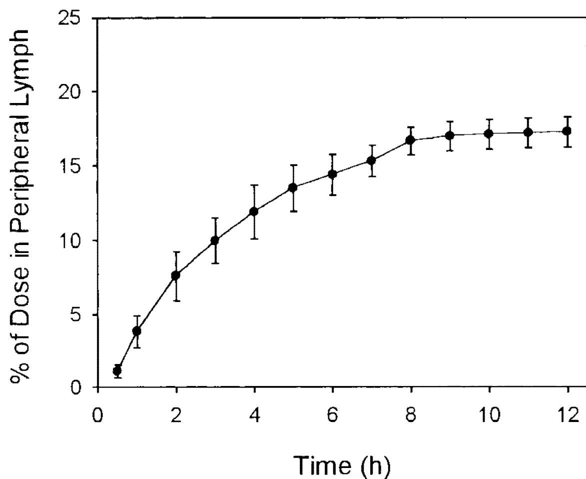

Fig. 3. Cumulative percentage of the insulin dose collected in peripheral lymph (mean ± SEM) vs. time for sheep administered insulin (0.5 IU/kg) by bolus SC injection.

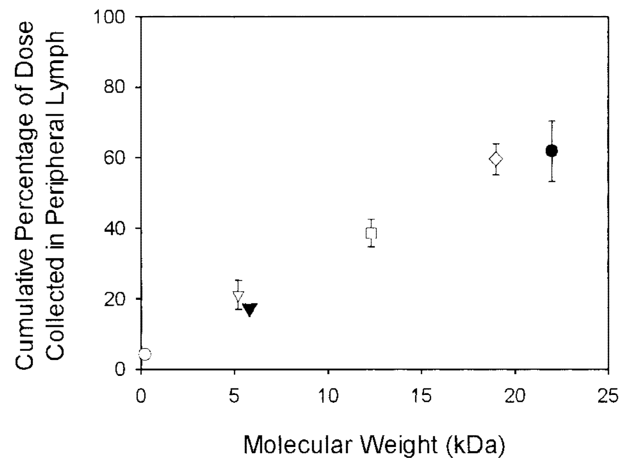

Fig. 4. Relationship between the proportion of the dose collected in peripheral lymph (mean ± SEM) and the apparent molecular weight for selected proteins and low molecular weight compounds. Data for fluorodeoxyuridine (○), inulin (▽), cytochrome C (□), and interferon-α-2a (◇) are from Supersaxo *et al.* (17), data for hGH (●) are from Charman *et al.* (18), and data for insulin (▼) are from this study.

importance (27). Inherent in our approach for estimating the fraction absorbed are the assumptions that (i) all of the lymph from the lower region of the leg was transported to the popliteal lymph node and collected through the efferent vessel cannula and (ii) there was no significant loss of insulin after the initial absorption and prior to the peripheral lymph collection. The first assumption was validated by confirming the absence of collateral lymphatic vessels during surgery and by comparing the postoperative lymph flows to normal values (20). The second assumption was more difficult to verify, however, the similarity between the total fraction absorbed in the lymph-cannulated group (35.7%) and the bioavailability in the SC control group (31.5%) suggests minimal loss of insulin upon transport through the lymphatic system.

The extent of lymphatic uptake of insulin (17.3% of the dose) was comparable to that for inulin (approximately 20% of the dose) (17) determined previously using a similar sheep model. However, expressing the lymphatic uptake as a percentage of the dose may substantially underestimate the lymphatic contribution to absorption if the fraction absorbed is low. Relative to the fraction absorbed, the lymphatic route contributed approximately 48% to the overall SC bioavailability of insulin in this study. Given the similar size of inulin and the insulin monomer (5.2 kDa for inulin, 5.8 kDa for monomeric insulin), and assuming complete absorption of inulin, these results could indicate that insulin was absorbed via the lymph in a form larger than the monomer. Previous studies have suggested the subcutaneous diffusion rate of insulin to be five to ten times lower than that for inulin (28). The authors concluded that insulin could be at least partially transported through the subcutaneous interstitium in a self-associated form. Further studies using a pig model have shown that non-dissociating cobalt-insulin hexamers can be absorbed from the subcutaneous tissue (6), providing further evidence for the potential absorption of insulin in an associated form.

Based upon the data presented here and the numerous studies of insulin absorption reported in the literature, several prototype models can be proposed to describe the SC absorption of insulin (Fig. 5). In Model A, dissociation of the insulin hexamer and dimer are the rate limiting steps and are required prior to absorption of monomer via either the blood or the lymph. Even though our results are not inconsistent with this model, the literature data demonstrating the absorption of the non-dissociating insulin-cobalt hexamer (6) would sug-

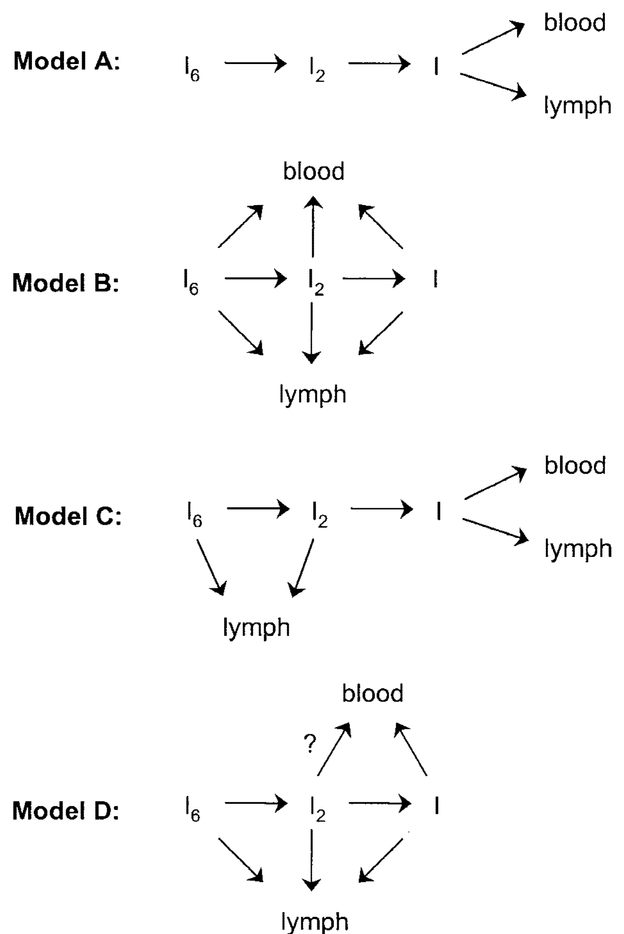

Fig. 5. Prototype models for the SC absorption of insulin based on results reported here and in the literature. (Key: I_6 = insulin hexamer; I_2 = insulin dimer, I = insulin monomer)

gest a potential for the absorption of associated forms. Model B represents the other extreme (compared to Model A) in which each of the different associated states can be absorbed via either the lymph or the blood. Given the previous molecular weight/size relationship for the predominance of the lymphatic route for larger molecules (17), it is likely that a molecule the size of the insulin hexamer would be taken up, in part, by the lymph. Model C illustrates a more restricted model in which the dimeric and hexameric forms are absorbed via the lymph and the monomer is absorbed exclusively via the blood. This model can most likely be ruled out on the basis of previous results for inulin (17), which suggest that a molecule the size of monomeric insulin is partially absorbed via the lymphatic capillaries. Model D likely represents a more realistic model in which the monomer, and possibly the dimer, are absorbed predominantly via the blood whereas both the monomer and associated forms can be absorbed via the lymph. The delayed rate of absorption of hexameric insulin previously reported (6) could be a function of slow diffusion through the interstitium followed by lymphatic absorption and transport to the blood circulation. The lymphatic contribution to the absorption of monomeric insulin (e.g. insulin analogues) is not currently known and future studies will be conducted to address this aspect and provide further insight into the mechanisms of insulin absorption.

Notwithstanding the potential differences in the rate and extent of absorption of insulin in the sheep model and in humans, demonstration of the potential for lymphatic absorption is significant for a number of reasons. First, a lymphatic contribution would help to explain variations in insulin absorption with exercise, heat, or massage as these factors are known to dramatically stimulate lymph flow in addition to their possible effects on subcutaneous blood flow (29,30). Second, lymph flow in different anatomical sites would be expected to vary widely, which may partially account for the injection site-dependence commonly observed for the absorption of insulin. Finally, understanding the factors that contribute to uptake via the blood and the lymph is critical to a thorough assessment of the absorption characteristics for insulin analogs that differ in their association states. It is only with an appreciation of the complex absorption process that pharmacokinetic-pharmacodynamic relationships can be realistically defined and fully utilized.

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