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Evidence for lymphatic transport of insulin by topically applied biphasic vesicles

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Abstract

The cutaneous delivery pathway through the lymphatics of a novel transdermal lipid-based delivery system (biphasic vesicles), which was previously shown to deliver sustained physiological levels of basal insulin in a pain-free manner across the skin, was evaluated in a diabetic rat model. Transdermal patches (one per rat) containing insulin in biphasic vesicles (1–10 mg recombinant human insulin dose) were applied to the shaved abdominal skin of streptozotocin-induced diabetic rats for 73 h. Blood glucose was monitored approximately every 2–10 h using a Lifescan glucose meter. Inguinal lymph node insulin levels were analysed by ELISA. Insulin in the lymph nodes increased in a dose- and time-dependent manner. Maximal transdermal insulin concentrations in the lymph nodes were observed with both 140 IU (5 mg: $43.0 \pm 18.0\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)) and 280 IU (10 mg: $48.0 \pm 19.6\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)) doses of recombinant insulin at t = 73 h. The level of insulin in the lymph nodes after subcutaneous injection of 1 mg insulin at the peak blood glucose response was 35.8 μIU mg^{-1} (n = 2), before falling to 0.35 μIU mg^{-1} by t = 48 h (n = 2). The lymphatics is involved in the transdermal insulin delivery by biphasic vesicles. This is the first report on the lymphatic transport of a protein after non-invasive topical application on the skin.

Introduction

After subcutaneous injection, drug molecules and drug carriers may be absorbed into blood capillaries or the lymphatics. From subcutaneous sites a proportion of the injected molecules or delivery system is taken up into the lymph mostly due to the inability to cross the continuous structure of the capillary endothelium. The absorption of proteins into the lymph is molecular-weight dependent, with increasing molecular weight resulting in higher recovery from peripheral lymph (Supersaxo et al 1990; Charman et al 2000). In addition to size, several other factors may also play a role in lymphatic uptake, such as solubility, molecular shape and physiological factors (Xie & Hale 1996; Chen et al 2000). The lymphatic absorption of liposomes after subcutaneous injection is also size dependent, but here only the small vesicles, < 100 nm, are able to access the lymphatic capillaries. The larger liposomes remain at the injection site and act as a depot (Oussoren & Storm 2001).

Insulin for diabetes is typically administered via subcutaneous injection. Once injected, the insulin can pass into the systemic circulation, either rapidly peaking within 30–60 min or slowly delivering its dose over hours from different formulations. The route of entry into the systemic circulation from a subcutaneous injection site appears to be primarily direct, with apparently only minor amounts being taken up by the lymph drainage system (Binder 1969). More recent data in sheep indicate that about 17% of the subcutaneously injected insulin (either neutral, soluble or protamine suspension) was taken up into the peripheral lymph (Porter & Charman 2000; Charman et al 2001), which is greater than previously thought.

Current understanding is that low-molecular-weight drugs penetrating through the skin undergo a series of partitioning/diffusion steps and are taken up into the blood vessels in the dermis. It is unlikely that large-size molecules would be able to diffuse into the microvasculature the same way, and further, we have no information on the

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fate of macromolecules en route from the surface of the skin, mainly because to date there was no delivery system that was able to transport macromolecules into the skin.

We have developed a novel lipid-based delivery system, biphasic vesicles, as a needle-free topical administration technology for macromolecules. Our previous studies with this system have indicated that diabetic rats responded to transdermally delivered insulin (King et al 2002). We showed a steady-state glucose response within 6–8 h that lasted for up to 75 h (King et al 2002). Steady-state serum insulin levels reached $20.08 \pm 5.44\ \mu IU\ mL^{-1}$ (mean ± s.e.m., n = 13), by 12–36 h.

The objective of this study was to evaluate the involvement of the lymphatic pathway for insulin by topical administration of biphasic vesicles in a diabetic rat model. Here we show that insulin delivered through the skin using a needle-free approach was absorbed into the regional lymph nodes and this pathway may be responsible for the achievement of therapeutic levels of insulin in the systemic circulation.

Materials and Methods

Materials

Sprague-Dawley rats (150–225 g) were obtained from Charles River Laboratories (Quebec, Canada). Streptozotocin (STZ: mixed anomers; 75% α-anomer) was obtained from Sigma (St Louis, MO). Recombinant human insulin (USP) was purchased from Cansera (Rexdale, ON) and insulin ELISA kits from Mercodia (ALPCO, Windham, NH). One Touch Profile blood glucose monitoring system and glucose test strips were obtained from Lifescan (Milpitas, CA). Opsite was obtained from Smith & Nephew (UK) and vinyl tape from Canadian Tire. Insulin was originally dissolved in 3 mM HCl, pH 3–3.5, then adjusted to formulation pH with 1 M NaOH (approximately pH 4.5).

Formulations

Insulin was encapsulated into biphasic vesicles (Biphasix) as described in Foldvari, US Patent No. 5,853,755. Briefly, the present formulation (code 1C) was selected after previous screening of various formulation compositions. The lipid-phase components (w/w%) (14% soya phosphatidylcholine (Natterman Phospholipide GmbH, Cologne, Germany), 4% cholesterol (Croda Canada Ltd, Toronto, ON), 13% propylene glycol (Wiler Fine Chemicals London, ON) and 2% *N*α-capryloyl-*N*ε-lauroyl L-lysine ethyl ester) were hydrated with the microemulsion aqueous phase (w/w%) (2% linoleamidopropyl-PG-d imonium chloride phosphate (MONA Industries Inc., Patterson, NJ), 1% olive oil (Natural Oils International, Inc., Arleta, CA), 0.15% methylparaben and 0.05% propylparaben (BDH, Toronto, ON)) containing the required concentration of insulin to provide 1, 2, 5 or 10 mg g^{-1} in the finished product. The final preparation was incorporated into a patch (Foldvari, US Patent No. 5,718,914) of 2.5-cm i.d., for 1 g of formulation, for application to rat abdominal skin.

STZ-diabetic induction in Sprague-Dawley rats

Rats were treated in compliance with the regulations of the Canadian Council for Animal Care. The rats had free access to rat chow, except where otherwise indicated, and water at all times. Before STZ exposure, rats were fasted for 18–24 h to increase pancreatic β-cell sensitivity to STZ-mediated destruction. The rats were injected intraperitoneally with STZ (55 mg kg^{-1}) in 100 mM sodium citrate buffer, pH 4.5. Control rats received no injection or were injected with citrate buffer. Diabetic induction was confirmed by monitoring blood glucose levels with a Lifescan blood glucose meter (see below), with blood glucose above 8 mmol L^{-1} (144 mg dL^{-1}) as the cut-off for diabetic state. Blood glucose, body weight and general health were monitored daily. Insulin treatments were initiated after allowing the diabetic state to stabilize over 3–4 days.

Blood sampling of Sprague-Dawley rats

Blood glucose was measured with a Lifescan blood glucose meter using approximately 50 μL (one drop) of fresh whole tail-vein blood. The tail was warmed in water to dilate the blood vessels, dried with a tissue, and nicked with a no. 11 scalpel blade to extract blood.

Pre-treatment of Sprague-Dawley rats

Rats receiving transdermal but not subcutaneous insulin were shaved before patch application. The rats were anaesthetised with isoflurane and warmed with a lamp during anaesthesia. The abdomen was shaved first with a pair of electric clippers, then with a Braun electric razor for a smooth finish. The skin was allowed to recover for 24 h before insulin treatment. Rats whose skin showed evidence of damage due to shaving were not included in transdermal insulin treatment groups.

Insulin treatment

Before the start of insulin treatment, the rats were divided into groups and individually labelled according to treatment type by colour coding with a strip at the base of the tail. Typical experimental group divisions were as follows, and the results are expressed as the mean ± s.e.m.: control (diabetic, untreated); control (diabetic, subcutaneous insulin (1 mg; 28 IU) solution); transdermal insulin treatment (diabetic, biphasic-insulin formulation).

Subcutaneous insulin injections were administered to conscious immobilised rats. Rats receiving transdermal insulin were lightly anaesthetised during patch application as described above to lessen the overall stress. The patches contained 1 g formulation. To prevent removal or chewing by the rats, patches were additionally secured with Opsite and vinyl tape.

Blood glucose was followed throughout the experimental period (every 2–10 h). Transdermal insulin treatment

periods lasted up to 73 h. Rats subjected to subcutaneous injected insulin treatments were followed for either 2 h (terminal bleed) or throughout the experimental time period (terminated at 73 h). At the end of the treatment period, the rats were anaesthetised and the skin cleaned and sampled. The skin was stored at −20 °C until used. The chest cavity was opened via the diaphragm and blood samples were taken by cardiac puncture. Inguinal lymph nodes were excised from the rat at the time of the terminal bleed and stored at −20 °C until used.

Sample handling

Lymph nodes or skin samples (approximately 100 mg) were homogenised in 3–5 volumes of 20 mM Tris/HCl, pH 7.4, containing 1 mM EDTA, 20 $\mu g\,mL^{-1}$ soybean trypsin inhibitor and 2 $\mu g\,mL^{-1}$ leupeptin per gram weight of tissue. Crude tissue homogenates were clarified by centrifugation (14 000 *g*) at 4 °C for 5 min and the homogenate supernatants were removed for insulin and protein analysis. Protein concentration was determined by Bradford reagent. Tissue homogenate insulin content was determined by ELISA. The kit was designed to detect human insulin from 5–25 μL sample through the use of a superconjugate. The standard curve range was 0.15–100 $\mu IU\,mL^{-1}$ (5.36–357 $pg\,mL^{-1}$ insulin). The ELISA kits had a cross-reactivity with rat insulin of < 0.67%.

Data analysis

Insulin levels were expressed as μIU (mg protein homogenate)$^{-1}$. Insulin levels were converted from $\mu IU\,mL^{-1}$ to $\mu IU\,mg^{-1}$ as described in equation 1.

$$\text{Lymph or skin insulin levels } (\mu IU\,mg\text{-}1) = \frac{\text{Insulin concentration } (\mu IU\,mL^{-1})}{\text{protein concentration } (mg\,mL^{-1})} / \quad (1)$$

Homogenate insulin levels achieved after subcutaneous injection and transdermal patch treatment were compared using the Kruskal–Wallis test (non-parametric) and the Nemenyi's test for the individual differences between treatments. Untreated diabetic rats were used as controls. Other calculations were as described in King et al (2002).

Results and Discussion

Blood glucose response to transdermal insulin encapsulated in biphasic vesicles

In this study, the blood glucose response to transdermal insulin was observable within hours, reaching an approximate steady state around 7 h and was still evident at the termination of the experiment at 73 h for the highest insulin dose as compared with untreated diabetic rats. The blood glucose response was dose dependent, where increasing the dose of insulin (1–10 mg (g formulation)$^{-1}$) increased the duration and magnitude of the blood glucose response (Figure 1A). Compared with the subcutaneous insulin treatment the pharmacodynamic bioavailability of insulin from the biphasic vesicles was 64.5% and 60.4% (as determined by the AUC of the blood glucose response curves) for the 1 mg and 10 mg dose of transdermal insulin, respectively. Thus the topically applied biphasic vesicle system was able to deliver a significant amount of bioactive dose of insulin lasting 60 to > 70 h.

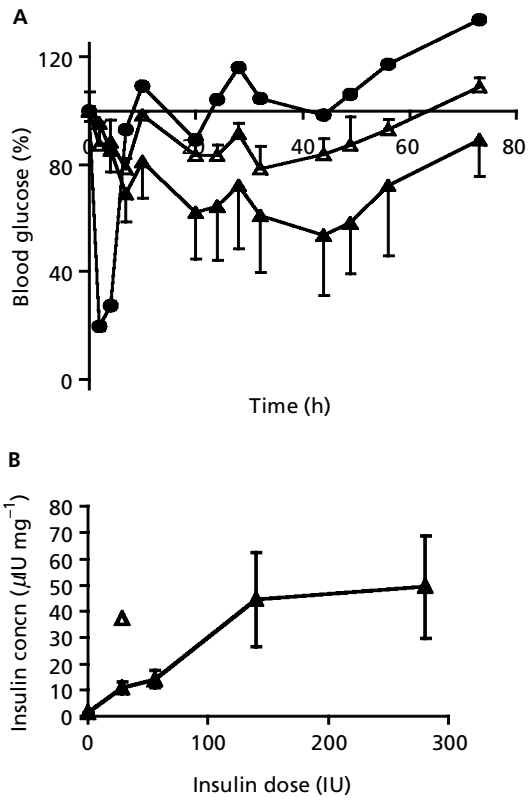

Figure 1 Dose-dependent transdermal absorption of insulin in inguinal lymph nodes. Diabetic Sprague-Dawley rats were treated with a single transdermal patch containing recombinant human insulin in biphasic vesicles (1, 2, 5 or 10 mg) or subcutaneous recombinant human insulin (1 mg). A. Blood glucose was determined for 1 mg (△) or 10 mg (▲) insulin in biphasic vesicles or subcutaneous recombinant human insulin (1 mg: ●). The results are expressed as the % as compared with the untreated diabetic controls (mean ± s.e.m., n = 4). B. Insulin absorption was determined in inguinal lymph nodes by ELISA from transdermal (t = 73 h, ▲) or subcutaneous (t = 2 h, △) treatment groups of diabetic rats. The results are expressed as the mean ± s.e.m. (n = 4).

Time-dependent transdermal absorption of insulin encapsulated in biphasic vesicles into lymph nodes

The transdermal patch containing the insulin in biphasic vesicles covered an approximately 5-cm^2 area from the shaved section of the lower abdomen up to the lower thorax. To standardise the range of peripheral lymph drainage the transdermal patch was applied to approximately

the same area in each rat. The application of a transdermal patch containing insulin in biphasic vesicles (10 mg (g formulation)$^{-1}$) to diabetic rats led to a steady time-dependent increase in the level of insulin in the inguinal lymph nodes throughout the experimental time (Figure 2). Under these conditions, insulin was discernable in the inguinal lymph nodes within 6 h ($7.96 \pm 2.98\ \mu$IU mg^{-1} (mean ± s.e.m., n = 3)) but not significant until 48 h (($35.02 \pm 6.36\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)) as compared with the diabetic control ($1.34 \pm 0.19\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)) ($P < 0.02$). At 73 h the level of insulin in the inguinal lymph nodes was still apparently rising with the 10 mg insulin (g formulation)$^{-1}$ treatment (($49.34 \pm 19.34\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)). Previously we have determined that insulin in the serum was evident, albeit at low levels, within 2 h (approximately $3.4 \pm 2.4\ \mu$IU mL^{-1} (mean ± s.e.m., n = 3)), but did not reach a steady state until 6–12 h. Serum insulin levels for those diabetic rats treated with transdermal insulin in biphasic vesicles reached $20.08 \pm 5.44\ \mu$IU mL^{-1} (mean ± s.e.m., n = 13) during the steady-state delivery of insulin. Serum insulin in rats receiving subcutaneous insulin solution (28 IU) reached $47.00 \pm 12.56\ \mu$IU mL^{-1} (mean ± s.e.m., n = 6) at 2 h, the time of the peak blood glucose response (King et al 2002).

Dose-dependent transdermal absorption of insulin encapsulated in biphasic vesicles into lymph nodes

Within hours of the start of transdermal treatment, insulin was observed in the inguinal lymph nodes, rising steadily throughout the period of treatment. Indeed, even a low transdermal dose of insulin (1 mg insulin (g formulation)$^{-1}$) gave detectable insulin in the inguinal lymph ($10.94 \pm 2.20\ \mu$IU mg^{-1} (mean ± s.e.m., n = 4)) after 73 h of treatment, indicating that insulin was still being delivered to the lymph, as compared with the diabetic controls. At this stage the apparent blood glucose response was no longer evident (Figure 1A). Maximal lymph node insulin was observed at the two highest doses of transdermal insulin tested (5 and 10 mg (g formulation)$^{-1}$, Figure 1B).

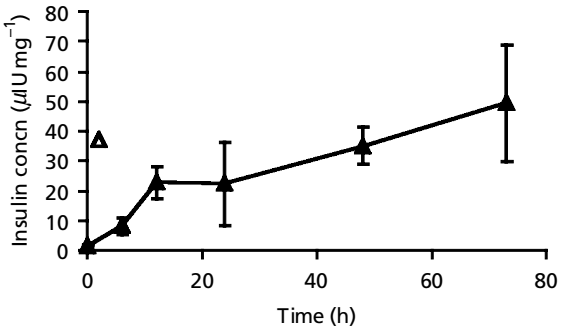

Figure 2 Time-dependent transdermal absorption of insulin into inguinal lymph nodes. Diabetic Sprague-Dawley rats were treated with a single transdermal patch containing insulin in biphasic vesicles (10 mg recombinant human) or subcutaneous insulin (1 mg recombinant human). At specific times over the next 73 h, at each time point 4 rats were terminated and the inguinal lymph nodes removed. The lymph nodes were analysed by ELISA from transdermal (▲) or subcutaneous (△) treatment groups of diabetic rats. The results are expressed as the mean ± s.e.m.

After 48 h of treatment, the skin contained high levels of insulin, 35.1 ± 9.3 mIU mg^{-1} (mean ± s.e.m., n = 3) (results not shown), approximately 1750 times higher than the insulin serum steady state ($P < 0.005$) and 1000 times higher than the level of insulin in the inguinal lymph nodes at the same time (48 h, $P < 0.02$). Thus the insulin could be pooled in the skin, potentially acting as an insulin reservoir, before being slowly delivered to the systemic circulation via the lymph drainage. Whether the pool of insulin is free insulin, biphasic vesicle-associated insulin or biphasic-vesicle-encapsulated insulin, as postulated in Figure 3, remains to be determined. Irrespective of this, the insulin depot could then deliver insulin for some time to ensure a constant supply of at least basal levels of insulin. A similar system has been proposed by Radwanski et al (1998) using interleukin-10 (IL-10), where the first rapid appearance of IL-10 in the plasma after subcutaneous treatment was attributed to the direct delivery to the blood and the second delayed and prolonged delivery was due to the uptake of IL-10 in the lymph before systemic delivery. Whether the successful delivery of insulin requires the breakdown of the formulation for absorption into the vasculature or it enters the lymphatics directly is uncertain. Theoretically, the lymph could take up the insulin in any form, encapsulated, hexameric or free monomeric.

Although our studies clearly demonstrate the entry of insulin into the lymph drainage, the relative amounts of insulin in the lymph versus the systemic circulation is harder to gauge. Indeed, the inguinal lymph nodes may not account for all the insulin — some of the transdermal patch area may drain directly into the axillary lymph nodes, thus the level of insulin in the lymph may be underestimated.

Mechanism considerations

The active form of insulin is the monomeric form (5.6 kDa). In solution, as the concentration of insulin increases so does the dimeric (11.2 kDa) and hexameric forms of insulin (33.6 kDa). Insulin is encapsulated into biphasic vesicles using an aqueous phase containing high starting concentration of insulin resulting in the major form of insulin in the formulation likely to be the 33.6 kDa hexameric insulin and is likely to be a mixture: fully encapsulated insulin; partially encapsulated insulin or insulin associated with the vesicle surface; and soluble insulin or free insulin. From the results, and the knowledge of insulin action, a possible mechanism of systemic insulin absorption may be postulated (Figure 3).

Free insulin is unlikely to penetrate into the skin itself, so it would have a negligible role in the blood glucose response. Partially encapsulated insulin, or insulin adhering to the outer surface of the vesicles, would potentially

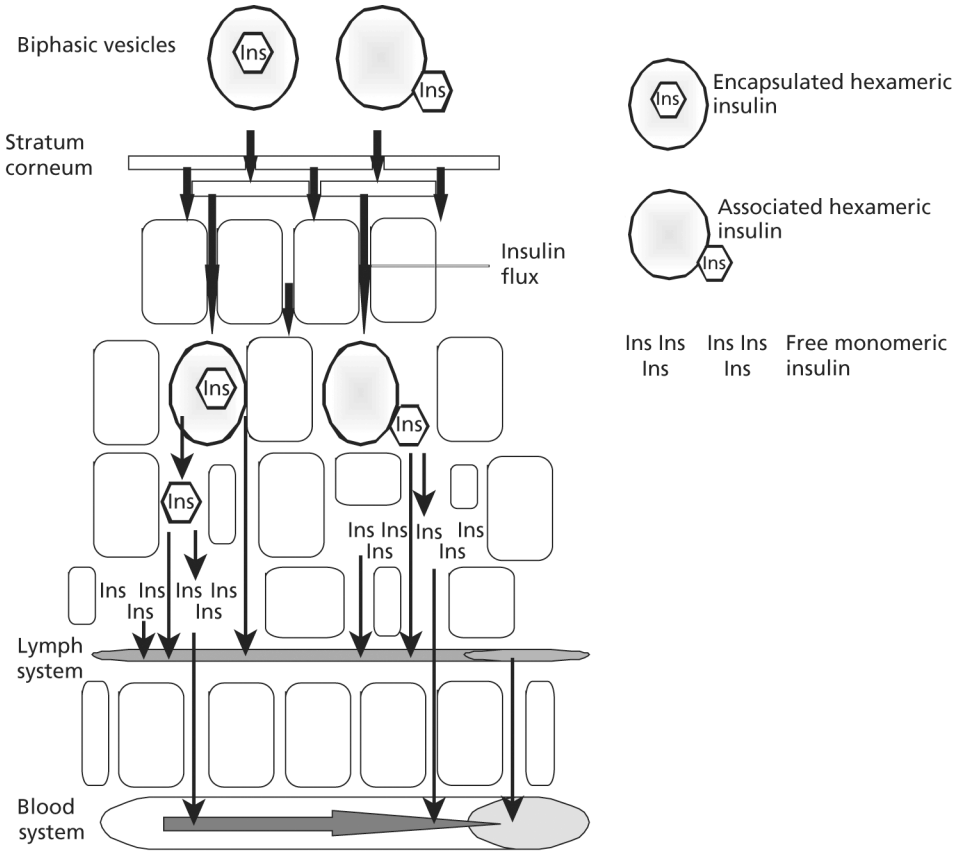

Figure 3 Postulated mechanism for biphasic-vesicle-mediated insulin delivery. Two possible mechanisms (blood circulation vs lymphatic circulation) are shown for the transdermal delivery of insulin: the direct systemic delivery of insulin after the disruption of the formulation and dissociation of the hexameric insulin; or the involvement of the lymph drainage. Biphasic vesicles, or fragments of vesicles, may either directly enter the lymph, or disrupt in the skin releasing the hexameric insulin to be taken directly into the lymph or again dissociation of the insulin hexamer before entrance into the lymph or systemic circulation.

require only the dissociation of the hexameric insulin, thus the potential availability (or potential response time) of the insulin for the blood glucose response should be more rapid. Encapsulated insulin should be somewhat retarded in its blood glucose response as it would require the dissociation, or release, of the insulin from the biphasic vesicles as well as dissociation from the hexameric form. Thus the early insulin responses could be due to insulin associated with the vesicles, absorbed through the stratum corneum and dissociating into monomers or dimers to be able to enter the systemic circulation directly. Vesicle- or vesicle-fragment-associated insulin from intercellular locations would presumably have to enter the lymph drainage before the systemic circulation and thus constitute the sustained phase of the blood glucose response. Several other transdermal delivery attempts using electrically driven methods, such as iontophoresis or ultrasound, resulted in the delivery of glucose-responsive serum insulin levels (20–50 μIU mL^{-1}) within 0.5–1 h after initiating the treatment, although the effect usually only lasts for a few hours (Tachibana 1992; Golomb et al 1993; Mitragotri et al 1995). Another delivery-system-based approach (e.g. Transfersulin applied in a non-occlusive manner) was shown to decrease blood glucose by 20–30% in mice within 2–4 h of application and by 15% within 6–8 h in man (Cevc et al 1998). Delivery by Transfersulin was described to occur through a mechanism related to the vesicles deforming and permeating through pores formed in the skin due to hydration gradient (Cevc et al 1998).

The delivery of insulin through the lymphatics after topical administration using the biphasic vesicles indicates a mechanism distinct from previous systems. The relatively short lag-response period of 2–8 h for the biphasic vesicles could be acceptable in practice but it still may be the combination of time required for: delivery through the stratum corneum barrier; a prerequisite breakdown of the vesicles and release of insulin in the skin, before the systemic uptake; and the uptake of the vesicles by the lymphatic system before the delivery of insulin to the systemic circulation.

Conclusions

Insulin administered subcutaneously or intramuscularly appears to be directly delivered into the systemic circulation,

with the lymphatic drainage accounting for 20% or less (Binder 1969; Supersaxo et al 1990). Here we showed, for the first time, that insulin, delivered through the skin using topically applied biphasic vesicles, utilizes the lymphatic pathway and the lymphatic drainage appears to be greater then after subcutaneous injection. The actual mechanism whereby the formulated insulin enters the skin remains to be determined.

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