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Insulin Detemir is Not Transported Across the Blood-Brain Barrier

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Abstract

Insulin detemir has a different profile of action on the central nervous system (CNS) than human insulin. It has been hypothesized that this is caused by an altered ability of insulin detemir to cross the blood-brain barrier (BBB). Here, we measured the permeability of the BBB to insulin detemir. We labeled insulin detemir with radioactive iodine (I-Det) and examined its ability to cross the BBB of the mouse. Permeation was assessed after intravenous injection and by brain perfusion in the presence or absence of excess insulin detemir. The ability of insulin detemir to inhibit human insulin transport across the BBB was also assessed. I-Det did not cross the BBB either after intravenous injection or when studied by brain perfusion, a method which removes or reduces the influence of circulating proteins. Unlabeled detemir was about 10 times less potent than human insulin at inhibiting the transport of radioactive human insulin across the BBB. The altered CNS profile of insulin detemir may be caused by its poor access to CNS receptors and by a block of human insulin from crossing the BBB.

Keywords

Blood-brain barrier; insulin; peptide; brain

Introduction

Insulin detemir is modified human insulin in that the threonine in position B30 has been deleted and the amino acid at B29 has been acylated with the C14 fatty acid myristic acid. These modifications facilitate oligomerization and binding to albumin which slows

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absorption from the site of injection and prolongs half-life in blood [18]. As a result, insulin detemir has a prolonged hypoglycemic action, an effect mediated through peripheral tissue insulin receptors.

Insulins also have effects on the central nervous system (CNS). These effects can be mediated through receptors in peripheral tissues, as exemplified by the hypoglycemic induction of hunger, seizures, and altered consciousness. But insulin also induces many effects through CNS receptors, such as its effects on brain development, cognition, and feeding [12,20,21,23,28]. The CNS clearly contains insulin, yet produces little or no insulin [34]. Therefore, CNS insulin is derived from the circulation. To enter the CNS, insulin must negotiate the blood-brain barrier (BBB). Insulin crosses the BBB largely because of the presence of a blood-to-brain saturable transport system [3,10,35]. This transport system is itself regulated or influenced by nitric oxide, inflammatory events, hibernation, glucose, triglycerides, obesity, and the diabetic state independent of glucose [2,4,13,22,25,33].

Most insulins in current therapeutic use are modified structurally or in regard to their vehicle compositions so that the duration of their peripheral action on glucose is altered[24]. What effect these modifications have on the access of these insulins to the CNS or on their CNS actions is seldom addressed. Since many of the actions proposed for insulin in the CNS affect parameters important in the treatment of diabetes, including glycemic control, body weight, and cognition, the question of whether and to what degree the various insulins enter the CNS is of great importance.

The effects of insulin detemir on the CNS differ from those of human insulin. Insulin detemir produces less hypoglycemia and less weight gain [17] apparently through dissociated mechanisms [14]. Although insulin detemir when compared to human insulin produces a similar time course of phosphorylation of peripheral insulin receptors, insulin signaling in brain tissues occurs sooner and is more robust [19]. Electroencephalographic activity is also increased with insulin detemir in comparison to human insulin in both mice [19] and humans [31]. Hypoglycemic thresholds and severity for adrenergic, neuroglycopenic, and cognitive symptoms have been reported to differ for insulin detemir [27,32]. These findings are consistent with an altered penetration of detemir across the BBB[19]. Here, we used state-of-the-art pharmacokinetic techniques to examine the ability of insulin detemir to cross the BBB of the mouse.

Research Design and Methods

Radioactive Labeling and Purifying

Recombinant insulin detemir obtained from a 3 ml disposable cartridge (Novo Nordisk, Inc, Princeton, NJ) was first purified of the other substances (zinc, mannitol, phenol, and disodium phosphate dihydrate) in the solution by adding 10 microl (140 microg) it to a G-10 Sephadex column and eluting in 100 microl fractions with phosphate buffer. Four fractions (400 microl) after the void volume were collected and combined which contained the detemir at an approximate concentration of 350 microg/ml. From this purified material, 20 microl (7 microg) was mixed with 2 mCi of ^{131}I and 40 microl of 0.2 M phosphate buffer (pH 7.5). Reaction was initiated by adding and mixing 10 microl of chloramine-T at a concentration of 0.25 M in phosphate buffer (pH 7.5). After 1 min reaction time, the mixture was added to a column of G-10 Sephadex and the purified radioactively labeled detemir (I-Det) eluted with phosphate buffer and 100 microl fractions collected.

Five microg of human insulin (Sigma Chemical Co, St. Louis, MO) was radioactively labeled with ^{131}I by mixing it with chloramine-T for 60 sec. Radioactive insulin (I-Ins) was purified on a column of G-10 Sephadex, eluting with phosphate buffer.

Acid Precipitation of Brain and Blood

Male ICR mice from our in-house colony (VA Medical Center-St. Louis) were anesthetized with urethane and the right jugular vein and left carotid artery exposed. All animal studies were done under protocols approved by our IACUC and in an AAALAC approved facility. Mice were given injections into the jugular vein of 300,000 cpm of I-Det in 0.2 ml of lactated Ringer's solution containing 1% bovine serum albumin (LR-BSA). Blood was obtained from a nick in the carotid artery, the mouse decapitated, and the brain harvested at 10, 30, or 60 min after the iv injection. Blood was centrifuged at 4500 g for 15 min, 50 microl of serum mixed into 0.25 ml of LR-BSA, and 0.25 ml of 30% trifluoroacetic acid (TCA) added and mixed. This solution was centrifuged for 15 min at 4500g and the supernatant (S) and pellet (P) separated and each counted in a gamma counter. Results were expressed as the percent of radioactivity which could be precipitated with acid (%Precip):

$$\%Precip = 100(P)/(S+P).$$

Brains were added to a hand-held glass homogenizer, 3 ml of LR-BSA added, and the brains homogenized with 12 strokes of the pestle. The homogenate was centrifuged at 4500g for min, the supernatant and pellet separated and counted, and %Precip calculated.

To correct for degradation that occurred during processing, I-Det was added to the bottom of a test tube into which arterial serum was collected or added to the bottom of a glass homogenizer to which a brain was added, the serum and brain being from an animal which had not received I-Det. These were processed as described for the biological samples and %Precip calculated. The values for %Precip from the biological samples was corrected by dividing them by the %Precip values for the processing controls and multiplying by 100 to yield the corrected value for %Precip (%Precip-c).

Determination of Peripheral Pharmacokinetics and Unidirectional Influx Rate into Brain After IV Injection

Male ICR mice anesthetized with urethane had their right jugular and left carotid artery exposed. At t = 0, 0.2 ml LR-BSA containing 300,000 cpm of I-Det was injected into the jugular vein. At either 2, 3, 4, 5, 7.5, 10, 15, 20, 25, or 30 min, the carotid artery was severed in a mouse, its arterial blood collected, and the mouse immediately decapitated. The arterial blood was centrifuged at 4500 g for 15 min and the level of radioactivity measured in 50 microl of serum.

The percent of the injected dose of I-Det present in a ml of serum (%Inj/ml) was calculated and its log value plotted against time. Regression analysis was used to compute the slope and intercept. The inverse of the slope was multiplied by 0.301 to yield the half-time clearance and the inverse of the antilog of the intercept was multiplied by 100 to give the initial volume of distribution in the body (Vd).

The brain was weighed and its level of radioactivity determined. The results were expressed as brain/serum ratios in units of microl/g and plotted against exposure time (Expt) in units of minutes:

$$Expt = [\int_0^t C_p(\tau) d\tau] / C_{pt},$$

where t is time and C_{pt} is the concentration in serum at time t. The slope of the line for the linear portion of the relation between brain/serum ratios and Expt measures the

unidirectional influx rate (K_i) in units of microl/g-brain and the intercept of that line measures the initial volume of distribution in brain (V_i) in units of microl/g.

Competition Determined by IV Injection

Male mice were anesthetized with urethane and the right jugular vein and left carotid artery exposed. Mice received an iv injection of 0.2 ml of LR-BSA containing 300,000 cpm/mouse of I-Ins. Some injections also contained either 1 microg/mouse of human insulin, 1 microg/mouse of detimer insulin, or 10 microg/mouse of detimer insulin. 10 min after the iv injection, carotid artery blood was obtained and the mouse decapitated. The levels of radioactivity in the arterial serum and whole brain were determined and the results expressed as brain/serum ratios in units of microl/g.

Unidirectional Influx Rate and Saturable Transport Determined by Brain Perfusion

Male CD-1 mice were anesthetized with ip urethane (4.0 g/kg). The thorax was opened, the heart was exposed, both jugulars severed, and the descending thoracic aorta clamped. A 26 gauge butterfly needle was inserted into the left ventricle of the heart and modified Zlokovic's [36] buffer (7.19 g/L NaCl, 0.3 g/L KCl, 0.28 g/L CaCl₂, 2.1 g/L NaHCO₃, 0.16 g/L KH₂PO₄, 0.17 g/L anhydrous MgCl₂, 0.99 g/L D-Glucose, and 10 g/L bovine serum albumin added the day of perfusion) containing I-Det (10)⁵ cpm/ml was infused at a rate of 2 ml/minute for 1–5 minutes [29]. The Zlokovic buffer was modified in that the erythrocytes were not added. The exact cpm infused was determined on a 10 microl aliquot of perfusion fluid. At the end of the perfusion time, the brain was removed and brain/perfusion ratios calculated. The unidirectional influx rate (K_i) was calculated by regressing the brain/perfusion ratio against perfusion time.

Other mice had 1 microg/ml of unlabeled insulin detemir or human insulin included in the perfusion buffer. These mice were studied after 2.5 min of brain perfusion/vascular washout and the results expressed as brain/perfusion ratios.

Statistics

Means are reported with their standard error terms and n. Two means were compared by Student's t-test. More than two means were first compared by analysis of variance followed by Newman-Keul's multiple comparison test. Regression lines were computed by the least squares method and compared with the program in Prism 5.0 (GraphPad, Inc., San Diego, CA).

Results

Table I shows the values for acid precipitation in brain and blood samples. These results have been corrected for processing controls which showed that 95% of brain and 96% of serum radioactivity precipitated with TCA. Because of significant degradation in the brain at 60 min, subsequent calculations for brain and blood were limited to the data collected up to and including 30 min.

Figure 1 shows the clearance of I-Det from blood after its iv injection. The relation between log(% Inj/ml) and time was statistically significant: $r = 0.583$, $n = 38$, $p < 0.001$. The half-time disappearance calculated from the slope was 64 min and the V_d was 1.47 ml.

Figure 2 shows the relation between brain/serum ratios and Expt time as calculated for I-Det by multiple-regression analysis. The results showed no significant correlation between brain/serum ratios and Expt, indicating no measurable blood-to-brain transport ($n = 36$).

Figure 3 examines the ability of I-Det to inhibit the BBB transport of insulin. ANOVA showed a significant overall effect ($n = 5-6/\text{group}$): $F(3,20) = 7.10$, $p < 0.005$. Newman-Keuls multiple comparison test showed that 1 microg/mouse of insulin and 10 microg/mouse of insulin detemir, but not 1 microg/mouse of insulin detemir, produced statistically significant ($p < 0.05$) inhibition of I-Ins transport across the BBB.

Figure 4A shows the results for brain perfusion of I-Det. There was no significant change in brain/perfusion ratios over time, indicating no measurable brain transport ($n = 17$). Figure 4B shows that neither insulin detemir nor human insulin had an effect on the brain/perfusion ratios of I-Det ($n = 12$ controls, 6 insulin detemir, 6 human insulin).

Discussion

The overall findings here show that insulin detemir does not readily cross the BBB. This is surprising in that most articles considering the BBB transport of insulin detemir have assumed that it would cross better than human insulin. This, in part, is because it was thought that the free fatty acid chain would increase lipid solubility and hence transmembrane diffusion across the BBB. Physicochemical characteristics, especially lipid solubility, can play an important role in determining the extent to which peptides cross the BBB [5]. A similar theory was advanced for ghrelin, which is acylated with octanol, but the presence of saturable transporters for ghrelin dominate its ability to cross the BBB [9]. Similarly, human insulin readily crosses the BBB not by virtue of its physicochemical characteristics, but because of a saturable transporter located at the BBB [3,11,34]. Blood-to-brain transporters typically increase the brain uptake rate of a substance by about 10–40 fold [15]. The lack of uptake of insulin detemir, therefore, probably is more related to an inability to be taken up by the insulin transporter located at the BBB than to its physicochemical characteristics.

Several factors are likely to contribute to the lack of insulin detemir transport across the BBB. First, its avid binding to serum albumin reduces the amount of free insulin detemir in the circulation; as a rule, free peptide crosses the BBB more readily than bound [6,8]. However, the brain perfusion studies found that insulin detemir does not cross the BBB when studied by this method, even though the concentration of albumin is much lower than in serum and there are no other serum proteins in the perfusion buffer. Therefore, protein binding may not be the sole reason for the lack of insulin detemir transport. There is evidence that another factor is at play, which is that insulin detemir is not well recognized by the insulin transporter. This is indicated in figure 3 where it is shown that it takes some 10 times more insulin detemir than human insulin to inhibit the BBB transport of radioactive insulin. This compares to about 3 times more insulin detemir being required to lower glucose, with 1 mg of insulin detemir possessing about 7 units of activity whereas 1 mg of insulin possesses about 24 units. This difference between insulin detemir and insulin is not based on molarity as their molecular weights are very similar at 5917 Da (insulin detemir) and 5808 (insulin). This relative dissociation between receptor sensitivity and transporter activity calls into question the long held assumption that it is the insulin receptor coupled to transcytotic machinery that is responsible for insulin transport across the BBB [16].

Our findings of poor penetration of insulin detemir across the BBB at first seem to contradict the findings of Hennige et al [19] who found a peak value of about 6 ng/ml in brain homogenate 2 min after the iv injection of 2 U/kg into mice. However, those studies did not correct for the vascular space of the brain, which constitutes about 1% of the brain by weight. Assuming a mouse of 20 g body weight, a volume of distribution in blood as measured here of 1.47 ml for iv injected insulin detemir, an average brain weight of about 450 mg, and that Hennige et al homogenized the brains in a volume of 1 ml, one would

calculate that 2 U/kg of iv detemir insulin would produce a brain homogenate containing about 12 ng/ml just from blood contamination. That only half that amount was found suggests that extraction of the insulin detemir from brain was less than 100%.

The finding that insulin detemir does not cross the BBB suggests that its altered CNS profile may be caused by a lack of stimulation of CNS receptors rather than because of an overstimulation of them. Additionally, as figure 3 shows that high doses of insulin detemir can block insulin transport, it may be that high therapeutic doses of insulin detemir may interfere with the ability of other insulins to cross the BBB and so further reduce CNS insulin activity. Insulin detemir may also be exerting its effects on the CNS by acting at circumventricular organs on vagal afferents as has been shown to occur with amylin and cholecystokinin [26,30].

These studies examined the ability of insulin detemir to rapidly cross the BBB. Studies conducted in this manner for human insulin have shown that it crosses rapidly and into most regions of the brain [3,7]. We did not examine brain regions and it is possible that insulin detemir might access some small regions of the brain. Furthermore, human insulin has a short half-life in the circulation, whereas the half-life of insulin detemir is much longer. Substances with the pharmacokinetic characteristics of insulin detemir, notably a small volume of distribution and long half-life in blood, can eventually access the CNS by way of the extracellular pathways [1]. However, the steady state level reached in the CNS is low, typically similar to that of serum albumin, and such substances likely have to be very potent to exert a CNS effect.

In conclusion, we found that insulin detemir does not readily cross the BBB. This was not just caused by protein binding but by a low level of recognition of insulin detemir by the BBB's insulin transporter. Although insulin detemir did not cross the BBB, at high doses it blocked the ability of human insulin to cross the BBB. We conclude that the altered CNS profile of insulin detemir is likely caused by its inability to activate CNS receptors or even to block other insulins from accessing the CNS.

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Figure 1.

Clearance of Intravenously Administered I-Det from Blood. Pharmacokinetic parameters were calculated for the relation between $\log(\%Inj/ml)$ vs time. Calculations based on the slope yielded a half-time clearance of 64 min and based on the Y-intercept yielded an initial volume of distribution of 1.47 ml.

Figure 2.
Calculation of Unidirectional Influx Rate Based on Multiple-time Regression Analysis.
There was no correlation between brain/serum ratios and Expt, indicating that there was no measurable transport of I-Det across the BBB.

Figure 3.

Inhibition of I-Ins Transport Across the BBB. A dose of 1 microg/mouse of human insulin inhibited transport, whereas 10 microg/mouse, but not 1 microg/mouse, of insulin detemir was needed to inhibit transport.

Figure 4.

Upper Panel: Time Curve for Brain Perfusion. The lack of a statistically significant relation between brain/perfusion ratios and time indicates no measurable transport of I-Det across the BBB. Lower Panel: Inhibition of I-Det Uptake. Neither unlabeled insulin detemir nor human insulin at 1 microg/ml affected the brain/perfusion ratio of I-Det.

Table I

Percent of Radioactivity in Brain or Serum Which Precipitated with TCA. Values are means $\pm$ SE of n = 2/group.

	10 min	30 min	60 min
Serum	102 $\pm$ 0.1	101 $\pm$ 0.2	101 $\pm$ 0.4
Brain	100 $\pm$ 0.6	81.5 $\pm$ 5.5	48.2 $\pm$ 10.7