A Direct In Vitro Demonstration of Insulin Binding to Isolated Brain Microvessels

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SUMMARY

Recent in vivo autoradiographic studies have suggested that circulating insulin may bind to the capillary wall, i.e., the blood-brain barrier. In the present study the blood-brain barrier insulin receptor was examined directly by measuring [^{125}I]-iodoinsulin binding to capillaries isolated from fresh bovine cerebral cortex. Microvessels were prepared by gentle hand homogenization and trapping on nylon mesh. The binding was rapid, specific, and reversible with one-half maximal binding attained in 7 min and maximal binding achieved in 45 min at room temperature. The high affinity site has an affinity constant of 2.3 ± 0.3 nM^{-1}, and 50% displacement of [^{125}I]-iodoinsulin occurred at approximately 9 ng/ml. [^{125}I]-Iodoinsulin was not displaced by excess thyrotropin, prolactin, or growth hormone, and proinsulin was much less potent than porcine insulin. These studies confirm the presence of a specific insulin receptor on brain microvessels. Although insulin does not cross the blood-brain barrier, the presence of an insulin receptor provides a possible mechanism by which blood-borne insulin can influence brain cell metabolism. DIABETES *30*:757–761, September 1981.

The brain is not generally considered to be an insulin-dependent tissue, but recent studies have shown specific receptors for insulin in the central nervous system (CNS) of a variety of species.[1,2] Moreover, Van Houten and Posner have presented autoradiographic evidence that insulin injected via the left cardiac ventricle in rats binds to microvessels in the CNS[3] and also to nerve endings terminating in the circumventricular organs.[4] In the present investigation, a pure microvessel preparation is isolated from bovine cerebral cortex and [^{125}I]-iodoinsulin binding to these microvessels is demonstrated to be rapid, specific, and reversible.

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Received for publication 20 October 1980 and in revised form 28 May 1981.

MATERIALS AND METHODS

Porcine crystalline insulin and proinsulin were generously supplied by Dr. Mary Root of Eli Lilly Co. (Indianapolis, Indiana). [^{125}I]-Iodoinsulin (150–250 μCi/μg) was prepared by the chloramine-T method as modified by Freychet et al.[5] Bovine serum albumin (BSA) was Pentex Fraction V from Miles Biochemical Corporation (Elkhart, Indiana). Human growth hormone, thyroid-stimulating hormone, and prolactin were gifts from the National Institutes of Health. Protein determinations were performed by the method of Lowry.[6]

Isolation of bovine brain capillaries. Capillaries from fresh bovine brain were prepared by a modification of the method of Goldstein.[7] Fresh brains obtained from a local slaughter house were immediately placed in oxygenated Krebs-Ringer bicarbonate (KRB) with 10 mM Hepes buffer, pH 7.4, for transport to the laboratory. The pia arachnoid membrane was stripped off and approximately 40 g of cortical shell and underlying white matter were removed and diluted 1:10 (w/v) with iced-cold Krebs-Ringer buffer containing 1% bovine serum albumin (BSA). The material was then gently hand homogenized with 12 strokes in a teflon-glass homogenizer, specially milled to give 0.25-mm clearance. The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet resuspended in 25% BSA buffer. After centrifugation at 1000 × *g* for 10 min the myelin, which floats to the top, was removed. The pellet was resuspended in 1% BSA Krebs-Ringer and filtered through a 210-μm nylon mesh to separate capillaries from networks and larger vessels. Then the filtrate was passed over a 1.2 × 1.5-cm column containing 0.25-mm glass beads. The capillaries stick to the beads, but the cellular debris (mainly nuclei and red blood cells) pass through. Capillaries were removed from the glass bead by gentle agitation and separated by allowing the beads to settle and then decanting the supernatant. Finally, the capillaries are collected on a 44-μm nylon mesh and washed into a plastic tube. All subsequent steps were carried out in plastic containers to prevent sticking of the capillaries to vessel walls. To prepare the cells for assay, they were centrifuged at 50 × *g* for 5 min at 4°C and gently resuspended in 0.1% albumin KRB buffer to a final concen-

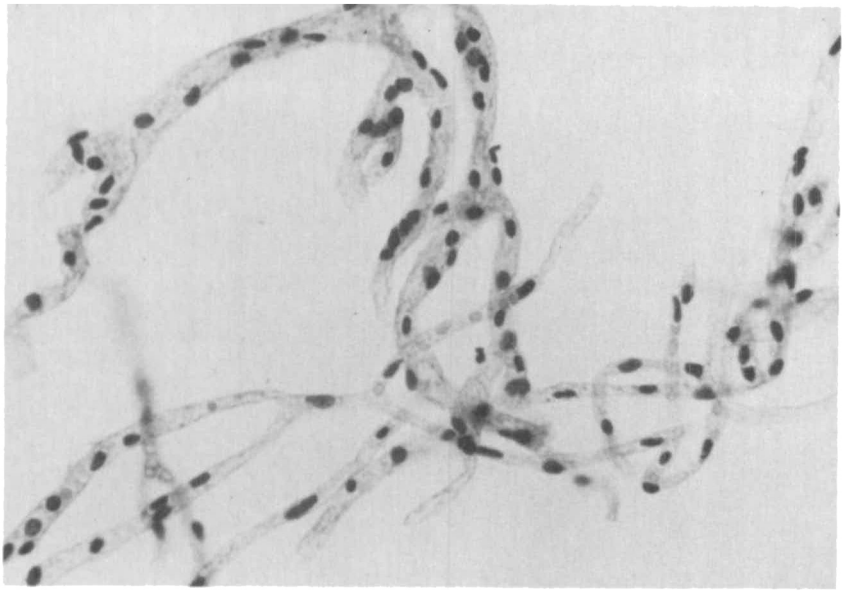


FIGURE 1. Photomicrograph of isolated bovine brain capillaries prepared by the method outlined in the text. This is an o-toluidine blue dye that stains the nuclei darkly. The other, lighter colored round objects are red blood cells trapped in the lumen.

tration of 500–800 μg protein/ml buffer. The final yield was approximately 1 mg of protein from 10 g of brain cortex. The purity of the preparations was monitored by phase contrast light microscopy. The only contaminating cell types seen were rare red blood cells that remained trapped within the capillaries (see micrograph, Figure 1).

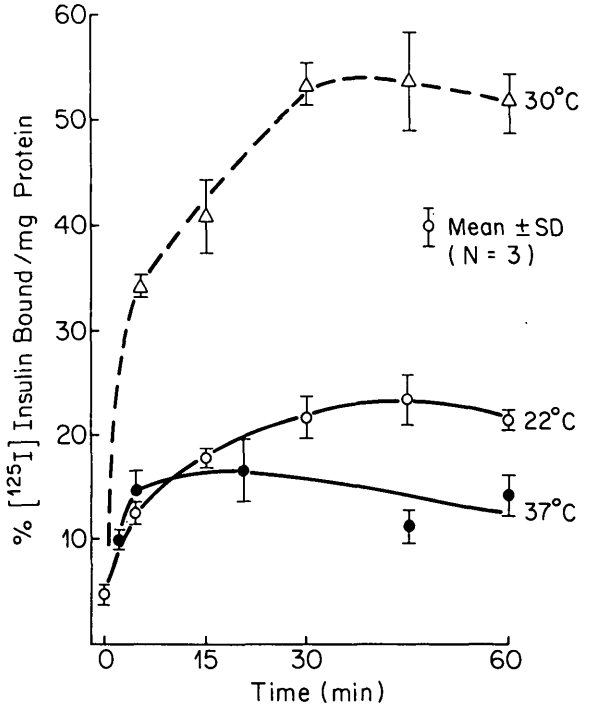
Recent studies reported by Williams et al.[8] and confirmed by us indicate that brain capillaries isolated by mechanical homogenization techniques are not viable on the basis of trypan blue dye exclusion tests. However, other investigators have shown that isolated brain microvessels demonstrate linear rates of glucose oxidation for at least 90 min of incubation,[9] actively transport potassium,[9] have almost identical amino acid transport K_m values as that observed in vivo,[10] and have high affinity catecholamine receptors.[11] Therefore, while the "metabolic viability" of the brain microvessel isolated by mechanical homogenization techniques is uncertain, this preparation maintains many cellular functions of the brain capillary that can be investigated in vitro.

Binding assay. For time course experiments, capillaries were suspended in a polypropylene Erlenmeyer flask at a final concentration of 500–800 μg protein/ml. The reaction was started by the addition of [^{125}I]-iodoinsulin in a final concentration of 0.3 ng/ml (or 0.05 μCi/ml). A parallel flask for determination of nonspecific binding was prepared identically except for the addition of 100 μg/ml of unlabeled insulin. All incubations were carried out at room temperature except as indicated. The flasks were gassed with 95% O_2/5% CO_2 at the beginning of the experiment and after each aliquot was removed. The capillaries were maintained in suspension by periodic shaking. Aliquots (0.4 ml) were withdrawn at 0, 5, 15, 30, 45, and 60 min, placed in iced microfuge tubes, and spun for 1 min at 10,000 × *g*. The supernatants were aspirated and saved, the pellet drained, and the tips cut off into counting tubes. After counting, the pellets were solubilized in 0.1 N NaOH at 50°C for 2 h, and then assayed for protein by the method of Lowry.[6]

The competitive displacement experiments were carried out in 5-ml plastic tubes containing 50 μl of [^{125}I]-iodoinsulin plus 50 μl of the appropriate dilution of unlabeled insulin. The incubation was started with the addition of 400-μl aliquots of capillaries. Incubations were carried out for 45 min at room temperature and terminated as described above. Unlabeled insulin was added in concentrations of 0, 1, 2, 10, 20,, 40, 100, 200, 1000, and 10^5 (nonspecific binding) ng/ml. Other hormones were added in place of insulin as shown in Figure 2.

Insulin degradation was monitored by precipitation in 10% TCA.[12] In all cases, the insulin was greater than 95% precipitable after incubation for 45 min at room temperature.

FIGURE 2. Time course of [^{125}I]-Iodoinsulin binding to brain capillaries at 22°C, 30°C, and 37°C. Steady-state binding is achieved in 45 min and one-half maximal binding in approximately 7 min at 22°C. Binding is most rapid at 37°C and most active at 30°C. Each point represents the mean ±SD (N = 3).



Binding was calculated as the percent of counts bound. Nonspecific binding was defined as binding in the presence of 100 μg/ml unlabeled insulin and was subtracted from the total binding to yield the specific binding. The nonspecific binding ranged between 12% and 25% of the total binding. Efforts to reduce this figure by spinning through oil, increasing the albumin in the incubation media, and changing the capillary concentration were not successful. Part of the nonspecific binding simply represented entrapment of extracellular isotope in the capillary pellet. In some studies, ^{3}H-inulin, an extracellular space marker, was added to the incubation medium. The average percent inulin radioactivity retained by the capillary pellet was 3.62 ± 1.09%/mg protein (mean ± SD, N = 18).

Scatchard plots of the competitive binding data were analyzed for the equilibrium binding association constants and the receptor site number assuming a two-site model, by an iterative process for nonlinear regression on an IBM 360 computer. All results were corrected per milligram protein.

RESULTS

Figure 2 shows the time course of [^{125}I]-iodoinsulin specific binding to isolated brain capillaries at room temperature (22°C), 30°C, and 37°C. At 22°C the binding is rapid, reaching one-half maximal at 7 min and peaking at 45 min. The maximum specific binding of 28%/mg protein is approximately equivalent to that for liver plasma membranes.[12] At 30°C the specific binding is higher than at 22°C and appears to reach steady state within 30 min. In binding studies in other cell types, some authors have reported higher binding at the lower temperature.[13] At 37°C the maximum binding was reduced to 16.7%/mg protein, but it achieved this peak in only 5 min.

The optimum pH was 7.6 (data not shown), similar to the alkaline pH optimum characteristic for insulin binding.[13] The specificity of the [^{125}I]-iodoinsulin binding for bovine brain capillaries is shown in the competitive binding curve of Figure 3. Half-maximal displacement occurs at approximately 9 ng/ml (1.7 nM). Prolactin and TSH show no displacement at molar concentrations of 30 and 2000 times tracer insulin concentrations, respectively. Human growth hormone displaces only minimally at concentrations 3 orders of magnitude above trace concentrations of insulin. The displacement curve for porcine proinsulin is shifted several orders of magnitude to the right, indicating that proinsulin is only about 4% as potent as insulin on a molar basis in its ability to displace [^{125}I]-iodoinsulin.

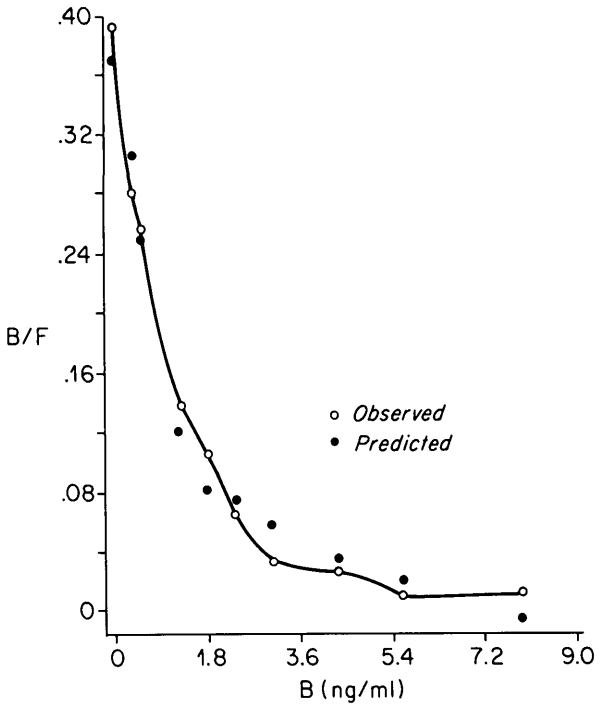


FIGURE 4. Scatchard plot of insulin binding to isolated brain capillaries. Open circles are the observed values and closed circles are the values predicted by nonlinear regression analysis based on Feldman's equation for two binding sites.[14] This analysis yields the following values:

High affinity, low capacity
K_a = 2.29 nM^{-1}
Capacity = 1.1 × 10^{11} sites/mg protein

Low affinity, high capacity
K_a = 0.05 nM^{-1}
Capacity = 8.4 × 10^{11} sites/mg protein

R_o = 9.5 × 10^{11} sites/mg protein

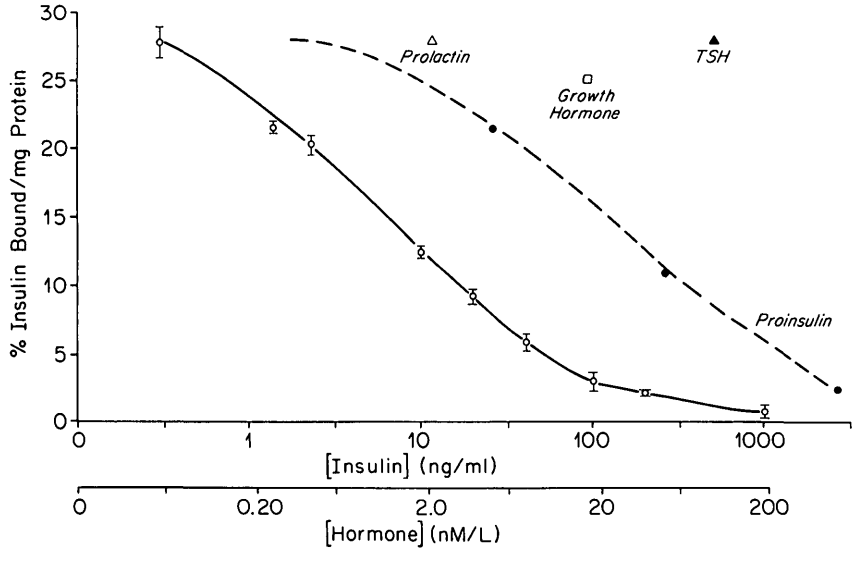


FIGURE 3. Competition binding curve of percent [^{125}I]-iodoinsulin specifically bound versus unlabeled hormone at 22°C. Each point represents the mean ± SEM (N = 3). A 50% decrease in binding is achieved at approximately 9 ng/ml (1.7 nM). Prolactin, TSH, and growth hormone do not compete with [^{125}I]-iodoinsulin for binding to the receptor; proinsulin competes poorly and is only 4% as active as insulin in displacing the [^{125}I]-iodoinsulin.

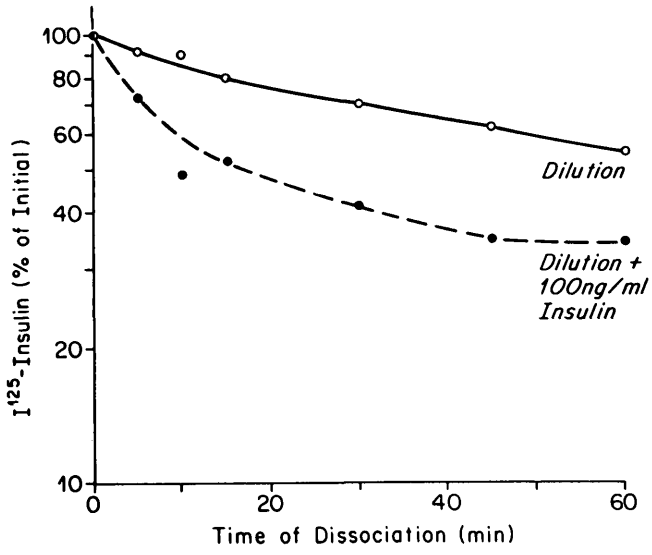


FIGURE 5. Time course of dissociation of [^{125}I]-Iodoinsulin previously associated with steady-state binding. Capillaries are then washed at 4°C and diluted (30-fold) into fresh media containing no insulin (open circles, solid line) or 100 ng/ml unlabeled insulin (closed circles, dashed line). Data represent the average of three experiments and are plotted as percent of initial insulin bound at beginning of dissociation experiment. Unlabeled insulin enhances the initial dissociation rate.

When the competitive binding results are replotted in the form of a Scatchard plot (Figure 4), a curvilinear, concave upward curve is produced. This curve can be fitted, assuming a two-site model by the equations of Feldman[14] and analyzed for the affinity constants, K_a, and receptor site number or capacity for each site. The high affinity site has a K_a of 2.3 ± 0.3 nM^{-1} and a capacity of 0.18 ± 0.05 pmol/mg protein; the low affinity, high capacity site has a K_a of 0.05 ± 0.02 nM^{-1} and a capacity of 1.4 ± 0.2 pmol/mg protein. The observed values are indicated by open circles and the predicted values by closed circles.

Figure 5 demonstrates the reversibility of the binding in a dissociation experiment. The capillaries were incubated to equilibrium (45 min at room temperature), then washed rapidly at 4°C and allowed to dissociate in fresh media by dilution alone or dilution in the presence of 100 ng/ml of unlabeled insulin. It can be seen that the initial rate of dissociation is enhanced by unlabeled insulin and then the curve becomes parallel to the dilution only curve. These results are in accord with the DeMeyts' model of negatively cooperative homeotropic receptors for insulin binding.[15]

DISCUSSION

There is now evidence that insulin may function in at least three different levels in brain: as a local neurotransmitter; as a neurotransducer, e.g., where circulating insulin may interact with cells of a putative satiety center in the hypothalamus; and at the microvessels which comprise the blood-brain barrier.

Havrankova et al.[2] first demonstrated that insulin receptors are widely distributed within the brain substance and further showed that insulin itself could be found in rat brain at concentrations up to 10-fold above plasma insulin levels.[16] These findings implied that insulin could act as a neurotransmitter within brain. However, Eng and Yalow[17] have recently reported that brain insulin levels in the dog and rabbit are only approximately equal to plasma insulin concentration. Van Houten and Posner[3] have presented autoradiographic evidence that [^{125}I]-iodoinsulin injected into the left cardiac ventricle binds widely to blood-brain microvessels that make up the blood-brain barrier. Our results support this conclusion and raise the argument that the high concentrations of insulin found in brain merely reflect endothelial receptor-bound insulin and do not imply synthesis of insulin by brain cells.

A rough calculation using our binding data and assuming a 50% occupancy of the high affinity receptor shows that the brain capillary receptors are capable of binding approximately 2–3 ng of insulin per gram of brain.* This value is intermediate between the concentrations reported by Eng and Yalow[17] and the higher levels observed by Havrankova et al.[16] However, the findings that insulin receptors can be found on cells cultured from neonatal rat brain that have minimal endothelial elements,[18] and the reports from Pacold and Blackard[19] and from Van Houten and Posner[4,20,21] of insulin binding and uptake by nerve endings terminating in the circumventricular organs (small regions of brain that lack a blood-brain barrier[22]) are compelling evidence that brain insulin does not originate simply from sequestration of circulating insulin by endothelial receptors. That is, insulin bound to brain capillaries may comprise only a portion of the total insulin in brain. This conclusion is further supported by the finding that plasma insulin levels can vary independently of brain insulin content.[23]

Our results now provide a direct demonstration of insulin binding to isolated brain capillaries, the blood-brain barrier. The binding is rapid, specific for insulin, and reversible. At room temperature, one-half maximal binding is attained in 7 min, and binding reaches steady state by 45 min. The reason for the relatively high proportion of nonspecific binding, approximately 25% of steady-state binding, is unclear, but it is interesting that this is approximately the same figure (30%) that Van Houten and Posner reported for nonspecific binding in their in vivo autoradiographic studies. The maximum specific binding of trace [^{125}I]-iodoinsulin, 28% per mg protein, is relatively high, but similar to that reported for liver membrane.[13] TSH, growth hormone, prolactin, and proinsulin interfere only minimally with the binding of [^{125}I]-iodoinsulin, demonstrating the specificity of the receptor for insulin.

When the competitive binding curve is replotted in the form of a Scatchard plot and analyzed assuming a two-site model, the high and low affinity binding constants are 2.3 and 0.05 mM^{-1}, respectively, and the capacities are 0.18 and 1.4 pmol/mg protein. The product of the binding capacity and affinity constant for the high and low affinity sites suggested that at 1 mg/ml of capillary protein, the bound/free ratio would be 0.41 from the high affinity site and 0.07 from the low affinity site.

The characteristics of insulin binding to this preparation are similar in many respects to the binding to human endothelial cells.[24] In human cells (at 20°C) cultured from umbili-

* Brain contains approximately 50 mg capillaries/g[8] or 5 mg capillary protein/g brain, assuming that the capillaries are 10% protein. If the high affinity receptor is 50% occupied, then 0.09 pmol of insulin is bound/mg capillary protein or about 0.5 pmol insulin bound to capillaries/g brain tissue. This would be equivalent to approximately 2–3 ng insulin/g brain.

cal veins, binding reached a maximum at approximately 60 min (cf. 45 min of brain capillaries), one-half maximal displacement was at ~6 ng/ml (cf~9 ng/ml), there was very little degradation of [^{125}I]-iodoinsulin, and the nonspecific binding, approximately 22%, was also similar.[24]

Goodner and Berrie have shown that insulin does not cross the blood-brain barrier,[25] yet circulating insulin is believed to have actions on the CNS. While insulin has been shown not to modulate glucose transport into brain,[26] Debons et al.[27] have shown that blood-borne insulin may alter appetite drive at a putative satiety center in brain in the vicinity of the circumventricular organs. In addition Daniel et al. have shown that insulin can influence net glucose uptake and glycogen formation by brain.[28] The presence of the blood-brain barrier insulin receptor provides a possible mechanism by which circulating insulin may modulate brain cell function without the peptide crossing the blood-brain barrier.[29] Presumably insulin binding at the blood-brain barrier results in the generation of metabolic signals in the brain endothelial cell, which are then transmitted to brain cells. These hypothetical pathways may be examined in future studies using the isolated brain capillary preparation.[29]

ACKNOWLEDGMENTS

This research was supported in part by USPHS grants AM-27301, NS-17701 and by American Diabetes Association Southern California Affiliate, Inc., and the UCLA Academic Senate. Dr. Frank is the recipient of a USPHS New Investigator Research Award and Dr. Pardridge of Research Career Development Award AM-00783. We would like to acknowledge the excellent technical assistance of Larry Mietus and Andrew Oppenberg and to thank Charlotte Limberg for her help in preparing this manuscript.

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