Enhanced Insulin Binding to Blood-Brain Barrier In Vivo and to Brain Microvessels In Vitro in Newborn Rabbits

H. J. L. FRANK, T. JANKOVIC-VOKES, W. M. PARDRIDGE, AND W. L. MORRIS

SUMMARY

Insulin is a known growth factor in nonneural tissue, and recent studies have shown that there are insulin receptors throughout the adult and fetal central nervous system. Since insulin has only limited access to the adult brain, this study was undertaken to determine if insulin has increased availability to the newborn brain where it may act as a neonatal brain growth promotor. In vivo brain uptake of ^{125}I-insulin after a single-pass carotid injection was measured in newborn, 3-wk-old and 11-wk-old (adult) rabbits. The brain uptake index (BUI) relative to a ^{3}HOH reference was 22.0 ± 1.1% (mean ± SEM) for newborn, 12.8 ± 0.6% for 3-wk-old, and 6.5 ± 0.1% for adults. Specific ^{125}I-insulin binding to isolated cerebral microvessels was similarly increased in the newborn (60.6 ± 3.3 %/mg protein) compared with the 3-wk-old (23.8 ± 1.7) and adult animals (13.6 ± 1.9). Scatchard analysis revealed that the difference was due to an increase in receptor number with only minimal changes in the affinity. The increased availability of circulating insulin to the newborn brain was further corroborated by elevated CSF/serum and brain/serum insulin ratios in the newborn versus adult. These results suggest that insulin has increased access to the newborn brain where it may function as a growth factor. DIABETES 1985; 34:728–33.

Insulin may be a growth factor for nonneuronal tissues as evidenced both by its stimulation of in vivo fetal growth[1] and its promotion of cell proliferation in tissue culture.[2–5] Although the importance of insulin in growth and development of the central nervous system (CNS) is unknown, recent reports suggest that insulin and its receptors are widely distributed throughout the CNS[6–9] and it does influence developing brain cells,[2–5] including the regulation of neuronal maturation.[10] Insulin has been shown to be sufficient and, in lieu of serum, necessary for the survival of peripheral neurons in culture.[2] Insulin is also known to stimulate synthesis of nucleic acids and proteins in cultured rat brain cells[3] and ornithine decarboxylase activity (the rate limiting enzyme of polyamine biosynthesis) both in vitro, in cell cultures from embryonic chick brains,[4] and in vivo in neonatal rat brains.[5] Moreover, Sara et al. have recently demonstrated the presence of insulin and insulin-like growth factor receptors in particulate fragments from human fetal brain.[11] The fetal brain insulin binding sites showed both higher affinity and receptor number than the same authors had previously reported for adult human brain.[12]

These findings suggest that insulin plays a role in the growth and development of the nervous system. However, since the access of insulin to the adult brain is limited,[13–15] if insulin is to have an effect on brain development its availability to the brain in the newborn should be significantly higher than in the adult.

In the present investigation the brain uptake index (BUI) of insulin, insulin binding to isolated brain microvessels, and brain, serum, and CSF insulin levels have been determined in newborn, 3-wk-old weanling, and 11-wk-old adult rabbits.

MATERIALS AND METHODS

Porcine crystalline insulin was generously supplied by Dr. Mary Root of Eli Lilly and Company (Indianapolis, Indiana), ^{125}I-iodoinsulin (100–150 μCi/μg) was prepared by the chloramine-T method as modified by Freychet et al.,[16] and ^{3}H-methoxyinulin and ^{3}HOH were purchased from New England Nuclear (Boston, Massachusetts).

Animals. New Zealand white rabbits were used for all studies. Newborn rabbits were used immediately upon delivery from the vendor (Irish Farms, Norco, California) and were less than 24 h old. The 3-wk-old and 11-wk-old animals were either used immediately or housed from 1 to 7 days and maintained on ad libitum water and Purina rabbit chow.

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From the UCLA Medical Center, Department of Medicine/Endocrinology, Los Angeles, California (H.J.L.F., W.M.P., W.L.M.); and the University of Chicago, Department of Medicine, Chicago, Illinois (T.J.-V.).

Address reprint requests to H. J. L. Frank, M.D., UCLA Medical Center, Department of Medicine/Endocrinology, Los Angeles, California 90024.

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Measurement of brain uptake index (BUI). New Zealand white rabbits were anesthetized by i.m. injection with ketamine (100 mg/kg) and Acetpromazine (1 mg/kg). The common carotid artery was surgically exposed and cannulated with a 30-gauge needle for newborn and a 27-gauge needle for older rabbits. The needle did not occlude the vessel, assuring free arterial flow throughout the procedure. The injection mixture contained approximately 5 μCi/ml of ^{125}I-labeled insulin and 50 μCi/ml of ^{3}H-labeled reference substance. Two reference substances were employed: (1) ^{3}HOH, which is a freely diffusible internal reference, and (2) ^{3}H-methoxyinulin. Inulin was chosen because its molecular weight (5000 daltons) is similar to that of insulin (6000 daltons), thus excluding the possibility that increased uptake of insulin could be explained by "leakiness" of the blood-brain barrier (BBB) and also because as a poorly diffusible extracellular space marker[17] it was more suitable as a reference for the uptake of insulin, which also is poorly diffusible. ^{14}C-inulin and ^{14}C-sucrose (both from New England Nuclear) were also used as extracellular space markers in some experiments.

The injection mixture was delivered as a rapid bolus and the animal was decapitated after 15 s, sufficient time for a single passage through the brain.[18] The ipsilateral hemisphere was then rapidly removed, homogenized by passage through an 18-gauge needle, dissolved in soluene (Packard Instruments), and simultaneously counted for ^{125}I and ^{3}H in Riafluor (New England Nuclear) in a Beckman LS-1800 liquid scintillation counter, as reported previously.[19] An aliquot of the injection mixture was also counted and the brain uptake index (BUI) was calculated from the following equation:

$$\text{BUI} = \frac{(\text{DPM } ^{125}\text{I-insulin/DPM } ^{3}\text{H-reference [brain]})}{(\text{DPM } ^{125}\text{I-insulin/DPM } ^{3}\text{H-reference [mixture]})} \times 100\%^{20}$$

The BUI is thus equivalent to the fractional extraction of ^{125}I-insulin relative to the extraction of ^{3}H-reference.[20]

Isolation of cerebral microvessels. Cerebral microvessels were isolated from the brains of newborn, 3-wk-old and 11-wk-old rabbits by a modification of the method previously described for bovine brain.[21] These vessels have been characterized by several investigators as being metabolically active and possessing hormone receptors.[21-24] After decapitation, the brains are rapidly excised and the choroid plexus is peeled away. The cerebral cortex is then scraped away from the underlying white matter and gently hand-homogenized with a specially milled (0.25-mm clearance) teflon homogenizer: seven strokes for newborns and 13–15 strokes for older animals. The newborns require fewer strokes because the basement membrane is less well-formed than in the older animals[25] and the additional strokes would result in disruption of the capillaries. The capillaries are then separated by flotation on a 25% albumin gradient, filtered through 210 μm and 85 μm nylon mesh filters, and then filtered through glass beads to remove nuclei and RBCs. To prepare the capillaries for binding assays, they were removed from the glass beads by gentle agitation, centrifuged at 500 × *g* for 5 min, and resuspended in 0.1% BSA in Hepes-KRB buffer (10 mM, pH 7.4). The final concentration was 500–800 μg protein/ml buffer.

These preparations do not always exclude trypan blue, but they are metabolically active and have been studied with respect to glucose, lactate, and fatty acid metabolism in vitro.[25,26] They are composed of a mixture of endothelial cells and pericytes. The lumens are patant and allow the entry of microspheres.[24]

Binding assay. The competitive displacement experiments were carried out in 5-ml plastic tubes containing 50 μl of ^{125}I-insulin (0.5 ng/ml final concentration) plus 50 μl of the appropriate dilution of unlabeled insulin. The incubation was started with the addition of 400-μl aliquots of capillaries and was carried out at room temperature (22°C) for 45 min, sufficient time to reach equilibrium binding.[21] The capillaries were maintained in suspension by periodic shaking. After 45 min, 400-μl aliquots were withdrawn, placed in iced microfuge tubes, and centrifuged for 1 min at 10,000 × *g*. The supernatants were aspirated and an aliquot was saved to check insulin degradation by TCA precipitation. The pellets were dried by inverting the tubes on a paper towel and the tips containing the pellets were cut off into counting tubes containing 0.1 N NaOH for later protein determination by the method of Lowry.[27] All tubes were counted in a gamma counter (Nuclear Chicago).

Insulin degradation was monitored by precipitation of the postincubation supernatants in 10% TCA.[28] The ^{125}I-insulin counts were >90% precipitable and there was no difference in TCA precipitation between brain microvessels from rabbits of different ages. Unlabeled insulin was added in final concentrations of 0, 1, 3, 10, 30, 100, 200, 500, and 10^{5} ng/ml. Binding was calculated as percent of counts bound. Nonspecific binding was defined as binding in the presence of 100 μg/ml (10^{5} ng/ml) unlabeled insulin and was subtracted from the total binding to yield specific binding. All results were corrected per milligram protein.

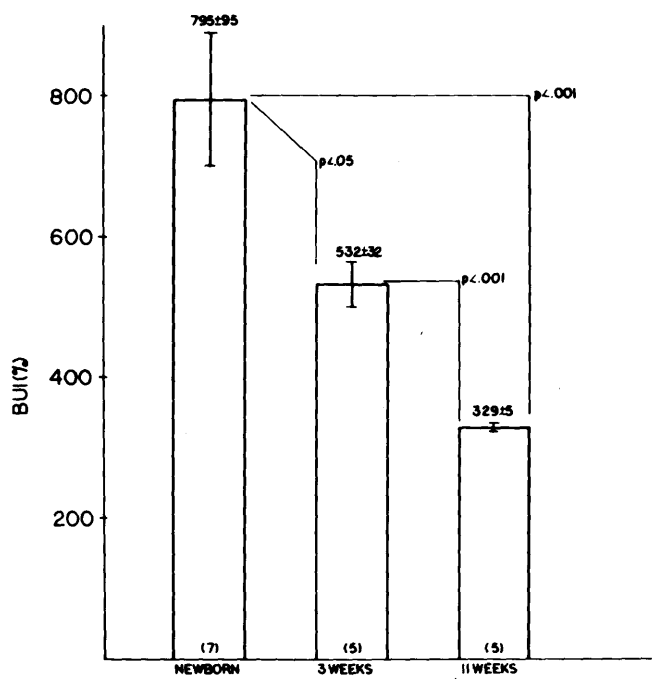

FIGURE 1. Brain uptake index (BUI) of ^{125}I-insulin with ^{3}H-inulin as a reference in newborn, 3-wk-old, and 11-wk-old rabbits. The BUI is calculated as described in the text. In this and all subsequent graphs, the results represent the mean ± SEM, with the number of determinations in parentheses for each age group. The BUI is highest for the newborn and decreases progressively with age.

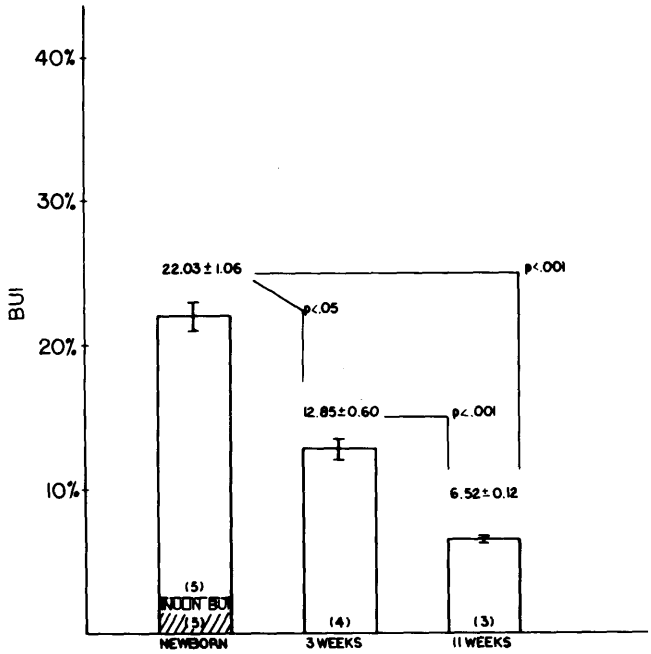

FIGURE 2. Brain uptake index (BUI) of ^{125}I-insulin with the highly diffusible ^{3}HOH as a reference. The crosshatched area in the newborn bar, which represents the uptake of ^{14}C-inulin relative to ^{3}HOH, is only 2.0 ± 0.3%. This value is the nonspecific uptake and it is below the ^{125}I-insulin uptake, thus demonstrating that the newborn blood-brain barrier is intact.

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 as previously described.[21,29]

Insulin RIA was done as reported by Baskin et al.[6] using porcine insulin standards. The high-affinity first antibody was a generous gift of Dr. Daniel Porte (Seattle, Washington) and was used at a 1:175,000 dilution. The assay could reliably measure 0.4 μU/ml of insulin in the sample using porcine insulin standards. CSF was obtained in anesthetized animals by making a 1-cm incision in the midline and then inserting a 27-gauge needle connected to polyethylene tubing into the subarachnoid space in the newborn or the cisterna magna in the 3-wk-old and adult rabbits. Only samples that contained < 100 RBCs/cc were accepted for analysis. Blood from the same animals was then obtained by cardiac puncture. All samples were frozen at −20°C until analysis.

To make the CSF samples approximately equal to the assay standards, they were adjusted to 0.5% protein by the addition of appropriate proportions of 0.1% or 1.0% bovine serum albumin in borate buffer at the first antibody step.[6] Glucose measurement was performed by the glucose-oxidase method using a Beckman glucose analyzer II.

Brain insulin levels were determined by extruding freshly isolated cortex through an 18-gauge needle and then homogenizing 1-g aliquots in 10 vol of 0.18 M HCl/75% ethanol in a polytron homogenizer. Between samples, the homogenizer was cleaned by two washings at full power for 30 s in fresh acid/alcohol. Control samples from each age group were spiked with 10,000 cpm of ^{125}I-insulin to determine the percent recovery through the isolation procedure. The brain samples were shaken overnight at 4°C, centrifuged at 30,000 × *g* for 20 min and then concentrated on Sep-Pak filters as described by Eng and Yalow.[30] After elution from the columns with 0.01 M HCl/75% ethanol, the samples were assayed as above using porcine insulin standards prepared in the same 0.01 M HCl/75% ethanol. The recovery of ^{125}I-insulin averaged 62% and the material was 95% TCA precipitable at the end of the procedure. Samples were stored at −20°C until assayed. Results were corrected for recovery and expressed in μU insulin/g wet wt of cortex.

RESULTS

Studies of brain uptake of insulin. The brain uptake of insulin was compared in rabbits of three different age groups: newborns, 3-wk-old (weanling) when many nutrition related metabolic changes occur, and 11-wk-old (adults). When the poorly diffusible ^{3}H-inulin was used as a reference (Figure 1), the BUI was 795 ± 95% in the newborn, 531 ± 32% in the 3-wk-old, and 329 ± 5% in the adult. That is, insulin was taken up into the brain approximately 8 times, 5 times, or 3 times as well as inulin in the newborn, 3-wk-old, and 11-wk-old animals, respectively. When ^{3}HOH was used as the reference in the same age group (Figure 2), the values were 22.0 ± 1.1% for newborn, 12.8 ± 0.6% for weanling, and 6.5 ± 0.1% for the adults.

To prove that the increased insulin uptake observed in the newborn animals was not due to nonspecific leakiness of the newborn blood-brain barrier (BBB), the BUI of ^{14}C-inulin relative to ^{3}HOH was determined (Figure 2). If the newborn BBB was leaky, one would predict a relatively large uptake of ^{14}C-inulin. In fact, the value for inulin was only 2.0 ± 0.3% (mean ± SEM, N = 5). In separate experiments in 4-wk-old rabbits, the BUI for ^{14}C-sucrose, another extracellular space marker, was 2.3 ± 0.1% (N = 3), not significantly different than the inulin space in the newborn.

Addition of excess unlabeled insulin (1.0 μg/ml) to the injection mixture decreased the BUI of ^{125}I-insulin relative to ^{3}HOH *from 26.2 ± 3.3% to 7.6 ± 1.2% in the newborn* (mean ± SEM, see Figure 3). This difference was highly sig-

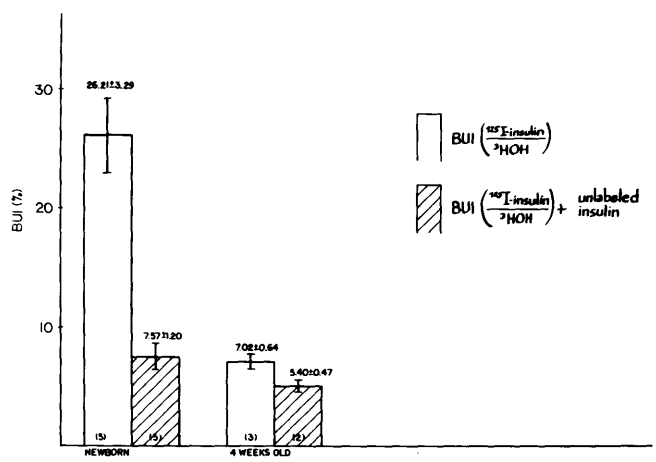

FIGURE 3. Brain uptake index of ^{125}I-insulin relative to ^{3}HOH in the presence (hatched bar) or absence (clear bars) of excess unlabeled insulin (1 μg/ml). The fact that the unlabeled insulin does not suppress the BUI for insulin to the inulin space (Figure 2) implies that there is a nonsaturable component to the insulin uptake.

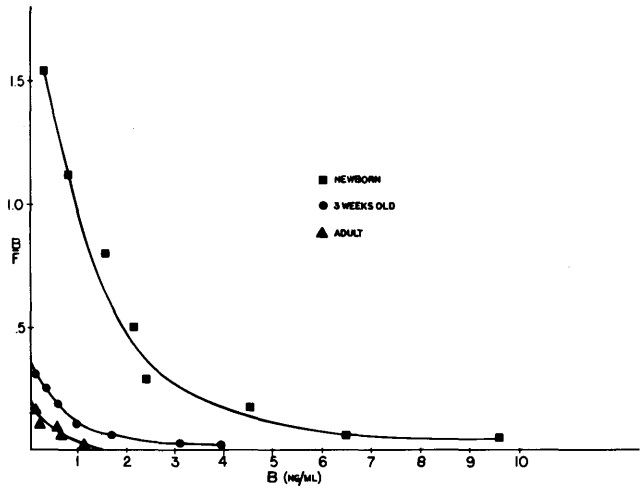

FIGURE 4. Scatchard plot of the binding data to isolated capillaries from newborn, weanling, and adult rabbits. The curves have approximately the same shape but differ in their intercept with the x-axis. Values for affinity constants and receptor numbers are summarized in Table 1.

nificant, $P < 0.01$. The BUI for insulin in the newborn was not only saturable, but also specific in the sense that glucagon and somatostatin did not compete with ^{125}I-insulin for brain uptake (data not shown). When the same experiment was done in 4-wk-old rabbits, there was some decrease in the BUI of ^{125}I-insulin by addition of unlabeled insulin, but the difference was not statistically significant (Figure 3).

Insulin binding to cerebral microvessels. To further investigate the nature of the increased BUI in newborn animals, ^{125}I-insulin binding to isolated cerebral microvessels was measured.

Competitive binding curves of ^{125}I-insulin binding to isolated brain capillaries from newborn, weanling (3-wk-old) and adult (11-wk-old) rabbits was performed. Nonspecific binding was 20–30% of total (maximal) binding. When these results were plotted in the form of a Scatchard plot (Figure 4), curvilinear, concave upward curves were produced. These curves could be fitted assuming a two-site model by the equations of Feldman[29] and analyzed for affinity constants K_a and receptor site number R_o or capacity for each site. The K_a and R_o (total number of receptors) values for the three groups studied are shown in Table 1. The major differences between the three groups is in the total number of receptors, which were highest for the newborns and lower for the other groups. The newborn rabbit brain capillaries had 1.4 times as many receptors as the 3-wk-old and 2.5 times as many as the adults.

Table 2 is a comparison of serum, CSF, and brain insulin levels in the three age groups. Since the newborn animals could not be fasted without risking dehydration, the values in all groups are from ad libitum-fed animals. The newborns had significantly lower serum glucose levels (99.6 ± 10.5 mg/dl versus 194.0 ± 17.7 for weanlings and 206.8 ± 11.5 for adults) and higher CSF and brain insulin levels. Although the adults tended to have higher values, the serum insulin levels were not significantly different in the three groups. The CSF/serum and brain/serum insulin ratios were highest in the newborn and lower in the 3-wk-old and adult animals.

DISCUSSION

Insulin may function as a growth factor or metabolic regulator of brain function, but it is generally acknowledged that insulin uptake by the adult brain, if it is present, is too slow to reliably measure by standard techniques.[13–15,31] Because of this, two methods were employed to address the question of whether the blood-brain barrier (BBB) has the ability to take up circulating insulin from the blood and thus potentially play a role in the movement of insulin from blood to brain. First, the BUI or brain uptake index of ^{125}I-insulin injected into the carotid artery of the rabbit was used as an in vivo measurement of the initial binding of insulin by brain capillaries. Second, ^{125}I-insulin binding to isolated brain capillaries was used as an in vitro measurement of binding by the BBB.

The brain uptake of insulin using a single-pass carotid injection technique was compared in newborn, weanling, and adult rabbits. The results demonstrated that the BUI of insulin was about three times higher in the newborn than the adult, and declined progressively with age. Due to the short duration of the BUI measurement, only 15 s (sufficient time for a single pass of the injected bolus[18]), it is likely that the increased BUI in the younger animals was due to higher initial binding of insulin to brain capillary endothelial cells rather than uptake or transport through the BBB. The increased BUI of insulin in the newborn brain was not attributable to a nonspecific "leakiness" of the newborn BBB, because the ^{14}C-inulin BUI was only 2.0%, similar to the 2.3% ^{14}C-sucrose space in the 4-wk-old rabbit and less than one-tenth of that for insulin (Figure 2). This verifies the findings of previous investigators who have shown that the permeability properties of the BBB are present at birth,[32,33] and is similar to the BUI for these substances reported by Oldendorf in the rat.[34] The fact that addition of unlabeled insulin did not decrease brain uptake of ^{125}I-insulin to the ^{14}C-inulin value of 2%, but only to 7.6%, suggests that part of that uptake in brain is nonsaturable and probably nonspecific. This component increased with age and was not reduced by the addition of free iodine in the injection mixture.

TABLE 1
Insulin binding to brain capillaries

Animals	K_1 (nM^{-1})	K_2 (nM^{-1})	C_1	C_2 (pmol/mg protein)	R_o
Newborn	2.90 ± 0.74	0.036 ± 0.028	0.32 ± 0.06	1.59 ± 0.06	1.81 ± 0.06
3-wk	1.87 ± 0.27	0.021 ± 0.017	0.17 ± 0.10	1.07 ± 0.45	1.24 ± 0.45
11-wk	2.30 ± 0.91	0.028 ± 0.011	0.14 ± 0.03	0.43 ± 0.13	0.57 ± 0.13

Results of affinity constants and capacities obtained from analysis of the Scatchard plots as described in the text. All values represent mean ± SEM of three separate experiments.

TABLE 2
Serum, brain, and CSF insulin levels

Age (N)	Brain insulin (μU/g)†	Serum insulin (μU/ml)	CSF insulin (μU/ml)	Insulin ratio (CSF/serum)	Insulin ratio (brain/serum)
Newborn (11)	17.5 ± 0.9*	26.2 ± 3.5	3.15 ± 0.21*	0.13 ± 0.02*	0.67
3-wk (5)	—	25.2 ± 6.0	2.29 ± 0.40	0.07 ± 0.02	—
Adult (10)	9.6 ± 3.4*	42.5 ± 7.1	1.77 ± 0.27	0.04 ± 0.004	0.22

Serum, brain, and CSF insulin values in newborn, 3-wk-old and 11-wk-old rabbits. The newborns have higher CSF insulin levels and higher CSF/serum and brain/serum insulin ratios than the older animals. All values represent mean ± SEM.
*$P < 0.001$ versus adult.
†Brain insulin levels are per gram wet weight of tissue.

Since the BUI measurements reflect events that occur during a single capillary transit of about 1 s, it is likely that the BUI reflects only binding of insulin to the luminal membrane of brain capillaries and not transport through the BBB. However, if insulin is transported across the BBB, binding to specific receptors on the luminal surface must be a first step of the transport process. Thus, BUI measurements may parallel the overall rate of insulin transport through the BBB, a process that may take many minutes to complete.[35]

The second technique, ^{125}I-insulin binding to isolated cerebral microvessels, was used in an in vitro attempt to define the functional mechanism responsible for the observed age-related differences in brain uptake. The insulin binding was highest in the newborn animals and decreased with age. Scatchard analysis of the binding data revealed that the major change was a reduction in the number of receptors with age (Table 1 and Figure 4). The isolated capillary preparation used in these studies has been partially characterized by the present authors[21] and others.[25,26] The preparations do not always exclude trypan blue,[21] but they are active with respect to glucose uptake and metabolism[26] and lactate and fatty acid uptake.[25]

The insulin binding to cerebral microvessels paralleled the BUI studies with the highest binding observed in newborns and a progressive decline with age (Figure 4 and Table 1). This may represent another part of the maturation process. Figure 5 shows the relationship between insulin binding to cerebral microvessels and brain uptake of insulin in the three age groups studied. There is a positive, nonlinear correlation between the two variables, implying that the decreased uptake parallels a decrease in brain capillary insulin binding.

If the increased brain uptake (BUI) and cerebral capillary binding of insulin in the newborn actually reflects an increased transport of insulin from the blood into the brain, one would predict higher CSF and brain insulin levels and, therefore, higher CSF/serum and brain/serum insulin ratios in the newborn than in the older animals. In Table 2 there is a comparison of CSF, brain, and serum insulin levels in newborn, 3-wk-old, and adult animals. It is clear from this experiment that not only are the CSF/serum and brain/serum insulin ratios higher in the newborn than in the adult ($P < 0.001$), but the absolute values of the CSF and brain insulin levels are also higher in the younger animals. This implies selective transport of insulin from blood to brain in newborn animals. The CSF insulin values in the 3-wk-old animals are not significantly different from the newborns or adults, but do lie between the two other age groups. The serum insulin values tended to be higher in the adult animals than in the newborns, but these differences were not statistically significant.

Amtorp and Saunders have pointed out that elevated CSF proteins in neonates may result from a decreased production rate of CSF and, hence, a relative decrease in bulk flow of peptides and proteins out of CSF.[36,37] Thus, the elevated CSF/serum ratio of insulin in the newborn may reflect decreased exodus from CSF rather than enhanced influx. Whereas CSF levels of insulin reflect insulin transport through the choroid plexus and the CSF outflow tracts, brain insulin levels reflect either de novo synthesis of insulin or transport of the peptide through the BBB.[38] Therefore, assuming de novo synthesis of insulin in brain, if it occurs, is not increased in the newborn, the increased brain/serum insulin ratios in the newborn brain (Table 2) corroborate the BUI and microvessel insulin binding results. Taken together, these diverse studies all favor the proposal that BBB transport of insulin is increased in the newborn.

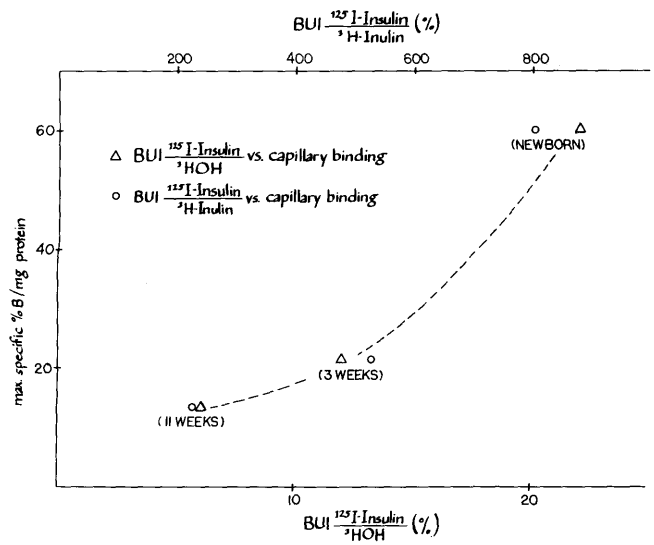

FIGURE 5. Maximum percent ^{125}I-insulin binding to brain capillaries versus brain uptake index for ^{125}I-insulin relative to ^{3}HOH (triangles, bottom scale) or ^{3}H-inulin (circles, top scale). There is a nonlinear relationship between the capillary binding and BUI in the younger compared with the older animals.

The increased binding observed in newborn brain microvessels may be part of a general phenomenon of increased insulin binding that has been previously reported for many tissues from newborns[39–42] and young red blood cells.[43] While it is possible that the large number of newborn insulin receptors in a consequence of the immaturity of the receptor, it is more likely, from a teleologic perspective, that the developing fetus and newborn require the continued anabolic effects of insulin for fuel storage and organ maturation and growth. The functional significance of this phenomenon in endothelial cells is unclear. However, our observation that increased insulin binding to brain microvessels in vitro parallels increased brain uptake of insulin in vivo leads to speculation that increased binding of insulin to microvessels may enable more insulin to be transported across the capillaries to underlying tissues, as proposed by Bar et al. for cardiac vascular endothelia[44] and by Jialal et al. for large vessel endothelium.[45] This may be true not only for insulin, but possibly for other insulin-like growth factors that have not yet been investigated.

Note added in proof. While this manuscript was being reviewed, an article by J. F. Haskell, E. Meezan, and D. J. Pillion (Am. J. Physiol. 1985; 248:E115–25) appeared, which showed that neonatal porcine cerebral microvessels have a greater number of insulin receptors/unit protein than the adult.

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