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VARYING BRAIN INSULIN CONCENTRATIONS DIFFERENTIALLY REGULATE THE FETAL BRAIN INSULIN RECEPTOR+

Sherin U. Devaskar*, Lynn Karycki and Uday P. Devaskar

Division of Neonatal-Perinatal Medicine,
Department of Pediatrics/Adolescent Medicine,
St. Louis University School of Medicine
and the Pediatric Research Institute,
Cardinal Glennon Children's Hospital,
1465 So. Grand Blvd. St. Louis, MO 63104

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We investigated the downregulating effect of varying states (physiologic and pharmacologic) of systemic and intracranial hyperinsulinism on the 28 to 30 day fetal rabbit brain insulin receptor.

Alloxan-induced maternal diabetes (n=5) produced mild fetal hyperinsulinemia (D) (plasma insulin concentrations = 59.80 ± 8.10 μU/ml, control = 26.25 ± 3.70; $p < 0.01$), whereas systemic administration (IMI) of 1.0 U (n=4) and 2.0 U (n=4) of insulin to the fetus resulted in moderate (103.13 ± 34.63 μU/ml) and severe (288.3 ± 51 μU/ml) fetal hyperinsulinemia respectively. All three states of systemic hyperinsulinemia neither altered the fetal brain insulin content nor the brain insulin receptor number and affinity. 0.01 U (n=4) of intracranial insulin administration (ICI) increased the brain insulin content four-fold ($p < 0.01$) but did not alter the brain insulin receptor number or affinity. 0.1 (n=5) and 2.0 U (n=7) of intracranial insulin increased the brain insulin content to supraphysiologic concentrations ($p < 0.01$) and decreased the fetal brain insulin receptor number ($p < 0.01$), the affinity remaining constant. We conclude that 1) regardless of the ability of insulin to cross the blood brain barrier, the downregulation of the brain insulin receptor is insulin dose-dependent and 2) the downregulation of the fetal brain insulin receptor is not a physiologic but a pharmacologic effect of insulin.

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*To whom reprint requests should be addressed.

Abbreviations: HI, Hyperinsulinemia; BPM, brain plasma membrane; D, fetus of the alloxan-diabetic mother; C, 30 day control; ICI, intracranial insulin; ICS, intracranial saline; IMI, intramuscular insulin; IMS, intramuscular saline.

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Insulin receptors have been demonstrated in the fetal rat (1) and rabbit brain (2). The downregulating effect of insulin on these receptors is controversial. High circulating insulin concentrations in the adult rat (3,4) and neonatal rabbit (5) failed to downregulate the brain insulin receptors. It was therefore suggested that the blood-brain barrier was impermeable to plasma insulin. Direct administration of a pharmacologic dose of insulin downregulated the neonatal rabbit brain insulin receptor in vivo (5), whereas in vitro more physiologic doses of insulin failed to downregulate the fetal rat brain cell and neonatal rat neuronal cell insulin receptors (6,7). To sort out whether the downregulation of the brain insulin receptor is dependent on the permeability of the blood brain barrier to insulin or on the hormonal concentration that is in contact with the receptor, we studied the effect of varying levels (physiologic and pharmacologic) of systemic and intracranial hyperinsulinism on the fetal rabbit brain insulin receptor.

Methods

I. Animals: New Zealand White pregnant rabbits of known gestation (~31 days) were obtained from Isaacs Rabbitary and caged individually for 3 to 5 days prior to any surgical or chemical manipulation. The animals were arbitrarily assigned to three major groups. Two groups of rabbits underwent surgery while the remaining group was chemically rendered diabetic.

II. Experimental Design:

A. Systemic Hyperinsulinemia (HI):

1. Chronic studies (chemical diabetes):

a. Chronic Mild HI (D): (n=5) Pregnant does at day 14 to 16 of gestation received alloxan (Sigma Chemical Co.) at 110 mg/kg intravenously along with susphrine (1:200) at 0.005 ml/kg intramuscularly (Berlax Lab Inc). 6 to 8 hours later, a dose of susphrine was repeated. Subsequent to the administration of alloxan, 25% glucose water was provided for drinking over 24 hours. Starting at 48 hours following the alloxan treatment, maternal blood glucose was monitored. Animals with blood glucose greater than 400 mg/dl received 2 U of regular insulin and 2 U of a long acting (NPH) insulin once a day till their blood sugars were less than 400 mg/dl. Two of the five animals required insulin therapy. Arterial blood gas analysis revealed no acidosis. This group mimicked a state of mild prolonged HI.

b. Control group (C): (n=5) received an equal volume of saline instead of alloxan and susphrine at the same period of gestation. In addition they consumed 25% glucose water for the initial 24 hours.

The animals in these two sub-groups were killed on the 30 day of gestation.

2. Acute studies:

Surgery: Initially does underwent surgery on the 29 day of gestation in order to study the fetuses on the 30 day. Most of these animals aborted within 12-18 hours following surgery. Therefore surgery was performed at an earlier gestation (27 d) under ketamine (35 mg/kg), Rompun (5 mg/kg) and 1% lidocaine local anesthesia. Both uterine horns were exteriorized through an abdominal incision and the fetuses received insulin or saline via a Hamilton syringe through the uterine wall. The fetuses within the intact uterus were returned into the abdominal cavity and the incision closed. The animals recovered from the anesthesia within 3 to 5 hours and were eating and drinking normally thereafter.

a. Moderate HI (IMI - 1.0 U): (n=4) These fetuses received 1.0 U of NPH insulin IM in their hind limbs (8,9).

b. Severe HI (IMI - 2.0 U): (n=4) These fetuses received 2.0 U of NPH insulin IM in the hind limb.

These doses of insulin were administered to study the pharmacologic effect of systemic HI on the fetal brain insulin receptor. Administration of higher doses (3.0 or 4.0 units) resulted in fetal demise.

c. Control group (IMS): (n=5) These fetuses received 0.02 ml of saline intramuscularly.

All the fetuses in these three sub-groups were killed on the 28 day of gestation.

B. Intracranial hyperinsulinism (ICI):

Surgery was performed as described above and the fetuses received either insulin or saline through the anterior fontanelle directly into the cranium on the 27 day of gestation.

1. Acute studies:

a. Mild hyperinsulinism (ICI - 0.01 U): (n=4) These fetuses received 0.01 U of NPH insulin intracranially.

b. Moderate hyperinsulinism (ICI - 0.10 U): (n=5) These fetuses received 0.10 U of NPH insulin intracranially.

c. Severe hyperinsulinism (ICI - 2.0 U): (n=7) 2.0 U of NPH insulin was administered intracranially in this group of fetuses.

Different doses of insulin were administered to study the direct effects of a low dose versus a pharmacologic dose of insulin on the fetal brain insulin receptor.

d. Control group (ICI): (n=5) received 0.02 ml of saline intracranially.

All the fetuses in these four sub-groups were killed on day 28 of gestation.

III. Blood samples: Maternal and fetal blood was collected in chilled glass tubes with EDTA and aprotinin (1000 KIU/ml). The plasma was separated by centrifugation at 4°C. Insulin concentrations were determined by radioimmunoassay and glucose by the glucose oxidase method using the Yellow Springs Instrument 27A (2,5).

IV. Brain insulin content: Mirsky's technique (10) was employed for extracting insulin from the fetal brains and insulin content was determined by the method of Havrankova et al (3). To calculate the percent recovery of the extraction, ^{125}I-insulin was added to the brain homogenate as a marker. The percent recovery in our hands was 41.54 ± 2.14. The brain insulin content was determined by a specific radioimmunoassay. The sensitivity of the radioimmunoassay is 0.5 μU per tube. The specificity of the assay is as follows: cross reactivity with pro-insulin is 33% and with C-peptide 0.02%. The interassay coefficient of variation is 9.3% at the low end of the

standard curve and 8.0% at the high end. The intra-assay coefficient of variation is 9.9% (low values) and 7.2% (high values).

V. Brain plasma membrane preparation (BPM): Brain plasma membranes were prepared as described previously (5). Protein concentration was determined by the method of Lowry (11) and DNA content was measured by Zamenof's modification (12) of Burton's technique (13). 5'-nucleotidase activity in the brain homogenate and BPM was determined as previously described (14).

VI. ^{125}I-insulin binding assay: The ^{125}I-insulin binding assay was performed as described before (5). Binding was specific for insulin, the non-specific binding being less than 2% of the total radioactivity. Monoiodinated ^{125}I-insulin used as a ligand in the assay was prepared by the method of Sodoyez et al (15) to a specific activity of ~150 mCi/mg. Scatchard plots of the specific insulin binding data (total binding - non-specific binding) were used to determine total binding capacities (Ro) and mean association constants (Ke) for receptors (16). The curvilinear Scatchard plots were resolved into two components, a high affinity, low capacity (R_1) and a low affinity, high capacity (R_2). The association constant for these two components K_1 and K_2 respectively, were also calculated (17) using the program described by Thakur and Rodbard (18). The first five points on the Scatchard plots (insulin concentrations ranging from 10^{-10} to 250^{-10} M) were employed to calculate the number and affinity of the high affinity receptor sites. Binding capacities in moles per liter were converted into number of receptors per milligram of BPM protein. Receptor number was also expressed per microgram DNA.

Statistics

All data are represented as means ± SEM. Statistical significance between a treated and control group was determined by the Student's two-tailed "t"-test. One-way analysis of variance was employed when more than two groups were compared.

Results

Fetal body and brain weights were no different in all the treatment groups compared to their respective control groups. In addition brain protein and DNA content did not change in any of the treatment groups. 5'-nucleotidase enzyme activity in the BPM from the treatment and control groups was two-fold the enzyme activity in the whole brain homogenate.

Table 1 depicts the maternal and fetal plasma glucose, fetal insulin concentrations and fetal brain insulin content. Maternal alloxan therapy resulted in maternal hypoinsulinemia and hyperglycemia. This was associated with fetal hyperglycemia and a two-fold increase in fetal plasma insulin concentrations. Systemic administration of 1.0 U of insulin produced an eight-fold increase in fetal plasma insulin concentrations

TABLE 1: Plasma Glucose and Insulin Concentrations and Brain Insulin Content

Groups (n)	Plasma glucose (mg/dl)		Plasma insulin (μU/ml)		Fetal brain insulin content (ng/g)
D					
(n=5) $\bar{X}$	332.0***	274.50	3.25*	59.80**	10.18
SEM	20.7	27.20	0.25	8.10	0.89
C					
(n=5)	111.38	57.96***	8.00	26.25	11.77
	2.74	8.42	1.52	3.70	1.03
t-test	<0.001	<0.001	<0.02	<0.01	NS
IMI					
1 U	127.0	64.25	-	103.13*	4.68
(n=4)	16.20	6.09	-	34.63	0.78
2 U	102.0	34.3**	-	288.3***	4.58
(n=4)	3.21	3.94	-	51.0	0.64
IMS					
(n=5)	119.0	62.70	-	13.72	4.44
	13.5	6.87	-	1.60	2.39
p(ANOVA)	NS	<0.01		<0.01	NS
ICI					
0.01 U	139	69.0	-	36.5	16.06**
(n=4)	4.5	2.0	-	13.5	2.76
0.1 U	113	64.6	-	36.33	133.07**
(n=5)	1.5	2.64	-	8.10	35.76
2 U	103.50	26.83**	-	891.67**	5820.0**
(n=7)	2.50	9.73	-	180.47	1009.5
ICS					
(n=5)	118.0	77.0	-	22.25	3.74
	10.0	10.70	-	1.27	1.17
p(ANOVA)	NS	<0.01		<0.01	<0.01

t-test: p * < 0.05, ** < 0.01, *** < 0.001, when compared to the respective control. NS = Not significant

without an associated change in plasma glucose concentrations. 2.0 U of insulin administered systemically resulted in a 20-fold increase in fetal plasma insulin concentrations and concomitant hypoglycemia. Fetal brain insulin content doubled ($p < 0.01$) from the 28 day to the 30 day gestation during development. In all the three states of systemic hyperinsulinemia, brain insulin content remained the same as the controls. Intracranial administration of insulin (0.01 and 0.1 U) increased the brain insulin content four-fold and thirty six-fold respectively, but did not alter the

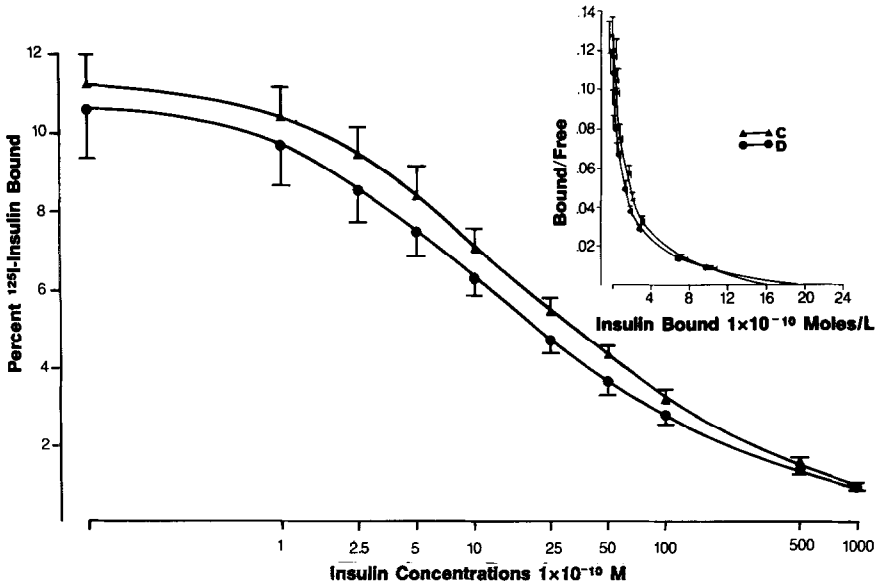

Figure 1: ^{125}I-insulin specific binding curves (Mean ± S.E.M.) of brain plasma membranes obtained from 30 day fetuses of alloxan-induced diabetic (D) and control (C) mothers. The inset represents the respective Scatchard plots (mean ± SEM). t-test = not significant.

plasma insulin concentrations. 2.0 U of insulin raised the fetal brain insulin content and plasma insulin concentrations to supraphysiologic concentrations as observed previously (5).

The ^{125}I-insulin binding curves of the various groups are shown in figures 1, 2 and 3. The concentration of unlabeled insulin that inhibits half the specific binding (ED_{50}), an approximate measure of affinity remained unchanged in all the treatment groups. The affinity remaining constant, a change in the percent ^{125}I-insulin binding is mainly due to a change in the receptor number. Curvilinear Scatchard plots shown in figures 1, 2 and 4, suggest either the presence of a heterogeneous receptor population or one class of sites exhibiting negative cooperativity. Using the two site model (15), R_1 and R_2 were identified and calculated. Although the calculated receptor number for the latter site may be an approximation, the change is the total receptor number (R_0) was in the same direction as the change in percent ^{125}I-insulin binding. The relative changes in receptor number in a particular treatment group (when compared

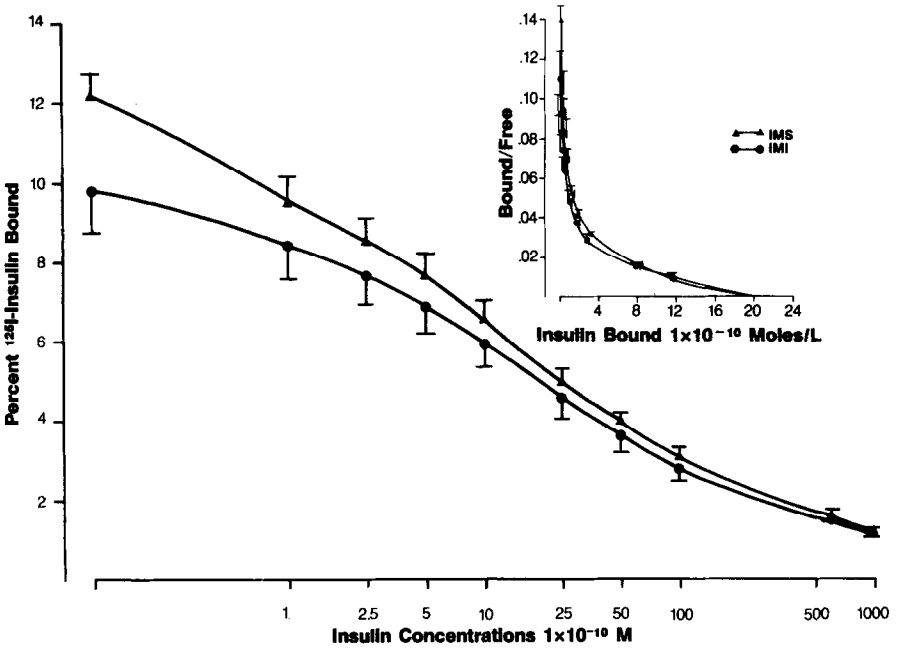

Figure 2: ^{125}I-insulin specific binding curves (mean ± SEM) of the intramuscular insulin (IMI - 1.0 and 2.0 U pooled) and saline (IMS) treated 28 day fetal brain plasma membranes. The inset demonstrates the respective Scatchard plots (mean ± SEM) t-test = not significant.

to the control) are useful in estimating the presence or absence of downregulation of the receptors.

The calculated number and affinity of the fetal brain insulin receptors are demonstrated in table 2.

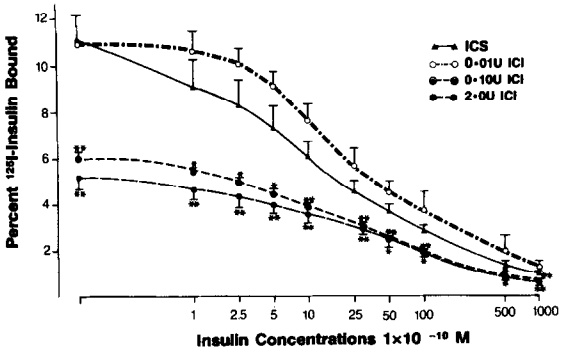

Figure 3: ^{125}I-insulin binding curves (mean ± SEM) of intracranial insulin (ICI - 0.01 U, 0.1 U and 2.0 U) and saline (ICS) treated 28 day fetal brain plasma membranes.
ICI - 0.01 U versus ICS = not significant
ICI - 0.1 U versus ICI-2.0 U = not signficant
ICI 0.1 U or 2.0 U versus ICS = p * <0.05, ** p <0.01

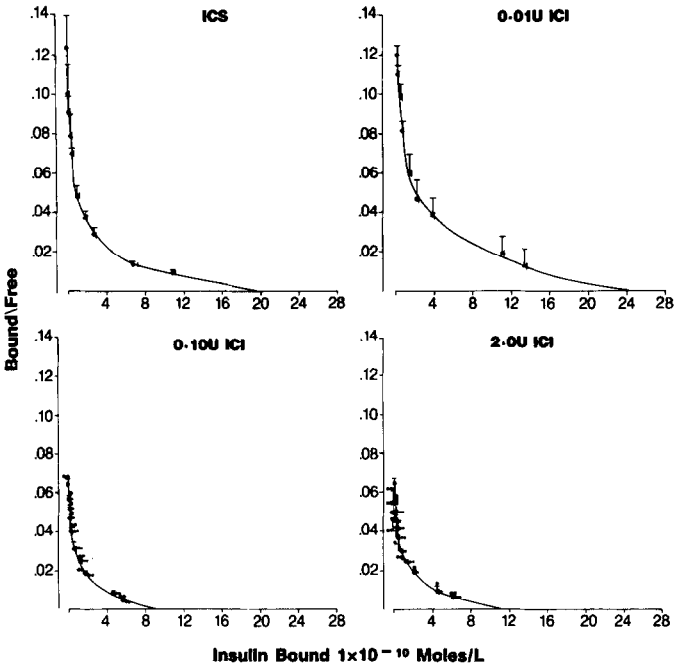

Figure 4: Scatchard plots (mean ± SEM) of the ^{125}I-insulin binding data of the intracranial insulin (ICI - 0.01 U, 0.1 U and 2.0 U) and saline (ICS) treated 28 day fetal brain plasma membranes.
ICI - 0.01 U versus ICS = not significant
ICI - 0.1 U versus 2.0 U = not significant
ICI - 0.1 U or 2.0 U versus ICS = p * $<$0.05, ** $<$0.01

The percent specific ^{125}I-insulin binding, R_0, R_2 or R_1 were unaffected by maternal diabetes or systemic insulin administration to the fetus (Since there was no difference between the 1.0 and 2.0 U, the data has been pooled). Intracranial administration of 0.01 U of insulin failed to alter the brain insulin receptor characteristics as well. However, a dose of 0.1 U of intracranial insulin decreased the percent specific ^{125}I-insulin binding, R_0, $R_1$and R_2. A pharmacologic dose of 2.0 U decreased the brain insulin receptor number to the same extent as the 0.1 U dose. The receptor affinity remained constant in all the treatment and control groups.

Discussion

Varying plasma insulin concentrations failed to alter the fetal brain insulin receptor characteristics. The observation is akin to the observations in adult rats exhibiting a physiologic hyperinsulinemic

TABLE 2: Brain Insulin Receptor Characteristics

Groups	% Specific binding per 200 µg BPM	Receptor No x 10^{10} mg protein			Receptor No x 10^{10} µg DNA			Affinity Constant x 10^{8}M		
		R_0	R_2	R_1	R_0'	R_2'	R_1'	Ke	K_2	K_1
D										
(n=5) $\bar{X} \pm$	10.63	198.58	188.99	9.58	2.48	2.36	0.12	0.63	0.11	11.48
SEM	1.25	9.72	9.88	0.89	0.19	0.19	0.01	0.07	0.02	2.10
C										
(n=5)	11.28	180.45	165.19	15.26	2.10	1.92	0.18	0.76	0.13	8.81
	0.75	12.45	12.84	3.54	0.15	0.13	0.05	0.09	0.04	1.47
t-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
IMI										
1.0 + 2.0 U	9.65	210.81	201.87	8.96	2.08	1.99	0.09	0.54	0.11	11.22
(n=4+4)	1.44	5.16	4.22	1.77	0.11	0.12	0.01	0.08	0.01	2.28
IMS										
(n=5)	12.22	210.81	203.95	6.84	2.12	2.06	0.06	0.74	0.13	18.63
	0.52	10.04	10.20	0.98	0.29	0.29	0.01	0.04	0.01	1.96
t-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
ICI										
0.01 U	9.73	229.99	218.25	11.72	2.28	2.16	0.12	0.58	0.11	10.0
(n=4)	0.64	22.98	21.55	1.41	0.02	0.02	0.002	0.06	0.008	2.22
0.1 U	6.06**	98.38**	92.15**	6.23	1.07**	1.02**	0.05*	0.71	0.12	10.84
(n=5)	0.19	6.68	6.41	1.12	0.13	0.14	0.006	0.07	0.01	2.01
2 U	5.20**	116.24**	109.91**	6.33	0.92**	0.87**	0.05*	0.53	0.16	8.13
(n=7)	0.56	9.74	9.71	1.11	0.14	0.13	0.01	0.08	0.05	1.89
ICS										
(n=5)	11.08	107.01	196.70	10.31	2.29	2.18	0.12	0.64	0.10	12.21
	1.04	8.08	8.82	2.09	0.26	0.25	0.03	0.07	0.02	1.90
(ANOVA)	<0.01	<0.01	<0.01	NS	<0.01	<0.01	<0.01	NS	NS	NS

t-test: p * <0.05, ** <0.01, when compared to the respective control. NS = Not significant

response (3,4) and neonatal rabbits with pharmacologic concentrations of plasma insulin (5). Systemic hyperinsulinemia (physiologic and pharmacologic) failed to increase the brain insulin content perceptibly. Even the sustained HI present in the fetuses of the alloxan diabetic mother did not increase the brain insulin content. Examination of the pancreati revealed that the sustained HI in this group was secondary to endogenous production of insulin as evidenced by β-cell hypertrophy (19) and the positive staining of the islets for β-granules (20) and insulin-specific immunoperoxidase (21). The question as to whether insulin crosses the

fetal rabbit blood-brain barrier has not been answered, however Frank et al performing acute experiments observed that radioactive insulin crossed the neonatal rabbit blood-brain barrier (22). If one assumes that insulin would similarly cross the fetal rabbit blood-brain barrier, the amount of hormone that entered the brain from the blood of both the chronic (mild) and acute (moderate and severe) hyperinsulinemic fetuses was not significant enough to cause an increase in the brain insulin content as measured in this study.

Direct intracranial injections of insulin (0.01 to 2.0 U) on the other hand significantly increased the brain insulin content. In addition the intracranial insulin injection studies revealed that the brain insulin content has to be significantly high to decrease the brain insulin receptor number. A four-fold increase in brain insulin content secondary to the administration of 0.01 U of NPH insulin did not alter the number of brain insulin receptors whereas a thirty-six fold increase in the brain insulin content decreased the brain insulin receptor number. A further increase in the brain insulin content (2.0 U of NPH insulin) decreased the brain insulin receptor number to the same extent as a thirty-six fold increase in the brain insulin content. This phenomenon has been observed in other tissues as well (23). In vivo occupancy of the brain plasma membrane receptors by the NPH insulin can result in an underestimation of the available receptor sites by the ^{125}I-insulin binding assay. The brain tissue was extensively washed during the procedure of plasma membrane preparation prior to the binding studies, thereby dissociating any previously bound hormone.

Using fetal rat hypothalamic cells in culture (mixed origin), Ciaraldi et al demonstrated no downregulation of the brain insulin receptors in response to physiologic concentrations of insulin (6). Boyd et al using rat neuronal cells in culture also failed to observe an insulin-induced downregulation of the insulin receptors (7). There is preliminary evidence in vitro that insulin downregulates the rat astrocyte glial cell insulin

receptor (24). In vivo, although pharmacologic concentrations of insulin administered directly into the neonatal rabbit brain have been observed to downregulate the brain insulin receptor (5), the effect of a more physiologic concentration of insulin on the brain insulin receptor has not been studied. Using whole brain plasma membranes we demonstrated no downregulation of the insulin receptor in the presence of a four-fold increase in brain insulin content.

In summary we have demonstrated that regardless of the permeability of the fetal blood-brain barrier to insulin, the brain insulin receptor downregulation is dependent primarily on the insulin concentrations in direct contact with the brain. The downregulation of the fetal brain insulin receptor is a pharmacologic and not a physiologic effect of insulin.

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