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INSULIN DOWNREGULATES NEONATAL BRAIN INSULIN RECEPTORS [1]

Sherin U. Devaskar* and Nicholas Holekamp

Division of Perinatal Neonatal Medicine, Department of Pediatrics
Saint Louis University School of Medicine

Pediatric Research Institute
Cardinal Glennon Memorial Hospital for Children
St. Louis, Missouri

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Insulin (2U-regular) was administered intracerebrally or intraperitoneally in newborn rabbit pups, to study the effect of the hormone on brain insulin receptor characteristics. Intraperitoneal insulin treatment produced an increase in plasma insulin concentrations from a control of ~ 24 μU/ml to 132 ± 36 ($p < 0.02$) and a decrease in plasma glucose from ~ 83 to 27 ± 10 mg/dl ($p < 0.01$). No change in brain insulin receptor characteristics was observed. On the other hand, insulin injected intracerebrally raised the plasma insulin to > 3000 μU/ml and lowered the plasma glucose to 32 ± 8 mg/dl ($p < .05$). In addition a decrease in brain insulin receptor sites from 262 ± 9 x 10^{10}mg protein^{-1} to 159 ± 6 ($p < 0.001$) was noted. When the data was expressed per μg DNA, a decrease from 4 ± 0.4 x 10^{10} to 2.5 ± 0.2 ($p < .01$) in receptor sites resulted. No change in the receptor affinity was observed. We conclude that a direct exposure of the brain to excess insulin results in a downregulation of the brain insulin receptors.

Insulin has been detected within adult neuronal cells (1), but the source of this hormone is controversial (2). In addition insulin receptors are widely distributed within the central nervous system (3). Unlike the receptors in other organs (4), adult brain insulin receptors are neither downregulated by systemic hyperinsulinemia nor upregulated by the hypoinsulinism associated with diabetes (3,5). In the neonate, hyper-

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*Reprint Request:

Sherin U. Devaskar, M.D., R. No. 519, Glennon Hall, Cardinal Glennon Memorial Hospital for Children, 1465 South Grand Boulevard, St. Louis, Missouri 63104.

ABBREVIATIONS

CNS, Central Nervous System; KIU, Kallikrein International Units; EDTA, Ethylene diamine tetra-acetic acid; BPM, Brain Plasma Membrane; TCA, Trichloro-acetic acid; DNA, De-oxyribonucleic acid; IP, Intraperitoneal; IC, Intracerebral; IC_{50}, Concentration that inhibits binding by 50%.

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insulinemia results in an increase in peripheral tissue insulin receptor concentrations (6,7), a phenomenon that is distinct from the adult. Although brain insulin receptors have been demonstrated in the neonatal rat (8), the influence of high circulating insulin concentrations on brain insulin receptors has not been studied. We therefore investigated the effect of excess insulin on the neonatal brain insulin receptor using the rabbit as our animal model.

MATERIALS AND METHODS

Animals: Pregnant New Zealand white rabbits were obtained from Isaacs Rabbitary and housed individually. The does were allowed to deliver, and the neonatal pups between days one and two of life were arbitrarily assigned to one of the following four experimental groups. The symbol "n" represents a mean of 2.1 ± 0.2 neonatal pups which were treated in the same fashion.

1) Group I (n=5): received 2U of Regular Insulin (U-100, Eli Lilly Co.) intraperitoneally.
2) Group II (n=4): served as a control group and received an equal volume (20 µl) of saline intraperitoneally.
3) Group III: (n=4) were administered 2U of regular insulin through the anterior fontanelle into the brain, and
4) Group IV (n=5): received 20 µl of saline intracerebrally in the same manner and served as a second group of controls.

Following the treatment, the pups were returned to their mothers to resume feedings. Six hours later, the neonates were killed by decapitation, the blood and brain tissue pooled separately prior to further processing.

Blood samples: Blood was collected after decapitation, in chilled glass tubes containing EDTA and aprotinin (1000 KIU/ml). Plasma was separated at 4^0C, plasma insulin quantitated by radioimmunoassay (10) and plasma glucose determined by the glucose oxidase method using the Yellow Springs Instrument 27A (10) as previously described.

Brain plasma membranes (BPM): The brains were quickly removed from the crania and pooled to yield adequate tissue. BPM were prepared by differential centrifugation using the technique described by Havrankova et al (3). The final membrane pellet was suspended in one volume of 50mM Tris buffer (pH 8.0) for every gram of original brain weight. Using bovine serum albumin as standards, protein concentration in brain homogenate and membrane was determined by the method of Lowry (11). DNA content in the brain was estimated by Zamenof's modification (12) of Burton's technique (13). BPM purity was ascertained by determining the activity of 5'-nucleotidase, a membrane marker enzyme (14).

^{125}I-insulin radio-receptor assay: Mono-iodinated insulin was prepared by the technique of Sodoyez et al (15), to a specific activity of ~ 150 µCi/µg using carrier free ^{125}NaI (New England Nuclear Corp.). This was used as the ligand. In preliminary experiments, optimal pH, temperature and time, where maximal ^{125}I-insulin binding to BPM occurs, were determined. The concentration of ^{125}I-insulin that saturates the receptor, the amount of BPM protein which is within the range of linearity to percent specific insulin binding, bacitracin concentration that inhibits degradation of insulin from ~ 9% to 3% were used in the assay. Therefore, in a final volume of 0.35 ml of 50mM Tris, ~ 70,000 cpms of ^{125}I-insulin along with 0.2mg BPM protein, 25 µg bacitracin (Sigma Chem. Co.) and native insulin ranging in concentrations

from 0 to 10^{-6}M were incubated for 16 hours at a pH of 8.0 and a temperature of 4°C. Non-specific binding, the maximal binding of ^{125}I-insulin that occurred in the presence of 10^{-6}M native insulin, was substracted from each point on the dose response curve to obtain specific binding. The dose response curves were subjected to Scatchard analyses (16) which generated curvilinear plots. These plots were subjected to the programme described by Thakur and Rodbard (17) and resolved into two components, the high affinity, low capacity (R_1) and the low affinity high capacity (R_2). The affinity constants K_1 and K_2 respectively, for these two components were also calculated. In addition the total binding capacity (R_o) in moles/liter were converted to number of receptors and the mean affinity ($K\bar{e}$) derived from the Scatchard plots.
Statistics: All data is represented as mean ± SEM. Differences between two groups were determined by using Student's "t" test.

RESULTS:

Mean body weight, brain weight, brain protein content, percent recovery of BPM protein, brain DNA content, plasma glucose and insulin concentrations are depicted in Table 1. No statistical difference was observed between the two insulin treated groups (Group I and III) and their respective controls (Group II and IV) in body and brain weight, brain protein and DNA content. Regardless of the type of treatment, the percent recovery of protein in all brain plasma membranes was constant, denoting a similar degree of purification of the BPM. This was further confirmed by a

TABLE 1: GENERAL CHARACTERISTICS OF THE BRAIN AND PLASMA CONCENTRATIONS

Groups (n)		Body Wt. (g)	Brain Wt. (g)	Brain Protein Content (mg/g)	% recovery of BPM protein	Brain DNA Content (mg/g)	Plasma Glucose (mg/dl)	Plasma Insulin (μU/ml)
Group I: IP Insulin (5)	$\bar{X}$ ±	58.96	1.28	55.22	27.10	0.91	26.5*	132.3*
	SEM	4.82	0.07	2.12	2.48	0.06	9.92	36.47
Group II: IP Saline (4)		51.09	1.24	57.19	27.39	1.01	89.25	19.80
		7.70	0.08	1.04	2.17	0.04	15.48	5.59
Group III: IC Insulin (4)		62.63	1.33	59.96	26.13	0.98	32.17^{x}	>3000†
		6.62	0.09	1.12	1.39	0.02	8.17	
Group IV: IC Saline (5)		62.40	1.34	58.88	27.23	1.04	77.50	28.33
		4.79	0.07	1.08	1.00	0.04	20.62	8.44

t-test: $^{x}p < 0.05$ (one tailed)

*$p < 0.02$ (two tailed)

† plasma was diluted 10 fold, the insulin concentration was still higher than the standard curve.

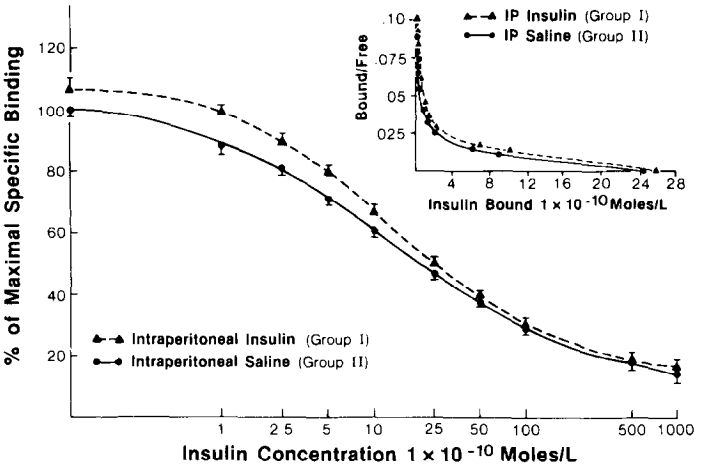

FIGURE 1: ^{125}I-insulin dose response curves of the IP insulin (group I) and saline (group II) treated neonatal BPM. Scatchard plots of the respective groups are shown in the inset.

consistent two-fold enrichment of 5'-nucleotidase enzyme in all BPM over that of the homogenates (data not shown). Plasma glucose concentration in the group that received insulin intraperitoneally (group I) decreased from a control of 89.25 ± 15.48 to 26.5 ± 9.92 mg/dl ($p < 0.01$), in response to an increase in plasma insulin concentrations from 19.80 ± 5.59 to 132.3 ± 36.47 μU/ml ($p < 0.02$). Intracerebral administration of insulin (Group III) resulted in a similar decline in plasma glucose from a control of 77.5 ± 20.6 to 32.17 ± 8.2 mg/dl ($p < 0.05$, one tailed t-test) in response to much higher plasma insulin concentrations (> 3000 μU/ml versus a control of 28.33 ± 8.44). The neonatal plasma glucose levels varied markedly within a group depending on the time interval from the last feeding to blood sampling.

Figure 1 represents the ^{125}I-insulin dose response curve for the intraperitoneal insulin (group I) and saline (group II) treated groups. The Scatchard plots are shown in the inset. No apparent difference between the two curves is observed. On the other hand, figure 2 denotes the ^{125}I-insulin dose response curves and Scatchard plots of the IC treated insulin (group III) and saline (group IV) groups. The

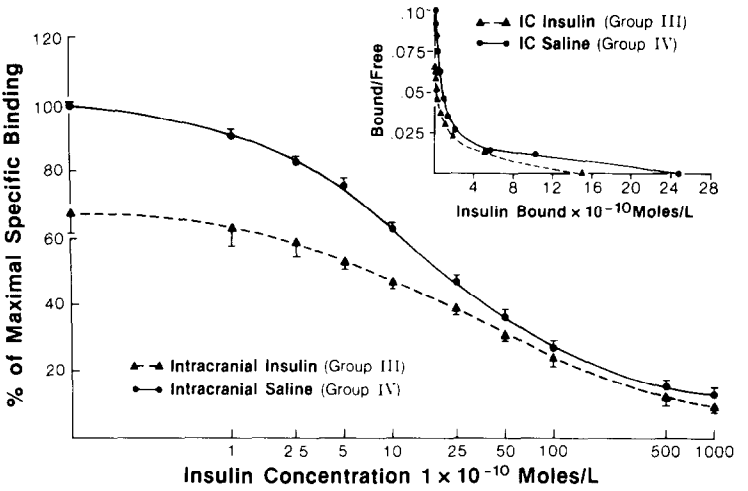

FIGURE 2: ^{125}I-insulin dose response curves and Scatchard plots of IC insulin (group III) and saline (group IV) treated neonatal BPM.

maximum specific binding for the control group was represented as 100% and the maximum specific binding for the insulin treated group expressed relative to the control group was 66.98 ± 5.94% ($p < 0.001$). The total binding capacity in moles /liter, derived from the Scatchard plots, decreased in group III to 15.1 ± 0.53 x 10^{-10} from a control (group IV) of 24.88 ± 0.82 x 10^{-10} ($p < 0.001$).

Figure 3 demonstrates the ^{125}I-insulin dose response curves in all four groups and the mean concentration of native insulin that inhibited the binding of the radio-ligand by 50% (IC_{50}). The IC_{50} in group I was 2.2nM, in group II, 1.9nM, in group III 4.5nM and in group IV 2.3nM, the mean being 2.73 ± 0.60nM.

The derived number and affinity of the neonatal brain insulin receptors in all four groups are shown in Table 2. In the IP treated groups I and II, no change in R_0, R_2 and R_1 was noted. When data were expressed per µg DNA, again no change in the receptor site concentration was noted. In the IC treated groups, however, administration of insulin resulted in a decline in the receptor number from a control of 262.24 ± 8.68 x 10^{10}mg protein^{-1} to 159.16 ± 5.54 ($p < 0.001$). This decline was solely due to a decrease in the low affinity receptor sites (R_2) from 251.97 ± 9.96 to

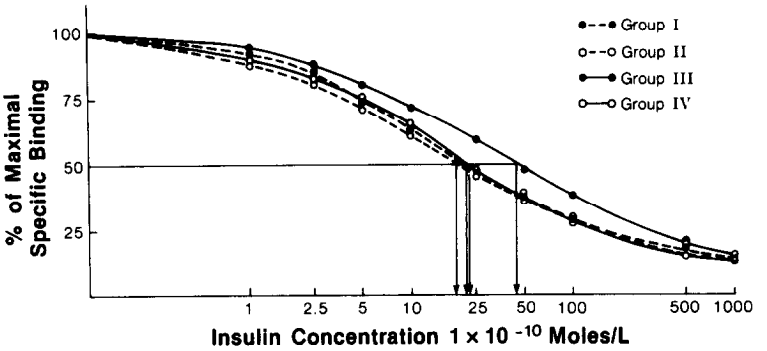

FIGURE 3: IC_{50} of Group I, II, III and IV.

150.71 ± 5.17 ($p < 0.001$), since the high affinity sites (R_1) remained unchanged. Receptor number (R_o') when quantified per µg DNA also decreased from 4.12 ± 0.43 x 10^{10} to 2.56 ± 0.16 ($p < 0.01$) and R_2' from 3.97 ± 0.44 to 2.42 ± 0.11 ($p < 0.01$), while R_1' remained unaltered. In all four groups, the mean affinity $K\bar{e}$, K_2 and K_1 were constant.

An estimate of brain insulin content was attempted following the acid-ethanol extraction (18) and measurement by radioimmunoassay (3) as previously described. A 1000-fold increase was noted in the IC insulin treated group III versus the control group IV. In addition, the IP insulin

TABLE 2: BRAIN RECEPTOR NUMBER AND AFFINITY

Groups (n)		Receptor No. X 10^{10}mg protein^{-1}			Receptor No. x 10^{10}µg DNA^{-1}			Affinity x 10^8M		
		R_o	R_2	R_1	R_o'	R_2'	R_1'	$K\bar{e}$	K_2	K_1
Group I: IP Insulin (n=5)	X ±	271.94	264.11	7.83	4.20	4.08	0.12	0.32	0.070	9.19
	SEM	10.75	9.94	1.09	0.30	0.30	0.01	0.011	0.007	1.21
Group II: IP Saline (n=4)		257.71	248.72	8.99	3.99	3.85	0.14	0.29	0.06	7.37
		10.55	12.08	1.93	0.28	0.29	0.03	0.014	0.012	1.05
Group III: IC Insulin (n=4)		159.16**	150.71**	9.94	2.56*	2.42*	0.17	0.35	0.10	6.41
		5.54	5.17	4.15	0.16	0.11	0.08	0.026	0.03	1.48
Group IV: IC Saline (n=5)		262.24	251.97	10.27	4.12	3.97	0.16	0.33	0.05	7.35
		8.68	9.96	1.68	0.43	0.44	0.02	0.036	0.007	0.61

two-tailed t-test *$p < 0.01$
**$p < 0.001$

treated group I had a similar amount of brain insulin as both the control group II and IV.

DISCUSSION

We characterized the neonatal brain insulin receptor in the rabbit and observed that these receptors, as in the adult (6), are not amenable to modulation by systemic hyperinsulinemia. Direct exposure of the CNS to insulin decreases the brain insulin receptor concentration, suggesting an effect of the hormone when it gains access to the brain. In adult dogs, Margolis et al observed insulin to cross the blood brain barrier slowly only when consistently high circulating insulin concentrations were present (19). Studies revolving around substrate utilization by the brain demonstrate that intraperitoneal insulin administration does not influence the brain glucose uptake, but intracisternal insulin augments brain glycogen formation in the adult rat (20). In the neonate during development, similar to the adult, insulin possibly may not cross the blood brain barrier.

A spuriously low assessment of brain insulin receptor sites can potentially be secondary to previous occupancy of receptors by the 2U of IC insulin administered in vivo. However,repeated washings and dilutions with buffer over a period of approximately two hours are involved in the preparation of BPM. These conditions lead to dissociation of previously bound hormone from receptors(22,21)prior to the radio-receptor assay. We therefore believe that the decrease in the insulin receptor sites in the IC insulin treated group is real and due to a downregulation. This decrease is unlike the response of insulin receptors in other neonatal organs (6,7), but similar to that of adult tissues (4). In this respect, the neonatal brain insulin receptor may be different from the insulin receptors in other peripheral tissues.

Although the same dose of insulin was administered both intraperitoneally or intracerebrally, in the intracerebral experiments higher plasma insulin concentration resulted. When administered intraperitoneally, the systemic

clearance of insulin may have been more rapid than when the hormone was administered intracerebrally. Insulin within the brain has to be cleared by the ventricular fluid and gain access to the blood prior to the onset of any metabolic clearance mechanisms. On the other hand, the ventrolateral hypothalamus has been observed to be sensitive to the direct injection of insulin within the brain, resulting in a stimulation of insulin secretion by the beta islet cells of the pancreas (23,24). The hypoglycemia secondary to this insulin secretion can be abolished by vagotomy (25) suggesting a central neuroregulatory role for insulin. We cannot sort out these events in our study, but either of the above mechanisms could be operating in the neonate.

Regardless of the plasma insulin concentrations, the decline in the plasma glucose in both the insulin treated groups was similar. This possibly could be due to the hepatic autoregulation of glucose, which comes into effect after an initial decline in plasma glucose concentrations, preventing a further decline (26,27).

In conclusion, we have demonstrated a downregulation of the neonatal brain insulin receptor in the presence of excess insulin, suggesting a functional role for the hormone in the developing CNS.

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