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INSULIN EFFECT ON [^{14}C]-VALINE INCORPORATION AND ITS RELATION TO HEXOKINASE ACTIVITY IN DEVELOPING BRAIN

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Summary: Using minced brain cortex from fetal and postnatal rats , we studied the incorporation of [^{14}C]-valine into protein in the presence of insulin. We also assayed the "particle bound" and soluble hexokinase in these tissues. Insulin significantly stimulated the incorporation of [^{14}C]-valine into brain proteins from fetal stage upto 2 days of life . After this period the insulin effect was minimal, with no effect by day 5. The "particle bound" (40,000g pellet) brain hexokinase, on the other hand, remained low till about 2 days of life and then increased to almost adult level by 5 days . Our results show that there is an inverse relation between this anabolic effect of insulin and the "particle bound" hexokinase activity in the cortex of developing rat brain.

Although , insulin-like proteins are present in the brain (1,2) , insulin receptors are widely distributed in the central nervous system(CNS) (3), and the interaction of insulin with the receptors in brain has been widely studied (4,5,6) , a metabolic role of insulin in CNS still remains unclear. Stimulation of brain ornithine decarboxylase activity during development in neonatal rats (7) as well as in cell cultures has been reported (8). A role for insulin in neuronal maturation in brain cell cultures has been demonstrated (9). Metabolic effects of insulin have been observed in brain cell cultures, not in brain tissue preparations. We decided to look at fetal brain tissue , for primary culture of brain cells may behave as immature population; we assumed that any effects of insulin on brain tissue might occur in similar circumstances of maturity. The present report reveals the first direct in vitro stimulatory effect of insulin on protein synthesis in cortical tissue . The effects are seen only during late fetal and early postnatal period. The theory that insulin acts by binding the isozymes of hexokinase to mitochondria (10, 11) explained the inability of insulin to affect brain metabolism , because the hexokinase is almost totally bound to mitochondria thereby preempting any effect of insulin. When preliminary experiments showed a clear effect of insulin on immature brain, we measured the soluble and mitochondrial bound (particle bound) enzyme activities during the same period. The present report also shows that the insulin effect on protein synthesis is only observed when the "particle bound " hexokinase is very low.

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Methods

Sprague Dawley timed pregnant rats of about 250g body weight were used. Incorporation of [^{14}C]-valine into protein was studied in the cerebral cortex of fetal, newborn and adult rats. The cortex was dissected out and chilled on ice. It was then sliced, minced and dropped into the incubation flask containing Krebs-Henseleit bicarbonate buffer (13) pH 7.4. All incubations were carried out in stoppered 25 ml. erlenmeyer flasks , using Krebs-Henseleit bicarbonate buffer pH 7.4, equilibrated with 95%O_2-5%CO_2 gas mixture. This medium contained the 20 natural amino acids at final concentrations of 0.1mM. After addition of the tissue to the incubation medium , all flasks were flushed with the gas mixture and incubated at 30^o in 95% O_2- 5% CO_2 in a Dubnoff metabolic shaker (60 oscillations/min). Insulin at a final concentration of 10mU/ml was added 5 mins. after the addition of the minced tissue. Reaction was started by adding [^{14}C}-valine (1 μCi / flask). After 60 min. of incubation perchloric acid was added to final concentration of 1M. The precipitate was washed twice with trichloroacetic acid, heated at 90^o for 15 min, washed once with ethanol : ether(1:1 v/v), twice with acetone and twice with ether, air dried and dissolved in 0.1N NaOH (14) and an aliquot was counted in a liquid scintillation counter .The incorporation of radioactive amino acid into the protein precipitate was linear throughout this incubation period. For hexokinase studies subcellular fractions were prepared(15) from litter mates. The cerebral cortex was homogenized in 0.25M sucrose-1mM 2-mercaptoethanol. The homogenate was centrifuged at 1000g for 15 minutes and the pellet was discarded. The supernatant was centrifuged at 40,000g for 20 minutes. The hexokinase in the supernatant is defined as the "soluble activity". The pellet was rehomogenized and suspended in 0.25M sucrose-1mM 2-mercaptoethanol and was treated with tritonX-100 to final concentration of 0.4% v/v to ensure exposure of all particulate activities. Since the "particle bound" hexokinase is actually "mitochondria bound"(16), these two terms are used interchangebly in this report. Hexokinase activity was measured spectrophotometrically (17,18). The incubation medium (1ml) contained (final concentration): 3.3mM glucose, 6.6mM ATP, 6.6mM MgCl, 0.3mM NADP, 40mM hepes buffer,pH 7.5, and one unit of glucose 6-phosphate dehydrogenase(or glucose 6-phosphate NADP oxidoreductase). One unit is defined as the amount of enzyme catalyzing the formation of 1μmole glucose-6-phosphate/min. Protein was also assayed(19), as a reference basis for both enzyme activity and [^{14}C] valine incorporation.

Results and Discussion

Fig.1 shows that insulin at low concentrations (0.38 μg/ml or 10mU/ml) increases the incorporation of [^{14}C]-valine into protein of brain cortical tissue significantly . This increase is most pronounced during the fetal stage up to 2 days of postnatal life. After this the effect falls on the 3rd day and has disappeared by the 5th. day. Yang and Fellows (20) using 5μg/ml of insulin in tissue cultures have shown an increase in [^{14}C]-valine incorporation into cell proteins, after a lag period of 1 hour, whereas the effect we report is seen within the first hour. Insulin binding to brain cell membranes has been shown by several investigators(21-24) . Lowe et al (24) using crude membrane preparations at various stages of development have shown that maximum specific binding of insulin at 19 days gestation is 7.2%, 1 day old is 10.9% and in adults it is 4%. In the light of our finding it is interesting that when the anabolic effect of insulin is maximum, the binding of insulin to membranes is also maximum, suggesting that insulin may be playing a major role in the intrauterine stages of brain development.

Studies on the hexokinase activity revealed (table 1) that the total activity of the soluble form of the enzyme remains more or less constant during the period of development we studied. It is the particulate or mitochondrial bound enzyme activity which increases during development. Other investigators have reported increases in the particulate bound hexokinase from the 8th. day (15). In our studies the "particulate bound " hexokinase is about 30-40% of the adult levels (figure 1) from 18 days gestation till the second day of life. The most striking observation is that

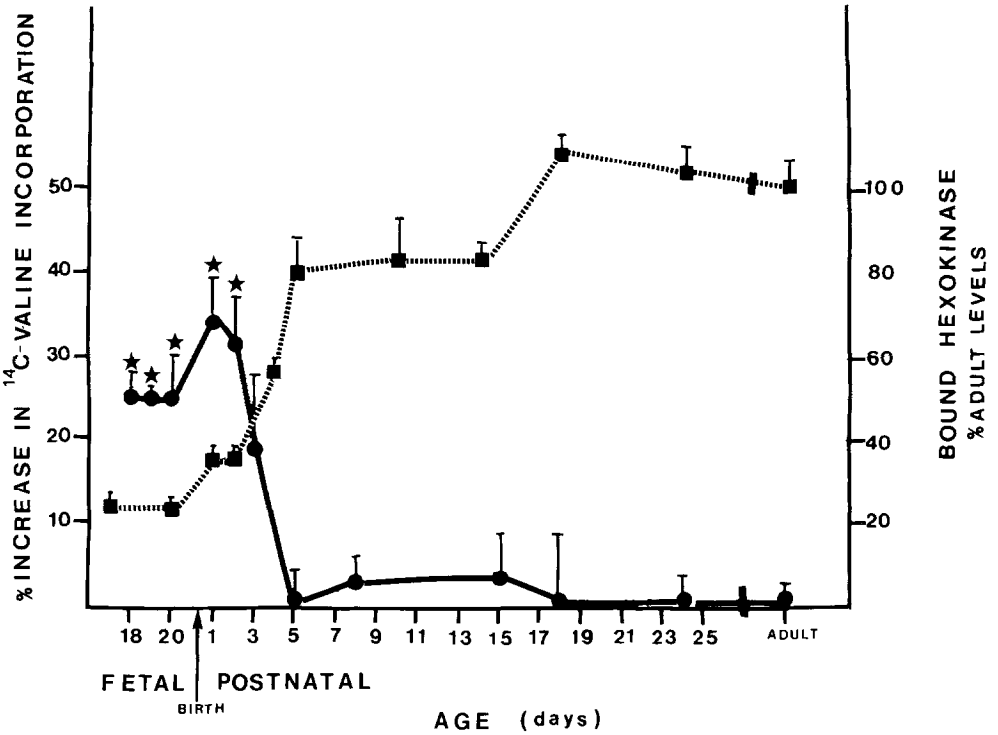

Figure 1.Effect of Insulin on Brain Protein Synthesis Related to the Bound Hexokinase Activity in the Developing Rat Brain Cortex

● % Increase in the [^{14}C] -valine incorporation into brain protein with insulin (10munits/ml) ± S.E.M.

■ Total mitochondria bound hexokinase, expressed as percent adult levels ±S.E.M.

★ Statistically significant P<0.01

Each point represents data from at least 4-6 animals.

it is only during this period that the insulin effect on [^{14}C]-valine incorporation into brain protein is significant. By the 5th. day of life the bound hexokinase activity attains about 80% of the adult levels (figure 1).The insulin effect is no longer observed after this period. In our studies we find that when this anabolic effect of insulin is maximal, the total bound hexokinase (figure 1) is 30% of the adult levels. There is no relation of this effect to the soluble hexokinase levels. It has been reported that the effect of insulin on a tissue is inversely related to the content of hexokinase isozyme II (25,28). This is the isozyme that binds to the mitochondria. Bessman and Geiger (25) proposed that the role of insulin in stimulating metabolism is to connect hexokinase to the appropriate sites on the mitochondria . It has also been reported that insulin has a stimulatory effect on the mitochondrial Kreb's cycle (26, 27). Our results also seem to support the idea that the anabolic effect of insulin in the fetal and early postnatal period may be observable because the mitochondrial bound (particulate bound) hexokinase is so low. Wilson (18) has related the soluble vs. bound hexokinase to a control of glucose metabolism and the differential effect of the product glucose 6-phosphate upon the binding of hexokinase. It would appear here is another example in which binding of hexokinase to particles, determines the effectiveness of insulin. The mitochondrially bound hexokinase has a preferential access to the intramitochondrial ATP

Table-1

Hexokinase Activity in Developing Rat Brain Cortex

Age (days)	Hexokinase Activity (U/100mg protein) Mitochondrial Bound	Soluble
Fetal		
17	2.4 ± 0.3	1.6 ± 0.05
20	2.4 ± 0.19	1.4 ± 0.1
Postnatal		
1	3.4 ± 0.19	1.8 ± 0.14
2	3.4 ± 0.15	1.5 ± 0.11
4	5.6 ± 0.3	1.5 ± 0.15
5	8.0 ± 0.35	1.7 ± 0.09
10	8.3 ± 0.5	1.6 ± 0.1
14	8.3 ± 0.6	2.0 ± 0.1
18	10.9 ± 0.5	2.5 ± 0.3
24	10.4 ± 0.4	2.6 ± 0.3
Adult	10.0 ± 0.45	2.3 ± 0.3

Experimental conditions are described in the text. Each value represent data from 4-6 animals and is expressed as mean ± SEM . One Unit is defined as the amount of Enzyme catalyzing the formation of 1μmole of glucose-6-phosphate per min.

generated (29) and thus makes the system more efficient. Knull et al (30) found that insulin injections in chicks (5 days old) resulted in an increase in the particulate hexokinase in brain, similar to the results of Borrebaek (31) and others (32) in studies on other tissues. The hexokinase acceptor theory of insulin action (10) interlinks insulin with mitochondrial hexokinase and cellular metabolism. If, indeed , insulin effects brain mitochondria in the fetus it might help to throw light on the problems of the fetus of the diabetic mother. These results support the general view that the prenatal management of the pregnant diabetic should include fairly strict maintanance of normal blood glucose levels.

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