

Effect of Different Thyroid States on Mitochondrial Porin Synthesis and Hexokinase Activity in Developing Rabbit Brain

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Voltage-dependent anion-selective channel proteins (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotes and in brain post-synaptic membranes. VDACs regulate anion fluxes of a series of metabolites including ATP, thus regulating mitochondrial metabolic functions. Hexokinase binds to porin. The mitochondrially bound hexokinase can greatly increase the rate of aerobic glycolysis. The activities of hexokinase and protein levels of mitochondrial porin were determined in brains of hypothyroid rabbits and in hypothyroid rabbits administered with thyroxine. Proteins were separated by electrophoresis, and the proteins of interest were quantified. Western blotting analysis revealed a significant decrease (~50%) in the relative amount of porin in the hypothyroid compared with euthyroid rabbits. The changes in the developmental pattern of hexokinase activity in the brain of hypothyroid rabbits and the effect of T₄ on this enzyme activity have been investigated. Hypothyroid rabbits showed lower activity than their corresponding age-matched normal neonates. Administration of thyroxine to the hypothyroid neonates at birth abolished the effects of methimazole [1-methyl-2-mercaptoimidazole (MMI)]. These findings apparently indicate that the synthesis of the pore-forming protein and the hexokinase enzymes are under thyroid control during the fetal and the early post-natal period.

Key words: hypothyroidism, methimazole, thyroxine, VDAC.

Thyroid hormone is a main regulator of growth, development, and homeostasis in higher organisms. Lack of adequate levels of thyroid hormone during the fetal and neonatal periods leads to mental deficiency and profound neurological deficits, including deafness and movement disorders in humans and multiple brain abnormalities in experimental animals (1). These abnormalities include biochemical, metabolic, cellular, and behavioral alterations through the brain, including a reduction in the mean size of neuronal cell bodies (2). There is a critical period during which appropriate thyroid hormone levels are essential for normal brain development. In humans, this period was considered to begin late in gestation and to extend through 1–2 y of age. In rodents, this critical period was thought to occur during the first 15 days after birth. This is an important period for neuronal differentiation, maturation, and development of neuronal processes.

Thyroid hormone acts by regulating target gene expression through binding to its high-affinity receptor, which belongs to the superfamily of nuclear hormone receptors that function as ligand-regulated transcription factors (3).

In the last few years, a number of genes coding for cytoskeletal proteins, transcription factors, enzymes, cell-adhesion molecules, neurotrophins and their receptors, or other proteins have been found to be under thyroid control in the rodent brain during the neonatal period (4). It is, however, clear that additional thyroid hormone

target genes must exist, and that their identification will increase our understanding of the molecular events underlying cerebral function and brain development.

Many studies dealing with the control of energy metabolism by thyroid hormones have been devoted to enzymes of the oxidative pathway; fewer studies have been devoted to glycolytic enzymes. The present study was undertaken to determine the influence of thyroid hormone on hexokinase binding protein (mitochondrial porin) synthesis and the functional properties of the hexokinase enzymes in the rabbit brain.

Mitochondrial porins are encoded by nuclear genes and synthesized by cytosolic ribosomes. Porin, also known as voltage dependent anion channel (VDAC-1), is the most abundant protein of the mitochondrial outer membrane. It is a large conductance anion-selective channel that is thought to be important in various aspects of mitochondrial function, possibly including apoptosis (5). Hexokinase has been reported to bind specifically to the voltage-dependent anion channel. Four isoforms of hexokinase exist in mammalian tissue (6). Only hexokinases I and II bind significantly to mitochondria. Hexokinase I is found predominantly in the brain (7). By binding to VDAC, hexokinase gains preferential access to mitochondrially generated ATP (8). Hexokinase catalyzes the first and a rate-controlling step in glycolysis. By having preferential access to mitochondrial ATP production, the mitochondrially bound hexokinase can greatly increase the rate of aerobic glycolysis (9).

The results presented here show a drop in the relative amount of mitochondrial porin and hexokinase activity in hypothyroid neonates brain, which is corrected after a

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treatment with thyroid hormone. Thus, these results suggest the requirement of thyroid hormones for the normal regulation of the hexokinase enzymes and mitochondrial porin synthesis in developing rabbits.

EXPERIMENTAL PROCEDURES

Animals and Treatments—Timed-pregnant rabbits weighing ~3.5 kg were kept under controlled housing conditions. The international criteria for the use and care of experimental animals in research were observed. Maternal and fetal hypothyroidism were induced by administration of 0.02% methimazole [1-methyl-2-mercaptoimidazole (MMI)] (w/v) in the drinking water of pregnant rabbits from day 14 of gestation. At least three animals were studied per experimental group to obtain representative values.

To study the effect of thyroid hormone supplementation, hypothyroid newborns were injected intraperitoneally with 5 µg/100 g, body weight, of thyroxine (T_4), at the time of delivery. T_4 was used for the *in vivo* hormonal treatments because it crosses the blood-brain barrier more efficiently than 3,5,3'-triiodothyronine (T_3) and is converted to T_3 in the brain (10). Control animals received the same volume of vehicle (10 mM KPi, pH 8.3, 0.9% NaCl).

Preparation of Rabbit Brain Mitochondria and Isolation of VDAC—Brains from 0, 24, and 48-h-old rabbits were removed, and placed on ice. Tissues were homogenized in 0.32 M sucrose at a ratio of 20 ml/g weight using a Teflon pestle in a motor-driven homogenizer. The homogenate was centrifuged at 1,500 ×g for 10 min at 2°C. The supernatant was carefully removed and centrifuged at 17,500 ×g for 20 min. The pellet was resuspended in 10 ml 0.32 M sucrose and rehomogenized as before (11).

A discontinuous sucrose gradient was formed in 36 ml polycarbonate tubes containing 16 ml of 1.2 M sucrose over which was layered 16 ml of 0.8 M sucrose followed by 4 ml of the resuspended pellet fraction. The gradient was centrifuged at 25,000 rpm for 2 h. The pellet at the bottom of the tube, corresponding to brain mitochondria, was suspended at a protein concentration of 25 mg/ml in 10 mM Tris-HCl, pH 7.2 and stored at -20°C until use. Protein concentrations were determined by the method of Lowry *et al.* (12) using crystalline bovine serum albumin as standard.

To prepare rabbit brain VDAC, mitochondria were lysed by osmotic shock; after centrifugation, the pellet, mainly composed of mitochondrial membranes, was solubilized by 3% Triton X-100, 10 mM Tris-HCl, pH 7.2, and 0.1 mM EDTA at a final concentration of 7 mg of protein/ml. After 30 min at 0°C, the sample was centrifuged at 40,000 ×g for 15 min, and the supernatant was loaded onto a dry hydroxyapatite/celite column (0.6 g, ratio HTP to celite 2:1, w/w). Elution was performed with the solubilization buffer. A volume identical to that of the solubilized protein was eluted from the column (13).

Assay of Glucose Phosphorylating Activities—Brain tissues from rabbits aged 0, 24, and 48-h were homogenized in 10 mM Tris-HCl, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Homogenate was centrifuged

at 4°C for 5 min at 1,250 ×g and the supernatant was further centrifuged at 20,800 ×g for 1 h at 4°C. The resulting supernatant was used to measure enzyme activity.

Hexokinase activity was measured as the total glucose-phosphorylating capacity using a glucose-6-phosphate dehydrogenase/NADP⁺-coupled spectrophotometric assay (14).

Glucose phosphorylating activity analysis involved assays at two glucose concentrations: 0.5 mM for hexokinase activity, a concentration at which glucokinase is essentially inactive and 25 mM glucose, a concentration at which all phosphotransferase activities were measured.

Assay for the Mitochondrial Binding Capacity of Hexokinase—This was carried out by the procedure described by Arora and Pedersen (9). Isolated rabbit brain mitochondria (0.5 mg protein) extensively depleted of indigenous hexokinase activity by pretreatment with glucose 6-phosphate for up to a total of three consecutive times (15), were pre-incubated with variable hexokinase concentrations (0–800 mU) of hexokinase enzyme for 30 min at 4°C. The mitochondria were pelleted in a microfuge at 10,000 relative centrifugal force for 5 min, and the pellet was washed with 250 µl of 250 mM sucrose/10 mM Tris-HCl, pH 7.5 medium and resuspended. Hexokinase assays were performed on the resuspended pellet and the supernatant fractions.

SDS-PAGE and Immunoblotting of VDAC—Electrophoresis on a 16% polyacrylamide slab gel of acetone-precipitated samples was performed in the presence of 0.1% sodium dodecyl sulfate according to the procedure of Laemmli (16). After electrophoresis, the gels were either stained, or used for Western blotting. Gels were placed in 10% trichloroacetic acid for 15 min to limit protein diffusion then stained with Coomassie blue solution consisting of equal volumes of 0.3% Coomassie Blue R-250, in 90% ethanol and 1% copper sulfate in 20% glacial acetic acid. Gels were destained in a solution of 0.5% copper sulfate, 10% glacial acetic acid, and 25% methanol. For western blotting, proteins from the SDS-PAGE gels were transferred onto polyvinylidene fluoride (PVDF) membrane. After being blocked in Tris-buffered saline (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 0.2% Nonidet P40 and 5% nonfat dry milk for one hour, the membrane was incubated with rabbit anti-rat VDAC anti-serum (10 µg/ml), for 1 h at room temperature. Excess antibody was then washed off, and the membrane was reblocked in Tris-buffered saline containing 5% nonfat dry milk and 0.2% Nonidet P40 for 60 min at room temperature. Primary antibody binding was detected with an ECL Western blotting kit (Amersham) and quantified by densitometry.

Planar Lipid Bilayer Measurement—The reconstitution of pore-forming activity in artificial membranes was performed by using black lipid membranes (17). The membranes were formed across circular holes (surface area about 0.1 mm²) in the thin wall separating two aqueous compartments from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane. The current through the membrane was measured with two calomel electrodes connected in series to a voltage source and a current amplifier. The amplified signal was monitored on a digital oscilloscope and recorded on strip chart.

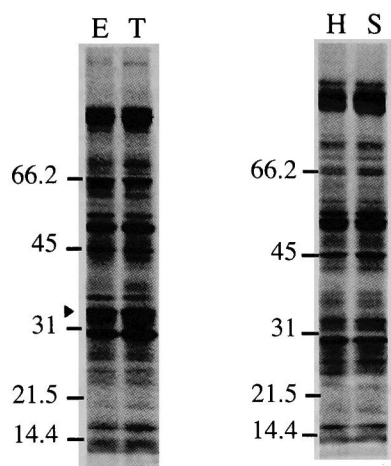


Fig. 1. Comparison of protein profiles of brain mitochondria isolated from euthyroid, hypothyroid, T_4 , and salt-treated hypothyroid rabbits at 48-h after delivery. Identical amounts of mitochondrial brain extracts from euthyroid (E), hypothyroid (H), and hypothyroid neonates treated with T_4 (T) or salt (S), at the indicated age were fractionated by SDS-PAGE on 16% gel. Protein molecular mass standards are given in kDa at the left side. The position of porin is shown by an arrowhead to the left of lane 1. Identity of porin bands was confirmed by western immunoblotting analysis, showing a high decrease in porin content in hypothyroid rabbits.

Reagents—Unless otherwise stated, reagents used were of analytical grade and supplied by Sigma Chemical Co.

RESULTS AND DISCUSSION

The rapid switch in metabolic pathways relevant for energy provision in the mammalian liver, from the predominant fetal anaerobic glycolysis to the efficient neonatal oxidative phosphorylation in the first hours-old neonate, relies entirely on the rapid postnatal mitochondrial increase in the relative amount of those molecular components that define mitochondrial bioenergetic and metabolic functions (18). Several of the enzymatic activities in the glycolytic and oxidative pathways of glucose exhibit thyroid hormone-dependent increases during brain development (19), but it is unlikely that all of these changes are mediated by direct thyroid hormone regulation of their gene expression. Brain mitochondria have been shown to bind hexokinase I selectively and reversibly in a glucose 6-phosphate sensitive manner (15). The mitochondrially bound hexokinase can greatly increase the rate of aerobic glycolysis. As the processes leading to or resulting from differentiation are energy-consuming, the coordination of metabolism by mitochondrially bound hexokinase could be of significant importance in the regulation of development.

Thyroid hormone is essential for normal postnatal growth and development of the nervous system (4). Administration of MMI to pregnant rodents before the onset of fetal thyroid function is known to prevent both maternal and fetal thyroid function (20). Neonatal hypothyroidism impairs development of virtually all tissues, but the most prominent deficits are observed in brain.

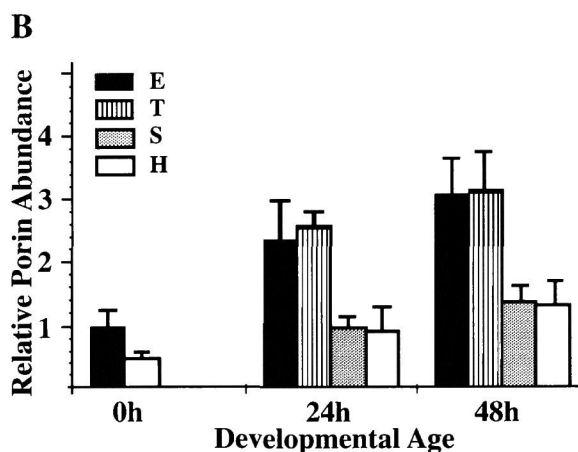
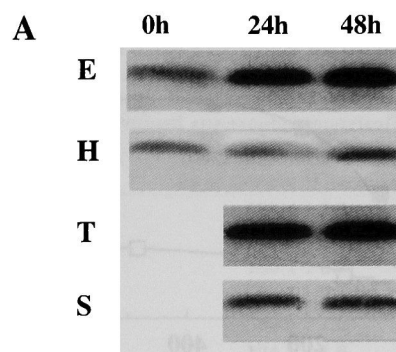


Fig. 2. Postnatal development of the amount of porin protein in isolated mitochondria of euthyroid and hypothyroid rabbit neonates. A: Representative gels obtained using three separate preparations each from euthyroid (E), hypothyroid (H), and hypothyroid neonates treated at birth with T_4 (T) or salt (S) isolated at various times after delivery. After centrifugation, 0.6 ml portions of supernatants were applied on hydroxyapatite/celite columns. Porins were eluted with 3% Triton X-100, and 30% of the eluted protein was subjected to SDS-PAGE 16% as described in experimental procedures. B: Estimation of relative amounts of purified mitochondrial porin. Densitometric quantitation of brain mitochondrial porin levels at 0, 24, and 48-h postnatal of coomassie blue-stained protein bands or Western blots are shown. The values are means \pm SE of three different mitochondrial preparations, presented as the relative porin abundance in hypothyroid rabbits (H), hypothyroid T_4 -treated (T) and hypothyroid salt-treated (S) compared with control rabbits (E) after assigning a value of 1 to the control average.

In order to investigate the possible effects of thyroid status on brain postnatal mitochondrial differentiation, and proliferation, the pore-forming protein of the outer mitochondrial membrane was selected as a marker of the process of mitochondrial proliferation and differentiation for two main reasons: (i) it is a major component of the outer mitochondrial membrane and also of brain mitochondrial proteins; and (ii) it is the hexokinase binding protein, potentially important for the regulation of glucose metabolism (21).

Brain mitochondria were isolated from normal, hypothyroid, T_4 , and salt-treated hypothyroid neonates after 0, 24, and 48-h of postnatal development, and the concentration of the outer mitochondrial membrane porin protein was measured. Comparison of the protein patterns of euthyroid and hypothyroid mitochondria

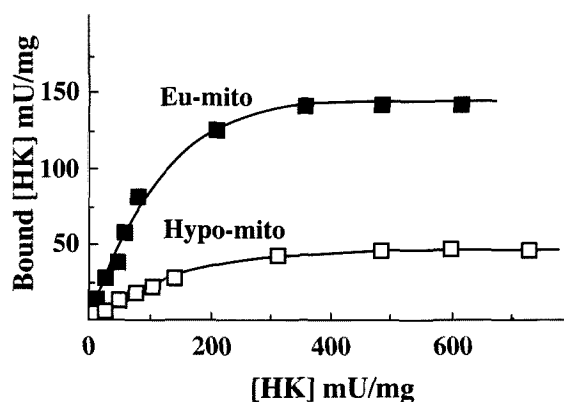


Fig. 3. Effect of hypothyroidism on the hexokinase binding capacity of brain mitochondria from newborn rabbits. Brain mitochondria (0.5 mg protein) isolated from euthyroid or hypothyroid (MMI) fetuses (0 h) extensively depleted of endogenous hexokinase activity were incubated with various hexokinase concentrations and the binding was assessed. The results are plotted as bound hexokinase versus free hexokinase.

revealed clear differences. In particular, the band corresponding to 33 kDa showed the most conspicuous difference between hypothyroid and euthyroid mitochondria. As shown in Fig. 1, at 48-h after delivery the amount of porin protein was more abundant in brain mitochondria from euthyroid rabbits. The protein profile of mitochondria isolated from T_4 -treated hypothyroid brains was essentially similar to that from euthyroid brains.

The relative levels of purified porin in euthyroid and hypothyroid rabbit brains were determined by quantitative immunoblotting using a specific antibody for VDAC. The amount of porin protein measured in isolated mitochondria of hypothyroid neonates accounted only for 50% of that found in normal age-matched neonates (Fig. 2, H and E, respectively). Salt treated hypothyroid neonates showed a lack of increase in the amount of porin protein (Fig. 2, S). Administration of T_4 to hypothyroid neonates promoted a 2-fold increase in the amount of isolated porin protein. No significant difference in the relative amount of porin protein was evident between mitochondria from T_4 -treated hypothyroid brains and euthyroid brains (Fig. 2, T, and E, respectively). These results indicate that the relative amount of this protein in mitochondria of normal and T_4 -treated hypothyroid neonates doubles during the first postnatal 24 h. Progressive and parallel increases were seen in mitochondrial porin concentration after administration of thyroxine on alternate days to hypothyroid rabbits (not shown).

The results reported herein indicate that the fetal brain of hypothyroid rabbits contains approximately half the amount of porin of normal fetal brain. Treatment of hypothyroid rabbit neonates at birth with T_4 but not with NaCl restored the normal increase in the porin protein levels after one day of hormone administration (Fig. 2). It is thus concluded that fetal thyroid hormones, and/or the maternal supply of these hormones to the fetus, are needed to achieve the normal level of mitochondrial porin in brain cells during rabbit fetal development.

Postnatal differentiation of pre-existing mitochondria is a key regulatory process that is accomplished very rap-

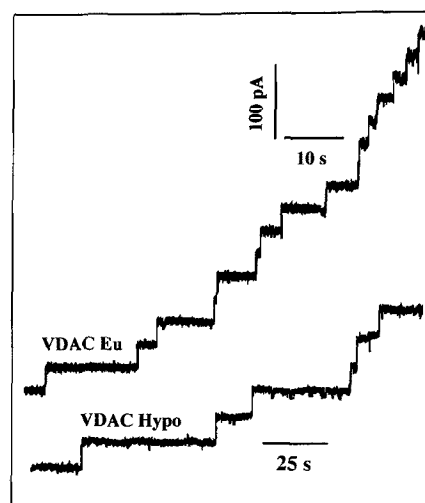


Fig. 4. Reconstitution of VDACs into planar lipid bilayer. The membrane current was increased after addition of the individual VDAC. Upper current trace, VDAC-Euthyroid; lower current trace, VDAC-Hypothyroid. The lipid bilayer was formed from 1% (w/v) diphytanoyl phosphatidylcholine in *n*-decane. The bath solution was symmetrical 1 M KCl, 10 mM HEPES, pH 7.2. The purified VDACs (50–100 ng) were added to the cis side. The membrane voltage was +10 mV.

idly after birth, as a result of a rapid increase in the rates of protein synthesis for mitochondrial proteins. Fetal hypothyroidism prevents the postnatal surge in mitochondrial porin, thus also impairing mitochondrial differentiation after birth. The rapid increase in porin protein after thyroid hormone administration to hypothyroid neonates in isolated mitochondria clearly indicates the necessary role of thyroid hormones for postnatal mitochondrial differentiation. These results are the first experimental evidence for a hormonal and rapid regulation of the outer mitochondrial porin by thyroid hormones. Further identification of the molecular background of this regulative mechanism of VDAC should yield insights into the physiological mechanism and related malfunctioning of this protein in the brain.

In order to determine the binding capacity of brain mitochondria for hexokinase, a fixed amount of mitochondria, extensively depleted of endogenous hexokinase activity by pretreatment with glucose 6-phosphate (15) was incubated with increasing concentrations of hexokinase. The results plotted as bound hexokinase versus free hexokinase (Fig. 3) indicate that brain mitochondria are saturated with hexokinase in both conditions. As demonstrated in Fig. 3, a marked difference was observed in the binding of hexokinase with hypo- and control brain mitochondria. Namely, in the case of hypo-mitochondria, about 50 mU of hexokinase binds per mg mitochondrial protein. In contrast, the saturated binding level of hexokinase to normal brain mitochondria was about 146 mU·mg⁻¹ protein. The data clearly indicate that the low hexokinase-binding capability of hypo-mitochondria was due to a quantitative difference in binding sites.

Mitochondrial VDAC proteins are known to form large conductance channels when reconstituted into a planar lipid bilayer. To determine whether the hypothyroidism-

Table 1. Glucose phosphorylating activities in soluble fractions from developing rabbit brain. Total glucose phosphorylating activity (TGPA) and hexokinase (HK) activities are given as mean \pm SE values for triplicate determinations ($n = 3-4$). TGPA was determined at 25 mM glucose, whereas hexokinase activities were determined at 0.5 mM glucose. Glucose phosphorylating activities in soluble fractions from normal, hypothyroid, hypothyroid T_4 -treated, and hypothyroid NaCl-treated rabbit neonates were determined spectrophotometrically.

Rabbit neonates	Time (h)	No. of rabbits	TGPA (pmol NADPH \cdot min $^{-1}$ \cdot μ g of protein $^{-1}$)	HK (pmol NADPH \cdot min $^{-1}$ \cdot μ g of protein $^{-1}$)
Euthyroid	0	4	136.6 \pm 0.2	102.4 \pm 3.2
	24	3	155.1 \pm 0.9	116.3 \pm 2.5
	48	3	246.1 \pm 0.2	185.1 \pm 3.3
Hypothyroid	0	4	80.4 \pm 0.7	64.4 \pm 2.7
	24	3	104.8 \pm 0.5	83.9 \pm 1.5
	48	4	155.3 \pm 1.6	124.5 \pm 1.6
Hypothyroid T_4 -treated	0	3	83.7 \pm 2.0	65.7 \pm 2.0
	24	3	127.1 \pm 1.3	99.8 \pm 3.7
	48	4	250.8 \pm 1.9	197.1 \pm 0.9
Hypothyroid salt-treated	0	3	75.8 \pm 1.4	62.2 \pm 2.1
	24	3	102.5 \pm 1.7	84.1 \pm 1.7
	48	3	158.1 \pm 1.4	127.8 \pm 2.4

related changes in the content of brain mitochondrial porin could alter the function of these proteins, they were added to the cis side of a planar lipid bilayer at a concentration of 50 to 100 ng/ml and an electrical potential difference of +10 mV was applied to the membrane. At this voltage, VDAC is mainly in its fully open state, and each insertion of a channel into the membrane yielded a discrete current step as illustrated in Fig. 4. Uniform stepwise increases in current flow across the membrane were observed and interpreted as the insertions of single channels. In the presence of 1 M KCl these single channel insertions represented conductance increments of 3.7 ± 0.1 nS ($n = 77$) and 4.0 ± 0.1 nS ($n = 81$) for VDAC-Eu and VDAC-Hypo, respectively. These conductances correspond to the fully open state of a single channel and are consistent with those reported for VDAC from brain (22).

Maturation of structure and function in the mammalian brain is normally accompanied by profound increases in local rates of glucose utilization (23, 24), but in rats made cretinous by radiothyroidectomy within the first 2 days after birth and studied later in adulthood, local cerebral glucose utilization in all brain regions examined was found to be depressed below values in euthyroid controls by 24–58% (25).

Hexokinase is a key enzyme in glucose metabolism. Binding of hexokinase to the outer mitochondrial membrane is tissue specific and developmentally regulated, the enzyme activity being markedly elevated in several cancer cells. In fact, approximately 70% of human brain hexokinase I is bound to mitochondria, presumably in a tetrameric form, in association with mitochondrial porin. Hexokinase I might act as a coordinator enzyme, preventing an imbalance of glycolytic reactions over mitochondrial oxidative reactions, which could lead to the accumulation of neurotoxic lactate.

Given the importance of glucose metabolism during gestation and early postnatal growth, the changes in the developmental pattern of hexokinase activity in the brain of hypothyroid rabbits and the effect of thyroxine hormone on this enzyme activity have been investigated. Three sets of experiments were performed to verify that hexokinase enzymes are under thyroid hormone regula-

tion. Table 1 shows the glucose phosphorylating activities determined in soluble fractions of different brain homogenates from normal, hypothyroid, T_4 , and salt-treated hypothyroid neonates at 0, 24, and 48-h of postnatal development using the spectrophotometric assay. Glucose phosphorylating activity analysis involved assays at two glucose concentrations, 0.5 mM for hexokinase activity, a concentration at which glucokinase is essentially inactive and 25 mM glucose, a concentration at which all phosphotransferase activities were measured. The contribution of hexokinase to the total glucose phosphorylating activity was 75% in euthyroid, 80% in hypothyroid, 78% in hypothyroid T_4 -treated, and 82% in the hypothyroid salt-treated neonates.

At birth, brain homogenates from neonates of MMI-treated rabbits showed significantly lower levels of hexokinase activity than their corresponding age-matched normal neonates. These were normalized within 24 h, when T_4 was injected at zero time (Table 1). The developmental profile of the hexokinase activity in brain homogenates of hypothyroid NaCl-treated neonates showed no postnatal increase (Table 1).

As a significant difference in enzyme activity was only observed between the hypo- and euthyroid group, the importance of the hormone action within the physiological range is apparent. The rapid response of hexokinase activity after administration of T_4 hormone to hypothyroid neonates in the hexokinase activity, provide evidence for a rapid thyroid hormone-mediated regulation of the hexokinase enzymes at either a transcriptional and/or post-transcriptional level.

The present results confirm that thyroid hormone is involved in the development of processes in brain that regulate glycolysis or require the energy derived from the metabolism of glucose. The effects of different thyroid status on various aspects of glucose metabolism can partly be explained by coordinated reductions in hexokinase binding protein concentration and hexokinase activity in developing rabbits.

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