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Circulating Glucose, Insulin and Ketone Bodies and Enzymes of Ketone Body Utilization in Brain Mitochondria from Suckling Rats Treated with High L-Thyroxine Doses

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The neo-T_4 syndrome was induced by subcutaneous administration of a total dose of (150 μg) L-thyroxine (T_4) to rats from their first day of live.

Neo-T_4 animals and their controls were sacrificed at 2, 4, 8, 11, 14, 22 and 25 days of age. A decrease in body weight was observed from the second day of life, and a decrease in brain weight from the eighth day of life in the neo-T_4 animals. Blood glucose and plasma insulin levels were decreased from 2nd day through 22nd day of life. Total plasma ketone bodies and β-OH butyrate levels increased in the neo-T_4 animals with respect to controls. until 8th day, although acetoacetate increased only until 4th day. The activity of key enzymes in the ketone bodies utilization pathway (3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase) were also measured in the animals brain. We found an activation of 3-hydroxybutyrate dehydrogenase until 11th day and 3-oxoacid CoA-transferase until 14th day, but no change in acetoacetyl CoA-thiolase was observed. Ketone bodies play a key role as energy substrates and precursors of brain lipids during the period of intense growth and myelination of the CNS. Considering the alterations described in this paper it seems that neo-T_4 syndrome could be an interesting model for studying metabolism of those substances in brain.

The administration of large doses of thyroid hormones to rats with immature central nervous system (CNS), produces an abnormal development of this system and of the endocrine system known as neo-T_4 syndrome (2). The main alterations are a decrease in brain and body weight (11), a decrease in pituitary TSH (1) and GH content (19) and a permanent histological damage of the cerebral cortex which have been related to abnormalities in the behaviour of adult animal. The syn-

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drome is irreversible if it is produced at an early age (9).

It has been proved that neo-T_4 rats show drastic alterations in their carbohydrate metabolism: persistent hypoglycemia, a marked decrease in liver glycogen and a decreased insulin response to glucose loads (11). These phenomena take place during a period in which there is a growth spurt of the brain, and, consequently, the energetic and biosynthetic requirements of this organ are increased. The possible relationship of the perturbed carbohydrate metabolism and the abnormal brain development during this syndrome has been suggested.

Ketone bodies —3-hydroxybutyrate and acetoacetate— are widely used by neonatal brain both as energy substrates and as precursors of brain lipids (10, 18). The high lipid content of the mother's milk (6) leads to high blood ketone body levels in the suckling rat. During this period, the enzyme activities related to ketone body utilization are increased in the brain while they decrease after weaning (16).

There are few studies referring to the effect of an altered thyroid state on the metabolism of ketone bodies in the brain of the immature animal. PATEL (21) showed that the activity of enzymes of ketone body utilization was decreased in hypothyroid suckling rats. On the other hand, GRAVE *et al.* (12) found an increase in 3-hydroxybutyrate dehydrogenase in suckling hyperthyroid animals.

This work shows the circulating levels of the main brain metabolic fuels, glucose and ketone bodies, in suckling neo-T_4 rats of different ages and in their controls. In the same stage, the activity of three mitochondrial enzymes involved in the conversion of ketone bodies into acetyl-CoA —3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), 3-oxoacid CoA-transferase (EC 2.8.3.50) and acetoacetyl-CoA thiolase (EC 2.3.1.9)— has been studied in the brain.

Materials and Methods

Animals. Wistar rats from our own laboratory were used. The mothers were fed a commercial diet containing 1-2 μg of iodine/g of diet. We considered 0 as the moment of birth of the animals, which was carefully observed, day 1 being 24 hours later. The number of pups in each litter was evened out to 8. In all cases, the litters were divided into two groups; one of them was treated with thyroxine, the other serving as control.

Treatment, sacrifice and sampling. L-thyroxine sodium pentahydrate from Sigma (T_4) was dissolved to a concentration of 0.3 mg/ml in slightly alkaline 0.9% NaCl. Animals were treated with a total of 150 μg T_4 in 4 injections administered between the first and the 8th day of life. Control animals received an equal volume of the solution used for dissolving the hormone. Animals were sacrificed by decapitation at 2, 4, 8, 11, 14, 22 and 25 days of age. A series of normal 300 g adult rats were also sacrificed. Part of the blood was collected for the determination of glucose and ketone bodies. Another part was collected in heparinized tubes, and plasma was obtained for insulin assay. The brain was also obtained.

Assay procedures. Glucose was assayed with glucose oxidase (13) in Ba $(OH)_2$-SO_4Zn deproteinized extracts. 3-hydroxybutyrate and acetoacetate were determined according to WILLIAMSON *et al.* (27) in ClO_4H deproteinized extracts, neutralized with KOH. Plasma insulin was assayed by a specific rat RIA, with reagents from the Novo Research Institute (Copenhagen, Denmark). All plasma samples were analyzed in the same run.

Brain was homogenized as described by PATEL (21) and part of the homogenate was used for obtaining crude mitochondria, according to SMITH *et al.* (23). The homogenates were centrifuged for 10 min in a refrigerated centrifuge at 800 *g*. The

supernatant was centrifuged again for 30 min at 2.500 *g*. The resultant precipitate was diluted in the same buffer used to homogenate and centrifuged again 30 min at 2.500 *g*. We repeated this procedure two new times. The last precipitate had over 70 % of 3-hydroxybutyrate dehydrogenase activity initially measured in the homogenate.

3-hydroxybutyrate dehydrogenase was assayed in samples sonicated for 30 s at 15 Khz and determined by the SWIATECK procedure (25). The incubation medium contained 1 mM KCN, 2 mM NAD, 30 mM DL-hydroxybutyrate in 50 mM Tris at pH = 8.1. The reaction was started by addition of the sample, and it was followed by the increase in optical density to 340 nm.

3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase were analyzed in samples treated with Triton X-100 to a final concentration of 0.5 % (w/v). 3-oxoacid CoA transferase was measured spectrophotometrically by the disappearance at 303 nm of acetoacetyl-CoA-Mg^{+2} complex in a mixture containing 10 mM $MgCl_2$, 5 mM iodoacetamide, 0.1 mM acetoacetyl-CoA in 50 mM Tris at pH = 8.5 and the enzymatic sample (28), after which the changes in optical density were examined in order to check the possible «deacylase activity» which was substracted in all cases. The specific reaction was started with the addition of 50 μmoles of succinate (28).

Acetoacetyl-CoA thiolase was assayed spectrophotometrically at 313 nm according to WILLIAMSON (28). The three enzymes were measured 24 h after the animals were killed. The enzymatic activities did not decrease when preserved for 4 weeks at —20°C, the initial rates of enzyme reactions were proportional the amount of added protein. Protein was determined as described by LOWRY *et al.* (15). One unit of enzymatic activity is defined as 1 μmol of substrate consumed or product formed per minute at 37°C.

Results

Table I shows body and brain weights, as well as blood glucose and plasma insulin levels in neo-T_4 and control rats of different ages. After the first dose of T_4 (at 2 days of life), there is a decrease in body weight, glycemia and plasma insulin in T_4-treated animals. Brain weight is significantly decreased from the 8th day of life. Brain and body weights of neo-T_4 animals remain below controls through the study period. Glycemia and plasma insulin levels reached control values after weaning —25 days of age—.

Fig. 1 shows 3-hydroxybutyrate and acetoacetate levels in plasma, as well as the sum of both —ketonaemia— during

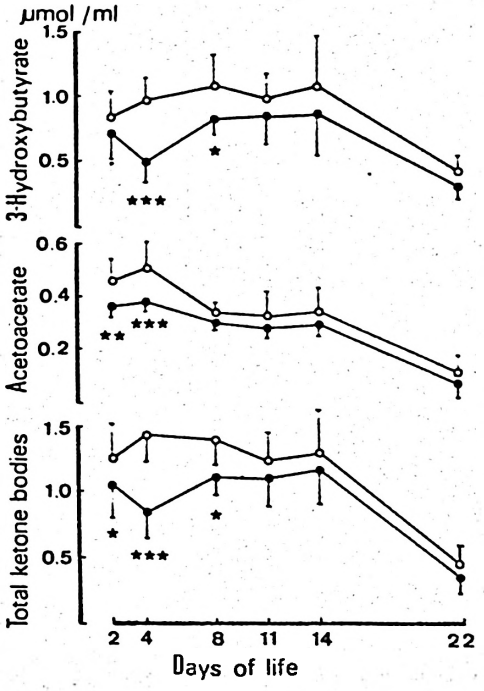

Fig. 1. *Blood 3-hydroxybutyrate and acetoacetate levels and ketonaemia in neo-T_4 and control rats.* Onset of T_4 administration was on the first day of life and the animals were killed at different stages of life. Each point is the mean ± SD of 12-15 animals (o——o) neo-T_4 rats, (●——●) controls. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table I. *Body and brain weights, glycemia and plasma insulin in neo-T_4 and control rats.* Each value ($\bar{x}$ ± S.D.) corresponds to 12-15 animals.

Parameters		Days of life						
		2	4	8	11	14	22	25
Body weight (g)	control	6.5±0.2	10.8±0.5	17.4±1.8	26.5±2.5	28.9±2.1	48.1±3.2	63.0±2.1
	neo-T_4	6.1±0.3	9.3±0.2	12.7±2.1	17.8±2.7	21.4±1.9	37.5±5.0	44.1±2.1
	p	*	***	***	***	***	***	***
Brain weight (g)	control	0.19±0.09	0.33±0.02	0.60±0.03	0.78±0.03	0.89±0.02	1.01±0.03	1.03±0.04
	neo-T_4	0.20±0.01	0.33±0.01	0.53±0.04	0.66±0.03	0.76±0.02	0.88±0.04	0.85±0.03
	p	N.S.	N.S.	***	***	***	***	***
Blood glucose (mg/100 ml)	control	80.5±5.5	80.4±3.3	85.0±5.1	96.4±6.9	115.0±12.0	116.4±8.3	117.5±7.3
	neo-T_4	72.4±4.7	67.6±5.4	57.2±6.6	81.3±8.5	85.4±17.6	98.5±9.1	116.6±9.3
	p	*	**	***	***	***	***	N.S.
Plasma insulin (ng/ml)	control	1.65±0.11	1.35±0.45	1.23±0.19	1.45±0.55	1.85±0.55	1.15±0.26	1.71±0.28
	neo-T_4	0.89±0.33	0.64±0.12	0.70±0.10	1.01±0.30	1.04±0.14	0.94±0.08	1.41±0.52
	p	**	***	***	**	***	*	N.S.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, N.S. = no significant.

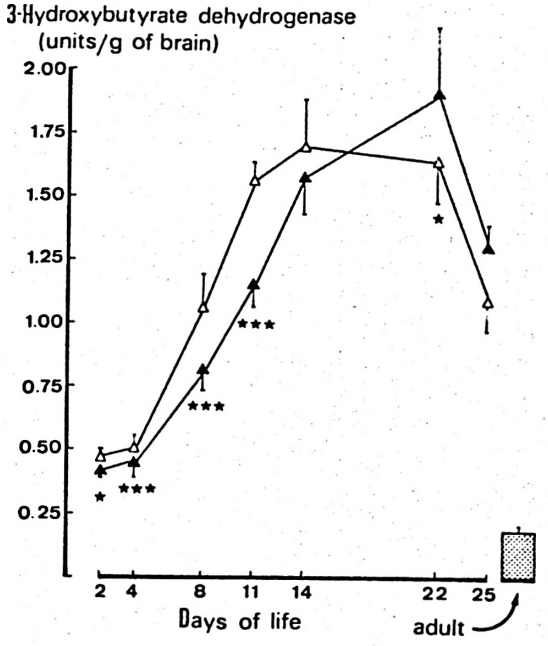

Fig. 2. *Changes with age in 3-hydroxybutyrate dehydrogenase activity of brain crude mitochondria from neo-T_4 and control rats.*
The onset of T_4 treatment was at first day of life and the animals were sacrificed at different stages of development, as indicated. Enzyme activities are expressed as units/g brain (wet weight). The results are mean ± SD for 12-15 animals. (△——△) neo-T_4 rats, (▲——▲) controls. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

the period which has been studied. Neo-T_4 rats present a significant increase in 3-hydroxybutyrate levels on days 4 and 8, and of acetoacetate levels on day 2 and 4. Ketonaemia remains significantly above control values on day 2, 4 and 8. In any case, plasma ketone bodies are relatively high —with respect to adult levels— in both neo-T_4 and control rats during suckling, decreasing after weaning —at 22 days of age—.

Fig. 2 shows the pattern of development of 3-hydroxybutyrate dehydrogenase in the brain of neo-T_4 and control rats, activity being expressed in units per gram of wet brain weight. This activity is significantly increased in neo-T_4 rats at 2, 4, 8 and 11 days of age, while it is decreased at 22 days of age. On the other hand, 3-hydroxybutyrate dehydrogenase activity is considerably higher during suckling in both neo-T_4 and control animals, with respect to the activity found in adult rats.

Fig. 3 shows the pattern of development of 3-oxoacid CoA-transferase, expressed in the same way as the preceding enzyme. This activity is increased in neo-T_4 animals at 4, 8, 11 and 14 days of age; it is also higher in suckling rats than in adults.

The pattern of development of aceto-

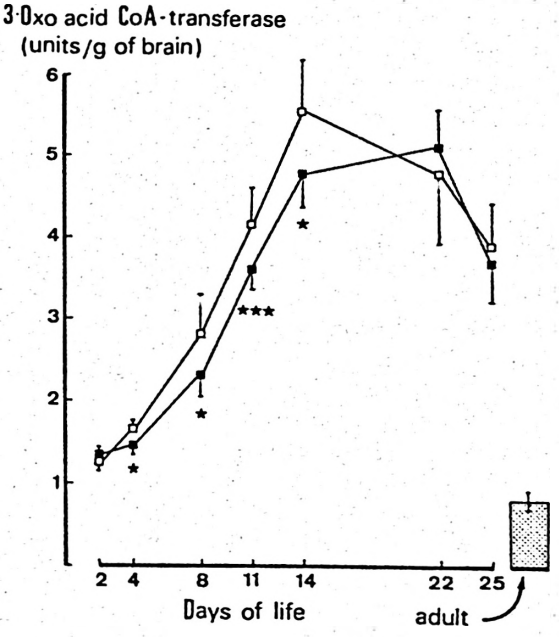

Fig. 3. *Changes with age in 3-oxoacid CoA-transferase activity of brain crude mitochondria from neo-T_4 and control rats.*
The treatment began at first day of life and the animals were sacrificed at different stages of development, as indicated. Enzyme activities are expressed as units/g brain (wet weight). The results are mean ± SD for 12-15 animals. (□——□) neo-T_4 rats, (■——■) controls. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table II. *Specific activity of 3-hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and Acetoacetyl-CoA thiolase in brain mitochondria from neo-T_4 and control rats.*

Activity is expressed as units/mg mitochondrial protein. Each value ($\bar{x} \pm$ S.D.) corresponds to 12-15 animals.

Parameters		Days of life						
		2	4	8	11	14	22	25
3-hydroxybutyrate dehydrogenase	control	0.016±0.001	0.014±0.002	0.025±0.004	0.029±0.002	0.037±0.003	0.045±0.002	0.038±0.002
	neo-T_4	0.017±0.001	0.016±0.001	0.026±0.005	0.032±0.002	0.038±0.004	0.041±0.003	0.036±0.002
	p	N.S.	**	N.S.	**	N.S.	*	N.S.
3-oxoacid CoA-transferase	control	0.050±0.004	0.046±0.002	0.081±0.004	0.098±0.005	0.115±0.011	0.131±0.001	0.124±0.012
	neo-T_4	0.049±0.003	0.048±0.001	0.087±0.004	0.102±0.005	0.127±0.011	0.128±0.012	0.120±0.015
	p	N.S.	N.S.	**	N.S.	*	N.S.	N.S.
Acetoacetyl-CoA thiolase	control	0.121±0.006	0.099±0.016	0.128±0.012	0.115±0.013	0.087±0.006	0.056±0.004	0.061±0.001
	neo-T_4	0.121±0.004	0.100±0.016	0.122±0.012	0.102±0.010	0.085±0.011	0.057±0.007	0.060±0.003
	p	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, N.S. = no significant.

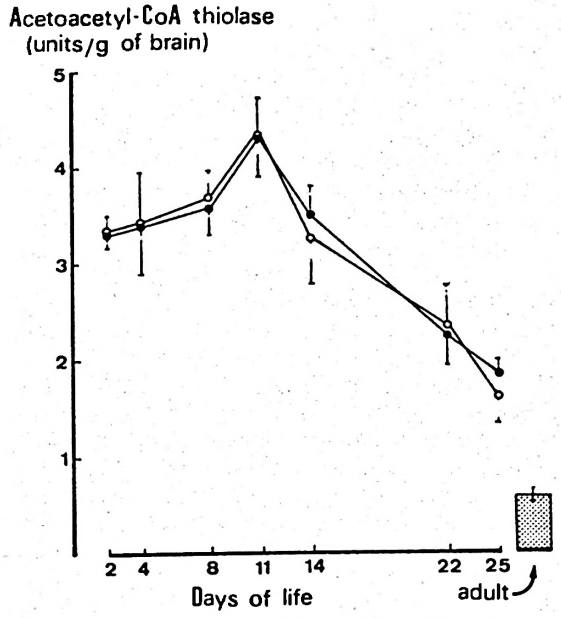

Fig. 4. *Changes with age in acetoacetyl-CoA thiolase activity of brain crude mitochondria from neo-T_4 and control rats.*

The onset of T_4 treatment was at first day of life and the animals were sacrificed at different stages of development, as indicated. Enzyme activities are expressed as units/g brain (wet weight). The results are mean ± SD for 12-15 animals. (o——o) neo-T_4 rats, (●——●) controls. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

acetyl-CoA thiolase is expressed in the same fashion as the preceding enzymes (fig. 4).

No difference has been observed between neo-T_4 and control animals at the stages which have been studied. Nevertheless, activities were significantly higher in suckling animals than in adult rats. Table II shows the activities of these same three enzymes in the same stages of life, but expressed as units per mg of mitochondrial protein. 3-hydroxybutyrate dehydrogenase appears significantly increased in neo-T_4 rats at 4 and 11 days of life, while it is decreased at 22 days of life. The specific activity of 3-oxoacid CoA-transferase is increased in these same animals at 8 and 14 days of life. There is no difference between neo-T_4 and control rats at any of the studied stages with respect to acetoacetyl-CoA thiolase specific activity.

Discussion

Thyroid hormones exert a strong influence on the development of the CNS (8). It is well known that hypothyroidism at an early age leads to a deficient maturation of that system (4); on the contrary, the presence of an excess of thyroxine during an early stage of the development of the rat —although producing an early maturation in certain types of conduct (22)— produces a retardation in growth, with decrease in body and brain weight (Table I). Adult neo-T_4 rats also show a decrease in correct responses in the Hebb-Williams test (7). The correlation between these observations and the biochemical alterations responsible for them is scarcely known. It has been observed that during genesis of the neo-T_4 syndrome there is a deficit of carbohydrate substrates —liver glycogen and blood glucose— precisely when the energy needs of the brain are greatest, as it is going through a stage of intense myelination and growth (5). Our results show that neo-T_4 rat is hypoglycemic during the whole period of suckling and is also deficient in circulating insulin. Many of the alterations in neo-T_4 syndrome are similar to those caused by prolonged fasting. We have demonstrated in another study (in press in *Biology of Neonate*) that during the first 8 days of the syndrome there is a decrease in milk intake in the studied animals returning to normal after this period.

Ketone bodies play an important role as energy substrates and precursors of lipid biosynthesis in the brain during its critical growth period (26). Neo-T_4 rats suffer a significant increase in ketonae-

mia during the first 8 days of life, higher than the normal high levels throughout suckling. This increased ketonaemia must be favoured by the low plasma insulin levels. On the other hand, neo-T_4 rats react to glucose loads much like diabetic animals (11). It has been described that during fasting or alloxan diabetes, the rate of utilization of ketone bodies is in proportion to their plasma levels (28).

Our results show that 3-hydroxybutyrate dehydrogenase is increased in neo-T_4 rats, in agreement with the results of GRAVE *et al.* (12) in hyperthyroid rats; 3-oxoacid CoA-transferase is also increased during approximately the first two weeks of life in neo-T_4 animals. When these activities are expressed per mg of mitochondrial protein, their increase is significant in a certain number of age groups. In contrast, the activity of acetoacetyl-CoA thiolase did not change in any of the studied stages. This could be related to the fact that acetoacetyl-CoA thiolase has two different localizations, in cytoplasm and in mitochondria (16). The correlation between the activities of the first two enzymes is in good agreement with the data reported by several authors (3, 14, 21).

From these results, it can be concluded that neo-T_4 rats present a desequilibrium of circulating energy and biosynthetic substrates —a decrease in blood glucose and an increase in ketone bodies— during a critical period in brain development. In suckling rat and at phisiological concentrations of both substrates, ketone bodies seem to be better precursors of brain lipid biosynthesis than glucose (26). Nevertheless, it has also been shown that glucose increases the production of CO_2 as well as lipid synthesis from 3-hydroxybutyrate and acetoacetate (20). The reciprocal influence of glucose and ketone body metabolism in the brain is still not well known (17). We think neo-T_4 syndrome can be an interesting model for studying the interrelationship of glucose and ketone body utilization in neonatal brain.

Resumen

Ratas con síndrome neo-T_4 inducido por administración subcutánea de tiroxina (150 μg/5 dosis) desde el primer día de vida, se sacrifican, así como sus controles, a los 2, 4, 8, 11, 14, 22 y 25 días de edad. En los animales neo-T_4, se observa decrecimiento del peso del cuerpo desde el 2.º día de vida y disminución en el peso cerebral desde el día 8. La glucosa en sangre y la insulina plasmática decrecen desde el día 2 hasta el día 22 de vida. Los cuerpos cetónicos totales en plasma, y los niveles de β-OH-butirato se incrementan en los animales neo-T_4 con respecto a los controles hasta el día 8, aunque el acetoacetato se incrementa solamente hasta el día 4. Se mide en el cerebro de estos animales la actividad de las enzimas clave de la utilización de cuerpos cetónicos observándose activación de 3-hidroxibutirato deshidrogenasa hasta el día 11, de 3-oxoacido-CoA-transferasa hasta el día 14 y ningún cambio en acetoacetil-CoA tiolasa.

En períodos de intenso crecimiento y mielinización cerebral los cuerpos cetónicos juegan un papel clave como substratos energéticos y como precursores de lípidos cerebrales. Las alteraciones reseñadas en este trabajo parecen mostrar que el síndrome neo-T_4 puede ser un modelo interesante para el estudio del metabolismo cerebral de estas sustancias.

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