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Neonatal Hyperthyroidism Alters Hepatic Epidermal Growth Factor Receptor Ontogeny in Mice

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ABSTRACT. Epidermal growth factor (EGF) liver receptor ontogeny and somatic growth were studied in mice from day 7 to day 70 postnatally to assess long-term effects of short-term postnatal thyroxine treatment. The mice were given 0.4 μg thyroxine/g body weight/day for the 1st wk of life. EGF receptor binding in liver tissue was studied on days 7, 15, 20, 30, and 70 postnatally. Treated animals had accelerated eyelid opening and tooth eruption, and permanent growth retardation was obvious from the second week of life. Hepatic EGF receptor-binding capacity increased markedly in control mice with increasing age in contrast to a very slow increase in treated mice, making the difference statistically significant ($P < 0.01$) from day 30. The affinity of EGF receptor binding initially was similar in the two groups of animals (1.09×10^9 M^{-1} and 1.02×10^9 M^{-1}) and increased by day 30 in controls (2.57×10^9 M^{-1}), an increase that was not observed in treated animals either at day 30 or 70. These results suggest a sensitive period of imprinting during the first 7 days postnatally, a period when thyroxine can exert a permanent effect on later growth and later hepatic EGF receptor number. (*Pediatr Res* 23: 557-560, 1988)

Abbreviations

EGF, epidermal growth factor
T_4, thyroxine
TSH, thyroid-stimulating hormone

Thyroid hormones are necessary for postnatal growth and development in rodents as in humans although the mechanism of the thyroid hormone effect is not entirely clear (1). Recent studies suggest that thyroid hormone interaction with specific growth factors may be important for normal postnatal growth and development (2). An interaction with the growth hormone-somatomedin axis has been described (2). Several other growth factors have been studied, but to date the interaction between thyroid hormones and EGF is the best characterized (3, 4). EGF is a polypeptide (mouse EGF mol. wt. 6045) first described in extracts from mouse submandibular glands (5–7). *In vivo*, EGF elicits precocious tooth eruption and eyelid opening in rodents, accelerates lung maturation in lambs and reduces gastric acid secretion in man (4–9). EGF binds to specific plasma membrane receptors that are found in many tissues and significant EGF binding to embryonal tissues has been reported (7, 10–14).

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An early indication of a close relation between thyroid hormones and EGF was found by Hamburgh *et al.* (15) who showed that the delay of eyelid opening and tooth eruption observed in hypothyroid animals was reversed by either thyroxine or EGF administration. Further studies suggest that synthesis of both EGF and its receptor protein are influenced by thyroid hormones (3, 4, 12, 16–20). Recent studies of early postnatal T_4 treatment (0–6 days) of newborn mice have shown a prolonged effect of the T_4 on tissue and urine EGF levels and a prolonged effect on growth (21). This effect was not observed in mice treated only during the 2nd wk of life (21). The present study was undertaken to relate the disturbances in somatic growth to the postnatal ontogeny of liver EGF receptors in mice given exogenous thyroxine during the first week of life.

MATERIALS AND METHODS

Experienced female time-mated Swiss Webster mice were purchased from Simonsen Laboratories, Gilroy, CA. Each litter was set to eight pups immediately after delivery. Half of the litter was treated with T_4 0.4 μg/g body weight once daily sc; the other half served as controls and were injected with alkaline saline vehicle. Injections were begun on the day of delivery (day 0) and continued to day 6. The animals were weaned on day 24 and maintained four animals per cage thereafter. They were weighed periodically and eyelid opening and tooth eruption were recorded. The mice were killed by CO_2 narcosis on day 7, 15, 20, and 30 or day 70; only female pups were included for study. There were eight treated and eight control pups in each age group.

Liver tissues were collected, immediately frozen on dry ice, and stored at −70°C until further processing. Protein measurements were done according to Petersen (22). Liver samples were processed for receptor studies according to Hoch and Hollenberg (23). In short, the tissue was homogenized with a glass Teflon homogenizer in (wt/vol 1/10) 0.25 M sucrose, 25 mM Tris-HCl buffer with addition of trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) 1 mg/ml. The homogenate was spun at 600 × *g* for 10 min. The pellet was discharged, the supernatant was respun at 10,000 × *g* for 30 min, and the second pellet was discarded. The supernatants were made 0.1 M in NaCl and 0.2 mM in $MgSO_4$ [by adding 1/10 (vol/vol) of a solution of 0.25 M sucrose, 25 mM Tris-HCl, 1.01 M NaCl, 2.02 mM $MgSO_4$], and spun at 48,000 × *g* for 45 min. The pellets were resuspended in Tris-HCl buffer, respun at 48,000 × *g* for 45 min and finally rehomogenized in 0.05 M phosphate buffer (1 ml/250 mg of original liver tissue). These preparations were stored at −70°C for EGF receptor analysis.

EGF used for radioreceptor analysis was purchased from Collaborative Research (Lexington, MA). EGF was iodinated using the Chloramine-T method (24) to achieve a specific activity between 250 and 300 cpm/pg of EGF. The specific activity of labeled EGF was determined using a double antibody, liquid

phase EGF radioimmunoassay previously described (3). EGF binding studies were carried out in 12 × 75 polystyrene tubes in a 200–300 μl reaction volume containing: 0.05 M phosphate buffer with 1% (wt/vol) bovine albumin, 100 μg of liver membranes, and ^{125}I-EGF. Incubations were carried out at 24°C for 45 min. Incubation time below 35 min and above 60 min decreased EGF binding. Samples were diluted with 4 ml of ice cold buffer [0.05 M phosphate buffer with 1% (wt/vol) bovine albumin], filtered through a Millipore filter (GF/C 1.2 μm) using a multiple manifold apparatus and rapidly washed with two 4-ml aliquots of ice cold buffer under reduced pressure (100 mm Hg). The radioactivity retained in the filter was measured in a gamma scintillation counter.

Radioactivity bound in the presence of an excess (5μg/tube) of cold EGF was defined as nonspecific binding (1–2% of added label). Specific binding was calculated by substracting nonspecific binding from the total amount bound. EGF binding in liver tissues from individual animals was assessed in duplicate with 100 g of membrane protein in 200 μl reaction volume with labeled EGF to achieve a free concentration of labeled EGF of 0.6 nM. For estimating affinity by the method of Scatchard (25), 100 μg of membrane protein in triplicate were added to a final volume of 300 μl with increasing amounts of cold EGF (0.09–50 nmol) in the presence of a tracer amount of labeled EGF (25,000 cpm). A best fit regression line was computer generated for the individual scatchard data. The affinity constant was calculated from the slope of the regression line. Maximal binding capacities were calculated as the intercept of the regression line and the horizontal axis.

The Student's *t* test was used for comparison of individual binding data. Results are recorded as mean and SD unless indicated otherwise.

RESULTS

Tooth eruption and eyelid opening were significantly accelerated in treated animals compared to controls. Tooth eruption occurred on day 6.6 ± 0.49 (mean ± SD) in treated animals *versus* day 9.9 ± 1.1, for controls ($p < 0.01$). Eyelid opening occurred on day 12.0 ± 0.81 in treated animals *versus* day 13.6 ± 0.74 for controls ($p < 0.01$). The decreased growth rate in treated animals resulted in a significantly lower body weight from day 8 in treated animals ($p < 0.01$) compared to controls (Fig. 1).

Hepatic EGF binding data from control and treated animals are shown in Figure 2. The binding of EGF to liver membranes was similar in control and treated animals on days 7, 15, and 20 and significantly different ($p < 0.01$) on days 30 and 70 in treated compared to control mice (Fig. 2). Scatchard analyses were conducted on liver samples from day 7 (samples from all animals in each group pooled), and days 30 and 70 (animals pooled two and two) (Fig. 3 and 4). On day 7 binding capacity and binding affinity were of the same magnitude in control and treated animals (1.09 *versus* 1.02 × 10^9 M^{-1} and 0.64 *versus* 0.76 ng/mg protein, respectively, Table 1). Among control animals an increase in high affinity receptor binding was observed with age, an increase not observed in treated animals (Table 1). Thus, EGF binding affinities in control animals were significantly higher than in treated animals at 30 days (1.06 *versus* 2.57 × 10^9 M^{-1})(Fig. 4; Table 1). The statistically higher B_{max} values ($p < 0.05$) in control animals on day 30 (Table 1) are evident as well on the horizontal axis of Figure 4. When binding was assessed in individual 70-day-old animals, there was significantly reduced

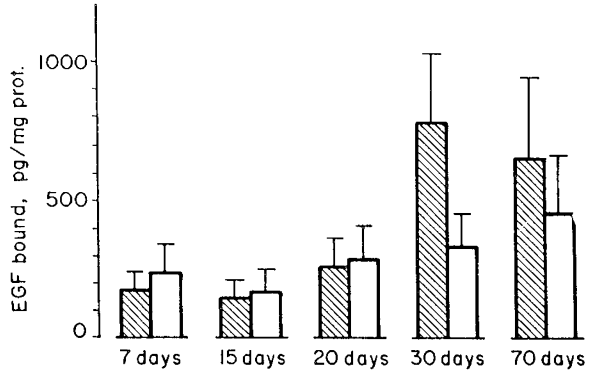

Fig. 2. EGF binding in liver membrane homogenates from individual animals at 7, 15, 20, 30, and 70 days of age. *Open bars*, treated animals; *hatched bars*, control animals. Values plotted as mean and SD.

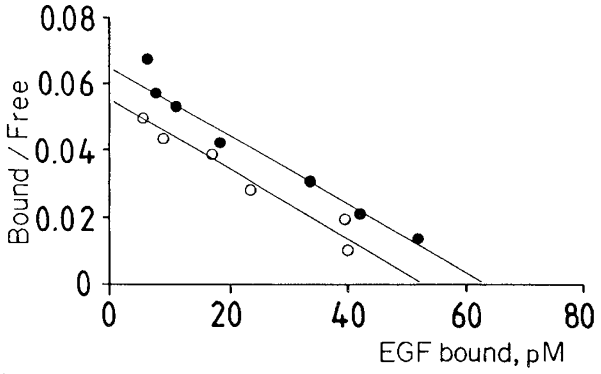

Fig. 3. Scatchard analysis of control animals at 7 days of age (*open circles*) and of treated animals at 7 days of age (*closed circles*).

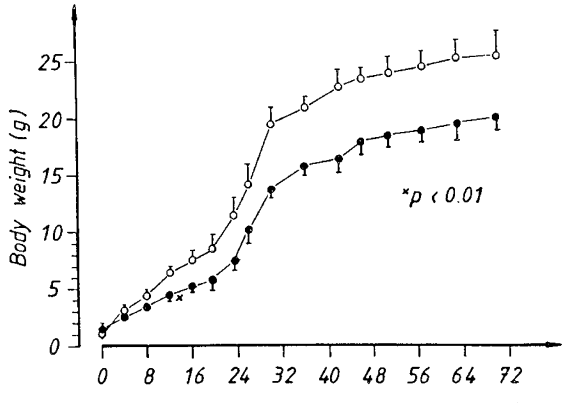

Fig. 1. Body weight in eight treated and eight control animals (*open circles*) followed from birth to 70 days of age. Age in days is plotted on the *horizontal axis*. Values are shown as mean and SD. The difference in weight becomes significant ($P < 0.01$) at 12 days (x).

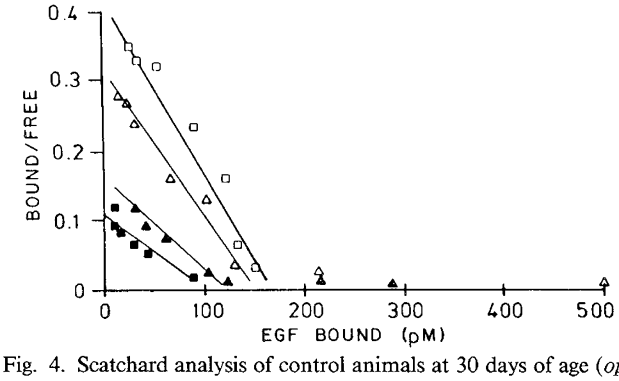

Fig. 4. Scatchard analysis of control animals at 30 days of age (*open squares*) and 70 days of age (*open triangles*), and of treated animals at 30 days of age (*solid squares*) and of 70 days of age (*solid triangles*).

Table 1. *Average hepatic EGF receptor maximal binding capacities (B_{max}) and affinity constants (Ka)**

	B_{max} (ng/mg protein)		Ka (10^9 M^{-1})	
Age (days)	Controls	Treated	Controls	Treated
7	0.64	0.76	1.09	1.02
30	2.07 ± 0.31	1.32 ± 0.29†	2.57 ± 0.39	1.06 ± 0.22†
70	1.93 ± 0.40	1.45 ± 0.38	2.02 ± 0.31	1.12 ± 0.37

* Values as mean and SD. See text for details; 7-day values represent pooled samples.

† $p < 0.05$ treated *versus* controls.

binding in treated as compared to control animals. However, when these animal samples were pooled (two and two) to develop data for Scatchard plots, there were no differences in affinity and binding capacity (2.02 *versus* 1.12 and 1.93 *versus* 1.45 × 10^9 M^{-1}, respectively, Fig. 4, Table 1).

DISCUSSION

Herein we observed the expected effect of thyroxine treatment during the 1st wk of life on eyelid opening and tooth eruption. Also, the treated mice showed a long-term growth retardation as reported earlier (Fig. 1) (21). In addition, hepatic EGF-binding capacity and affinity were significantly reduced on day 30 compared to values in control animals. In control animals there was an increase in maximal binding capacity and binding affinity of hepatic EGF receptors between 20 and 30 days (Table 1; Figs. 2 and 4). In the treated animals at 30 days both parameters remained at lower, immature levels (Table 1; Fig. 4). In other studies of normal control animals two populations of hepatic EGF receptors have been observed (11, 26). We were not able to show the presence of two receptor populations in our study. However, the increase in affinity observed with age is most probably due to an increase of a high affinity receptor and not due to a change of individual receptor affinity.

There is considerable evidence supporting a thyroid hormone dependency of EGF receptors. EGF receptor binding has been shown to increase in response to thyroid hormone treatment in mouse skin and rat liver (12, 18, 19), and congenitally hypothyroid mice fail to show the postnatal increase in hepatic EGF binding normally observed between 20 and 30 days (26). In the present control animals the expected increase of hepatic EGF receptor binding was observed, whereas the animals treated neonatally with thyroxine had no increase (Figs. 2 and 4). In this regard they resemble hypothyroid animals.

Other late abnormalities were observed in mice treated with thyroxine for the first 6 days of life. Growth retardation was obvious in our animals (Fig. 1) and it has been shown that this effect persists in adulthood (27–29). Impaired pituitary growth, decreased pituitary TSH concentrations, increased hypothalamic thyroid-releasing hormone levels, decreased TSH responsiveness to propylthiouracil challenge, and delayed puberty also have been reported (28) as well as a significantly reduced serum TSH level and increased pituitary iodothyronine 5′ monodeiodinase activity (29). Thus, permanent dysfunction at both the hypothalamic and pituitary levels occurs (28, 29). An altered set point for T_4 feedback control has been postulated based on the altered level of pituitary iodothyronine 5′ monodeiodinase activity. Serum EGF concentrations also are elevated at 30 days in mice treated neonatally with T_4 although submandibular gland EGF concentrations are reduced (21). Whether the altered EGF metabolism/ontogenesis is related to the growth retardation is not clear.

The present observations in combination with earlier studies indicate that neonatal T_4 treatment of mice or rats permanently alters growth and alters tissue EGF ontogenesis and EGF receptor metabolism (21, 28, 29). These parameters seem to be imprinted by the prevailing T_4 level during early postnatal life. Hormonal imprinting during a critical, usually perinatal, period of development is a well-characterized phenomenon. In the rodent, neonatal androgen administration produces permanent alterations of hypothalamic regulation of pituitary gonadotropin secretion, adult behavior, and adult sexual activity as well as the patterns of growth hormone secretion, body growth, and hepatic steroid metabolism (30–33). Neonatal administration of alloxan or insulin to rats has been reported to produce permanent alterations of glucose tolerance, and neonatal vasopressin treatment alters adult vasopressin responsiveness (34–37). The mechanism(s) of hormonal "imprinting" however is not clear. Permanent effects on brain structure have been demonstrated (32), and a permanent alteration of developing, transiently "plastic" receptors has been proposed (33, 37). Interestingly, the imprinting effect in some instances has been observed to be transmitted to subsequent generations of rodents (28, 34, 37).

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