

Thyroxine Increases Submandibular Gland Nerve Growth Factor and Epidermal Growth Factor Concentrations Precociously in Neonatal Mice: Evidence for Thyroid Hormone-Mediated Growth Factor Synthesis

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ABSTRACT. Thyroxine (T₄) administration to adult female mice significantly increases submandibular gland (SMG) nerve growth factor (NGF) and epidermal growth factor (EGF) concentrations and does so in a time- and dose-dependent manner. Postnatal maturation of the SMG can be markedly accelerated by T₄ treatment. We, therefore, performed a series of experiments to examine the effect of T₄ on SMG NGF and EGF content and concentration as a function of postnatal age in neonatal mice. In experiment 1, male and female neonatal Swiss-Webster mice received daily subcutaneous injections of T₄ (0.4 µg/g body weight) for 6, 13, or 20 days and were sacrificed 24 h after the last injection. Vehicle injected mice served as controls. SMG NGF and EGF content and concentration were measured by specific double antibody radioimmunoassay systems. Pools were made using either female or male SMGs. Since no significant differences were noted for NGF or EGF content using sex of the animal as the determining variable, the values were combined. At 7 days of age, mean SMG NGF and EGF content and concentration of control mice significantly exceeded those of T₄-treated animals ($p < 0.05$). At 14 days of age, mean SMG NGF and EGF content in T₄-treated mice significantly exceeded those in control mice by 39- and 22-fold, respectively ($p < 0.001$). At 21 days of age, these increases were 4100- and 2400-fold, respectively. In order to determine more precisely the time of onset of responsivity of the SMG to thyroid hormones, a second series of experiments was performed. Vehicle and T₄ (0.4 µg/g body weight daily) injected mice were sacrificed at 7, 9, 11, and 14 days of age. SMG EGF concentration was measured by radioimmunoassay. T₄ treatment was without significant effect at 7 and 9 days of age. At 11 days of age, mean SMG EGF content and concentration in the T₄-treated mice exceeded that in control mice by a factor of 2 ($p < 0.05$). Subsequently, SMG EGF content and concentration increased exponentially. Daily treatment of neonatal mice from birth or from the 7th postnatal day resulted in similar SMG EGF content and concentration when measured at 14 days of age. These data indicate that T₄ administration to neo-

natal mice significantly increases SMG NGF and EGF concentrations and markedly accelerates their appearance in the gland as early as 11 days of age. Onset of SMG EGF responsiveness to thyroid hormone appears to commence at some time on or after the 7th postnatal day. The observation of exponential increases in SMG growth factor concentrations strongly suggest thyroid hormone mediated synthesis. The use of the neonatal SMG as a model will allow further insights into the biosynthesis of NGF and EGF and also into the mechanism of thyroid hormone action. (*Pediatr Res* 20: 281-284, 1986)

Abbreviations

EGF, epidermal growth factor
NGF, nerve growth factor
SMG, submandibular gland
T₃, triiodothyronine
T₄, thyroxine

The SMG of the adult mouse of both sexes contains high concentrations of NGF (1) and EGF (2). Levels in males exceed those in females by two orders of magnitude, reflecting the known sexual dimorphism of the SMG. NGF and EGF concentrations can be significantly modulated by androgens (3, 4) and thyroid hormones (5-7) in the adult animal. In the neonatal mouse, the SMG is histologically and biochemically immature and contains very low concentrations of NGF and EGF (8). Thyroxine administration to neonatal mice accelerates histological and biochemical maturation of the SMG (7, 9-12). Aloe and Levi-Montalcini (13), and Gresik and Barka (9) have shown that thyroxine treatment of immature mice results in the appearance of measurable NGF and EGF, respectively, in the SMG as early as 15 days of age. In previous studies, we have shown that in normal intact neonatal mice, SMG NGF and EGF concentrations increase exponentially after 17-18 days of age (8) in agreement with previous reports (13, 14), and the increases appear to correlate with increasing concentrations of T₄ and T₃ (8). Since the time course of action of triiodothyronine requires a lag time of approximately 40 h prior to the appearance of newly accumulated EGF in the SMG of adult intact female mice (15), it became of interest to examine the effect of thyroxine on SMG NGF and EGF concentrations and the time of onset of thyroid hormone action in the neonatal mouse.

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MATERIALS AND METHODS

Female Swiss-Webster mice were mated at the supply house (Simonsen Laboratories, Gilroy, CA) and were shipped to the CHUL vivarium on the 12th day of pregnancy. Pregnant mice were housed in individual plastic cages on a bedding of wood shavings in a temperature- ($22 \pm 1^\circ\text{C}$) and light- (14 h light) controlled room with free access to laboratory food and water. Humidity was not controlled.

At birth, the litters were reduced to 10 pups per dam. Male and female pups were distributed randomly within and across litters. Litters were then randomly chosen for T_4 or for vehicle injection. Pups received daily subcutaneous injections of $0.4 \mu\text{g } T_4/\text{g}$ body weight in 0.005 N NaOH , $0.9\% \text{ NaCl}$; control mice received equivalent volumes of vehicle alone. Pups were sacrificed 24 h after the last T_4 injection at 7, 14, and 21 days of age in experiment 1, and at 7, 9, 11, and 14 days of age in experiment 2. In a third experiment, neonatal mice were injected daily with T_4 ($0.4 \mu\text{g}/\text{g}$ body weight) from birth through 13 days or from the 7th through the 13th postnatal day and were sacrificed on the 14th postnatal day, 24 h after the last injection. In order to yield sufficient material, SMGs were pooled for analysis. Pups were pooled according to sex and were pooled randomly across litters in order to avoid maternal influences. Pools comprised six animals each at all ages except for 21-day-old mice when SMGs from three mice were pooled. SMGs were removed on ice and were homogenized in 10 vol 0.05 M phosphate buffered saline, $\text{pH } 7.2$ at 4°C . The supernatant was recovered after centrifugation at $25,000 \times g$ for 30 min at 4°C and was frozen at -20°C pending radioimmunoassay.

SMG NGF and EGF concentrations were measured by sensitive and specific radioimmunoassays (5, 6). The samples were measured in duplicate and at two dilutions within the same assay run. Supernatant protein concentrations were measured by the method of Lowry *et al.* (16) using bovine serum albumin as standard.

Statistical analyses were performed using Student's *t* test for unpaired observations.

RESULTS

Experiment 1. Body weights of T_4 -treated neonatal mice were consistently lower than those of vehicle injected mice (data not shown). SMG weights did not differ significantly between the vehicle and T_4 injected mice at 7 or 14 days of age. At 21 days of age, there was a significant increase in SMG weight in T_4 -treated mice compared to controls ($p < 0.001$).

Since no significant sex differences were noted for NGF and EGF concentrations at 7, 14, and 21 days of age in both control and T_4 -treated mice, the results were combined (Table 1). In control mice, SMG NGF and EGF contents and concentrations declined at 14 days of age ($p < 0.05$) and subsequently increased to levels similar to those observed at 7 days, at 21 days of age. SMG NGF and EGF content remained stable through 14 days

and increased significantly between 14 and 21 days of age. T_4 treatment resulted in a significant decrease in SMG NGF and EGF content and concentration at 7 days of age compared to vehicle injected control mice ($p < 0.05$). Subsequently, SMG NGF and EGF concentrations in T_4 -treated mice increased significantly and exceeded those measured in controls. The increases were exponential; at 14 days of age, the mean fold increases for SMG NGF and EGF concentrations were 31 and 23, respectively, those of control mice; at 21 days of age, 2100- and 1500-fold increases, respectively, were noted. The fold increases in NGF and EGF content at 14 days of age were 39 and 22, respectively, increasing further to 4100 and 2400, respectively, at 21 days of age.

Experiment 2. The results of experiment 1 indicated that the neonatal SMG begins to respond to thyroid hormones during the 2nd postnatal wk. In order to determine the time of onset of responsiveness of the gland to T_4 , vehicle and T_4 ($0.4 \mu\text{g}/\text{g}$ body weight daily) injected mice were sacrificed at 7, 9, 11, and 14 days of age. Body weights of T_4 -injected mice were generally, but not significantly, lower than those of control mice (data not shown). As observed in experiment 1, there was no significant hypertrophic effect of T_4 on SMG weight between 7 and 14 days of age.

T_4 administration had no significant effect on SMG EGF content or concentration at 7 or 9 days of age (Table 2). At 11 days of age, mean SMG EGF content and concentration in the T_4 -treated mice exceeded that in the control mice by a factor of 2 ($p < 0.05$). Subsequently, as in experiment 1, the increase in SMG EGF concentration was exponential.

Experiment 3. Mean body weight of 14-day-old mice treated with T_4 daily from the 7th to the 13th postnatal day was slightly but significantly greater than that of mice treated with T_4 daily from birth through 13 days of age ($p < 0.05$; data not shown).

Table 2. Effect of T_4 administration on SMG EGF content and concentration during early neonatal life

Age (days)	T_4	EGF	
		ng/gland	ng/mg protein
7	-	0.095 ± 0.002	0.092 ± 0.005
	+	0.078 ± 0.003	0.084 ± 0.007
9	-	0.102 ± 0.017	0.099 ± 0.007
	+	0.110 ± 0.011	0.079 ± 0.006
11	-	0.080 ± 0.007	0.061 ± 0.002
	+	$0.150 \pm 0.019^*$	$0.120 \pm 0.017^*$
14	-	0.132 ± 0.005	0.057 ± 0.002
	+	$55.30 \pm 9.50^\dagger$	$24.80 \pm 3.09^\dagger$

* $p < 0.05$.

† $p < 0.001$.

Table 1. Effect of T_4 administration on SMG NGF and EGF content and concentration in neonatal mice

Age (days)	T_4	NGF		EGF	
		ng/gland	ng/mg protein	ng/gland	ng/mg protein
7	-	0.151 ± 0.029	0.085 ± 0.013	0.086 ± 0.013	0.048 ± 0.006
	+	0.099 ± 0.007	$0.052 \pm 0.003^*$	$0.063 \pm 0.006^*$	$0.036 \pm 0.002^*$
14	-	0.129 ± 0.010	0.049 ± 0.002	0.102 ± 0.011	0.037 ± 0.003
	+	$5.059 \pm 1.220^\dagger$	$1.523 \pm 0.395^\dagger$	$2.259 \pm 0.464^\dagger$	$0.867 \pm 0.153^\dagger$
21	-	0.595 ± 0.192	0.142 ± 0.057	0.760 ± 0.244	0.144 ± 0.040
	+	$2444.70 \pm 796.80^\dagger$	$293.80 \pm 61.0^\dagger$	$1807.30 \pm 763.50^\dagger$	$216.20 \pm 59.0^\dagger$

* $p < 0.05$.

† $p < 0.001$.

Table 3. Effect of T_4 administration from birth to 13 days or from 7 to 13 days of age on SMG EGF content and concentration at 14 days of age

Group		EGF	
		ng/gland	ng/mg protein
T_4	0-13	19.1 ± 2.2	5.6 ± 0.5
T_4	7-13	21.6 ± 3.0	5.7 ± 0.8

No significant differences were noted, however, for mean SMG weight or mean SMG EGF concentration or content (Table 3).

DISCUSSION

The SMG of the neonatal mouse is immature histologically and biochemically. At birth, the granular tubule cells, the site of NGF and EGF synthesis (17, 18), are absent but begin to make their appearance toward the end of the 2nd postnatal wk (19). The factors responsible for histological and biochemical maturation of the SMG are unknown. In the adult mouse, granular tubule cells are more developed in male mice than in females, and in immature mice a clear sexual dimorphism in the histological appearance of the gland is evident by 20 days of age (19). These sexual differences in adult mice, and presumably in immature mice, are androgen dependent (3, 4, 19).

Recently, attention has focused on the potential role of thyroid hormones as modulators of SMG maturation. Thyroxine has both hyperplastic and hypertrophic effects on the adult SMG (20); the changes are most marked in the granular tubule cells. T_4 administration to adult mice significantly increases SMG NGF (5, 6, 13), EGF (6, 7), and renin (21) concentrations. SMG protease activity also is increased by T_4 administration (7). Further, the ontogenetic profiles of SMG NGF and EGF concentrations correlate temporally with those of serum T_4 and T_3 (8).

The present study confirms and extends previous observations on the time course of appearance of NGF and EGF in the SMG of neonatal mice in response to thyroid hormone administration. Aloe and Levi-Montalcini (13) showed that administration of T_4 to postnatal mice significantly increased SMG NGF concentration at 14 days of age when treatment was instituted at 7 days of age. Gresik and Barka (9) reported that thyroxine treatment from birth was associated with a marked increase in SMG EGF content at 15 days of age. In both cases, continued administration of T_4 led to further exponential increases in SMG NGF and EGF content. The present study indicates that concentrations of both NGF and EGF increase significantly in the SMG of T_4 -treated neonatal mice by 14 days of age and that EGF increases significantly as early as 11 days of age. In both cases, the increases were exponential and the 2400-plus fold increases in SMG NGF and EGF contents between 14 and 21 days of age in the T_4 -treated mice strongly suggest thyroid hormone-mediated synthesis of both growth factors. The exponential increase in SMG NGF and EGF concentrations in T_4 -treated mice between 14 and 21 days of age, moreover, is reminiscent of the increase seen in normal animals after 21 days of age (8).

Kumegawa *et al.* (10, 22) have shown that T_4 administration to neonatal mice increases precociously the number of secretory granules and the appearance of esteroprotease activity in the granular tubule cells. Unlike androgens, for which no response was noted prior to 15-21 days postnatally (12, 23), T_4 increases mitochondrial α -glycerophosphate dehydrogenase activity, a known biochemical marker for thyroid hormone action (24), and glucose-6-phosphate dehydrogenase activity, a granular tubule cell marker (12), by the 5th postnatal day (11, 12). Protease activity, however, was not modified by T_4 treatment prior to 15 days postnatally (12). In the present study, a clear response was noted for EGF as early as 11 days of age.

Responsiveness of the SMG to thyroid hormone administration implies the presence of specific nuclear T_3 receptors in the SMG. Although T_3 receptors have not, as yet, been demonstrated in the neonatal SMG, they have been shown in the adult gland (15). The α -glycerophosphate dehydrogenase and glucose-6-phosphate dehydrogenase responses of the SMG at 5 days of age, before the appearance of the NGF, EGF, or protease responses, imply the presence of T_3 receptors in the gland prior to the appearance of mature granular tubule cells. The failure of the gland to respond to T_4 administration in terms of NGF or EGF accumulation suggests postreceptor immaturity of intracellular biosynthetic mechanisms in the granular tubule cells of newborn mice. This sequence of events would be compatible with initial thyroid hormone responsiveness being localized to granular tubule cell precursor during early neonatal life and thyroxine accelerated appearance of mature granular tubule cells and their biosynthetic products. After completion of these studies, Lakshmanan *et al.* (25) reported that T_4 treatment during the first postnatal week increased SMG RNA, protein, and lipid contents but had no significant effects on SMG NGF concentration.

Studies in adult female mice indicate that following administration of a saturating dose of T_3 , SMG EGF concentration remains unchanged for approximately 40 h before increasing to maximum levels 96 h after T_3 injection (15). If the neonatal granular tubule cell, once having begun to mature histologically, behaves as does the adult, the present data suggest that the granular tubule cells may begin to respond to T_4 exposure as early as 7-8 days of age. The results of experiment 3 indicate that SMG EGF concentration measured at 14 days of age in mice which had received daily T_4 injections from 7 days of age was not statistically different from that of mice injected from birth. This observation is compatible with the hypothesis that SMG EGF responsiveness to thyroid hormones occurs at some point on or after the 7th postnatal day. Moreover, the results suggest that exposure to thyroid hormones prior to 7 days of age is not necessary for induction of accelerated maturation of the SMG using EGF or NGF as the end-point determinant of such maturation. These results, however, differ slightly from those reported by Lakshmanan *et al.* (25) who showed a significant increase in SMG EGF content in 15-day-old mice treated from birth compared to mice treated from day 7. The differences are difficult to reconcile since Lakshmanan *et al.* (25) used essentially the same protocol employed in the present study.

A further point of interest pertains to the observation of significant, albeit low concentrations of both NGF and EGF in the neonatal mouse SMG and is in agreement with previous observations (8, 25), but not with those of other groups (9, 13, 26). The reason for this discrepancy is not clear, particularly since the sensitivities of the assay systems are similar. Extensive cross-reactive studies for both NGF and EGF antisera used in these studies have failed to identify molecules which result in significant displacement of either radioligand. Nexø *et al.* (27) have reported by radioimmunoassay and by radioreceptor assay the presence of significant amounts of EGF in fetal mouse tissues. Discrepant results in the two systems suggested that the fetal EGF molecule, while retaining its ability to bind to placental membrane receptors, was immunologically different from adult SMG EGF. It is possible, therefore, that the anti-EGF antiserum used in these studies may recognize the putative fetal EGF. A similar argument can be advanced for NGF. However, evidence in favor of a fetal NGF is lacking. An additional possibility is that SMG EGF may reflect EGF derived from maternal milk (28). Of interest is the observation that T_4 administration to neonatal mice significantly decreases SMG NGF and EGF content at 7 days of age, suggesting that thyroid hormones accelerate disappearance and/or catabolism of the growth factors from the gland in the early postnatal period. Whether this reflects catabolism of glandular or circulating NGF and EGF is not known.

In sum, the present observations demonstrate clearly that T_4 administration to neonatal mice exponentially increases SMG

NGF and EGF concentrations in a manner compatible with T₄-induced synthesis of the growth factors. The use of the neonatal mouse SMG will allow further insight into the mechanism of thyroid hormone action.

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