bition of HK activity by 2,3-DPG may also have contributed to the decrease in the concentration of G-6-P and other intermediates distal to this step. The magnitude of the increase in the 2,3-DPG concentration was probably further enhanced by the increasing plasma Pi concentration. After the first 10–14 days of life all glycolytic intermediates fell simultaneously. At this time other control mechanisms became operative since glycolytic enzyme activities were also decreasing, as demonstrated by the significant correlation between the decreasing activity of red cell DPGM and corresponding decrease in the red cell 2,3-DPG concentration.

These studies have demonstrated that red cell PFK plays a key regulatory role in postnatal life in lambs. Thus, the fetal lamb can serve as a model for the study PFK activation and inhibition *in vivo* and the regulation of 2,3-DPG synthesis.

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Epidermal Growth Factor Binding to Neonatal Mouse Skin Explants and Membrane Preparations—Effect of Triiodothyronine

S. B. HOATH,[1] J. LAKSHMANAN, AND D. A. FISHER

Department of Pediatrics, Harbor-UCLA Medical Center, UCLA School of Medicine, Torrance, California 90509

ABSTRACT. Daily treatment of newborn Swiss-Webster mice with triiodothyronine (T3, 500 ng/day) increased epidermal growth factor (EGF) content in whole skin (epidermis + dermis). Separation of the epidermis using 0.01 M dithiothreitol followed by processing for radioimmunoassay measurement reveals levels of EGF 2-to 3-fold higher in epidermis than in whole skin. *In vitro* flotation of circular skin sections from control and T3 treated neonatal mice in medium containing [I^{125}]EGF showed increased uptake of label following 5 days of *in vivo* T3 treatment. Mouse skin membrane preparations exhibit saturable, specific binding of [I^{125}]EGF. T3 treatment for 5 days *in vivo* significantly increased EGF binding capacity in skin membrane preparations but did not alter EGF receptor affinity (Kd 4.5 nM). Protein, RNA, and DNA concentrations were significantly increased in whole neonatal mouse skin following T3 administration. These results suggest one mechanism by which thyroid hormones increase skin EGF concentration is augmentation of skin EGF receptor binding. (*Pediatr Res* 19: 277–281, 1985)

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Reprint requests Dr. Steven B. Hoath, Department of Pediatrics, University of Cincinnati Medical Center, Mail Location 541, Room 6263, 231 Bethesda Avenue Cincinnati, OH 45267.
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[1] Current address: Department of Pediatrics, ML 541, University of Cincinnati College of Medicine, Cincinnati, OH 45267.

Abbreviations

EGF, epidermal growth factor
T3, triiodothyronine
SMG, submandibular gland
PBS, phosphate buffered saline
BSA, bovine serum albumin

Among the many well known developmental effects of thyroid hormones is the promotion of early incisor eruption and eyelid opening in neonatal rodents (17, 18, 28). These integumental effects are similar to those produced by EGF (7). Recently, we presented evidence linking hyper- and hypothyroidism in neonatal mice with changes in concentrations of EGF in whole skin (15). These findings suggested mediation of hormone action by modulation of endogenous tissue growth factor levels.

The present report refines our previous study. Levels of immunoassayable EGF in T3 treated and control neonatal mouse epidermis are presented and contrasted with those obtained from whole skin. An effect of *in vivo* T3 treatment to enhance uptake of [I^{125}]EGF by whole neonatal mouse skin *in vitro* is described. Membrane binding data are presented suggesting T3 induction of EGF receptor number in skin. Finally, measures are given of total protein, RNA and DNA in the skin of control and T3-treated neonatal mice.

MATERIALS AND METHODS

Preparation of epidermal samples. Newborn Swiss Webster mice were randomly crossfostered into litters of eight pups containing four control (bobbed tails) and four T3-treated animals. Treatments consisted either of 10 μl 0.9% saline (controls) or 500 ng T3 in 10 μl 0.001 N NaOH given subcutaneously. Injections began on the day of birth (day 0) and continued daily for 4 consecutive days. Animals were sacrificed by decapitation 24 h after the last injection on the 5th postnatal day. The dorsal and upper flank skin was dissected free of subcutaneous tissue, washed in 0.9% saline, and sections were floated dermal side down on 20 ml of medium in a 60 × 15 mm tissue culture dish (Costar Plastics, Cambridge, MA) using the dish cover as a lid. The medium consisted of 0.05 M phosphate buffered saline, pH 7.3, 5% glucose, and 0.01 M dithiothreitol DTT (Cal Biochem, La Jolla, CA). After 60 min of incubation in room air at 23° C, the skin was carefully removed, placed dermal side down on absorbent paper, and the epidermis gently peeled off as a continuous sheet. The epidermis was washed for 2 min in 0.9% saline. Individual epidermal samples weighing 7–15 mg were diluted in 500 μl ice cold 0.05 M PBS containing 0.5 mg/ml sodium azide. Homogenates were prepared using 20 strokes of a tight-fitting automated glass/Teflon pestle homogenizer. The homogenates were clarified by centrifugation at 30,000 × *g* for 30 min at 4° C prior to assay. Whole skin sections from the above animals weighing approximately 75 mg were prepared as described previously (15). Total homogenate protein concentrations were determined by the method of Lowry *et al* (19).

Whole skin [I^{125}]EGF uptake. Dorsal and ventral skins were dissected from 5-day-old neonatal mice following hormone treatment as described for the epidermal preparations. Skin specimens were placed dermis down on a clean glass plate and circular sections were cut from each specimen using a no. 6 cork borer. Each section, measuring approximately 48 mm in diameter, was floated in 1 ml 0.05 M PBS + 5% glucose in 48-well plastic cluster plates (Costar). Incubations for binding assays were begun by transferring the skin sections to adjacent wells containing 400 μl of the same buffer + approximately 220,000 cpm [I^{125}]EGF at 23° C. Sections were floated on the incubation mixture with the epidermis uppermost and dry. The reaction was terminated by swirl-washing the section in 0.9% saline for 20 s, then blotting away excess water with absorbent paper. The sections were weighed, placed in the bottom of 12 × 75 mm polystyrene tubes, and counted in a gamma counter.

Preparation of skin plasma membranes. Preliminary studies were performed on crude skin membrane preparations following the protocol of O'Keefe *et al* (21). In brief, individual skins from 5-day-old T3-treated and littermate control mice were homogenized 1:10 (w/v) in 0.05 M PBS using a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) at 95% high speed for 60 sec. Samples were spun at 1000 × *g* at 4° C and the fat-free supernatant respun at 40,000 × *g* for 30 min. The membrane pellet was resuspended in 500 μl PBS and respun at 40,000 × *g*. The final pellet was resuspended in 500 μl PBS and frozen at −20° C until assay.

Skin plasma membranes were isolated by differential centrifugation according to the method of Hock and Hollenberg (16) for isolating the EGF plasma membrane receptor from human placental tissue. In summary, dorsal skins were homogenized in 0.25 M sucrose and 25 mM Tris-HCl, pH 7.4. Following the initial Tissumizer disruption, samples were further homogenized with 15 strokes in an automated glass/Teflon pestle apparatus. The homogenate was filtered through nylon mesh and centrifuged at 4° C for 10 min at 600 × *g*. The supernatant was aspirated and recentrifuged at 10,000 × *g* for 30 min. The resulting supernatant was made 0.1 M in NaCl and 0.2 mM in $MgSO_4$, then respun at 50,000 × *g* for 40 min. The pellet was resuspended in 50 mM Tris-HCl, pH 7.6, then harvested by recentrifugation a 50,000 × *g* for 30 min. The final pellet was resuspended in 50 mM Tris-HCl to yield approximately 1 mg/ml membrane protein. Protein determinations were performed by the method of Peterson (22) using BSA as standard.

I^{125}EGF binding assay. Mouse EGF was purified from testosterone treated adult male mice by the method of Savage and Cohen (23). EGF was radiolabeled with I^{125} as previously described. The specific activity of the [I^{125}]EGF employed for binding assays was determined by self-displacement assay of the tracer hormone in a homologous mouse EGF-radioimmunoassay (15).

Typically, measurements of peptide binding were performed with 50–100 μg of membrane protein suspended in 0.05M PBS, pH 7.3, containing 250 μg BSA in a final reaction volume of 200 μl. Binding was initiated in 12 × 75 mm polystyrene tubes at 23° C by the addition of 220,000 cpm [I^{125}]EGF with and without the presence of 2 μg unlabeled EGF. After the designated incubation period the reaction was terminated by diluting the samples with 3 ml ice-cold PBS and passing of the mixture through Whatman glass fiber filters (Whatman, Inc, Clifton, NJ) on a multiple manifold filtration apparatus. The filters were washed with 12 ml cold buffer and radioactivity retained on the filters was counted in a gamma counter.

Biochemical analysis of mouse skin. Approximately 100 mg of dorsal skin (epidermis and dermis) were minced with fine scissors on an ice-cold glass plate. The tissue mince was solubilized 1:9 (w/v) in 2N KOH using a glass/Teflon motor-driven pestle homogenizer at 4° C. The homogenate was then centrifuged at 25,000 × *g* for 20 min and transferred to 12 × 75 mm glass tubes. The pellets were rehomogenized in 0.5 ml of 2N KOH at 4° C and recentrifuged as described above. The supernatants were combined and processed as follows: aliquots equal to 50 mg tissue were treated with excess perchloric acid to precipitate macromolecules. The tubes were kept in ice for 20 min and centrifuged at 500 × *g* for 30 min. The supernatants were discarded and the pellets were resuspended in 2 ml ice-cold 0.2N perchloric acid and centrifuged as described above. The supernatants were discarded and the washing procedure was repeated twice.

The pellets were resuspended using glass rods in 2 ml 0.3N KOH and incubated at 37° C for 16–18 h for hydrolysis and extraction of RNA. The DNA and protein were reprecipitated with perchloric acid to a final concentration of 10% followed by centrifugation at 500 × *g* for 30 min. The pellets were washed once with 0.2N perchloric acid, recentrifuged and the superna-

tants combined with the previous supernatants containing the RNA hydrolysate. The precipitate was then digested with 10% perchloric acid at 90° C for 30 min to extract the DNA hydrolysis products. The samples were cooled and centrifuged at 1000 × *g* for 30 min. The supernatants was used for DNA estimation as described by Giles and Myers (12). The protein pellet was dissolved in 0.1N NaOH and protein concentration measured by the method of Lowry *et al.* (19) using BSA dissolved in 0.1N NaOH as standard. RNA was determined by ultraviolet absorption at 260 nm according to the method of Fleck and Munro (10). Calf thymus DNA (Sigma) and *Escherichia coli* RNA (Sigma) were used as standards.

RESULTS

Epidermal EGF levels. Postnatal treatment of neonatal mice with T3 significantly elevated endogenous EGF levels in epidermis and whole skin (Fig. 1). Absolute values for epidermal EGF (mean ± SEM) were 159 ± 10 and 307 ± 25 pg EGF/mg protein for control and T3 groups, respectively ($p < 0.001$). Whole skin values from the same animals were 70 ± 6 *versus* 93 ± 10 pg/mg protein ($p < 0.05$). Experimental groups consisted of 16 control and 16 T3-treated littermates drawn from a total of five litters. Equal numbers of control and treated pups were taken from each litter. No significant difference in mean body weight of control and treated pups was noted. (All statistical analyses were with Student's *t* test.)

Serial dilutions of mixed epidermal supernatant from T3-treated and control mouse pups (range 2.5–200 μl) were tested for parallelism with the EGF standard curve in the RIA system. After log-logit transformation, the slopes of the regression lines (supernatant vs standard curve) were statistically similar. Recovery of standard EGF (50–100 pg) added to 25 μl epidermal supernatant (equivalent to 50 pg EGF) was assessed in quadruplicate specimens. Mean (± SEM) recovery was 92 ± 2%.

[I^{125}]EGF uptake in whole skin. Dorsal skin from 5-day-old T3-treated mice exhibited an increased rate of uptake of [I^{125}] EGF from the medium when floated for up to 150 min in room air at 23° C (Fig. 2*B*). Values shown are normalized to 100 mg wet weight of tissue. Mean weights of individual skin sections were similar in the two groups (control = 48.1 ± 1.2 mg; T3 = 45.7 ± 1.3 mg; $p > 0.05$). Ventral skin sections exhibited a similar pattern of T3-induced label uptake, but total values were approximately 1.5 times greater than those for dorsal skin at 60 min (data not shown). In contrast, dorsal skin from 4-day-old mouse pups showed a small but nonsignificant increase in [I^{125}] EGF uptake following T3 treatment (Fig. 2*A*). Ventral skin uptake was significantly greater than dorsal skin uptake in both groups ($p < 0.01$).

[I^{125}]EGF membrane binding. Specific binding of [I^{125}]EGF was increased in crude skin membrane preparations from 5-day-old T3-treated mouse pups *versus* littermate controls (Fig. 3).

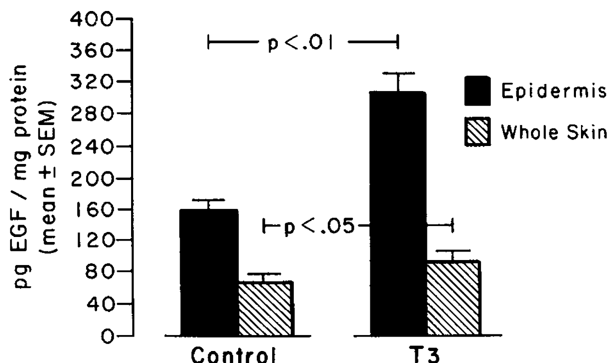

Fig. 1. Effect of T3 on EGF content in neonatal mouse epidermis and whole skin. Values represent 16 control pups *versus* T3-treated littermates sacrificed on the 5th postnatal day. Significance testing by Student's *t* test.

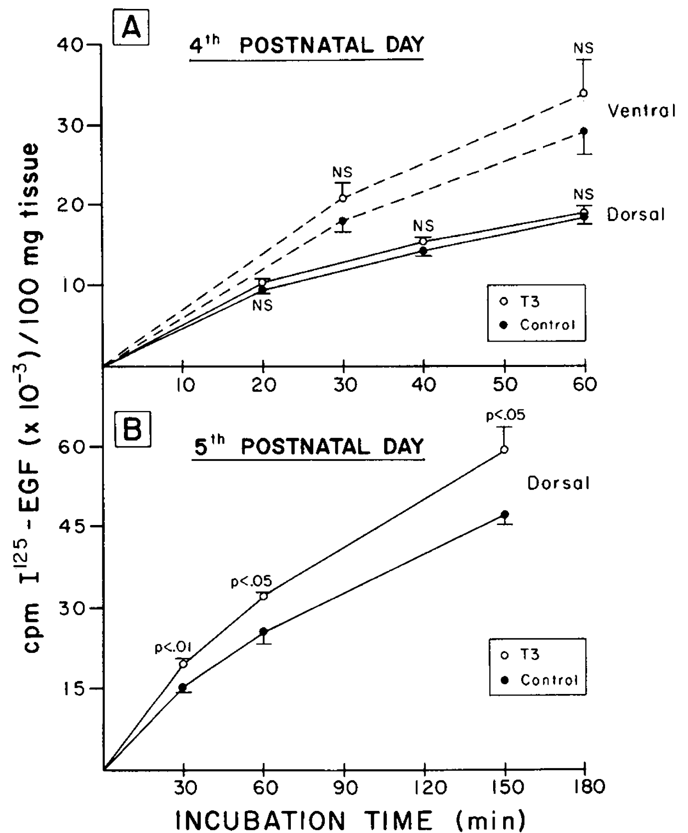

Fig. 2. Effect of *in vivo* T3 treatment on *in vitro* uptake of [I^{125}]EGF by neonatal mouse skin (*A*) uptake of label by ventral and dorsal mouse skin on the 4th postnatal day. Each skin section incubated at 23° C in 400 μl buffer containing 100000 cpm [I^{125}]EGF (360 pg). See text for details (*B*) uptake by dorsal mouse skin on the 5th postnatal day. Similar conditions to above except sections incubated with 200,000 cpm [I^{125}] EGF. All *time points* represent the mean ± SEM of six to eight individual animals.

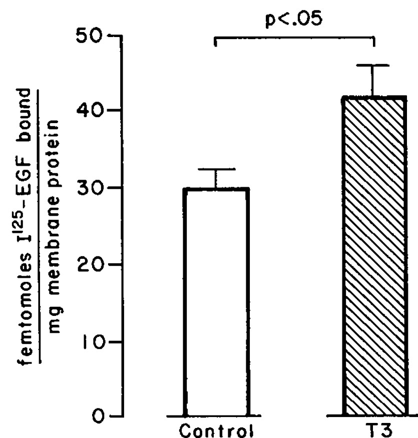

Fig. 3. Increase in [I^{125}]EGF specific binding to crude skin membrane preparations following T3 treatment. Approximately 100 μg membrane protein were incubated for 30 min at 23° C in a total reaction volume of 250 μl containing 280,000 cpm [I^{125}]EGF (1000 pg). Specimens were assayed in duplicate and corrected for nonspecific binding by subtraction of counts bound in the presence of 3 μg unlabeled EGF. Results are given as means ± SEM. Significance testing was performed using Student's 2-tailed *t* test for unpaired observations.

Each specimen represents ventral skin pooled from two animals for a total of 12 animals in each group. Mean body weight was similar in the two groups of animals. Equilibrium binding curves for dorsal skin membrane preparations (Fig. 4) showed a similar

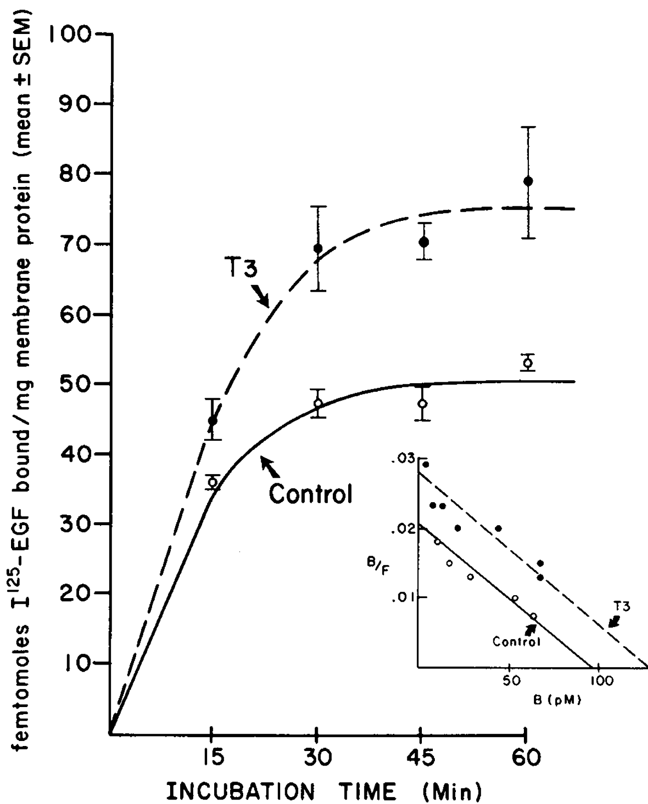

Fig. 4. Binding of [I^{125}]EGF to skin plasma membrane preparations. Membranes were prepared according to the method of Hock and Hollenberg (16) using dorsal skin samples pooled from 16 control and 16 T3-treated 5-day-old neonatal mice. Approximately 40 μg membrane protein were incubated for 30 min at 23° C with 225,000 cpm [I^{125}]EGF (690 pg) in a total reaction volume of 225 μl. Results are means ± SEM of triplicate determinations corrected for nonspecific binding as described in text. The *inset* shows Scatchard analysis of subsequent equilibrium binding data using increasing amounts of [I^{125}]EGF. Analysis was performed under the assumption of linear correlation (correlation coefficients = −0.90 and −0.95 for T3 and control groups, respectively).

Table 1. *Effect of T3 on macromolecular content of neonatal mouse skin**

	Protein (mg)	RNA (μg)	DNA (μg)
Control	3.6 ± 0.1	223 ± 9	143 ± 9
T3	4.3 ± 0.2†	285 ± 13†	242 ± 11†

* Values are means ± SEM and are normalized to 100 mg wet weight of whole skin.

† $p < 0.01$.

increase in binding in the T3-treated group with a plateau reached by approximately 30-min incubation. Scatchard analysis of subsequent equilibrium binding data (Fig. 4, *inset*) is consistent with a single order of binding sites with a Kd of 4.5 nM. Maximum binding capacities for control and T3-treated membranes were 0.59 and 0.70 pm [I^{125}]EGF/mg membrane protein, respectively. Binding was improved using freshly isolated membrane preparations with >10% specific binding achieved in the T3-treated group using 50 μg membrane protein; no difference in equilibrium binding of [I^{125}]EGF was noted between ventral and dorsal skin membrane preparations within either group (data not shown).

Effect of T3 on macromolecular constituents of whole skin. Table 1 shows the effect of T3 treatment on protein, DNA, and RNA content of whole skin of 6-day-old mouse pups. Injections were begun on the day of birth (day 0) and continued daily until sacrifice on postnatal day 6 approximately 24 h after the last injection. Groups consisted of two litters containing six pups each. T3 treatment resulted in significant elevations in all constituents studied. In contrast to the previous groups, T3 produced a significant reduction in body weight in this experiment from 4.9 ± 0.3 g for controls to 3.7 ± 0.2 g ($p < 0.01$). EGF levels measured in dorsal skin homogenates from the same experimental animals increased following T3 treatment from 81 ± 4 to 290 ± 30 pg/100 mg wet weight ($p < 0.01$).

DISCUSSION

The present results confirm our earlier observation that T3 treatment of neonatal mice increases skin EGF concentrations. Moreover we confirm earlier reports by Frati *et al.* (11) (using an EGF radioreceptor system in adult mice) that epidermal levels of EGF exceed dermal levels several fold. As in whole skin, postnatal treatment with T3 augments EGF levels in epidermis. Previously, we speculated as to the possible sources of this EGF and showed that maternal sialoadenectomy did not significantly decrease skin EGF levels in mouse pups on the 2nd postnatal day (17), obviating an important maternal salivary gland source (15). Newborn mouse submandibular glands contain little EGF compared to adults (14). Another source of EGF in the suckling animal is maternal milk which has been shown in both humans and rodents to contain significant quantities of EGF (2, 3, 25). Finally, local (paracrine) growth factor production and action, as with somatomedin (8), is an unproven possibility.

Supporting milk as a possible source of skin EGF is the observation that oral administration of EGF elicits eyelid opening in neonatal mice (7). Recent data by Thornburg *et al.* (27) suggests that labeled mouse EGF given orally to suckling rats undergoes molecular processing and structural alteration during absorption with subsequent accumulation of the label in skin. Although it is known that antibodies and other macromolecules are absorbed by the neonatal rat intestine during the first 18 days of life (5, 6), the extent of EGF absorption is not clear.

To assess skin binding of EGF we first measured the uptake of [I^{125}]EGF by small skin sections floated in vitro under simple assay conditions (Fig. 2*B*). Carpenter *et al.* (4) had reported that iodinated EGF was biologically equipotent to unlabeled EGF as assessed by the acceleration of eyelid opening *in vivo*. In this study, *in vivo* T3 treatment markedly enhanced labeled EGF uptake by skin. We observed, however, that treatment for less than 5 days produced inconsistent results (Fig. 2*A*). In earlier studies measuring immunoassayable EGF levels in skin (15), we also observed a lack of effect of thyroid hormone in the early (day 2) postnatal period. These findings suggest a postnatal delay of several days in the onset of EGF-related thyroid hormone responsiveness in skin.

In order to examine whether the increased uptake of labeled EGF by whole skin *in vitro* was a specific receptor-related event, membranes were prepared from whole skin by two different methods (16, 21). In both preparations, we observed approximately 1.5-fold greater EGF binding activity/mg protein in membranes from the T3-treated animals. Scatchard analysis (Fig. 3) suggested that the increase in binding was due to increased binding capacity rather than increased receptor affinity (24). Green et al. (13) have presented data in rats showing an inverse relation between postnatal age and EGF receptor number in isolated epidermal basal cells. Although our data do not allow correlation between binding activity and cell number, we observed that T3 treatment increased macromolecular constituents of neonatal mouse skin including protein, RNA, and DNA at 6 days of age (Table 1). Exogenous EGF produces similar elevations at 5 days postnatal age (1).

Recently, Mukku (20) reported that thyroid hormone increases

EGF receptor levels in adult rat liver. The present study supports these observations and shows that the developing mouse responds to thyroid hormone with increased levels of endogenous EGF receptors in a known EGF target organ, the skin. Thus, one mechanism for modulation of developmental events by thyroid hormones is the modulation of growth factor receptors.

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Intrauterine Growth-Retarded Rat Pups Show Increased Susceptibility to Pulmonary O_2 Toxicity

LEE FRANK, PAMELA L. LEWIS, AND TERESA GARCIA-PONS

Pulmonary Research Division and Oak Asthma Research and Treatment Facility, University of Miami School of Medicine, Miami, Florida 33101

ABSTRACT. We used a nutritional deprivation model to produce intrauterine growth-retarded (IGR) rat pups (birth weight = ~75% of normal). The IGR newborns evidenced a marked reduction in tolerance to >95% O_2 exposure: 10-day survival = 10/47 (21%) *versus* 18/36 (50%) for control pups, and LT_{50} = 7.2 days *versus* 10 days for controls ($p < 0.01$). Various lung parameters at birth and during O_2 exposure were examined to try to define why prenatal undernutrition should compromise the survival of IGR rats in hyperoxia. We found decreased lung glutathione peroxidase and glucose-6-phosphate dehydrogenase activity (with normal superoxide dismutase and catalase levels) in the IGRs at birth; decreased lung disaturated phosphatidylcholine content (even more markedly decreased in 1-day premature pups); and decreased lung surface area/body weight. These factors and other features of newborn IGRs reported in the literature may help to explain how prenatal undernutrition compromises postnatal tolerance to prolonged high-O_2 exposure. (*Pediatr Res* 19: 281–286, 1985)

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Reprint requests: Dr. Lee Frank, Pulmonary Research R120, University of Miami, School of Medicine, P.O. Box 016960, Miami, FL 33101.
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