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Original article

Ontogeny of the liver nuclear T_3-receptor during the last days of incubation and posthatch in the chick embryo

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Summary — The characteristics of the nuclear T_3 receptor in the liver of the chick embryo were studied from incubation day 18 until day 1 posthatching. Treatment of the nuclei with 3 mol.l^{-1} $MgCl_2$, which removed the endogenously bound hormone, was used in order to determine the total amount of receptors. The affinity constant K_a decreased between incubation day 18 ($0.996 \pm 0.276 \cdot 10^9$ M^{-1}) and day 19 ($0.247 \pm 0.072 \cdot 10^9$ M^{-1}), remained the same thereafter until hatching and increased again on day 1 posthatching ($1.846 \pm 0.928 \cdot 10^9$ M^{-1}). The total amount of receptors tended to increase from incubation day 18 to day 20 non-pipping (np) (from 4.40 to 11.55 fmol/µg DNA) and decreased thereafter to 2.38 fmol/µg DNA on day 1 posthatching. The amount of free binding sites reached a maximum on day 19 (6.91 fmol/µg DNA) and then decreased drastically until posthatching (0.19 fmol/µg DNA). The maximal specific binding was found on day 20 (np), just prior to penetration of the air chamber. During the time at which the level of T_3 remains high in the plasma, a reduction in the amount of receptor was observed, which may be the consequence of a down-regulation by T_3 itself.

chick embryo / T_3-receptor / ontogeny

Résumé — Ontogénie des récepteurs nucléaires du foie à la T_3 pendant les derniers jours d'incubation de l'embryon de poulet. *Les caractéristiques des récepteurs nucléaires de la T_3 dans le foie de l'embryon de poulet ont été étudiées du 18^e j d'incubation jusqu'au jour suivant l'éclosion. Le traitement des noyaux avec $MgCl_2$ (3 mol.l^{-1}), qui libère l'hormone endogène liée, a été utilisé pour permettre une détermination des récepteurs totaux. Une diminution de l'affinité (Ka) est observée entre le 18^e et le 19^e jour de l'incubation (0,996 ± 0,276 et 0,247 ± 0,0725•10^9 M^{-1} respectivement), où elle se stabilise jusqu'à l'éclosion, pour augmenter de nouveau 1 jour après l'éclosion (1,846 ± 0,928•10^9 M^{-1}). Le nombre des récepteurs totaux tend à augmenter du 18^e j au 20^e j d'incubation* (non-pipping) *(4,40 à 11,55 fmol/µg ADN) et baisse jusqu'à 2,38 fmol/µg ADN le lendemain de l'éclosion. Le nombre de sites spécifiques de liaison libres pour la T_3 atteint un maximum au 19^e j (6,91 fmol/µg ADN), puis diminue fortement dès l'éclosion (0,19 fmol/µg ADN). La liaison spécifique maximale est obtenue au 20^e j* (non-pipping), *juste avant la pénétration de la chambre à air. Durant la présence de taux élevés de T_3 dans le plasma, le nombre de récepteurs est réduit. Ceci peut être la conséquence d'une régulation négative causée par la T_3 elle-même.*

embryon de poulet / récepteur de T_3 / ontogenèse

INTRODUCTION

Previous studies have shown the existence of a nuclear T_3 receptor in various tissues in rat, tadpole and rabbit (Surks *et al,* 1973; Yoshizato *et al,* 1975; Lindenberg *et al,* 1978). During ontogenesis, where thyroid hormones are known to play an important role in maturation and development of several tissues, T_3 binding sites have mainly been studied in rat tissues, such as liver (Degroot *et al,* 1977; Coulombe *et al,* 1979) and brain (Schwartz and Oppenheimer, 1978; Perez-Castillo *et al,* 1985; Hubank *et al,* 1990). Bellabarba and Lehoux (1981) and Bellabarba *et al* (1983, 1988) proved the existence of a T_3 receptor in liver, brain and lung of the chick embryo and determined the binding characteristics on different days during early and late ontogenesis. Brain and muscle tissue have also been examined in the prenatal stage by Haidar *et al* (1983) and Dainat *et al* (1986) respectively.

Large changes in concentrations and metabolism of thyroid hormones and in the levels of growth hormone and glucocorticoids (Scanes *et al,* 1987) occur during the last week of incubation. Thyroid hormone concentration in plasma changes rapidly during this period. There is a marked rise in thyroxine (T_4), reaching a maximum on the day of pipping and a gradual increase in 3,3',5-triiodothyronine (T_3) (Thomas and Hylka, 1977; Decuypere *et al,* 1979), which is due to the onset of the 5'-monodeiodinase activity during that time (Decuypere *et al,* 1986; Huybrechts *et al,* 1989).

Moreover, physiological events such as the transition from allantoic to pulmonal respiration, yolk sac retraction and the hatching process itself seem to be related to changes in thyroid physiology (Decuypere *et al,* 1990). Since the liver is the major site of peripheral T_4 conversion and T_3 production, while profound changes take place in these processes during the week prior to hatching, we investigated the properties of the T_3 receptor in the liver of chick embryos systematically during the last week of embryogenesis.

MATERIALS AND METHODS

Hisex White chick embryos were purchased from Euribrid, Aarschot (Belgium), and incubated at 37.8 °C in a forced-draught incubator with continuous lighting and turning once every hour through an angle of 45°.

Blood samples were taken by cardiac puncture (embryos) or by decapitation (1-day-old chicks) in heparinised tubes. Plasma was stored at –20 °C after centrifugation. The liver was removed and frozen at –20 °C until further determination (between 1–4 weeks). The tissue was only frozen and thawed once.

Plasma T_4 levels were measured by means of a commercial kit (Abbott Diagnostic Division) with intra- and inter-assay variabilities of 3.2 and 3.3% respectively. The T_3 RIA (antiserum Mallinckrodt Diagnostica, tracer Amersham IM-321), used for determining the T_3 concentration in plasma and homogenate, had an intra-assay variability of 2.9% (Huybrechts *et al,* 1989). A parallelism test was performed prior to the measurements by diluting the homogenate (crude homogenate minus nuclear pellets) in T_3 free serum.

DNA was measured according to the method of Maniatis (1989).

Preparation of nuclei

The technique of Bellabarba and Lehoux (1981) was applied in a slightly modified version for the preparation of the nuclei. In brief, livers were washed twice with 0.15 M NaCl and approximately 0.5 g hepatic tissue was homogenized in 2 ml SMT–EDTA buffer at pH 7.4 (0.32 M sucrose, 3.2 mM $MgCl_2$, 0.01 M Tris, 1.5 mM EDTA), using an Ultra Turrax homogeniser during 2 x 10'. After centrifugation for 10 min at 700 *g* (4 °C) (Sorvall Superspeed RC2-B), the supernatant was removed and the resulting pellet was purified on a sucrose discontinuous den-

sity gradient (2.4 M sucrose, containing 1 mM $MgCl_2$), as described by Surks *et al* (1973). The suspension was ultracentrifuged for 45 min at 53 000 *g* (Beckmann L8-M ultracentrifuge). The nuclear pellet was resuspended in SMT–EDTA using a Potter–Elvehjem handhomogenator so as 1 ml suspension contained nuclei from 0.25 g liver. Oppenheimer *et al* (1974), who used the same preparation technique, checked the purity of the nuclear preparation by phase microscopy and electron microscopy.

Incubation of isolated nuclei

Binding experiments were performed in glass tubes containing 50 µl labeled T_3 (± 35 000 cpm; 3 000 µCi/µg), 50 µl unlabeled hormone (100 pM – 1 000 nM) (or analogue or buffer) and 20 µl nuclear suspension. Tubes were first incubated for 1 h at 4 °C followed by another 30 min at 37 °C, as described by Bellabarba and Lehoux (1981). At the end of the incubation period, the samples were centrifuged for 10 min at 10 000 *g* (4 °C). The supernatant was removed and the nuclear pellet was counted in a gamma counter (LKB Wallac Rackgamma II) for 1 min. Total binding was determined by adding tracer and buffer to the nuclear suspension, while aspecific binding was determine by adding excess unlabeled hormone (1×10^{-6} M) instead of buffer. Aspecific binding was subtracted from total binding to measure the specific binding.

The total amount of receptors was determined after stripping of the endogenous bound T_3 (Kelly *et al,* 1979). The number of free binding sites indicated the amount of receptors that was not occupied by endogenous hormone. The occupied receptors were measured by subtracting free number from total number of binding sites. Binding capacities (B_{max}) and equilibrium association constants (K_a) for the specific nuclear binding sites were measured by Scatchard analysis using the LIGAND program (Munson and Rodbard, 1980).

Determination of the binding affinity for T_3-analogues

The relative affinities of the binding sites for L-T_3, L-T_4 and triiodothyroacetic acid (Triac) were determined by incubating the usual tracer amount with increasing amounts of each analogue (100 pM – 1 000 nM) and carrying out a Scatchard analysis. L-T_3, L-T_4 and Triac were obtained from Sigma Chemical Company.

Statistical analyses

These were performed by analysis of variance (ANOVA) followed by the least significant difference test when *F* was significant (Snedecor and Cochran, 1967). Values were expressed as mean ± SEM of 5 data sets (Scatchard plots).

RESULTS

Hormone concentration

Plasma T_4 levels increased slightly from day 18 to day 20 and reached a maximum at the moment of external pipping ($P < 0.01$). At 1 day posthatching there was a significant decrease in T_4 concentration ($P < 0.01$) (fig 1).

Plasma T_3 levels remained the same on day 19 and day 20, non-pipping (np) and day 20 internal pipping stage (ip). Plasma concentration became maximal on day 21

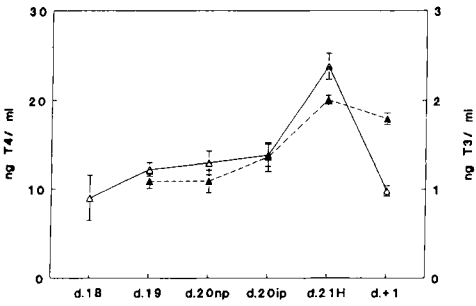

Fig 1. Plasma T_4 (Δ) and T_3 (▲) concentrations (ng/ml plasma) in the embryo from day 18 of incubation to day 1 posthatching (mean ± SEM). d 20 np: non pipping, d20 ip: internal pipping, d 21 H: hatching, d +1: first day after hatching.

(hatching). Then it decreased slightly but not significantly at 1 day posthatching (fig 1).

Within the cell, T_3 concentration followed a somewhat different pattern (fig 2). T_3 levels were unchanged during day 18 and day 19, increased on day 20 np and then decreased when the air chamber was penetrated (day 20 ip). At hatching cell T_3 content became maximal ($P < 0.01$) and decreased at day 1 posthatching, returning to day 18–19 levels.

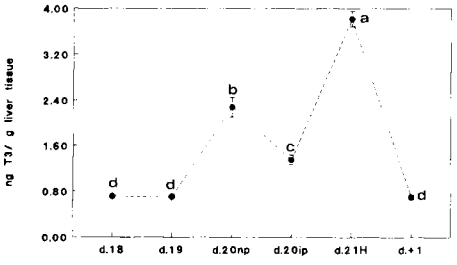

Fig 2. Concentration of T_3 (ng/g liver tissue) in the hepatic cells during the last week of incubation (mean ± SEM). Legend: see figure 1; a–d: groups sharing no common letter are significantly different ($P < 0.01$; ANOVA).

Receptor study

The specific binding (SB) of T_3 to its nuclear receptor, expressed in % SB of the total counts, showed an increase until day 20 (np) and a decline thereafter, both with the total (measured after treatment with $MgCl_2$) (fig 3) and free (measured without $MgCl_2$ treatment) receptors (data not shown). The specific binding of the total binding sites was slightly higher than of the free binding sites, indicating that the endogenous hormone really was removed from the receptor.

The affinity constant K_a (fig 4) showed a non significant decrease from day 18 to day 19 of incubation. Then it remained

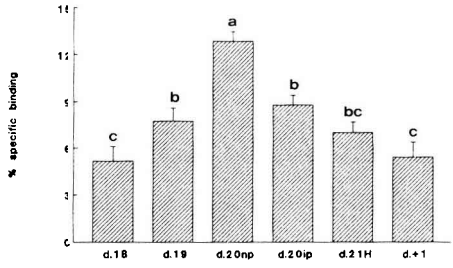

Fig 3. % specific binding of the total counts of T_3 to the total number of liver receptors during incubation of the chicken embryo (mean ± SEM; $n = 5$). Legend: see figure 1; a–d: groups sharing no common letter are significantly different ($P < 0.05$; ANOVA).

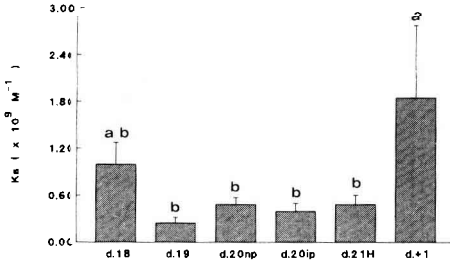

Fig 4. K_a during the ontogeny of the embryo (mean ± SEM; $n = 5$). Legend: see figure 1; a–b: groups sharing no common letter are significantly different ($P < 0.05$; ANOVA).

nearly unaltered for 3 days (days 19, 20 and 21) until it increased significantly on the first day posthatching ($P < 0.05$).

The total amount of receptors (B_{max}) (fig 5), measured in $MgCl_2$-treated nuclei revealed a pattern similar to that found in the percentage of specific binding; however, in the present case, differences between groups were not significant.

The free amount of receptor sites, *ie* not occupied by endogenous hormone (data

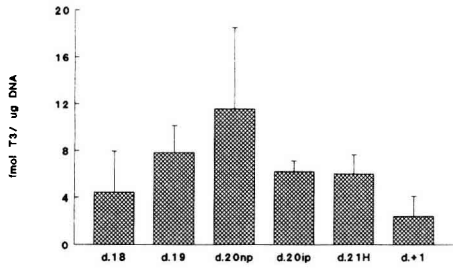

Fig 5. The total amount of receptors, determined after $MgCl_2$ treatment (n = 5). Legend: see figure 1; data are expressed as fmol/µg DNA (mean ± SEM). No significant difference between groups.

not shown), was highest on day 19, after which it decreased suddenly until there was nearly no free binding site left at day of hatching and day 1 posthatching. This resulted in an occupancy of T_3 receptors, as shown in figure 6.

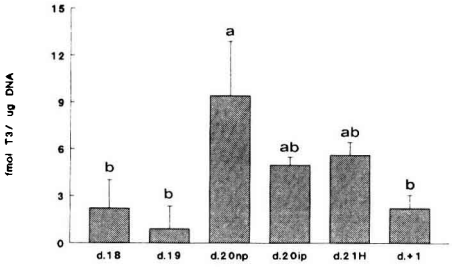

Fig 6. Occupied binding sites, measured as the difference between the amount of free and total receptors, during the last week of incubation (n = 5). Legend: see figure 1; data are expressed as fmol/µg DNA (mean ± SEM); a–b: groups sharing no common letter are significantly different ($P < 0.01$; lsd).

DISCUSSION

The plasma concentrations of thyroid hormones during the last week of incubation in chicken embryo in our studies are comparable to the data described by Scanes *et al* (1987).

The finding that the T_3 concentration in the liver cell did not parallel that in the plasma indicates that T_3 formation by peripheral deiodination and its transport to the circulation are to some extent independently regulated processes, as indicated by the time shift found in concentrations both in the liver and plasma during development. Galton and Hiebert (1987) found that the systems of deiodination in the liver appear to minimize the production or accumulation of T_3. They assume that the amount of T_3 required by the developing embryo is extremely critical, and the 2 enzyme systems (5-D and 5'-D) act in a coordinated manner to protect hepatic T_3-sensitive mechanisms from overexposure to T_3.

At day 21 (hatching) a simultaneous rise in concentration is perceptible in the cell and the plasma. There appears to be a high production of T_3 about that time, which might be due to a shift from 5 to 5'-D linked with an increased substrate availability (T_4) for the enzyme. Galton and Hiebert (1987) found dynamic changes in deiodinase systems during the late stages of embryogenesis. 5'-D Activity doubled between day 15 and 21 of incubation, while most of the 5-D activity decreased during the 48 h before hatching. Accumulation of T_3 in the liver cells generated from T_4 by 5'-D activity seems to be minimal until just before hatching, due to the high level of 5-D activity which converts T_4 to rT_3 and any T_3 that is formed by 5'-D activity to 3,3'-T_2.

After the peak value reached at day 20 np, which might be induced by the high level of corticosterone at day 19 (Kalliecharan and Hall, 1974), as it has been shown in amphibians that corticosteroids are able to increase the T_3 receptor capacity (Suzu-

ki and Kikuyama, 1983), the total amount of receptors decreased. However, the pattern of occupied receptors mainly corresponded to that of the T_3 concentration in the cell and the percentage occupancy remained very high, which might be caused by increasing T_3 concentrations. Samuels *et al* (1982) proposed a model to explain this hypothesis: the T_3–receptor complex may cause a decrease in the rate of appearance of newly synthetized receptor and a slow increase in the rate of receptor degradation. The investigators assume that the expression of the gene for the receptor protein is negatively controlled by the thyroid hormone–receptor complex and that consequently the production of receptor mRNA rapidly decreases. Sarapura *et al* (1991) found that in mice harboring thyrotropic tumors, the changes in mRNA after T_4 administration depend on the isoform of the receptor: the mRNA for α_1, α_2 and β_2 decreases, while that for β_1 increases significantly. Dependent on the receptor isoform that is most abundant at that time, it might be possible to observe an increase in B_{max} first and later on a decrease after increased thyroid hormone concentration prior to hatching. Halperin *et al* (1990) also found a decrease in receptor number once the occupancy of the receptors reached a certain value (69%). These observations seem to confirm our results and the hypothesis proposed by Samuel (1982).

The phasic pattern of receptor development can be linked to the complex sequence of events that occur at hatching: initiation of pulmonary respiration, pipping of the egg shell and emergence of the hatchling. These events are dependent on the proper development of the supporting musculature and its control mechanism, modifications in the circulatory and respiratory systems and the withdrawal of the yolk sac, in all of which T_3 is known to be involved (Decuypere *et al*, 1990). Since GH is known to increase in the embryo during the week prior to hatching (Scanes *et al*, 1987) and has been shown to augment the amount of T_3 receptors (Dewil *et al*, 1991), GH might therefore play a role in the increase in receptor capacity at that time. Other receptors, such as the hepatic GH receptor in the chicken and the human myometrical progestin receptors, are also known to display a phasic pattern (Giannopoulos and Tulchinsky, 1979; Vanderpooten *et al*, 1991).

The change in B_{max} on day 20 np and 20 ip corresponds to the shift in 5-D activity to 5'-D activity (Borges *et al*, 1980; Galton and Hiebert, 1987) and coincides with the transition from allantoic to pulmonary respiration (Decuypere *et al*, 1979). A direct role of the thyroid hormones in stimulating surfactant synthesis and hence in the onset of pulmonary respiration has been shown in mammals (Ballard, 1980; Redding and Pareira, 1974; Chopra, 1976). In birds, this role of thyroid hormones may be indirectly assumed by mediation of the glucocorticoid receptors (Morishige, 1982). A putative role of thyroid hormones in the balance of factors stimulating the production or degradation of ornithokinin (Decuypere *et al*, 1990), the latter being causally linked with changes in vascular resistance during pulmonary maturation, again may point to a connection between the onset of pulmonary respiration and the binding of T_3 to its receptor.

The binding studies with the T_3 analogues, T_4 and Triac (3,3',5-triiodothyroacetic acid), revealed relative affinities (T_3: 1.0, T_4: 0.1, Triac: 2.0) that corresponded to K_a values frequently found in previous studies (Koerner *et al*, 1974; Bellabarba and Lehoux, 1981; Weirich and Mcnabb, 1984). It was therefore concluded that in the present study we were dealing with a nuclear T_3 receptor similar to that described by other investigators.

In 1985, Bellabarba and Lehoux found a T_4 binding protein that showed a marked increase in B_{max} and decrease in K_a, while the T_3 receptor showed small changes up until day 19.

This suggests that T_4 may play a role during chicken embryogenesis before the complete development of 5'-D activity. The T_3 receptors seem to play a more important role in embryogenesis when this hormone is more abundantly present.

The difference observed in K_a during the late developmental stages may be due to the expression of several mRNAs with different affinities for T_3. Sjöberg *et al* (1991) found in the retina of a chick embryonic eye that TRβ_2 mRNA was relatively abundant only at day 6, whereas later in development by day 14 the more common β_1 predominated. Vennström *et al* (1991) found that TRα mRNA was expressed in all chicken tissues from the earliest stages with little variation in level. TRβ mRNA was found mainly in brain, lung and yolk sac and increased at late stages of development. The expression pattern of TRβ correlates well with known effects of thyroid hormones during development, and the investigators suggest that these effects are mediated by TRβ.

Analogous to lung and brain tissue changes in ratio of expression between TRβ/TRα in chick liver tissue may explain our findings of a change in K_a between day 18 and day 1 posthatch, assuming that the affinities of the α and β isoform are different. The expression of the different mRNAs for thyroid hormone receptors in the embryo during the last week of incubation still needs to be elucidated.

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