

Differences in hepatic growth hormone receptor binding during development of normal and dwarf chickens

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(Received 31 August 1990; accepted 12 November 1990)

Summary — The aim of this study was to compare the growth hormone (GH) receptor in liver microsomal fractions of normal chickens (Dw) and chickens carrying the dwarf gene (dw). Specific binding of GH to its hepatic receptor was significantly higher for Dw embryos from d 14 till d 20 of incubation than for dw embryos. The difference in binding was due to a decreased binding capacity but not affinity in the livers of the dwarf embryos. The same binding pattern was found in livers of adult chickens: lower binding was again caused by a lower number of GH receptors and at this stage the difference was even clearer than during embryonic development. Binding studies on livers of growing chicks demonstrated that binding was low for both genotypes, but a small though significant difference between them remained. The cause of this decrease in number of GH receptors in dwarf birds has yet to be determined but may be due to the primary action of the dwarf gene.

chicken / growth hormone receptor / dwarf

Résumé — Différence de liaison de l'hormone de croissance à son récepteur hépatique pendant le développement des poules normales et des poules naines. L'objectif de cette étude était de comparer le récepteur de l'hormone de croissance (GH) dans les fractions microsomales hépatiques des poules normales (Dw) et des poules portant le gène de nanisme lié au sexe (dw). La liaison spécifique de GH à son récepteur hépatique est plus élevée chez les embryons Dw que chez les embryons dw du 14^e j au 20^e j d'incubation. Cette différence est due à une diminution du nombre des récepteurs, et non de leur affinité, sous l'effet du génotype dw. Le même résultat, mais plus intense encore, est observé chez les poules adultes. Chez le poulet en croissance, la liaison est moins élevée que chez l'adulte quel que soit le génotype, mais il y a toujours une légère différence significative entre les génotypes. La cause de ce moindre nombre de récepteurs hépatiques de la GH chez les poules naines n'est pas connue. Elle peut résulter de l'action primaire du gène de nanisme.

poulet / nanisme / récepteur de l'hormone de croissance

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INTRODUCTION

The hormonal profile of chickens carrying the sex-linked dwarf gene (*dw*) has been well characterised. Recent results describing the endocrinological and metabolic effects of this dwarf gene have been reviewed by Tixier-Boichard *et al* (1989) and by Decuyper *et al* (in press). Circulating triiodothyronine (T_3) levels are depressed but growth hormone (GH) concentrations in the plasma are elevated during the fast growing period (Scanes *et al*, 1983; Huybrechts *et al*, 1987). In spite of the high GH levels, plasma immunoreactive insulin-like growth factor I (IGF-I) remains low during this stage (Huybrechts *et al*, 1987). It also has been shown that after GH injection the absolute increase in IGF-I levels is much smaller in dwarf birds than in normal birds (Huybrechts *et al*, 1988).

In the chick embryo it has been found that dwarfs have lower T_3 levels at the time of air-sac penetration and decreased IGF-I levels at the time of hatching, but the dwarf gene does not cause any changes in other hormonal parameters of the thyrotrophic or somatotrophic axis during this stage of development (Huybrechts *et al*, 1989). GH, which is able to stimulate the 5'-monodeiodinase (5'D) enzyme that converts thyroxine (T_4) into T_3 , is not effective when injected on d 18 of incubation into dwarf embryos, although it can increase circulating T_3 levels in normal embryos (Kühn *et al*, 1986).

All these results support the hypothesis that sex-linked dwarf chickens lack or have reduced numbers of GH receptors. A reduction in hepatic GH receptors was first demonstrated in 20-wk-old broiler and Leghorn chickens bearing the dwarf gene (Leung *et al*, 1987). This was confirmed by our group in adult dwarf broiler breeders (Kühn *et al*, 1989). Since no information concerning possible differences between

the genotypes was available during embryogenesis or during the period of fast growth where GH may play a crucial role, a comparative study of the hepatic GH receptor between normal and dwarf chickens was set up during these periods.

MATERIALS AND METHODS

All the animals used in this study issued from the mating between heterozygous sires and dwarf females of a medium-heavy laying strain, kindly provided by Dr Mérat (INRA, France). To distinguish dwarf and normal birds at an early stage, a linkage was made between the dwarf gene and the *S,s* gene for feather colour; this resulted in brown-feathered dwarf and white-feathered normal birds.

The eggs were incubated at 37.5°C in a forced-draught incubator. After hatching, the birds were housed under a long-day photoperiod of 16 h light and 8 h darkness (16 L:8 D). Food and water were available *ad libitum* until experimentation.

After the animals had been killed (adult birds at the age of 2 yr), the livers were excised and frozen at -20°C for analysis.

The hepatic GH receptors were determined as described earlier (Vanderpooten *et al*, 1989). Chicken GH (cGH) was purified from a crude pituitary extract by immuno-affinity chromatography using a monoclonal antibody. Purity was controlled by polyacrylamide gel electrophoresis and reversed phase HPLC (Berghman *et al*, 1988). This cGH preparation was iodinated to a specific activity of 100–200 $\mu\text{Ci}/\mu\text{g}$, with the Iodo-gen iodination reagent following the method of Fraker and Speck (1978).

Microsomal fractions from individual livers were prepared (Shiu *et al*, 1973) and stored at -20°C. Livers of embryos were pooled. In order to remove endogenous hormone from the receptors, membranes were treated with a 3 M MgCl_2 solution (Kelly *et al*, 1979), which resulted in a protein loss of \approx 40%. The treatment consisted of a 5-min incubation of 1 ml of microsomal fraction with 1 ml of 3 MgCl_2 at room temperature. After dilution with 2 ml ice-old Tris-HCl buffer (pH 7.5; 25 mM) the mixture was centrifuged at 2 000 g for 10 min at 4°C and the pellet washed

with 1 ml Tris-HCl buffer. Finally, after centrifugation, the pellet was resuspended in Tris-HCl buffer containing 10 mM CaCl₂ and 0.5% (w/v) BSA (assay buffer). Protein concentration was estimated on these membranes using the method of Lowry, modified by Peterson (1977).

In order to test the efficiency of this MgCl₂ treatment we incubated ¹²⁵I-cGH with untreated liver membranes and compared the remaining radioactivity which was specifically bound after the membranes had been treated with MgCl₂ or with H₂O. This preliminary experiment demonstrated that all specifically bound ¹²⁵I-cGH was removed by MgCl₂.

Specific binding of cGH was determined by incubating 100 µl ¹²⁵I-cGH (35 x 10³ cpm) with 100 µl membrane fraction, either in the presence or in the absence of an excess of unlabelled cGH (500 ng/100 µl/tube). Specific binding was calculated by subtracting nonspecific binding from total binding and expressed as % of total cpm added, per mg protein.

After incubation at room temperature for 20 h, 2 ml of ice-old assay buffer per tube was added. Following centrifugation for 10 min at 2 000 g (4 °C), the radioactivity in pellets was counted in a gamma counter (LKB-Packgamma II 1720).

Scatchard analyses were performed by incubating a fixed amount of membrane prepara-

tions with a fixed amount of labelled hormone and increasing amounts of unlabelled hormone.

From these results the receptor-binding parameters (capacity and affinity) were calculated using the LIGAND program (Munson and Rodbard, 1980). This resulted in the mean ± SEM of the affinity and capacity of Scatchard plots, performed in 3–8 replicates.

All results were expressed as the mean ± SEM; the number of animals are given in the figures. Statistical analyses were carried out by 1-way analysis of variance (ANOVA) followed by the least significant difference test (Lsd-test), when *F* was significant. Since no individual results of the replicates of the Scatchard plots were available, but only means ± SEM, affinity constants or binding capacities were compared using the Lsd-test.

RESULTS

The specific binding of ¹²⁵I-cGH to MgCl₂-treated liver microsomal fractions of normal and dwarf embryos from the 14th d of incubation till the first day after hatching is shown in figure 1. A significantly higher

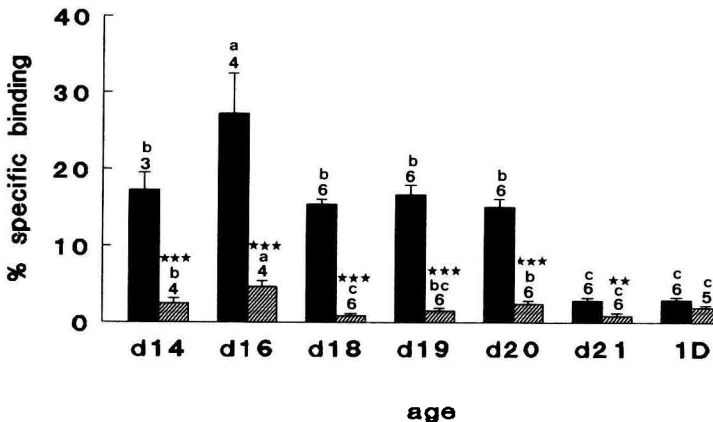


Fig 1. Percentage specific binding/mg protein of ¹²⁵I-cGH to MgCl₂ treated liver microsomal fractions of dwarf (▨) and normal (■) embryos. The amount of membrane proteins incubated varied from 0.48–0.72 mg/100 µl for normal and from 0.61–0.68 for dwarf embryos. Number of measurements on pooled livers is shown above bars. a–c: groups within the same genotype, sharing no common letter are significantly different (Dw: *P* < 0.001; dw: *P* < 0.05, ANOVA). ** *P* < 0.01, *** *P* < 0.001: significance between Dw and dw at each time point. Age: d = embryos; D = post-hatching.

binding was found in normal embryos compared to dwarfs from d 14 till d 20 of incubation ($P < 0.001$, except for d 21: $P < 0.01$). The specific binding to the GH receptor in normal embryos decreased prior to hatching ($P < 0.001$) to values which were not different from dwarf values 1 d after hatching.

Scatchard plots were calculated when specific binding was high enough to allow precise determination of the binding parameters, which was not always the case in dwarfs. These results are shown in figures 2a and b. Affinity constants of the hepatic GH receptor did not change significantly during embryonic development or between the 2 genotypes, following the least significant difference test (lsd test, fig 2a). The binding capacity (expressed in fmol/mg protein; fig 2b) of the hepatic GH receptor of dwarf embryos was significantly lower than the values found for the controls of the same age on the time points. Scatchard plots could be calculated for both genotypes (d 14, 16, 19 and 20).

Within the group of normal embryos we found a significant ($P < 0.01$) decrease in capacity of the hepatic GH receptor towards the end of incubation when compared to d 14.

After hatching, the specific binding to the hepatic GH receptor was determined in 2, 3, 5 and 8-wk-old normal growing and dwarf birds (fig 3). During this period a difference between the 2 genotypes re-

mained ($P < 0.01$), although binding was low for normal chickens when compared to values obtained in embryos and adult birds. Scatchard plots could only be calculated for the normal growing birds. Figure 4 shows a representative Scatchard plot for the 2-wk-old birds. The affinity constants and binding capacities are shown in table I. These parameters did not change significantly (lsd-test) between the ages examined during this rapid growth period.

In adult animals, the difference in GH binding between both genotypes was very pronounced: for the normal adults we found a specific binding of $33.41 \pm 5.35\%$ per mg protein ($n = 8$) while only 5 out of 7 dwarf adults showed positive specific binding: $0.75 \pm 0.23\%$ per mg protein ($n = 5$, $P < 0.001$, ANOVA) (fig 3). The low binding percentage did not allow calculation of the binding parameters in dwarf birds. In normal birds, an affinity constant of $4.93 \pm 0.83 \cdot 10^{10} \text{ M}^{-1}$ ($P < 0.05$ compared to K_a values obtained for growing chicks) and a capacity of 5.63 ± 0.51 fmol/mg protein ($P < 0.001$ compared to B_{\max} values obtained for growing normal chicks) were found.

DISCUSSION

The ontogenic study of the hepatic GH receptor in the normal (Dw) growing embryos and chicks indicates that binding to the GH

Table I. LIGAND calculated affinity (K_a) and capacity (B_{\max}) values for the hepatic GH receptors of 2, 3, 5 and 8-wk-old Dw chickens.

Age (wk)	2	3	5	8
K_a (10^{10} M^{-1})	1.64 ± 0.82	1.91 ± 1.11	2.28 ± 0.73	1.11 ± 0.35
B_{\max} (fmol/mg protein)	1.49 ± 0.61	0.97 ± 0.45	0.98 ± 0.25	1.92 ± 0.46

receptor decreases towards the end of incubation, is low at time of hatching, and remains relatively low during the first weeks after hatching, compared to the binding values obtained in 14–20-d-old embryos and adults. This decline towards the end of incubation and the low binding in post-hatch chicks has also been found in chickens of a commercial layer strain (Vander-

pooten *et al*, 1990). Comparing dwarf and normal chickens, it is obvious that as early as d 14 of embryonic development there is a difference in hepatic GH receptor binding, and this is due to a lower binding capacity in the liver of the dwarf embryos. This difference disappears transiently at the time of hatching when binding for the normal embryos decreases. The de-

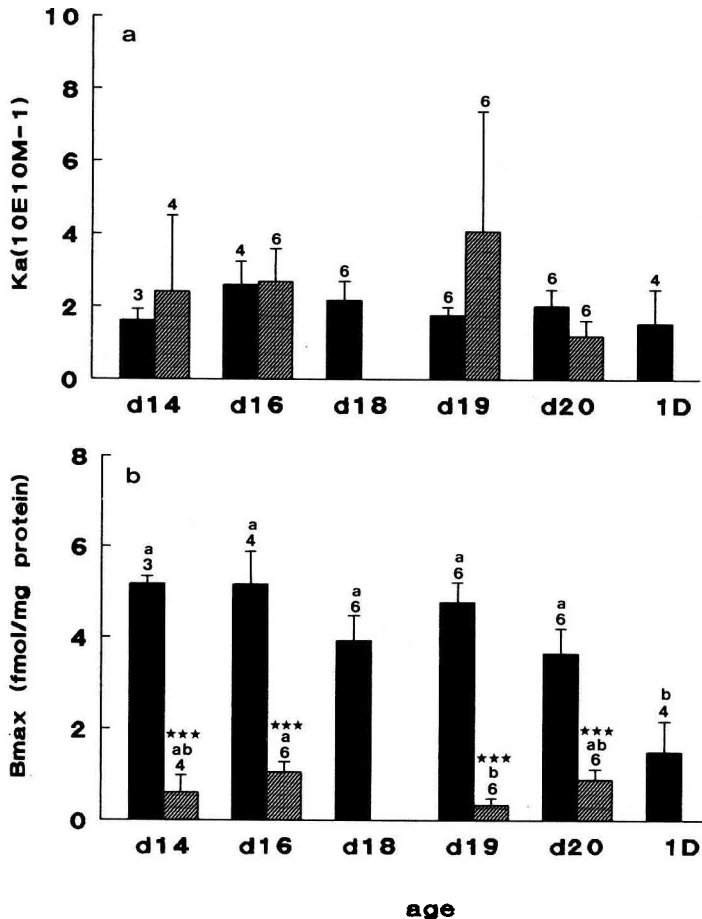


Fig 2. LIGAND calculated affinity (a) and capacity (b) values for hepatic GH receptors of dwarf (▨) and normal (■) embryos. Scatchard plots were calculated from experiments using increasing amounts of cGH. Number of measurements on pooled livers is shown above bars. **a:** No significant differences were found by lsd-test. **b:** a–b: groups within the same genotype sharing no common letter are significantly different ($P < 0.01$, lsd-test). *** $P < 0.001$: significance between Dw and dw at each time point. Age: d = embryos; D = post-hatching.

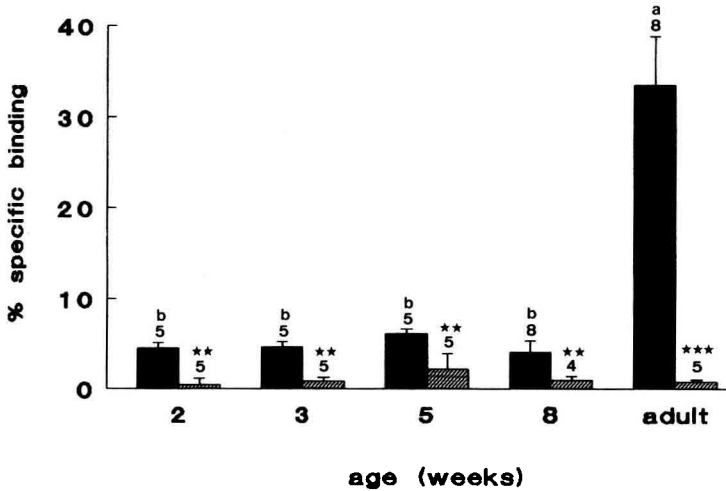


Fig 3. Percentage specific binding/mg protein of ^{125}I -cGH to MgCl_2 -treated liver microsomal fractions of dwarf (▨) and normal (■) chickens during postnatal development. The amount of membrane proteins incubated was 0.53 and 0.50 mg/100 μl for normal and dwarf adults respectively, and varied from 0.78–0.95 mg/100 μl for normal chicks and from 0.89–1.02 mg/100 μl for dwarf chicks. Numbers of animals per group are shown above bars. a–b: Dw groups sharing no common letter are significantly different ($P < 0.001$: ANOVA). No significant difference was found between dw groups. ** $P < 0.01$, *** $P < 0.001$: significance between Dw and dw at each time point (ANOVA).

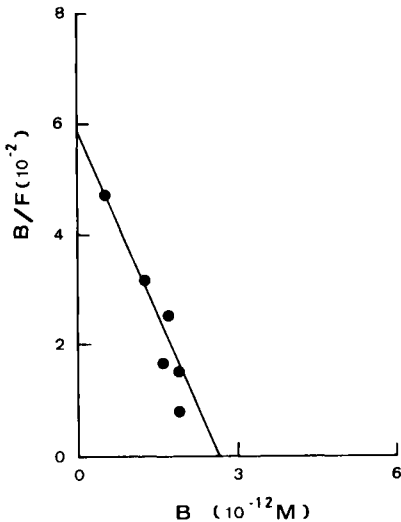


Fig 4. Scatchard plot for the hepatic GH receptor of 2-wk-old Dw chickens, calculated from a competition experiment using increasing amounts of cGH.

pressed GH receptor number in dwarfs can explain why GH could not stimulate the 5'-monodeiodinase enzyme to convert T_4 into T_3 in these embryos (Kühn *et al*, 1986). The effect of the dwarf gene may also play a major role in the low plasma T_3 as well as IGF-I levels observed in juvenile chickens (Scanes *et al*, 1983; Huybrechts *et al*, 1986) and hence be an important factor in determining the decreased growth during postnatal development (Tixier-Boichard *et al*, 1989).

During the prenatal period no difference in circulating GH was found between dwarf and normal birds; this only occurred from the 3rd wk posthatch till the 18th wk (Huybrechts *et al*, 1987). Therefore it is not likely that the depressed hepatic GH binding capacity in the dwarf embryos is caused by a GH induced down-regulation, since at that time of development, circulat-

ing GH levels are equal for both genotypes.

After hatching we found a low binding in dwarfs and in normal chicks, but the difference between the 2 genotypes remained, although the absolute difference was much smaller. Leung *et al* (1987) found similar binding characteristics in 6 and 8-wk-old broiler chicks: specific binding did not exceed 4% of total cpm. They also demonstrated a significantly lower binding for the 6 and 8-wk-old, but not the 2-wk-old chicks (Leung *et al*, 1984).

Circulating GH concentrations in the domestic fowl are elevated at the time of hatching and during the first weeks post-hatch. This period corresponds with the occurrence of low binding percentages found in the normal growing chicks in this study. However, the hypothesis that the GH receptors in the microsomal fractions are occupied to a great extent is excluded since they have been desaturated by the $MgCl_2$ treatment. A possible explanation is down-regulation by the high circulating GH concentrations. Arguments in support of this hypothesis have been found in studies on hypophysectomised chicks. Hypophysectomy resulted in a marked decrease of circulating GH, while an obvious increase in the number of hepatic GH receptors was found; this effect was reversed by acute GH administration. Since the number of available GH receptors depends on the balance between receptor internalisation and degradation on the one hand, and receptor recycling plus new synthesis on the other (Posner *et al*, 1981), the observed down-regulation in growing chicks may be the result of a shift in balance towards a higher receptor degradation, due to an increased receptor activity during this period of fast growth. This argument is supported by the findings of Husman *et al* (1988) who suggested that down-regulation of somato-

genic receptors in rat liver is the result of transport of internalised ligand-receptor complexes to lysosomes, probably to degrade the ligand and/or receptor and terminate the hormone action. In addition, Roupas and Herington (1989) also suggested that GH receptors undergo a ligand-binding induced internalisation and that subsequently 75% of these internalised receptors are degraded and not recycled.

In the adult stage, the expression of the dwarf gene was most evident: dwarf chickens did not show any specific binding at all (< 0.5%), while the controls of the same age showed a high specific binding to the GH receptor. In a previous report on adult dwarf broiler breeders, we still found some specific binding for the dwarfs. Calculation of the Scatchard plots demonstrated that the lower binding was due to a decreased capacity of the hepatic GH receptor (Kühn *et al*, 1989). These results confirm those of Leung *et al* (1987) who found the same difference in expression of the dwarf gene depending on genetic background.

The cause of the decreased number of hepatic GH receptors in the sex-linked dwarf chicken remains to be investigated but it may well be the consequence of a mutation in the GH receptor gene, as has been found in Laron dwarfs (Amselem *et al*, 1989; Godowski *et al*, 1989).

When the comparison between the 2 genotypes is examined, it appears that there is a better correlation between GH binding and growth than between circulating GH and growth, especially between strain comparisons, as has also been pointed out by Leung *et al* (1987). However, when the developmental pattern of GH binding in normal growing chickens is considered, this correlation no longer fits: GH binding is lowest when growth rate is high.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Fund for Scientific Research, Belgium (NFWO): FKFO 2.0039.90. A Vanderpooten was supported by the Instituut ter Aanmoediging van het Wetenschappelijk Onderzoek in de Nijverheid en de Landbouw (IWONL) and L Huybrechts by the National Fund for Scientific Research (NFWO). We are indebted to P M  rat for donating the fertilised eggs and livers of adult animals and to L Berghman for the affinity-purified cGH.

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