甲状腺ホルモンによる血中インスリン様成長因子結合タンパク質(IGFBP),および各組織のIGFBP-2mRNA発現の変化

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Effects of Thyroid Hormone on Serum Insulin-Like Growth Factor Binding Proteins (IGFBPs) and IGFBP-2 mRNA Expression in Chickens

Akira TSUKADA[1]*, Takeshi OHKUBO[2], Minoru TANAKA[3], Kunio NAKASHIMA[3], Masaaki WAKITA[1] and Sadao HOSHINO[1]

[1]Department of Animal Science, Faculty of Bioresources,
[2]Center for Molecular Biology and Genetics, and 3 Department of Biochemistry, Faculty of Medicine, Mie University, Tsu, Mie 514-8507, Japan

We examined serum IGF binding proteins (IGFBPs) and tissue (liver, kidney and brain) IGFBP-2 mRNA in 5-weeks-old cockerels with different thyroidal status to elucidate a possible role of thyroid hormone on IGFBPs in the chicken. Assay for serum IGFBPs by Western ligand blot detected three IGFBPs having molecular masses of 30, 36 and 41 kDa. The ^{125}I-IGF-I bindings to these IGFBPs were significantly decreased in propylthiouracil (PTU) induced hypothyroid cockerels, and restored to euthyroid control levels by thyroxine (T_4) supplement. T_4 supplement to euthyroid birds had no significant influence on ^{125}I-IGF-I binding to IGFBPs. The expression of IGFBP-2 mRNA in the liver was significantly reduced in hypothyroidism and restored to normal level by T_4 replacement, whereas T_4, injected to intact birds, failed to affect hepatic IGFBP-2 mRNA expression. In contrast, the expression of IGFBP-2 mRNA in the kidney was increased in hypothyroid birds and decreased both in T_4-treated intact and hypothyroid birds. In the brain, IGFBP-2 mRNA level was not influenced by the changes in thyroidal status. IGFBP-2 mRNA was detected in all tissues examined, with varying degree. The expression was higher in the testis, gizzard, brain, eye and bursa of fabricius, followed by kidney and digestive organs. In the liver, the expression was very low compared with other tissues.

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Key words : hypothyroidism, chicken, IGFBPs, IGFBP-2, thyroxine

Introduction

Insulin-like growth factors (IGFs) play an important role in growth regulation in birds as well as in mammals (HOSHINO *et al.*, 1982). IGFs (IGF-I and IGF-II) are synthesized in many tissues in fetus and adult. They act locally or after circulating to distant targets, and stimulate growth, differentiation, or the expression of differentiated functions by acting through their receptors. In all biological fluids, IGFs are bound to high-affinity IGF binding proteins (IGFBPs), six of which have been cloned in mammals (SHIMASAKI and LING, 1991). These IGFBPs regulate the actions of

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* Corresponding and off print requesting author : A. TSUKADA
Department of Animal Science, Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, Japan. Tel : 81-59-231-9491, Fax : 81-59-231-9640, E-mail : g5tsuka@bio.mie-u.ac.jp

IGFs by influencing their half-life, tissue distribution, and the interaction of IGFs with their receptors (CLEMMONS, 1991). Unlike mammals, IGFBPs have not been fully characterized in birds, though there is considerable evidence for the existence of IGFBPs in the circulation (ARMSTRONG *et al.*, 1989 ; MORISHITA *et al.*, 1993 a ; RADECKI *et al.*, 1997).

Although roles of these IGFBPs have not been clearly established in the chicken, their circulating concentrations in serum and binding activities are known to be modulated in response to hypophysectomy (MORISHITA *et al.*, 1993 b), nutritional condition (BRUGGEMAN *et al.*, 1997 ; KITA *et al.*, 1996 ; LEILI *et al.*, 1997 ; MORISHITA *et al.*, 1993 a), developmental change (RADECKI *et al.*, 1997 ; SCANES *et al.*, 1997) and thyroidal state (MORISHITA *et al.*, 1993 c). Recently, chicken IGFBP-2 cDNA has been cloned and characterized (SCHOEN *et al.*, 1995).

We have previously demonstrated that hypothyroidism reduces both serum levels of IGF-I and hepatic expression of IGF-I mRNA, and that this is closely associated with reduced hepatic expression of growth hormone receptor (GHR) mRNA. Thyroxine (T_4) supplement restores these changes induced by hypothyroidism (TSUKADA *et al.*, 1998). Although we did not assess a possible effect on IGFBPs in that experiment, it seemed to be of importance to investigate interrelationship between thyroid hormone/growth hormone and IGFBPs. In the present paper, we describe the effect of thyroid status on circulating IGFBPs and IGFBP-2 mRNA expression in various tissues of cockerels.

Materials and Methods

Animals

One-day-old White Leghorn (Babcock B-300) male chickens were obtained from a local hatchery (Tsu, mie pref.) and reared in a heated battery with free access to a commercial formula feed (21% crude protein, 2,950 kcal metabolizable energy/kg, Zennou) and water. At 5 weeks of age, 24 chicks were assigned randomly to the following four groups : control, T_4, propylthiouracil (PTU), and PTU plus T_4. Chicks were fed the diet without (control and T_4 groups) or with PTU at 0.1% (PTU and PTU plus T_4 groups) for 8 days. Chicks in the T_4 and PTU plus T_4 groups received a daily sc injection of T_4 (100 μg/kg body weight) at 17 : 00 for 4 days from the 5 th to 8 th day of treatment. Chicks in the control and PTU groups received the same alkaline solution (0.01 N NaOH) as that in which T_4 was dissolved. It was confirmed previously that hypo- and hyperthyroidism are induced successfully in chicks by the treatments with PTU and/or T_4 respectively (TSUKADA *et al.*, 1998). Twenty hours after the final injection of T_4 or vehicle, chicks were weighed, blood samples were taken by heart puncture, and then killed by decapitation to remove the various tissues. The tissues were quickly excised, frozen in liquid nitrogen, and stored at −80℃ until used for RNA extraction. Blood was allowed to clot at room temperature, and then centrifuged to collect serum samples. The sera were stored at −20℃ until analyzed.

Analysis of IGFBPs by Western ligand blotting and cross linking

Western ligand blotting of serum IGFBPs was performed by the procedure

described previously (MORISHITA *et al.*, 1993a). Briefly, 5μl of serum samples were roaded on 12% polyacrylamide gels containing sodium dodecylsulphate (SDS, 1%, w/v) after first being incubated with SDS and subjected to electrophoresis. Transfer of separated serum proteins onto nitrocellulose membrane was performed using a Tris-glycine buffer (25 mM, pH 8.3) containing methanol (20%, v/v). The nitrocellulose membranes (69×90 mm) were dried, soaked and incubated with ^{125}I-labeled IGF-I (3.5×10^6 c.p.m./membrane). The membranes were dried after being washed, then exposed at −80℃ to X-ray film (Fuji New RX, 165×216 mm) for 7 days. The amounts of IGFBPs were quantitatively determined by a Fuji BAS 1000 imaging analyzer (Fuji Photo Film Co, Tokyo, Japan).

To determine the amount of free IGFBPs in serum, cross linking of ^{125}I-IGF-I with IGFBPs was performed as described by HARDOUIN *et al.* (HARDOUIN *et al.*, 1987). In brief, ^{125}I-IGF-I (10μl, ca. 50,000 c.p.m.) was mixed with 20μl serum and the mixture was incubated for 16 h at 4℃. Then 80% disuccimydyl suberate solution (10μl) was added and the mixture was incubated for 15min at 4℃. During this incubation, ^{125}I-IGF-I was covalently bound to IGFBPs which had not been associated with endogenous IGF-I. To this mixture, 40μl SDS solution was added and incubated for 15 min at 60℃. A portion of the solution (40μl) was used for SDS-polyacryamide gel electrophoresis (PAGE). After electrophoresis, the gel was dried, the radioactivity was detected by autoradiography, and analyzed as mentioned above.

Northern blot analysis of IGFBP-2 mRNA

Total RNA was extracted from frozen tissues by the acid guanidium-phenol-chloroform method (CHOMCZYNSKI and SACCHI, 1987) and quantified by absorbency at 260 nm (1 optical density unit=40μg RNA/ml solution). Total RNA (20μg) were separated on a 1% (w/v) agarose gel containing 2.2 M formaldehyde. The integrity of the RNA and the accuracy of the spectrophotometric determinations were assessed by visual inspection of the ethidium bromide-stained 28 S and 18 S ribosomal RNA (rRNA) bands after the electrophoresis. The RNA was then blotted onto a nylon membrane (Gene Screen, Dupont, Boston, MA, USA) and hybridized with a radiolabeled chicken IGFBP-2 cDNA probe cording the region (454-929) of chicken IGFBP-2 (SCHOEN *et al.*, 1995). The 475 bp probe was generated by RT-PCR amplification of total RNA for 8-weeks-old cockerel liver using primers, 5'-AGAGCATCCTTGCTGAGAAC-3' (sense) and 5'-TGTATAAATCCAGCACCGGG-3' (antisense). The amplified fragment was subcloned into the pCR^{TM}2.1 vector (Invitrogen Corporation, San Diego, CA) and sequenced in a DNA sequencer (Applied Biosystems 373A) for dideoxynucleotide chain-termination method (SANGER *et al.*, 1977). The amounts of mRNA detected with this probe was quantitatively determined by the Fuji BAS 1000 imaging analyzer.

Statistical analysis

The data were statistically analyzed by analysis of variance and Student's *t*-test or Cochran-Cox test.

Results

The analysis of chicken serum by Western ligand blot detected three IGFBPs

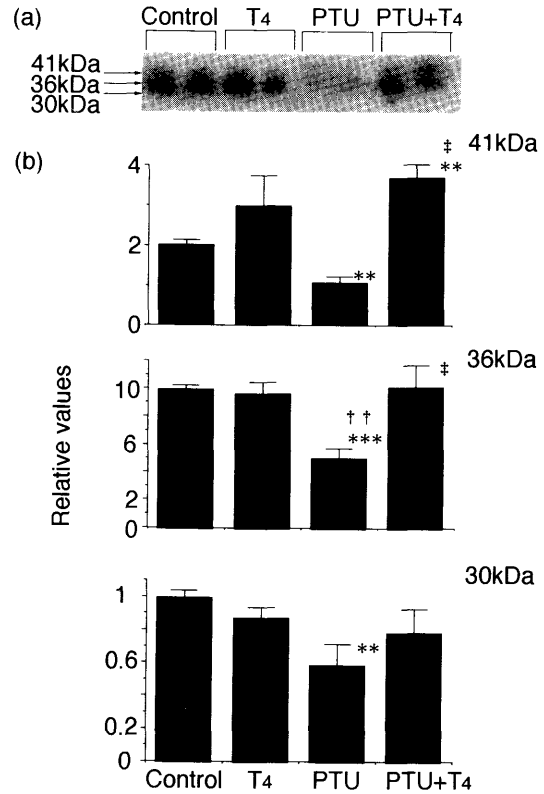

Fig. 1. SDS-PAGE of chicken serum IGFBPs detected by Western ligand blotting. (a) The autoradiograms of two representative samples in each experimental group are shown. Serum proteins were separated by electrophoresis and blotted with ^{125}I-labeled IGF-I. Proteins with molecular masses of approximately 30, 36 and 41 kDa were detected. (b) The IGFBPs were quantified by a Fuji BAS 1000 imaging analyzer and data are shown as arbitrary units. Values are means+S.E.M. (n=6).
**P<0.01, ***P<0.001 vs control. ††P<0.01 vs T_4. ‡P<0.05 vs PTU.

having molecular masses of 30, 36 and 41 kDa (Fig. 1 a). ^{125}I-IGF-I bindings to these IGFBPs were significantly lower in hypothyroid group than those in the controls, and were restored to the levels of control by T_4 supplement. T_4 supplement to intact birds had no influence on ^{125}I-IGF-I bindings to all of the IGFBPs (Fig. 1 b).

The cross-linking analysis revealed that two bands, at 150 and 39 kDa, are detectable in all chicken groups (Fig. 2 a). ^{125}I-IGF-I bindings to 150 kDa IGFBP being unsaturated with endogenous IGF-I were not affected in hypothyroidism. However, T_4 supplement significantly reduced the ligand bindings both in normal and hypothyroid birds. On the other hand, the ligand bindings to 39 kDa IGFBP were

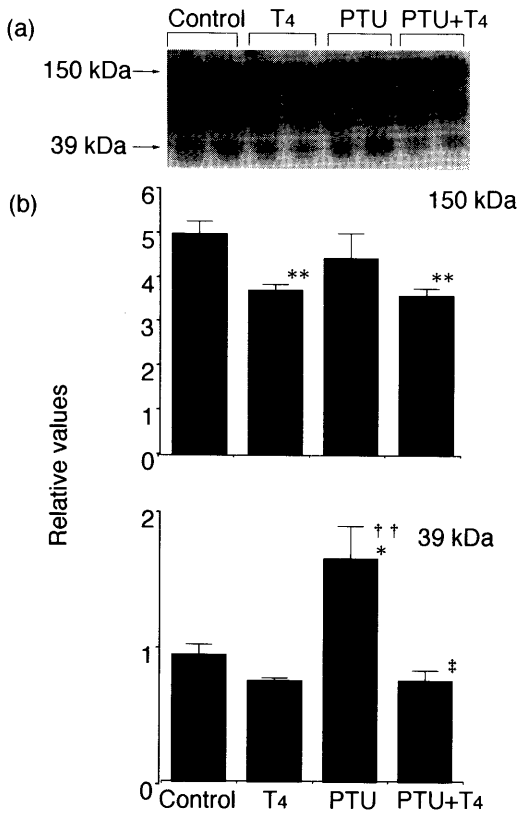

Fig. 2. SDS-PAGE of chicken serum IGFBPs covalently bound with labeled IGF-I (Cross-linking). (a) The autoradiograms of two representative samples in each experimental group are shown. Proteins with molecular masses of 39 and 150 kDa were detected. (b) The IGFBPs were quantified by a Fuji BAS 1000 imaging analyzer and data are shown as arbitrary units. Values are means+S.E.M.(n=6).
*$P<0.05$, **$P<0.01$ vs control. ††$P<0.01$ vs T_4. ‡$P<0.05$ vs PTU.

elevated with PTU treatment, and restored to control levels with T_4 treatment (Fig. 2 b).

The results of Northern blot analysis of IGFBP-2 mRNA expressions are shown in Figs. 3-5. The expression of IGFBP-2 mRNA in the liver was significantly reduced in hypothyroidism and restored by T_4 supplement, while T_4 supplement to intact birds had no influence on the IGFBP-2 mRNA expression (Fig. 3). In contrast to the liver, the expression of IGFBP-2 mRNA in the kidney was significantly increased in hypothyroidism, and decreased with T_4 supplement irrespective of thyroid status of birds (Fig. 4). In the brain, expression of IGFBP-2 mRNA was not influenced by the changes in thyroidal status employed (Fig. 5).

To consider a possible relationship between serum IGFBPs and IGFBP-2 mRNA

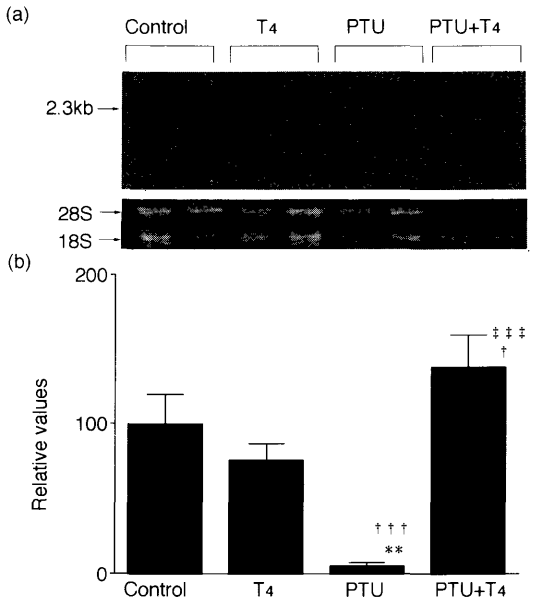

Fig. 3. Expression of IGFBP-2 mRNA in the liver by Northern blot analysis. (a) The autoradiograms of two representative samples in each experimental group are shown. The bands of 2.3 kb IGFBP-2 mRNA are indicated by an arrow on the left. The bottom panel shows the ethidium bromide stained 18 S and 28 S ribosomal RNA bands to demonstrate equal RNA loads in the different lanes. (b) Relative IGFBP-2 mRNA expression in livers of each experimental group. The radioactivities of 2.3 kb IGFBP-2 mRNA bands were quantitatively determined by a BAS 1000 imaging analyzer and expressed as a percentage of that of control (mean + S.E.M., n = 6).
** $P < 0.01$ vs control. † $P < 0.05$, ††† $P < 0.001$ vs T_4. ‡‡‡ $P < 0.001$ vs PTU.

expressions, the gene expression of IGFBP-2 was determined in various tissues. The results are shown in Fig. 6. The expression of IGFBP-2 mRNA was observed in all tissues tested : relatively higher levels of expression were found in testis, gizzard, optic lobe, brain and bursa of fabricius, followed by adipose tissue, kidney, crop sac, stomach and intestine. The expression levels were low in muscle, lung, heart, spleen, pancreas, and liver.

Discussion

Three IGFBPs with 30, 36, and 41 kDa molecular masses were found in the serum by Western ligand blotting (Fig. 1) consistently with our previous report (MORISHITA *et al.*, 1993 a). However, molecular masses of IGFBPs differ somewhat from other reports (ARMSTRONG *et al.*, 1989 ; BAXTER *et al.*, 1987 ; FRANCIS *et al.*, 1990 ; KITA *et al.*, 1996 ;

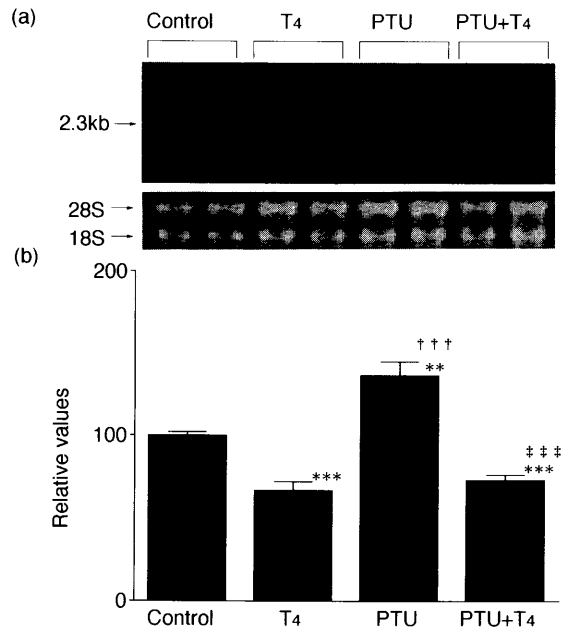

Fig. 4. Expression of IGFBP-2 mRNA in the kidney by Northern blot analysis. Signs and legends are the same as Figure 3.
$^{**}P<0.01$, $^{***}P<0.001$ vs control. $^{\dagger\dagger\dagger}P<0.001$ vs T_4. $^{\ddagger\ddagger\ddagger}P<0.001$ vs PTU.

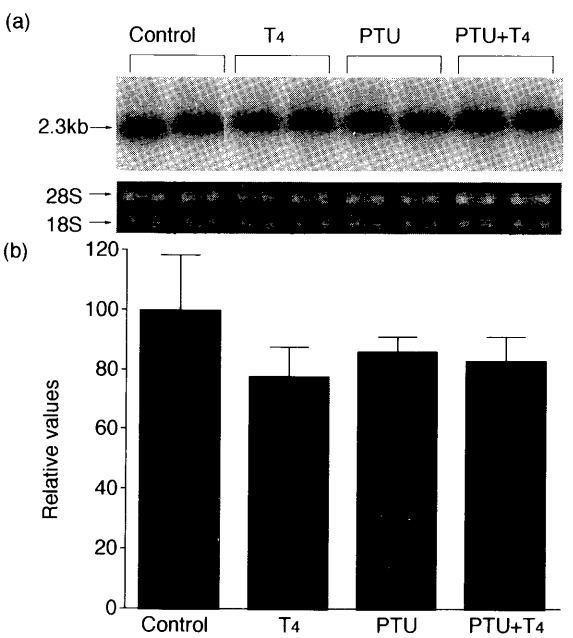

Fig. 5. Expression of IGFBP-2 mRNA in the brain by Northern blot analysis. Signs and legends are the same as Figure 3.

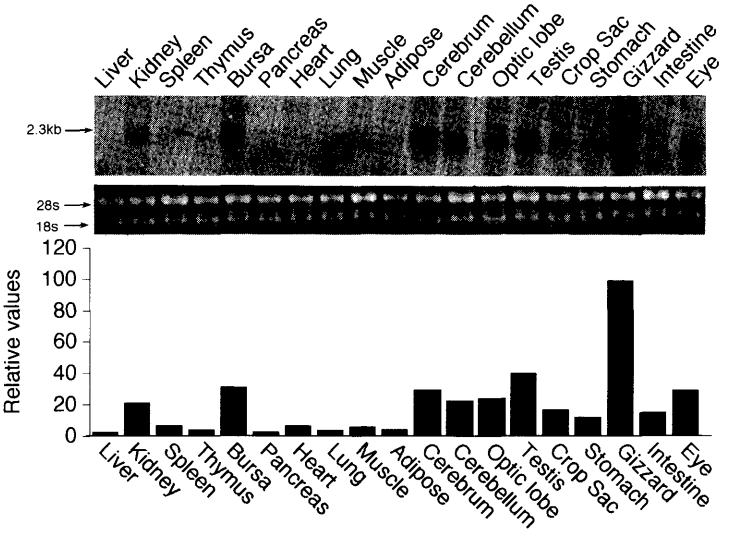

Fig. 6. Expression of IGFBP-2 mRNA in various tissues from 5-weeks-old cockerels by Northern blot analysis. (a) The autoradiogram of a representative sample from various tissues is shown. The bands of 2.3 kb IGFBP-2 mRNA are indicated by an arrow on the left. The bottom panel shows the ethidium bromide stained 18 S and 28 S ribosomal RNA bands to demonstrate equal RNA loads in the different lanes. (b) Relative IGFBP-2 mRNA expression in various tissues. The values are results of a representative sample from cockerels. The similar results were confirmed with samples from other two cockerels. The radioactivities of 2.3 kb IGFBP-2 mRNA bands were quantitatively determined by a BAS 1000 imaging analyzer and expressed as a percentage of that of gizzard.

RADECKI *et al.*, 1997), suggesting that molecular species vary according to the sources of serum samples obtained.

The result of cross-linking analysis showed that two bands at 39 and 150 kDa were found in all groups of chickens (Fig. 2). The 39 kDa protein seems to be the 36 kDa protein bound with 7 kDa IGF-I. The difference between the expected molecular mass of bound protein (36+7=43 kDa) and the observed one (39 kDa) may be due to the conformation of the complex as reported by BAXTER *et al.* (1987). The significant increase of the 39 kDa complex radioactivity in hypothyroidism is not due to increased serum concentration of 36 kDa protein, but probably due to the lowered circulating concentrations of IGF-I, which in turn increase vacant sites of IGF-I binding to IGFBPs. The result of Western ligand blot (Fig. 1), together with impaired IGF-I production (MORISHITA *et al.*, 1993 c ; TSUKADA *et al.*, 1998) in hypothyroid birds, supports this view.

The 150 kDa protein seems to be a major IGFBP in the chicken, because when chicken sera were chromatographed on a sephadex G-200 column, IGF-I binding activity is mainly found in 150 and 30 kDa proteins and most of circulating IGF-I are

distributed among 150 kDa protein fractions (MORISHITA *et al.*, 1993 b). However, it remains obsure whether or not the 150 kDa protein represents chicken IGFBP-3, a complex consisting of acid-labile and acid-stable subunits and IGF-I, and dissociates into subunits during SDS-PAGE. In addition, it is unknown whether the 41 kDa protein detected in Western blot is a part of 150 kDa protein.There were several protein bands between 150 kDa- and 39 kDa-protein in the cross-linking experiment (Fig. 2). However, the nature of these proteins remains to be elucidate because these proteins were not observed in Western blotting experiments.

We have confirmed that the 30 and 36 kDa IGFBPs crossreact with an antiserum raised against bovine IGFBP-2 (Upstate Biotech. Inc., New York, U.S.A.), and the 30 kDa protein increases significantly when chicks were fasted (MORISHITA *et al.*, 1993 a), or hypophysectomized (MORISHITA *et al.*, 1993 b). Dietary energy or protein restriction also increases the 30 kDa IGFBP in meat-type female chickens at 19 days of age (KITA *et al.*, 1996). In the present experiment the 30 kDa protein was detected only as a faint band and the 36 kDa protein band was ten times denser than the 30 kDa one (Fig. 1), indicating that the 36 kDa IGFBP is a substantial circulating IGFBP in the chicken. SCHOEN *et al.* (1992) found that the 33 kDa IGFBP of serum from 2-days-old chicks is immunoprecipitated with a IGFBP-2 antiserum produced against a 20 amino acid sequence near the carboxy-terminus of human IGFBP-2. Later they confirmed that chicken IGFBP-2 has 33,500 molecular mass and exhibits 68 and 66% identity to bovine and human IGFBP-2, respectively (SCHOEN *et al.*, 1995). Based on these immunochemical and molecular mass studies, it seems reasonable to assume that the 36 kDa protein in the present study is chicken IGFBP-2. However this does not exclude a possibility that the 30 kDa protein is chicken IGFBP-2, because of the crossreaction with the bovine IGFBP-2 antiserum and of a close molecular mass. More studies are clearly required to elucidate which protein is chicken IGFBP-2 in the circulation.

Nevertheless, it was clearly demonstrated that hypothyroidism reduces circulating IGFBPs including IGFBP-2 and T_4 replacement restores it completely (Fig. 1). Further, IGFBP-2 mRNA expression in hypothyroid birds was reduced in the liver, elevated in the kidney and unaltered in the brain (Figs. 3-5). This is the first report indicating that circulating IGFBP-2 level and its gene expressions are under thyroidal regulation in the chicken. In the previous study (TSUKADA *et al.*, 1998) we found that hypothyroidism reduced hepatic growth hormone receptor (GHR) mRNA expression, followed by reduced hepatic expression of IGF-I mRNA and decline in circulating IGF-I levels, though there were no significant effects on GHR and IGF-I gene expressions in the brain and testis, and T_4 treatment abolished the alterations found in the liver and circulation. Accordingly, it seems likely that thyroid hormone modulates hepatic IGFBP-2 gene expression indirectly by affecting numbers of GHR and thereby IGF-I production. This view is supported from the findings that thyroid status has no influence on IGFBP-2 gene expression in the brain (Fig. 5), where IGF-I mRNA is expressed in a GH-independent manner (TSUKADA *et al.*, 1998), and that GHR-lacking dwarf chicks exhibit no response on hepatic IGFBP-2 gene expression to varying thyroid status (Tsukada, unpublished). The treatment with IGF-I increases IGFBP-2

mRNA abundance and protein secretion in myoblasts (ERNST *et al.*, 1992). Circulating levels of IGFBP-2 are significantly reduced in hypothyroid mice which are characterized with lower serum IGF-I levels (SUGISAKI *et al.*, 1993). These findings present suggestive evidence that IGF-I is involved in IGFBP-2 production in chickens as well as in mammals. There is positive evidence that thyroid hormone regulates IGF-I production directly. For instance, thyroid hormone increases hepatic IGF-I gene expression (WOLF *et al.*, 1989) and circulating levels of IGF-I in hypophysectomized mammals (LATIMER *et al.*, 1993), and stimulates IGF-I mRNA expression in cultured rat hepatocytes (TOLLET *et al.*, 1990). However, this is unlikely in chickens, because thyroid hormone treatment of hypophysectmized chicks has no effect on circulating IGF-I levels (SCANES *et al.*, 1986). Taken together, all these reports suggest that thyroid hormone regulates hepatic IGFBP-2 expression indirectly through GH-dependent IGF-I production in the chicken.

IGFBP-2 mRNA was detected in all tissues as show in Fig. 6. Interestingly the expression was higher in the testis, gizzard, and brain than in other tissues, the expression in liver being relatively low. However, if we compare organ weight, the liver (12.71±0.61 g) was heaviest, followed by the gizzard (11.93±0.91) and kidney (4.30 ±0.26), the weight of testis being less than 0.12 g. Accordingly the liver and gizzard appear to be important organs that contribute circulating IGFBP-2 levels, if these organs secrete IGFBP-2 into blood in an endocrine fashion. In conclusion, the IGFBP-2 mRNA expression in the liver was parallel to the changes in circulating IGFBPs, suggesting a possible relationship between them, but the available data are not sufficient at present to warrant any conclusion on the source of IGFBP-2 protein in chicken circulation.

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甲状腺ホルモンによる血中インスリン様成長因子結合タンパク質(IGFBP)，および各組織のIGFBP-2 mRNA発現の変化

塚田　光[1]*・大久保武[2]・田中　実[3]・中島邦夫[3]
脇田正彰[1]・星野貞夫[1]

[1]三重大学生物資源学部，
[2]遺伝子実験施設，
[3]医学部，津市 514-8507

ニワトリのインスリン様成長因子結合タンパク質(IGFBP)に及ぼす甲状腺ホルモンの役割を明らかにするために5週齢の雄ヒナを用い，甲状腺機能を人為的に変え，血清中のIGFBPs，および肝臓，腎臓，脳のインスリン様結合タンパク質-2（IGFBP-2）mRNAに及ぼす影響を調べた。血清中のIGFBPはウェスタンリガンドブロット法で解析し，分子量30，36，41 kDaの3種類のIGFBPを検出した。これら3種類のIGFBPへの^{125}I-IGF-Iの結合は，プロピルチオウラシルで誘起した低甲状腺機能ヒナで有意に減少し，サイロキシン（T_4）投与によって正常レベルまで回復した。正常ヒナへのT_4投与はIGFBPへの^{125}I-IGF-Iの結合に有意な影響を与えなかった。肝臓でのIGFBP-2 mRNAの発現は，低甲状腺機能ヒナで有意に減少し，T_4投与によって正常値に回復した。しかし，正常ヒナへのT_4投与は肝臓のIGFBP-2 mRNA発現に有意な影響を与えなかった。肝臓とは対照的に腎臓でのIGFBP-2 mRNAの発現は，低甲状腺機能ヒナで増加し，T_4を投与した正常ヒナ，および低甲状腺機能ヒナで減少した。また脳ではIGFBP-2 mRANの発現レベルは甲状腺機能の影響を受けなかった。また，調査した全ての組織でIGFBP-2 mRNAの発現が見られたが，発現の程度は精巣，筋胃，脳，眼，ファブリシウス嚢などで高く，腎臓や消化管ではやや低く，特に肝臓では非常に低かった。

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