Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6943–6946, July 1995
Physiology

Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone

(bovine growth hormone/hepatocyte/amino acid uptake/RNase protection assay/18S rRNA)

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Communicated by Christian B. Anfinsen,¶ Johns Hopkins University, Baltimore, MD, April 10, 1995

ABSTRACT **Augmentation of vertebrate growth by growth hormone (GH) is primarily due to its regulation of insulin-like growth factor I (IGF I) and IGF II levels. To characterize the effect of GH on the levels of IGF I and IGF II mRNA in a teleost, 10 μg of bovine GH (bGH) per g of body weight was administered to juvenile rainbow trout (*Oncorhynchus mykiss*) through i.p. injection. The levels of IGF I and IGF II mRNA were determined simultaneously, by using RNase protection assays, in the liver, pyloric ceca, kidney, and gill at 0, 1, 3, 6, 12, 24, 48, and 72 hr after injection. In the liver, IGF I mRNA levels were significantly elevated at 6 and 12 hr (≈2- to 3-fold, $P \leq 0.01$), while IGF II mRNA levels were significantly elevated at 3 and 6 hr (≈3-fold, $P \leq 0.01$). In the pyloric ceca, IGF II mRNA levels were significantly elevated at 12, 24, and 48 hr (≈3-fold, $P \leq 0.01$), while IGF I mRNA was below the limits of assay accuracy. GH-dependent IGF mRNA appearance was not detected in the gill and kidney. Serum bGH levels, determined by using a radioimmunoassay, were significantly elevated at 3 and 6 hr ($P < 0.005$). In primary hepatocyte culture, IGF I and IGF II mRNA levels increased in a bGH dose-dependent fashion, with ED_{50} values of ≈45 and ≈6 ng of bGH per ml, respectively. The GH-dependent appearance of IGF II mRNA in the liver and pyloric ceca suggests important roles for this peptide hormone exclusive of IGF I.**

Vertebrate growth is primarily controlled by the availability of growth hormone (GH) and the insulin-like growth factors (IGF I and IGF II) to their respective receptors. The secretion of GH from the pituitary, and its subsequent binding to GH receptors, signals the production of IGF I mRNA and polypeptide by liver (endocrine production) and other tissues (autocrine/paracrine production). The activities of IGFs are modulated by a set of specific IGF binding proteins and the IGF type 1 receptor of target tissues. Mitogenic (1) and metabolic properties, such as the uptake of glucose and amino acids (2), incorporation of sulfate into cartilage proteoglycan (3), nucleic acid and protein synthesis, and osmotic and ionic regulation in teleosts (4), have been ascribed to IGF.

In the rat and mouse, the direct relationship between increase in the levels of circulating GH and IGF I mRNA has been established in the liver, lung, pancreas, kidney, thymus, spleen, heart, skeletal muscle, testes, and pituitary, but not the uterus and ovary (ref. 5 for review). The effects of GH on IGF I are thought to be due primarily to increased transcription (6) of the IGF I gene rather than effects on the stability of IGF I mRNA. In contrast, it has been suggested that endocrine synthesis of IGF II by the liver is not under the control of GH (7, 8) and is regulated by placental lactogen (9) during prenatal development. The influence of GH on the production of IGF II mRNA is apparently limited to the brain (10), heart, and skeletal muscle (11), which may explain the highly restricted nature of postnatal endocrine IGF II expression in these rodent species (7).

cDNA corresponding to IGF I (12) and IGF II mRNA (13) have been described in teleost species. In addition, the levels of four forms of IGF I and one form of IGF II mRNA in 10 tissues and two developmental stages of the rainbow trout have been determined (14). The responsiveness of IGF I mRNA to GH in the liver of coho salmon has also been reported (15). To determine the time course of GH-dependent IGF mRNA appearance in the organs of a teleost, IGF I and IGF II mRNA levels were determined simultaneously by using RNase protection assays (RPAs), in total RNA extracted from the liver, pyloric ceca, kidney, and gill of rainbow trout injected with bovine GH (bGH). A bGH-specific RIA was employed to determine the clearance rate of bGH in plasma samples from these fish. Trout primary hepatocytes treated with various doses of bGH were used to investigate the dose responsiveness of IGF mRNA appearance. The GH dose-dependent response of IGF I and IGF II mRNA levels in the liver, and only IGF II in the pyloric ceca, suggests important roles for IGF II in processes mediated by the endocrine and autocrine/paracrine pathways of GH and IGF.

MATERIALS AND METHODS

Injection of bGH and Tissue RNA Preparation. Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing ≈150 g were held at 15°C for 1 month and fed daily to satiation. After 5 days without feeding, the trout were anesthetized in MS222 and then given an i.p. injection of either 10 μg of bGH (USDA-bGH-B-1) per g of fish body weight or carrier solution (0.01 M $NaHCO_3$, pH 8/0.15 M NaCl). Tissues and blood samples were collected from three GH-treated and three control fish prior to injection (time 0) and at 1, 3, 6, 12, 24, 48, and 72 hr after injection. Tissue total RNA was extracted by using the acid phenol/chloroform method (16) and was further purified by using lithium chloride precipitation (17).

Primary Hepatocyte *in Vitro* Culture. Trout (≈350 g) were starved for 14 days prior to isolation of hepatocytes. Liver was perfused *in situ* with collagenase (Worthington) and cells were seeded at a density of 4×10^6 per well of a six-well culture plate coated with poly(L-lysine) (Sigma) as described (18). Cells were maintained in L15 medium (GIBCO) and allowed to

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Abbreviations: IGF, insulin-like growth factor; GH, growth hormone; bGH, bovine GH; RPA, RNase protection assay.

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attach for 24 hr at 15°C; then the medium was replaced and four plates each were supplemented with 0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 ng of bGH (USDA-bGH-B-1) per ml. After incubation for 6 hr at 15°C, cells were harvested and total RNA was extracted by using the acid phenol/chloroform method.

RPA and 18S rRNA Normalization. Radiolabeled antisense cRNA probes corresponding to trout IGF I and IGF II were synthesized and used in an RPA as described (14). Approximately 1×10^5 cpm each of IGF I and IGF II cRNA probes were used in a hybridization reaction with 50 μg of tissue RNA or serially diluted sense cRNA quantification standards at 48°C for ≈18 hr. The unprotected RNA was then digested with 30 units of RNase T2 per ml at 30°C for 1 hr. Samples were resolved on 6% acrylamide/8 M urea gels and autoradiographs were scanned on a video densitometer. Standard curves generated from sense cRNA were used to generate pg of IGF mRNA per μg of RNA. Levels of 18S rRNA were determined for each sample by dot blot analysis of 1 μg of tissue RNA and were used to generate normalized mRNA concentration units of pg of IGF mRNA per μg of 18S rRNA as described (14). Student's *t* tests were done to assess statistical significance.

RIA Determination of Serum bGH Levels. Approximately 1 ml of blood was centrifuged at ≈3000 rpm for 5 min and the serum then was stored at −20°C. Samples were assayed in duplicate as described (19) with initial volumes of 100 μl per determination. High-level bGH readings were confirmed by reassay at 5, 2, 0.25, or 0.05 μl per determination.

RESULTS AND DISCUSSION

An RPA was employed to simultaneously measure IGF I and IGF II mRNA levels in tissue and primary hepatocyte RNA. The IGF II antisense cRNA probe was as described (14) and protects a 496-bp fragment from the C domain to the 3′ untranslated region. The IGF I probe employed here was designed to measure the levels of all four IGF I mRNA forms and was generated by PCR. Briefly, oligonucleotide primers designed to the B domain (IB, 5′-GGGCCCGAGACCCTGTGT) and to the D domain (ID, 5′-TTAAGCTGCCTTGCCAGACTT) of IGF I were employed in a PCR with 1 ng of trout IGF IEa-2 cDNA as template. Nucleotide sequence analysis confirmed the identity of this construct as corresponding to a 213-bp region from the B to D domain of trout IGF IEa-2, which is common in nucleotide sequence to all four forms of IGF I except for four nucleotide differences present in IGF IEa-1. The cross-reactivity of the IGF I probe with all four forms of IGF I, but not IGF II, was confirmed by using sense cRNA corresponding to each of the IGF types in an RPA. The nucleotide substitutions present in IGF IEa-1 were insufficient to allow digestion by RNase T2, and all four IGF I forms produced a single protected fragment of the predicted size, while IGF II did not produce any sizable protected fragments (data not shown). Since the levels of 18S rRNA did not correlate statistically ($P > 0.05$) with bGH treatment in any of the tissues examined (data not shown), they were used to normalize IGF mRNA levels throughout all studies reported in here.

Appearance of GH-Dependent IGF I and IGF II mRNA in the Trout Liver. Relatively high levels of IGF mRNA are produced in the liver, which is the main endocrine source of serum IGF. Therefore liver is the organ of choice for an initial investigation of the GH dependency and temporal pattern of IGF mRNA expression. The level of IGF I mRNA increased significantly ($P < 0.01$) in response to i.p. injection of bGH at 6 hr and remained significantly elevated at 12 hr (Fig. 1*A*), while IGF II mRNA levels were significantly elevated at 3 and 6 hr (Fig. 1*B*). IGF I and IGF II mRNA levels responded with a 2- to 3-fold increase over mock injected controls. The peak mRNA level of IGF I (11.28 ± 1.28 pg/μg of 18S rRNA; mean ± SE) occurred at 6 hr and was significantly ($P < 0.01$) higher

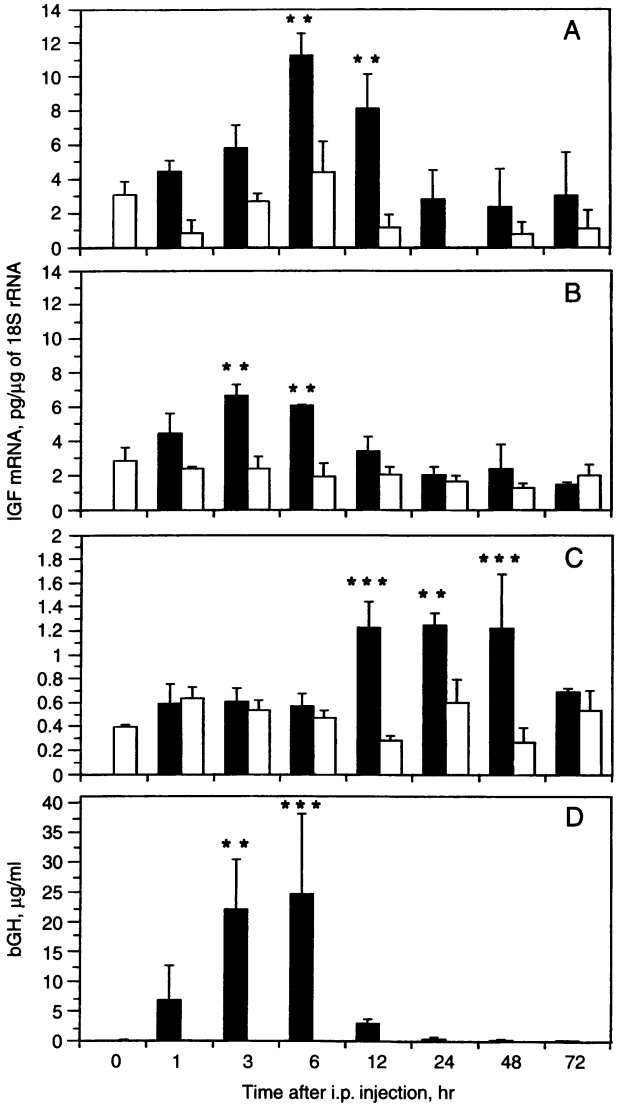

FIG. 1. GH-dependent appearance of IGF I (*A*) and IGF II (*B*) mRNA in the trout liver, IGF II mRNA in the pyloric ceca (*C*), and serum levels of bGH after intraperitoneal injection (*D*). The levels of IGF mRNA in *A–C* were determined simultaneously in RPAs and are normalized to levels of 18S rRNA. The levels of serum bGH in *D* were determined by a bGH-specific RIA. Mean levels ($n = 3$) and standard error of bGH- and mock treated fish are indicated by ■ and □, respectively. Significance values of $P \leq 0.01$ and $P \leq 0.001$ are indicated by ** and ***, respectively. The levels of bGH in mock treated fish were not detectable in *D*.

than that of IGF II (6.64 ± 0.68 pg/μg of 18S rRNA), which occurred at 3 hr. Throughout the 72 hr, the mean mRNA levels of IGF I (1.68 ± 0.78) and IGF II (2.04 ± 0.56) in mock injected fish did not differ significantly from each other ($P > 0.05$) or from IGF mRNA levels determined previously in untreated juvenile trout liver (14).

GH-Dependent Appearance of IGF II mRNA in the Pyloric Ceca. Where present, the pyloric ceca in fish are outpouchings of the anterior part of the intestine and function as sites of digestion and absorption of sugars, amino acids, and dipeptides (20). Additionally, pancreatic acinar cells are distributed in the connective tissues of the pyloric ceca in some teleost species, including the salmonids (21). In the mouse, the pancreas is second only to liver in IGF I mRNA levels. However, this level appears to be independent of GH (6).

As seen in Fig. 1*C*, the level of IGF II mRNA is GH-dependent in the trout pyloric ceca, being significantly ($P \leq$

0.01) elevated at 12, 24, and 48 hr by ≈4-, ≈2-, and ≈4-fold, respectively. Peak levels at these three time intervals are all ≈1.2 pg/μg of 18S rRNA and are ≈5-fold lower than maximal liver IGF II mRNA levels. The mean level of the mock injected controls throughout the 72 hr was 0.46 ± 0.10 pg/μg of 18S rRNA, which is not statistically ($P > 0.05$) different from IGF II mRNA levels determined previously in untreated juvenile trout pyloric ceca (14). The levels of IGF I mRNA were detectable but too low to be quantitatively interpreted and were apparently lower than those determined previously (14). It is not clear if the IGF mRNA levels observed are attributable to pancreatic acinar cells or the mucosal epithelia of the pyloric ceca. Localization of IGF I and IGF II mRNA in the pyloric ceca by *in situ* hybridization suggests the presence of IGF mRNAs in both cell types (M. Greene, M.J.S., and T.T.C., unpublished data).

Enhancement of intestinal amino acid uptake by GH has been demonstrated in teleost species including coho salmon and striped bass (22, 23). Although such enhancement has not been reported for the pyloric ceca, the GH-dependent synthesis of IGF II mRNA in the pyloric ceca suggests a role for IGF II in the digestive/absorptive processes of this tissue.

IGF mRNA in the Kidney and Gill. The gill and kidney are involved in osmotic and ionic regulation during freshwater and saltwater adaptation of salmonid fish. GH and IGF I have been implicated in these physiological processes (4, 24). In the present study, IGF I and IGF II mRNA were detected in the kidney but their levels were not measurably altered by bGH. The mean levels of kidney IGF I and IGF II mRNA throughout the 72 hr were 0.10 ± 0.03 and 1.10 ± 0.13 pg/μg of 18S rRNA, respectively. Kidney IGF I mRNA levels were not statistically ($P > 0.05$) different from levels determined previously in the kidney of untreated juvenile trout. The IGF II mRNA levels were slightly but significantly ($P < 0.05$) higher than the 0.57 ± 0.14 pg/μg of 18S rRNA value determined previously (14).

In the gill, IGF II mRNA was not elevated significantly ($P > 0.05$) by bGH, and IGF I mRNA was undetectable (<1 pg/50 μg of gill RNA). The mean level of IGF II mRNA throughout the 72 hr was 0.79 ± 0.15 pg/μg of 18S rRNA, which was not significantly ($P > 0.05$) different from IGF II mRNA levels determined previously in untreated juvenile trout gill. In the present study, IGF I mRNA levels were apparently lower than those determined previously (14).

The finding of GH-independent IGF mRNA synthesis in the gill and kidney is in apparent conflict with the reported GH-dependent synthesis of IGF I mRNA in rainbow trout gill and kidney, as measured by quantitative Northern blot analysis, following a total of three i.p. injections of 0.25 μg of recombinant chum salmon GH per g of body weight administered over 6 days (24). It is possible that the GH-dependent appearance of IGF mRNA in the above-mentioned study is a

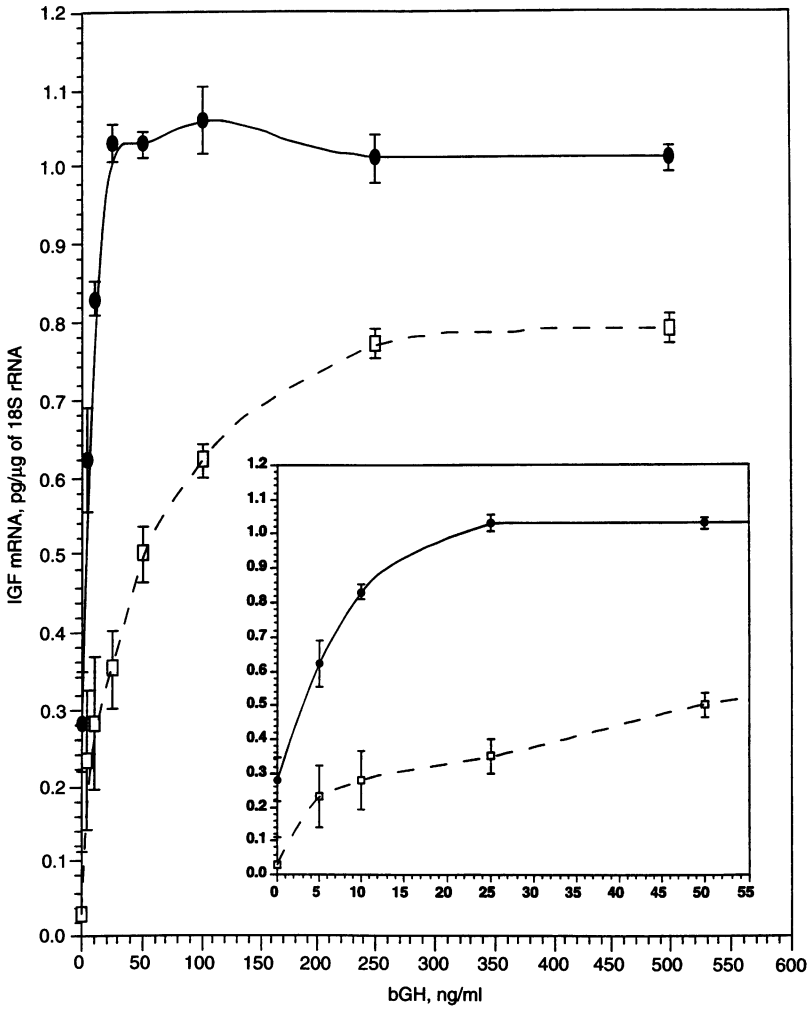

FIG. 2. Dose-dependent appearance of IGF I and IGF II mRNA in cultured trout primary hepatocytes in response to bGH treatment. The levels of IGF mRNA were determined simultaneously by an RPA and are normalized to levels of 18S rRNA. Mean levels (n = four flasks of cells, all cells prepared from a single liver) and standard error of IGF II and IGF I mRNA are indicated by ● and □, respectively. Identical symbols and axes to the larger figure are used in *Inset*. The ED_{50} values for IGF I and IGF II are ≈45 and ≈6 ng/ml, respectively.

consequence of the extended period of administration, a lower dosage level, or use of homologous hormone.

Determination of Serum bGH Levels in i.p. Injected Trout. To obtain a profile of the availability and clearance of the injected bGH, an RIA was employed to specifically measure serum bGH levels at each time point employed in tissue sampling for IGF mRNA. As seen in Fig. 1*D*, bGH reached significantly elevated levels after 3 hr ($P < 0.005$), remained significantly elevated at 6 hr ($P < 0.001$), when it reached the highest level of ≈24.5 μg of bGH per ml, and remained detectable, but not significantly elevated, through 72 hr. The time profile of serum bGH level coincides with or precedes the GH-dependent appearance of IGF mRNA in the liver and pyloric ceca (Fig. 1). This temporal profile is consistent with that found for the i.p. administration of recombinant salmon GH to coho salmon (25). The levels of bGH in this experiment exceed the endogenous GH levels in trout, which were determined to be ≤10 ng/ml of plasma (19). The level of bGH was either undetectable or extremely low (≤1 ng/ml) in mock injected fish. This suggests that the anti-bGH antibody employed in the RIA does not cross-react appreciably with endogenous trout GH, and the signals detected in mock treated fish are likely to be the result of contamination or noise.

Dose–Response of IGF mRNA to bGH in Trout Primary Hepatocyte Culture. One fundamental property of hormone action is dose dependency. While whole animal injection studies can identify the time course of the response to exogenous GH, determination of dose–response can be confounded by the highly variable nature of endogenous serum GH levels. To confirm that the levels of IGF I and IGF II mRNA respond to GH in a dose-dependent fashion, an *in vitro* serum-free trout primary hepatocyte culture (18) was employed. Primary trout hepatocytes have been shown to retain at least some of the normal functions of liver parenchymal cells, including GH-dependent appearance of IGF I mRNA (15).

As seen in Fig. 2, IGF I and IGF II mRNA levels respond in a dose-dependent fashion to the presence of bGH in the serum-free medium, with ED_{50} values of ≈45 and ≈6 ng/ml, respectively. Although the absolute magnitudes of IGF mRNA levels were approximately equal, a higher basal IGF II mRNA level resulted in a higher maximal level. The possibility that GH-dependent hepatic production of IGF II mRNA is an artifact of pharmacological or heterologous dosage is diminished by the fact that the ED_{50} for IGF II mRNA is lower than that of IGF I in these cells.

Physiological Implications of the GH–IGF Interaction. Liver is the primary source of endocrine IGF. The fact that IGF I and IGF II mRNA appeared in a bGH dose-dependent manner suggests that both of these peptide hormones respond to physiological pulses of GH. The finding that IGF II mRNA has a lower ED_{50} than that of IGF I may suggest different response thresholds. The appearance of high levels of GH-dependent hepatic IGF II mRNA suggests that trout, like humans but unlike mice and rats, retain high levels of serum IGF II into adulthood.

The nonhepatic organs were selected to further investigate the putative role of GH and IGF in osmoregulation and enhanced uptake of sugars and amino acids. These processes may utilize a local (nonhepatic) and GH-dependent production of IGF in order to supplement hepatic IGF. This would provide a means of attaining high or alternatively modulated peripheral IGF levels while maintaining the more generally available and tightly regulated pool of serum IGF. The GH-dependent production of IGF II mRNA, and presumably the corresponding polypeptide, in the pyloric ceca suggests the importance of this peptide hormone in the autocrine/paracrine regulation of growth and metabolism. To establish the distinct roles of IGF polypeptides, the mature forms of trout IGF I and IGF II have been biosynthetically produced in milligram quantities by secretion from the yeast *Pichia pastoris*. The ability of these two peptides equals or exceeds the ability of recombinant human IGF I (Promega) to stimulate the incorporation of [^{35}S]sulfate into gill cartilage proteoglycan (unpublished data).

We thank Drs. E. G. Grau and G. W. Litman for their critical reading of this manuscript as well as the Albert Powell trout hatchery, the University of Maryland Biotechnology Institute Aquaculture Research Center, and Dr. Yonathen Zohar for the generous supply of trout and aquaculture support, and Christina Råbergh for her indispensable help with the primary hepatocyte culture. This research was supported by grants from the National Science Foundation (DEB-91-05719 and IBN-93-17132) to T.T.C. This is contribution no. 247 of the Center of Marine Biotechnology, University of Maryland Biotechnology Institute.

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