Effects of chronic cysteamine treatment on growth enhancement and insulin-like growth factor I and II mRNA levels in common carp tissues

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Insulin-like growth factors (IGF) belong to a family of growth factors with structural homology to proinsulin. In our previous studies, we found that both IGF-I and IGF-II gene expression showed growth hormone (GH) dependence in the brain and liver of juvenile common carp when treated in vivo with GH for a short time. This present work aimed to study the effects of both the short-term and long-term GH induction of IGF gene expression using cysteamine (CSH) and fasting/re-feeding. CSH is an agent that can deplete somatostatin to increase circulating GH level. IGF mRNA levels in the flesh (muscle) and liver of common carp were determined using real-time PCR.

The chronic treatment of common carp with CSH was carried out for 63 d, with growth enhancement of the treated fish noted. Hepatic IGF-I and IGF-II mRNA levels increased in a dose-dependent manner with short-term CSH treatment, whereas IGF-I decreased and IGF-II increased in the liver after chronic CSH treatment. IGF-I and IGF-II mRNA levels in muscle were found to be elevated with the high-dose, long-term CSH treatment. Under the experimentally induced catabolic states of fasting, both hepatic IGF-I and IGF-II gene expression were significantly reduced, whereas they showed no change in muscle. After re-feeding, the hepatic expression of IGF-I in liver and muscle rebounded significantly. The hepatic IGF-II expression level showed no rebound after re-feeding, but the IGF-II level in muscle rebounded to the level of the fed group after re-feeding.

Cysteamine: Growth rate: Real-time quantitative PCR: Gene expression

Unlike the expression of insulin-like growth factor I (IGF-I), which is highly responsive to growth hormone (GH) stimulation (Bichell et al. 1992; Meton et al. 1999), hepatic IGF-II gene expression in mammals is generally believed to be independent of GH. Meanwhile, GH may stimulate the expression of IGF-II in the extrahepatic tissues (Sara & Hall, 1990; Jones & Clemmons, 1995). However, von Horn et al. (2002) recently suggested that human hepatic IGF-II gene is regulated by GH.

Conflicting results also exist concerning the effects of GH on IGF-II gene expression in different fish species. In rainbow trout, GH dose-dependent responses are shown in hepatic IGF-I and IGF-II mRNA levels (Shamblott et al. 1995), but in sea bream, only hepatic IGF-I gene expression is regulated by GH, and there is no change in hepatic and extrahepatic IGF-II mRNA levels after GH injection (Duguay et al. 1996). We have previously shown, in an acute in vivo study using ribonuclease protection assay, that both IGF-I and IGF-II exhibit GH dependence in the brain and liver of juvenile common carp (Tse et al. 2002). A differential expression pattern in response to the acute GH stimulation of IGF in various common carp tissues has also been studied using real-time PCR assay (Vong et al. 2003).

To study the effects of chronic GH treatment on IGF-I and IGF-binding proteins, different livestock species have been used. In adult male chickens, plasma concentrations of IGF-I and IGF-binding protein 3 were markedly elevated by the continuous administration of GH. In contrast to the increases in circulating concentration of GH and IGF-I, no changes in plasma concentration of thyroid hormones, reproductive hormones, glucose or NEFA were found during chronic GH treatment (Radecki et al. 1997). Circulating concentrations of IGF-I were elevated by the chronic administration of GH in pigs (Chung et al. 1985; Etherton et al. 1987) and lambs (Pell et al. 1990).

Interactions are also found between nutritional status and IGF. The extent of nutrient deprivation determines the degree to which IGF-I synthesis and, following periods of nutrient modification, IGF-I secretion will return to normal. Food deprivation for 5 d depresses circulating IGF-I concentration, which returns to near-initial values after re-feeding (Kim et al. 1991; Morishita et al. 1993).

It has also been reported that a complete return to normal IGF-I level was observed following restricted feeding (Kita et al. 1996). Conversely, IGF-II concentration increased following 24 h of food withdrawal in chickens (McMurtry et al. 1998).

The neuroendocrine regulation of GH secretion is affected by a number of stimulatory and inhibitory neurohormones.

Abbreviations: CSH, cysteamine hydrochloride; $\Delta Ct$, difference between the threshold cycles for target and reference; GH, growth hormone; HSI%, hepato-somatic index; IGF, insulin-like growth factor; MLI, muscle weight divided by length index; SRIF, somatostatin.

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Carp cysteamine-regulated IGF-I and IGF-II levels

Materials and methods

Animal and experimental design

Three hundred 3-month-old (weight 2–4 g) common carp (Cyprinus carpio) were kindly provided by the Agriculture, Fisheries and Conservation Department of the Hong Kong Government. Carp were acclimatised at a constant temperature (20 ± 2°C) with a controlled natural photoperiod of 16 h light–8 h dark in an aquarium room with a closed recirculation system for 1 week. In the CSH treatment study, carp (50 per group) with CSH for 63 d, the chronic effect of GH induction on the growth rate, and the gene expression levels of IGF-I and IGF-II were investigated in carp tissues. To our knowledge, reports of the effects of CSH on growth are scarce with regard to either fish or other vertebrates. This is also the first report linking CSH to IGF-I and IGF-II gene expression. The present study is the first report on the effects of long-term CSH treatment on mRNA levels for IGF in animals.

Total RNA extraction

The tissues of juvenile carp were homogenised with a Polytron PT3100 (Kinematica, Littau, Switzerland). The probe of the Polytron PT3100 was cleaned with diethylpyrocarbonate-treated water and chloroform before use. Tissue masses ranging from 0.1 to 0.5 g were homogenised with TriPure Reagent (Roche, Mannheim, Germany) solution in a ratio of 100 mg to 1 ml TriPure Reagent, and the homogenate was divided into 1 ml aliquots. Chloroform (0.2 ml) was added, and the solution was mixed vigorously for 15–30 s. The homogenate was placed on ice for 2 min and then centrifuged at 12000 g for 15 min at 4°C. An equal volume of isopropanol was added into the aqueous supernatant, and this was centrifuged at 12000 g for 10 min at 4°C. The RNA pellet was washed with 1 ml 75% C2H6O and then centrifuged at 12000 g for 10 min at 4°C. The RNA pellet was speed-vacced for 10 min at 65°C, resuspended in diethylpyrocarbonate-treated water, spectrophotometrically quantified and then inspected on a 1% formaldehyde gel to check for integrity (Ausubel, 1999). The gel was then photographed under UV transillumination.

First-strand cDNA synthesis

Total RNA (3 µg) was added to a nuclease-free microcentrifuge tube with 100 ng random hexamer, heated at 65°C for 10 min and quick-chilled on ice. The content of the tube was collected by brief centrifugation, and 6 µl 5X first-strand synthesis buffer, 3 µl 0.1 mM-dithiothreitol, 1 µl 10 mM-dNTP, 1 µl RNaseout inhibitor and 1 µl MMLV (Moloney Murine Leukemia Virus) reverse transcriptase were added. The tube was incubated at 37°C for 90 min, followed by heat inactivation at 65°C. The first-strand cDNA products were stored at −20°C.

Primer design and conditions for real-time PCR

Gene specific primers were designed using the ABI Primer Express program (PE; Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction, based on the DNA sequences reported for IGF-I cDNA (Liang et al., 1996), IGF-II (Tse et al. 2002) and 18S rRNA (GenBank accession no. U87963). For IGF-I gene amplification, the primers used were 5’-CGCTCGAGATGTTGTGCAAC-3’ and 5’-CTGTATGCGCTGTCCCTGTGTCAG-3’ with a 72 bp amplicon. For IGF-II gene amplification, the primers were 5’-ACCACCACAGGCCTCTTCATGC-3’ and 5’-CATCGACTCTGTGCAGA-3’ with an amplicon length of 66 bp. For 18S amplification, the primers used were 5’-AATCCCGCGCTCCTTCCTGTTCG-3’ and 5’-CTCGAGGCACCTCATAAACC-3’ with an amplicon length of 66 bp. HPLC-purified primers were used to minimise the effects of any side reaction products in an attempt to lead to more reliable DNA quantification.

The PCR was prepared by adding 12.5 µl 2X master mix (Brilliant SYBR Green QPCR master mix; Stratagene, La Jolla, USA), 2.5 pmol upstream primer, 2.5 pmol downstream primer, 0.375 µl 30 µm-reference dye and 1 µl cDNA to a final volume of 25 µl. Real-time PCR was performed on an ABI Prism 7700 machine (Applied Biosystems) with...
denaturation at 95°C for 2 min, followed by forty cycles of 30 s each at 95°C, of 30 s at 65°C (IGF-I and 18S rRNA) 62°C (IGF-II) and of 30 s at 72°C, and finally a dissociation programme with samples first melted at 95°C for 15 s and then equilibrated at 60°C before slowly reheating with a ramping time of 19 min 59 s back to 95°C. The PCR condition was optimised with no peak in the dissociation curve plot (data not shown), indicating neither primer–dimer formation nor template contamination. Data were collected during the annealing and extension period in each cycle.

All standard curves for quantification of IGF-I, IGF-II and 18S rRNA (normalisation) genes were linear, with a linear correlation ($R^2$) of about 1 (data not shown). The comparative $C_T$ method (the difference between the threshold cycles for target and reference) was used; before using the arithmetic formula $2^{-\Delta C_T}$ for quantification, an experiment was performed to validate the efficiency of the target amplification, the efficiency of the reference amplification being approximately equal to this (data not shown). Data were analysed using two-way ANOVA comparing the interactions of dose and time (d) as sources of variation. Means were compared using Bonferroni post-tests in Prism 4 on GraphPad (San Diego, CA, USA) software using a personal computer.

**Results**

**Body weight and fork length measurements of carp in this experiment**

Fish of a similar size were evenly distributed to different treatment groups. Growth varied between different CSH treatment groups, as indicated by the average body weight (biomass divided by number of fish; Fig. 1(A)) and fork length (Fig. 1(B)). Biomass was measured instead of individual body weight to minimise the variation in the amount of fish food taken up between individual fish within the same tank. Fish fed at higher dosages had significantly higher growth rates than fish fed at lower dosages or those not receiving CSH. The control group was the group constantly fed with unformulated pellets. The increases in biomass of the control (without CSH), dose 1 (1000 mg/kg CSH), dose 2 (2000 mg/kg CSH) and dose 3 (3000 mg/kg CSH) treatment groups between day 0 and day 63 were 61, 84, 115 and 180 %, respectively; whereas the increase in fork length of the control, dose 1, dose 2 and dose 3 treatment groups from day 0 to day 63 was 22·7, 20·3, 24·6 and 38·1 %, respectively.

**Organo-somatic indices of carp following CSH administration**

The hepato-somatic index (HSI%; percentage of liver weight divided by whole body weight) and the index of muscle weight divided by length (MLI) were the indicators of fish growth in this experiment. The HSI% increased in the common carp by 40 % (control), 30 % (dose 1), 14 % (dose 2) and 2 % (dose 3), respectively (Fig. 1(C)). A dose-dependent decrease in HSI% was observed in the treatment groups compared with the control group. The increases in MLI in the common carp were 6 % (control), 3 % (dose 1), 7 % (dose 2) and 13 % (dose 3), respectively (Fig. 1(D)). In contrast to the HSI%, a dose-dependent increase in MLI was
observed in the treatment groups compared with the control group. The decrease in HSI\% and the increase in MLI indicated that the body weight gain was mainly contributed by the weight gain in muscle rather than in the internal organs.

Validation experiments for the real-time PCR

Standard curves for relative quantification of IGF-I, IGF-II and 18S rRNA were generated, as shown in Fig. 2(A). All standard curves for the quantification of IGF-I, IGF-II and the 18S rRNA gene were linear with a linear correlation ($R^2$) of about 1. The comparative $C_T$ method was used instead of the standard curve method to eliminate the adverse effect of any dilution errors made in creating the standard curve samples.

Validation experiments were performed to demonstrate that the efficiency of the target amplification and the efficiency of the reference amplification were approximately the same (Fig. 2(B,C)). We assessed whether the two amplicons had the same efficiency by looking at how $\Delta C_T$ varied with template dilution. The slope of log dilution factor vs. IGF-I (Fig. 2(B)) and IGF-II (Fig. 2(C)) was 0.0937 and 0.0627, respectively. The slope was less than 0.1, which can fulfill the criteria of using the $\Delta\Delta C_T$ method.

The data were analyzed using the arithmetic formula $2^{-\Delta\Delta C_T}$ according to user bulletin #2 of the ABI Prism 7700 sequence detection system. Briefly, the average $C_T$ of the target gene and housekeeping gene, respectively, were calculated. We chose 18S rRNA as the housekeeping gene because it varies least compared with other housekeeping genes such as GAPDH (Bustin, 2000) and β-actin (Foss et al. 1998). The $\Delta C_T$ value was then determined by subtracting the average 18S rRNA $C_T$ value from the average target $C_T$ value. The standard deviation of the difference was calculated from the standard deviations of the target and 18S RNA values. Finally, $\Delta\Delta C_T$ was calculated by subtracting the $\Delta C_T$ calibrator value, and the fold-change was determined by evaluating the expression $2^{-\Delta\Delta C_T}$.

Quantification of IGF-I and IGF-II gene expression

IGF-I and IGF-II mRNA levels were determined in the liver and muscle of carp after CSH treatment using real-time PCR. As IGF-I and IGF-II mRNA may cross-react with each other due to a high degree of shared nucleotide identity of 56–64%, real-time quantitative PCR was employed in this study for its accuracy and high sensitivity. Amplification of an endogenous control, 18S rRNA, was performed to standardize the amount of sample RNA added to the reaction.

As shown in Fig. 3(A), the addition of supplementary CSH to the diet induced the expression of both hepatic IGF-I and IGF-II mRNA after 7 and 63 d of treatment. The increase in IGF-I was more evident than the increase in IGF-II expression. The expression of IGF-I and IGF-II gene expression was significantly increased after this time in the dose 3 treatment group, which received the highest dosage of CSH (Fig. 3(B), denoted by b).

The chronic CSH treatment induction profile in muscle was quite different from that in liver. In contrast to the situation for liver, both IGF-I and IGF-II gene expression showed no change after 63 d of feeding for the control, dose 1 and dose 2 treatment groups. For the dose 3 treatment group, however, it showed 9-fold and 4.3-fold increases in the IGF-I and IGF-II gene expression of muscle after CSH treatment.
mRNA levels (Fig. 4, denoted by c). This showed that long-term CSH treatment with the highest dosage significantly induced both IGF-I and IGF-II gene expression in muscle.

Growth decreased significantly during the fasting period (Fig. 5(A)). The dietary intake of CSH induced growth and restored growth from fasting to the extent of constant feeding by re-feeding dose 3 pellets. Hepatic IGF-I and IGF-II levels were suppressed in fasting (starved) carp (Fig. 5(B,C)). However, the level of IGF-I rebounded dramatically in the liver (210-fold) and muscle (540-fold) after re-feeding (Fig. 5(B,D)), whereas IGF-II showed no rebound in liver after re-feeding (Fig. 5(C)). IGF-II in muscle showed no response to fasting and rebounded to the level of the fed group after re-feeding (Fig. 5(E)).

Discussion

An improvement in fish growth performance for economic purposes can be achieved by enhancing growth rate. The growth rate of animals is modulated by their interactions with the environment, including food abundance and quality, temperature and photoperiod (Beckman et al. 1998). The effects of these on growth rate are in turn modulated by the endocrine system. The somatotrophic axis, which includes GH, GH receptor, IGF-I, IGF-I-receptor and IGF-binding proteins that are found in mammals and fish (Mommsen, 1998), is believed to play a key role in the regulation of growth (Kostyo, 1999).

SRIF is the major inhibitory peptide regulating GH secretion. SRIF antagonists have recently been synthesised successfully and shown to induce GH release (Baumbach et al. 1998). CSH has been generally used as a tool to antagonise SRIF (Kungel et al. 1996). SRIF antagonists may have applications as a food additive to promote GH release, and thus enhance cultured fish growth in intensive large-scale aquaculture. They enjoy the advantage of a lack of species specificity, simple chemistry, convenient oral administration, and in particular, a relatively low cost. Although GH and IGF-I have a more direct effect in the field of fish food enhancement to improve economic returns (Moriyama et al. 1990, 1993), cysteamine seems to be more applicable for farmed animals such as fish.

Carp (such as Cholymbi; Hanumanthappa et al. 2002) are commonly used as a model to study the effect of feeding additives because of their availability and wide distribution. It has been recently been proven that CSH supplementation can significantly increase serum GH level in grass carp (over 150 g; Xiao & Lin, 2003a,b). In animals, either exogenous GH administration (McLean & Donaldson, 1993) or GH transgenesis (Devlin et al. 1994) resulted in an increase in growth. Direct GH administration sometimes does not result in growth. Bauer et al. (2003) showed that the infusion of GH
to fetal sheep suffering intrauterine growth retardation restored fetal IGF-I levels but did not improve fetal growth, further reducing fetal kidney and intestinal weights.

In the present study, we examined the effects of daily CSH treatment both on IGF-I and IGF-II mRNA levels by real-time quantitative PCR and on growth rate in juvenile common carp. Neither serum GH level (by RIA) nor GH expression level (by extraction of the pituitary) was measured because of the small size of the juvenile fish. Nevertheless, our focus was on the regulation of growth and the GH–IGF axis by the long-term dietary intake of cysteamine. Body weight increases obviously in a dose-dependent manner (Fig. 1(A)). Although fish usually gain close to 1 g/d, restricted feeding (3% of body weight fed per day) was used in our experiments rather than feeding ad libitum because it allowed management to be consistent in the quantities of food delivered. This may
not have allowed the fish to demonstrate maximum growth rates, but the difference in weight gain between the different treatment groups was significant enough to demonstrate the increase in growth.

In the present study, chronic and continuous CSH treatment enhanced the growth rate in a dose-dependent manner. A dose-dependent increase in MLI and a decrease in HSI% were observed in the treatment groups compared with the control group. In muscle, an induction of IGF-I and IGF-II mRNA levels with peak orders of magnitude of 9-3 and 4-4, respectively, was obtained after 63 d of daily oral administration of CSH (3000 mg/kg). This is consistent with our previous hypothesis that IGF-II may be related to growth and development both in the early embryonic stage (Tse et al. 2002) and later in adults as IGF-II is expressed even more than IGF-I in most extrahepatic tissues (Vong et al. 2003). A recent report also supports our hypothesis by demonstrating that IGF-II mRNA levels were 3-3-fold greater in the muscle of fast-growing catfish compared with slow-growing catfish (Peterson et al. 2004).

It is well known that GH increases muscle growth (Machlin, 1972) and decreases fat deposition in pigs (Pursel et al. 1990; Wieghart et al. 1990), lambs (Nancarrow et al. 1991) and salmonids (McLean et al. 1994). It has also been suggested that the reduction of fat can be attributed to the direct action of GH (Etherton et al. 1987; Ebert et al. 1988). In mammals, GH is known to stimulate fat mobilisation and activate lipase to catalyse triacylglycerol hydrolysis. GH also induces absorbed nutrient repartitioning and shifts in the balance of protein deposition from fat cell to muscle cell. Smith et al. (1988) suggested that slower-growing genotypes of rainbow trout directed more dietary energy to fat deposition and less to protein synthesis. Later, Dunham et al. (2002) suggested that faster-growing transgenic common carp direct a greater proportion of their energy towards protein synthesis. It is therefore hypothesised that IGF-I and IGF-II may play a role in growth and development via their anabolic effect by stimulating GH. IGF stimulate amino acid uptake and protein synthesis in muscle and can greatly reduce the rate of protein breakdown within muscle fibres.

Nevertheless, in the present study, the induction in IGF-I and IGF-II mRNA levels was detected only for the highest dose of long-term CSH treatment. IGF-I and IGF-II mRNA levels did not differ in other treatment groups, either for the long-term or the short-term CSH treatment. This is consistent with the study carried out in catfish. The amounts of muscle IGF-I and IGF-II mRNA did not differ in recombinant GH-injected fish compared with controls, but neither differed from controls on days 0, 2, 7, 14 and 21. This further supports the idea that CSH is a good feeding additive in terms of food stability and long-lasting effect.

With long-term CSH treatment (Fig. 3), we found that hepatic IGF-I mRNA levels increased, whereas the hepatic IGF-I mRNA level decreased significantly with peak values of about two orders of magnitude. One possible explanation for this phenomenon is a negative-feedback regulation of GH secretion resulting from the higher serum GH levels after long-term CSH feeding. There is evidence to support the fact that IGF-I suppresses the hepatic GH receptor in sheep (Min et al. 1996), which in turn suppresses the synthesis of IGF-I. This may explain why there is a dose-dependent increase at day 7 but a decrease at day 63, except in the control group.

On the other hand, the increase in IGF-II was maintained at a dosage of 3000 mg/kg, probably due to the fact that IGF-II is more sensitive to GH. In other words, less GH is needed to induce IGF-II even though GH receptor is suppressed. Verification by measuring the gene expression level of GH receptor in common carp is needed to support this view. Another possible explanation for the differential expression of IGF-I and IGF-II is that they may respond to GH stimulation in a different manner.

A relationship between feeding, IGFB and growth in fish was also studied. Hepatic IGF-I and IGF-II mRNA levels were significantly decreased, with a shift towards catabolism during fasting. In chickens, however, food deprivation for 5 d depressed the circulating IGF-I concentration and increased the circulating IGF-II concentration after 24 h food withdrawal (McMurry et al. 1998). The difference might be due to the different period of food deprivation.

Hepatic IGF-I mRNA level but not that in the extrahepatic tissues was downregulated in fasted carp. Re-fed carp exhibited a profound rebound in hepatic IGF-I level. The IGF-I expression pattern of fasted and re-fed carp is consistent
with the results of studies in chickens (Kim et al. 1991; Morishita et al. 1993) and Masu salmon (Duan & Plisetskaya, 1993). In the present study, we also investigated the IGF-I expression level in the extrahepatic tissues after re-feeding. Similar to liver, IGF-I expression level in muscle also showed a drastic increase. On the other hand, hepatic IGF-II levels showed no response to re-feeding. This might suggest that hepatic IGF-I is tightly regulated and plays an important role in the physiological consequences of stressors such as fasting and adaptation to sea water (McCormick, 1996; Mancera & McCormick, 1998). The drastic rebound in both liver and muscle showed that the regulation of IGF-I expression might not be tissue specific. On the other hand, IGF-II may possess other functions such as regulation of somatic growth, and this function may be tissue specific and more related to autocrine controls. Further study is needed into the function of IGF in fish, especially the tissue-specific function(s) of IGF-II.

In summary, we demonstrated for the first time that long-term treatment with CSH enhanced the growth of carp, suggesting that CSH would be an effective tool to enhance growth. This is also the first report linking CSH to IGF in any species. Both IGF-I and IGF-II mRNA levels in the muscle of common carp increased with long-term, high-dose CSH treatment. Hepatic IGF-I and IGF-II mRNA levels increased with CSH feeding for 7 d. However, the IGF-I mRNA level decreased and the IGF-II mRNA level increased in the liver following CSH treatment. Under the experimentally induced catabolic states of fasting, both hepatic IGF-I and IGF-II mRNA levels were reduced significantly. After re-feeding, however, liver and muscle IGF-I appeared to rebound significantly to a greater extent than was seen with the constantly fed carp. The hepatic IGF-II expression level showed no response to re-feeding. The IGF-II expression level in muscle showed no response to fasting and rebounded to the level of constantly fed group after re-feeding.

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