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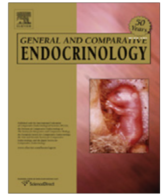

Effects of crustacean hyperglycemic hormone (CHH) on the transcript expression of carbohydrate metabolism-related enzyme genes in the kuruma prawn, *Marsupenaeus japonicus*

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ABSTRACT

Crustacean hyperglycemic hormone (CHH), a member of a neuropeptide family present only in arthropods, plays a pivotal role in the modulation of hemolymph glucose levels, molting, reproduction, and the stress response. Although it has been determined that hepatopancreas and muscle are the major tissues in which CHH regulates hyperglycemic activity, the molecular mechanism by which CHH regulates carbohydrate metabolism remains unclear. In this study, we analyzed the mRNA expression levels of enzymes involved in glycogen metabolism and gluconeogenesis in order to determine how CHH regulates hemolymph glucose levels. We first cloned cDNAs encoding four carbohydrate metabolism-related enzymes from the kuruma prawn, *Marsupenaeus japonicus*, glycogen phosphorylase (MjGP), glycogen synthase (MjGS), fructose 1,6-bisphosphatase (MjFBPase), and phosphoenolpyruvate carboxykinase (MjPEPCK). RT-PCR analysis showed that eyestalk ablation remarkably decreased *MjGP* and increased *MjGS* transcript levels in the hepatopancreas, but not in muscle. Considering the fact that various eyestalk factors, including MIH, are removed by eyestalk ablation, these results indicate that after eyestalk ablation the metabolic state proceeds towards glycogen accumulation in the specific tissues related to molting. In contrast, *MjFBPase* and *MjPEPCK* transcript levels were not significantly changed by eyestalk ablation, indicating that CHH and other eyestalk-derived factors might not induce gluconeogenesis. Quantitative real-time PCR analysis showed that exposure of hepatopancreas to recombinant CHH significantly changed the expression levels of *MjGP* and *MjGS*, but not *MjFBPase* and *MjPEPCK*. Collectively, these results indicate that CHH is involved in glycogen metabolism in hepatopancreas.

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1. Introduction

Energy balance in the body of an organism is maintained by a specific neuroendocrine system. In crustaceans, crustacean hyperglycemic hormone (CHH), a neuropeptide synthesized in and secreted from the X-organ/sinus gland (XO/SG) complex in the eyestalk, plays a central role in the regulation of energy metabolism [12]. This hormone was first described as a "diabetogenic factor" found in eyestalk extracts of the crab *Uca pugilator* [1], and CHH isolated from the crab *Carcinus maenas* was first sequenced by Kegel et al. [16]. Because CHH administration resulted in recovery of normal hemolymph glucose levels in the animalin severe hypoglycemic state caused by eyestalk ablation, CHH is thought to regulate carbohydrate metabolism. In addition, CHH is also involved in other important biological events, including lipid metabolism, molting, vitellogenesis, and the stress response [12].

CHH and its structurally related peptides, molt-inhibiting hormone (MIH), vitellogenesis/gonad-inhibiting hormone (VIH/GIH), and mandibular organ-inhibiting hormone (MOIH) in crustaceans, and ion transport peptide (ITP) in insects, form a neuropeptide family called the CHH-family [17]. The CHH-family peptides generally consist of 70–80 amino acid residues with six conserved cysteine residues that form three intramolecular disulfide bonds [13,21]. In crustaceans, the CHH-family peptides, as well as the chromatophorotropins family, are mainly produced by and secreted from the XO/SG complex. CHH-family peptides have also been found in the pericardial organ, foregut, hindgut, ventral nerve cord, and retina of several crustacean species, however, their function has not been clearly identified [12]. To date, the CHH-family members have only been isolated from arthropods, but a CHH-like sequence has been found in the genome of the nematode, *Caenorhabditis elegans* [5].

Abbreviations: CHH, crustacean hyperglycemic hormone; FBPase, fructose 1,6-bisphosphatase; GP, glycogen phosphorylase; GS, glycogen synthase; MIH, molt-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; PEPCK, phosphoenolpyruvate carboxykinase; SG, sinus gland; SGP, sinus gland peptide; VIH, vitellogenesis-inhibiting hormone; XO, X-organ.

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In the kuruma prawn, *Marsupenaeus japonicus*, eight CHH-family peptides have been identified [24,28,45–47]. Seven of these sinus gland peptides (SGPs) are expressed specifically in the eyestalk, termed SGP-I to -VII. SGP-IV is thought to function as MIH, while the other six SGPs function as CHH and VIH [42,47]. SGP-V and SGP-VI also show weak MIH activity. The eighth additional member, designated as MIH-B, is expressed in the brain and nerve cord rather than in eyestalk [28]. Although MIH-B shares structural similarity with SGP-IV, its MIH activity is much weaker, suggesting that MIH-B has an additional undetermined function. Because SGP-VII is the predominant SGP in the sinus gland extract of *M. japonicus* and exhibits relatively strong hyperglycemic activity, we have used SGP-VII as a representative CHH molecule to investigate the mode of action of CHH [22,23].

CHH is a pleiotropic hormone and may act on various tissues [8], primarily hepatopancreas and muscle. A number of studies have reported that the hyperglycemic effect of CHH is correlated with glycogenolysis in these tissues; both synthesis and degradation of glycogen are regulated by CHH [34]. Reddy and Kishori [32] and Sedlmeier [37] reported that eyestalk ablation caused activation of glycogen phosphorylase (GP; EC 2.4.1.1) in these tissues and simultaneous inactivation of glycogen synthase (GS; EC 2.4.1.11) in hepatopancreas, respectively. In addition, various stressors induced changes in glycogen and total carbohydrate concentrations in hepatopancreas and muscle [3,32], which was accompanied by an increase in CHH secretion [16,19,20]. Incubation of hepatopancreas with CHH reduced the incorporation rate of glucose into glycogen, which evidences that CHH suppresses GS activity [38]. Furthermore, it was demonstrated that injection of CHH decreased GS activity in the muscle of an eyestalkless crayfish to the normal level within 15 min [37]. These actions of CHH are mediated by an unidentified membrane-bound receptor and cyclic nucleotides [8,12].

Glucose is produced by glycogenolysis or gluconeogenesis, *de novo* synthesis of glucose from non-carbohydrate precursors, such as lactate, pyruvate, glycerol, and amino acids. Many studies have demonstrated that both activation of GP and inhibition of GS contribute to the glucose production induced by CHH, suggesting that CHH activates glycogenolysis. However, this regulation has not been investigated at the gene or protein level. Furthermore, whether CHH induces glucose production through gluconeogenesis has not been determined.

In the present study, to evaluate the effects of CHH on glycogen metabolism and gluconeogenesis, we focused on a part of glycogenolysis-related enzymes, GP and GS, and a part of rate-limiting enzymes in gluconeogenesis, fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) and phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32). It is known that the enzymatic activity of PEPCK is mainly regulated by its mRNA expression level [11,35,44] and that the activity of FBPase is regulated by the transcriptional mechanism and by two physiological inhibitors, AMP and fructose 2,6-bisphosphate in both vertebrates and invertebrates [11,15]. We measured mRNA expression levels of these four enzymes under the following two experimental conditions: (1) *in vivo* deprivation of endogenous CHH eyestalk-derived factors, including CHH, by eyestalk ablation and (2) exogenous CHH treatment of hepatopancreas *ex vivo*.

2. Materials and methods

2.1. Animals

Adult kuruma prawns, *M. japonicus*, approximately 20 g in body weight, were purchased at a fish market in Tokyo, Japan. Prawns were kept in a tank with natural seawater and a constant L12:D12 photoperiod at 20 °C, and provided daily with a commercial prawn diet (Goldprawn, Higashimaru Co., Kagoshima, Japan) at least one week until experiment. To exclude the effects of diet on energy metabolism, examined prawns were starved after eyestalk ablation. Most prawns (~95%) remained at the intermolt stage at 7 days after eyestalk ablation. The molting stage was judged by morphological appearance and by hardness of the shell. Only prawns at the intermolt stage were used for the experiments.

2.2. cDNA cloning and sequencing

Prawns at the intermolt stage were anesthetized on ice. Hepatopancreas, tail muscle and ovary were dissected out from the anesthetized prawns. Total RNA was extracted from the tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription (RT) was performed using total RNA (1 μg), SuperScript III Reverse Transcriptase (Invitrogen), and oligo (dT) primer, 5′-AAGGAGTGGTATCCAGTG TGCTGG$(T)_{30}$VN-3′.

The sequences of primers used in this study are listed in Table 1. PCR reactions to obtain partial cDNA fragments of *MjGP*, *MjGS*, *MjFBPase* and *MjPEPCK* were performed using the corresponding

Table 1
Sequence of primers used for cloning, RT-PCR and qPCR.

Primer	Sequence
GP-F1	5′-TAYGARAARCCNAARMG-3′
GP-F2	5′-TTYGAYATHCARGTNAARMG-3′
GP-F3	5′-GTGGAAGAGCCTGATGATTGGCTGCG-3′
GP-F4	5′-TCAGCAAGAGCTGGATTGCA-3′
GP-R1	5′-GGNCKNGCYTTYTCCCA-3′
GP-R2	5′-AARTTCAGRTTNCCNGTNCC-3′
GP-R3	5′-TGGCCTTGATGCGATTGTAC-3′
GP-R4	5′-CTCCAGCTGAATGACACTCATCC-3′
GP-5′RACE	5′-CATCACAGGCCGACTGAATGCCCAAG-3′
GP-5′RACE_nest	5′-GAGAGGTAGTACACGCGCTT-3′
GP-3′RACE	5′-GGCTGAACAGATCATCCCAGCTGCTGA-3′
GP-3′RACE_nest	5′-TCTCCACAGCTGGAACTGAA-3′
GP-qF	5′-GCTGGAACTGAAGCCTCTGG-3′
GP-qR	5′-CCTTGCCCATCTCTTCCATC-3′
GS-F1	5′-TTGGARGTNGCNAAYAA-3′
GS-F2	5′-TTYACNACNCAYGCNAC-3′
GS-F3	5′-GAGGAGCTGGGCGATCAGTAT-3′
GS-R1	5′-GRTTNCCRTCNACNARCCA-3′
GS-R2	5′-ATRTCNSCNCCYTTRTT-3′
GS-R3	5′-GCGCTCCATGCAGTACCTGT-3′
GS-5′RACE	5′-GGAGCGGATATTGTTAATGGCGGTTG-3′
GS-5′RACE_nest	5′-ACATACTGATCGCCCAGCTC-3′
GS-qF	5′-CCACCTGTCCAACACCTTCA-3′
GS-qR	5′-TGATAATGTCCGGCTTGCAC-3′
FBP-F1	5′-TGYTTYGAYCCNYTNGAYGG-3′
FBP-F2	5′-ATGACTTCCACAGGCCAG-3′
FBP-R1	5′-CKRTGNACRTCNGCNACCAT-3′
FBP-R2	5′-TAGGTTATGCTTCTTGTACAAGTCG-3′
FBP-5′RACE	5′-CTGCAAGGCATCGGATACGGATGGAA-3′
FBP-5′RACE_nest	5′-ATGGATCCTATGGAGACCAG-3′
FBP-3′RACE	5′-AACCTCTGGGATCCAGCGGTGTCTGAG-3′
FBP-3′RACE_nest	5′-GCTGGTGCACGTTACATTG-3′
FBP-qF	5′-GACATTCATCAGCGCACTCC-3′
FBP-qR	5′-TCACACTGGTGCAGGGAATC-3′
PEPCK-F1	5′-TTYTGGGARGGNYTNGARAARGA-3′
PEPCK-F2	5′-TCACCATCACCTCATGGCTTGGAGACAGC-3′
PEPCK-R1	5′-AACCARTTNACRTGRAADATYTT-3′
PEPCK-R2	5′-GTGCTGCAAGTAGTGGCCGAAGTTGTAGCC-3′
PEPCK-qF	5′-CTCGGCCATTCTAI I I GGAG-3′
PEPCK-qR	5′-GCATGATCACC I I I GCCTTG-3′
Actin-F	5′-TCCACGAGACCACATACAAC-3′
Actin-R	5′-CACTTCCTGTGAACGATTGA-3′
18S_rRNA-qF	5′-GTTCGTTCGTTCGTTCGTTC-3′
18S_rRNA-qR	5′-GTACGTCCACCGCTGCATTA-3′

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