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ScienceDirect
General and Comparative Endocrinology 153 (2007) 170–175
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Effect of insulin/IGF-I like peptides on glucose metabolism in the white shrimp *Penaeus vannamei*

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Received 15 September 2006; revised 2 March 2007; accepted 19 April 2007
Available online 27 April 2007

Abstract

The insulin-like hormone superfamily encompasses insulin, relaxin, and insulin-like growth factors I (IGF1) and II (IGF2). Insulin hormones regulate cell growth, metabolism, and tissue-specific functions. The presence of insulin has been demonstrated in various invertebrates, and their function as growth promoting or controlling factors has been established in molluscs and insects. In crustaceans, the presence of insulin/insulin-like growth factor (IGF)-like peptides has also been suggested and functional studies have been associated with metabolic control. The general aim of the current study was to elucidate the functional significance of insulin-like peptides in the white shrimp *Penaeus vannamei*. Because the primary structure of *Penaeus* insulin is yet unknown, we examined the effect of mammalian insulin/IGF-I on glucose metabolism in *P. vannamei*. Juvenile shrimps were injected with a single dose of recombinant human (rh) IGF-I or bovine insulin in intermolt stage. Glucose/glycogen levels in shrimp hemolymph and tissues (muscle, hepatopancreas and gills) were determined over a 5 h period by means of an enzymatic analysis. We showed that an injection of rhIGF-I induced a significant ($P < 0.01$) increase in glucose levels in hemolymph, 1 h after injection and followed by a decrease ($P < 0.05$) 5 h post-injection. In the hepatopancreas, an increase ($P < 0.05$) in the glycogen content was observed 3 h after insulin treatment. Finally, a significant elevation ($P < 0.01$) of glycogen content in the gills throughout the entire sampling period was detected. Our study suggests the presence of endogenous *Penaeus* insulin(s) that, just like its vertebrate counterparts, is likely to be involved in the regulation of carbohydrate metabolism in crustaceans.
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Keywords: *Penaeus*; Insulin; IGF-I; Glucose; Hemolymph; Hepatopancreas; Muscle; Gills

1. Introduction

Insulin and the insulin-like growth factors (IGF-I and -II) belong to a superfamily of polypeptides that play important roles in the regulation of cellular metabolism and growth of vertebrates. They are characterized by a high degree of sequence homology. From the point of view of evolution, IGFs and insulin stem from a common precursor molecule, which was encoded for by a gene that underwent duplication, million years ago and subsequently diverged into IGFs and insulin (Froesch and Zapf, 1985).

In recent years, increasing evidence has accumulated that invertebrates contain peptides that share substantial sequence similarities and/or biological actions with mammalian insulin and insulin-like growth factors. These peptides have been well characterized in the neurosecretory system of insects (Duve, 1978; Barret and Loughton, 1987; Masumura et al., 2000; Wu and Brown, 2006) and molluscs (Sevala et al., 1993; Lebel et al., 1996; Floyd et al., 1999; Gricourt et al., 2003).

In crustaceans, the presence of insulin/IGF like peptides has been also suggested. An insulin immunoreactive peptide has been identified from lobster hepatopancreas

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doi:10.1016/j.ygcen.2007.04.014

(Sanders, 1983a) where it was shown to stimulate the glycogenesis in lobster muscle (Sanders, 1983b), without effecting hemolymph glucose levels (Sanders, 1983c). Methanolic extracts from crab (Davidson et al., 1971) and lobster (Gallardo et al., 2003) have been demonstrated to contain insulin-like activity as they were able to stimulate glycogen synthesis and glucose oxidation to CO_2 in mouse/rat tissues. Furthermore, tyrosine kinase insulin-like receptors have been characterized in the hepatopancreas and the muscle of the shrimps *Penaeus monodon* (Lin et al., 1993) and *Penaeus japonicus* (Chuang and Wang, 1994).

While there is considerable information that supports the existence of insulin/IGF-like peptides in crustaceans, studies related to the functional significance of these peptides in decapods are limited. In an attempt to gain further insight into the functional role of insulin/IGF-like peptides in crustaceans, this study evaluated the effect of mammalian insulin/IGF-like peptides on glucose metabolism in the shrimp *Penaeus vannamei*.

2. Materials and methods

2.1. Peptides

Bovine insulin and recombinant human IGF-I (rhIGF-I) were obtained from Sigma (St. Louis, MO). Lyophilised peptides were reconstituted according by manufacturer's instructions and stored at a concentration of 1 mg/ml at −20 °C. For experimental use, insulin/rhIGF-I stocks were thawed and diluted in phosphate-buffered saline (pH 7.4).

2.2. Animals

Juvenile *P. vannamei* (10 ± 0.17 g) were obtained from stocks held in seawater ponds at CENAIM-ESPOL experimental station (Palmar, Ecuador). The animals were maintained in an 18 tons tank with 100% exchange rate seawater held at 25 °C. While in the tank, shrimps were exposed to 12 h light/dark cycles and fed based on 5% total biomass with artificial diet.

2.3. Insulin/IGF-I administration

Animals at intermolt stage were selected by microscopical examination of the edge of a uropod (Robertson et al., 1987). Shrimps were transferred to experimental set ups and randomly assigned to 2.5 l. glass vials three days before bioassay. Twenty-four hours before experimental use food was withdraw. For the administration of insulin/rhIGF-I, shrimps were removed from the vials and injected with insulin/IGF-I (0 or 0.1 μg/g BW of shrimp) at the second abdominal segment while folded. After injection, shrimps were promptly returned to their allocated vials and no food was given for the rest of the experimental period. At various intervals, (0 and 1–5 h post-injection) shrimps were removed for hemolymph sampling and then sacrificed by decapitation. Hepatopancreas, abdominal muscle and gills (100–200 mg) were then excised, quickly frozen in liquid nitrogen and stored at −80 °C until required for analysis. Hemolymph samples were processed immediately.

2.4. Glucose levels determination

Hemolymph was excised from the ventral region (arthropodial membrane) using a syringe rinsed with anticoagulant solution (10% sodium citrate). One hundred microliters of hemolymph were transferred to a microtube containing 300 μl of 95% ethanol. Tube contents were mixed by vortexing and centrifuged at 4000 rpm for 5 min at 4 °C. Forty microliters of supernatant were removed for glucose analysis. Glucose levels were determined according to instructions from the manufacturer (Sigma GAHK-20). Briefly, the supernatant was placed in a microtube containing 80 μl of enzymatic reagent solution (1.5 mM NAD, 1 mM ATP, 20 U hexokinase, 20 U glucose-6-phosphate dehydrogenase and 20 ml of deionised water). Tube contents were mixed by vortexing and allowed to incubate for 15 min at room temperature. Following this, the absorbance of samples was determined at 340 nm in a spectrophotometer (Thermo Spectronic-Genesys 20). The glucose levels in hemolymph samples were then calculated using a calibration curve made with known glucose standards (0.25–5 μg).

2.5. Biochemical analysis of glycogen content

Excised shrimp tissue was thawed on ice and homogenized in 1.5 ml of ice cold distilled water using a microhomogenizer (Ultra Turrax) at 20,000 rpm for 3 s then centrifuged at 13,000 rpm for 5 min at 0 °C. The supernatants obtained were used for glycogen determination (Dubois et al., 1956). Briefly, 100 μl sample of supernatant were placed in a microtube with 2 ml of 20% trichloroacetic acid and centrifuged at 3000 rpm for 20 min. The volumes obtained were transferred into tubes with 2 ml ether and centrifuged again. The upper layers were carefully discarded and the rest of the volumes were placed in tubes with 1.5 ml distilled water, 500 μl of 80% phenol and 5 ml concentrated sulphuric acid. The samples were allowed to incubate for 30 min at room temperature. Ten minutes before the end of the incubation period, 200 μl of the reaction mixture was transferred to a microplate. The absorbance was determined at 492 nm in a spectrophotometer (Lab Systems). Known glucose standards were processed in the same way as the samples and used to construct a calibration curve.

2.6. Statistical data analysis

Results are expressed as means ± standard error of the mean (SEM). Each value represents 8 replicates. Data were tested for normality and homogeneity of variances, and transformed to logarithm when necessary. A two-ways analysis of variance (ANOVA) followed by LSD multiples comparison test was applied to establish significant difference between sampling periods. All analyses were performed using the statistical package "Data desk" version 6.1 (Data Description, Inc., 1997). The level of significance was set at $p < 0.05$.

3. Results

3.1. Effects of insulin/IGF-I on glucose levels

In this study, rhIGF-I treated shrimps displayed elevated glucose levels, in comparison with PBS-injected controls. The glucose levels in the hemolymph of rhIGF-I-treated animals increased 37% over the PBS-injected control 1 h post-injection (Fig. 1), followed by a significant decrease ($P < 0.05$) 5 h post-injection compared to the PBS-treated control. However, these values were similar to those registered in non-injected animals at 0 h (Fig. 1). In insulin treated shrimps, no significant changes in glucose levels were observed comparable to PBS-injected control; nevertheless, an increase in glucose content was detected 5 h after injection in both insulin and PBS-injected shrimps.

3.2. Effects of Insulin/IGF-I on glycogen tissue content

Shrimp were examined for changes in glycogen content in hepatopancreas, abdominal muscle and gills. Samples

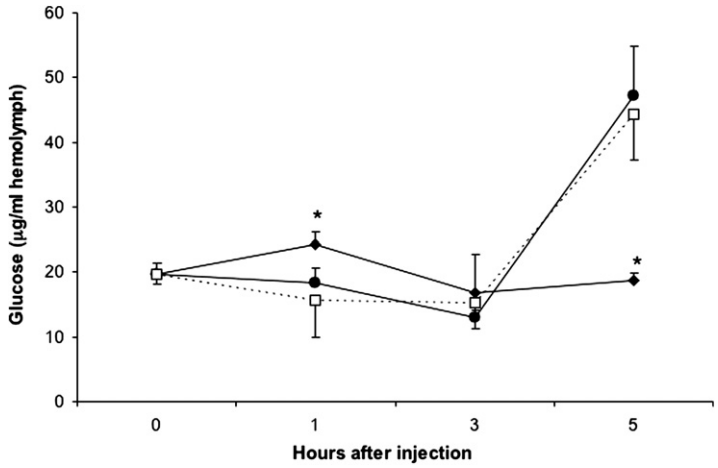

Fig. 1. Glucose levels in shrimp hemolymph after growth factors administration. Hemolymph samples were taken from rhIGF-I (◆), bovine insulin (●) or PBS (□) as control. Each value represents means ± SEM of 8 assays. For rhIGF-I-treated shrimps, an asterisk indicates that the value is significantly different ($P < 0.05$) from the PBS-treated control at the same time point. Under the conditions of this study, shrimps after insulin injection did not show any significant changes in glucose levels comparing with control values.

were taken at 0 h (non-injected animals) then 1, 3 and 5 h after treatment with insulin/IGF-I. One hour post-injection of rhIGF-I and PBS control, the glycogen content in the hepatopancreas fell significantly ($P < 0.05$) below the basal level (0 h, not showed in the figure). In insulin-treated animals, a significant increase ($P < 0.05$) of the hepatopancreas glycogen content was detected 3 h post-injection. The glycogen contents were again significantly ($P < 0.05$) decreased 5 h post-injection; in contrasts to the PBS-control glycogen levels that continued to increase (Fig. 2). Hepatopancreas from animals treated with PBS and rhIGF-I showed glycogen contents similar to the values registered in pre-injected animals (0 h) 3 and 5 h after injection.

In abdominal muscle, the glycogen content significantly decreased ($P < 0.05$) under the registered values in non-injected animals 1, 3 and 5 h post-injection of all treatments (Fig. 3). A very marked reduction ($P < 0.001$) in muscle glycogen content was observed after insulin injection compared to the PBS-injected control. In rhIGF-I-treated shrimp, a significant decrease ($P < 0.01$) in the glycogen content was observed only 3 h after injection when compared with to PBS-injected control (Fig. 3).

Glycogen contents in the gills of insulin-treated shrimps were significantly elevated ($P < 0.01$) over the PBS-control values 1 and 3 h after injection (Fig. 4). This increase was also significantly higher ($P < 0.01$) than the basal value (0 h) 3 and 5 h after treatment (not showed in the figure). Tissue glycogen contents of rhIGF-I-treated animals registered 1, 3 and 5 h post-injection were within the range measured in non-injected animals (0 h), although the glycogen

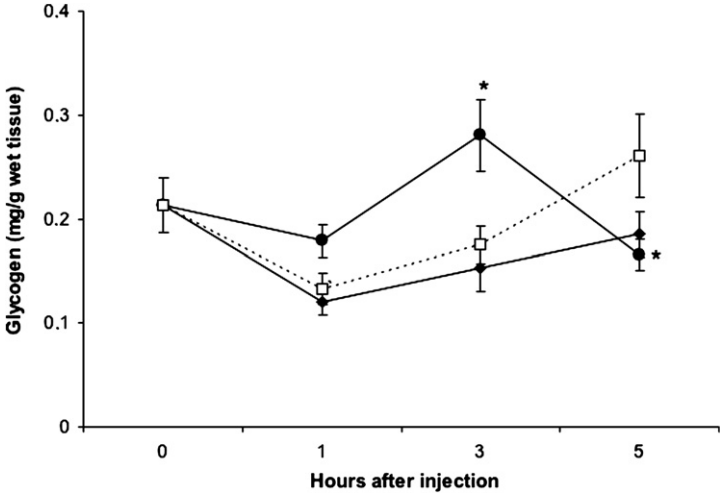

Fig. 2. Changes in hepatopancreas glycogen content after growth factors treatments. Tissue was excised from shrimp injected with rhIGF-I (◆), insulin (●) or PBS (□) as a control. Each value represents means ± SEM of 8 assays. For animals injected with insulin, an asterisk indicates that the value is significantly different ($P < 0.05$) from the PBS-treated control at the same time point. Under the conditions of this study, no significant increase was detected in hepatopancreas glycogen content after rhIGF-I injection.

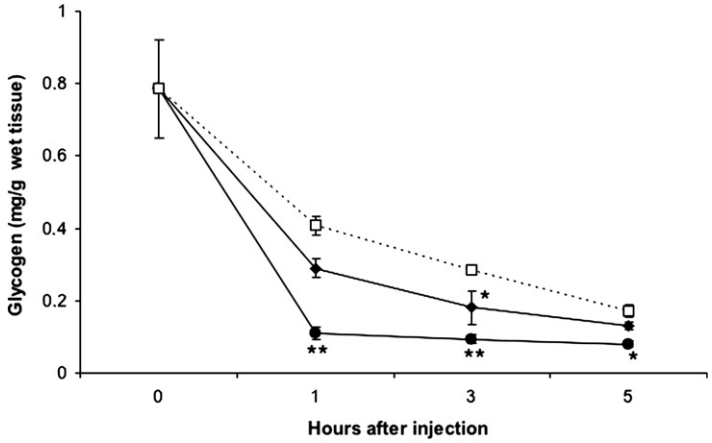

Fig. 3. Changes in abdominal muscle glycogen content after growth factors treatments. Tissue was excised from shrimp injected with rhIGF-I (◆), insulin (●) or PBS (□) as a control. Each value represents means ± SEM of 8 assays. For animals injected with insulin, two asterisk indicate that the value is significantly lower ($P < 0.001$) from the PBS-treated control at the same time point. For rhIGF-I treated shrimp, an asterisk indicates that the value is significantly lower ($P < 0.01$) than the PBS-treated control at the same time point.

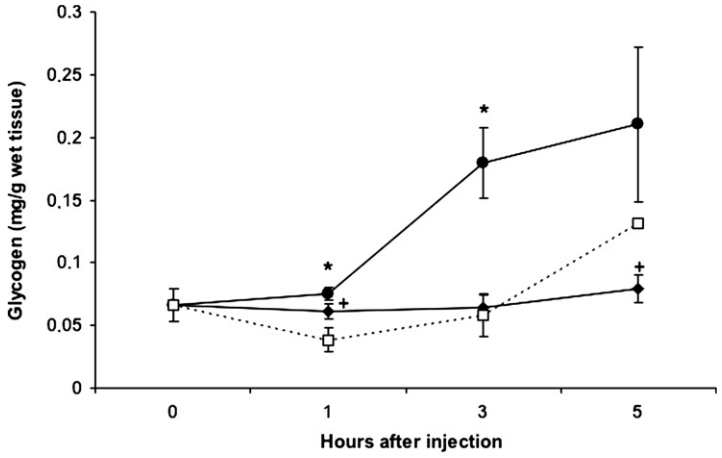

Fig. 4. Effect of insulin/IGF-I on gills glycogen content. Tissue was excised from shrimp injected with rhIGF-I (◆), insulin (●) or PBS (□) as a control. Each value represents means ± SEM of 8 assays. For animals injected with insulin, an asterisk indicates that the value is significantly higher ($P < 0.01$) from the PBS-treated control at the same time point. For rhIGF-I-injected animals, a (+) symbol indicates that the value is significantly different ($P < 0.05$) from the PBS-treated control at the same time point.

contents were slightly higher ($P < 0.05$) 1 h post-injection and lower ($P < 0.05$) 5 h post-injection compared to the PBS-treated control (Fig. 4).

4. Discussion

Glucose is the major component of hemolymph carbohydrates in crustaceans (Meenakshi and Scheer, 1961). One of important signalling molecules, playing a role in the regulation of hemolymph glucose levels is the well-known Crustacean Hyperglycaemic Hormone (CHH). This neuropeptide induces hyperglycemia and hyperlipidemia in the hemolymph, providing glucose and lipids in order to meet the energy requirements of the organs and tissues of decapods (Fanjul-Moles, 2006). The search for a molecule that could play a dual control mechanism with the CHH similar to the glucagon-insulin system to regulate blood sugar has recently gained attention. The presence of a vertebrate-type insulin-like peptide has been reported in some crustacean species (Sanders, 1983b; Davidson et al., 1971; Gallardo et al., 2003). In this study, we evaluated the effect of insulin/IGF on the hemolymph glucose levels and glycogen contents in the hepatopancreas, abdominal muscle and gills of the white shrimp *P. vannamei*.

In rhIGF-I injected animals, no significant variation of hemolymph glucose was revealed 1, 3 and 5 h post-injection compared with the basal value (Fig. 1). These glucose levels were similar to the kinetics of hyperglycemia in untreated animals (bled only) used as controls in crustacean hyperglycemic hormone bioassays. Furthermore, we did not observe significant difference between glucose levels of insulin and PBS-control injected shrimps, although a raise of glucose level was observed 5 h post-injection with both treatments (Fig. 1). Other studies have reported a

hyperglycemia peak 2 h post-injection in the hemolymph of saline/incubation medium-injected animals (controls) followed by a recovery to a baseline level 4 h post-injection as a response of handling stress (Sefiani et al., 1996; Lorenzon et al., 2004). The increase of glucose 5 h post-injection obtained in our study might be due to different responses from animal in intermolt and pre-molt stages. Even though intermolt shrimps were selected for this study, it seems that an animal group was undergoing intermolt–pre-molt transition. Leinen and McWhinnie (1971) observed glucose increase in hemolymph of crayfish in pre-molt stage 6 h post-injection with bovine insulin or control-vehicle, also did not find any significant difference between treatment and controls. Since, untreated animals (bled only control) were not included in this study to determine the effect of handling on glucose metabolism in this specie, we can not conclude that the difference observed between glucose levels of IGF-I injected animals from the PBS-controls was an effect of the injected hormone. Further studies are needed to establish if insulin/IGF family have no effect on the rate of glucose removal from the hemolymph in *P. vannamei* as some workers have showed in other crustacean species (Leinen and McWhinnie, 1971; Sanders, 1983b).

Although insulin-like peptides have been identified and purified in insects, only two studies exist which show that insulin-like peptides have no effect in insect carbohydrates metabolism as insulin in mammals (Wu and Brown, 2006). Bombyxin, an insulin-like peptide, has no effect on trehalose or lipid levels in hemolymph when injected in silkworm *Bombyx mori* adults (Satake et al., 1999). Similarly, in *Drosophila melanogaster*, insulin has no effect on glucose uptake or lipid synthesis in Kc cells, but it does increase glucose oxidation and lactate production (Ceddia et al., 2003).

Our data obtained in hepatopancreas of insulin-treated shrimps show higher glycogen contents 3 h post-injection and which 5 h post-injection fall to lower levels, compared to controls (Fig. 2). This indicates that the decrease of glycogen contents in hepatopancreas can be correlated with the increase of the glucose levels registered in the hemolymph at the same time (Fig. 1). Apparently, insulin induces glucose metabolization into glycogen in the hepatopancreas 3 h post-injection, indicating that this tissue is one of sites for glucose accumulation (Meenakshi and Scheer, 1961; Verri et al., 2001). However, the reduction in the glycogen contents and the increase of hemolymph glucose (Fig. 1) could be an indicative of glycogenolysis, the produced free glucose molecules are transported to target tissues such as abdominal muscle and gills (Glowik et al., 1997). In contrast to our results, Richardson et al. (1997) found no significant difference in percent of glucose uptake into glycogen in the hepatopancreas between rhIGF-I-injected animals and PBS-BSA-injected controls. Histological studies demonstrated the presence of β cells type (insulin producing cells) in hepatopancreas of two crustacean species, suggesting that this tissue could be an insulin secretion site since tissue extracts from the same species showed insulin-like bioactivity (Davidson et al., 1971). Additionally, Sanders (1983a) also determined insulin-like immunoreactivity in the hepatopancreas of *Homarus americanus* that could explain the effect of external insulin over the glycogen content of hepatopancreas after 3 h injection as showed our results.

Abdominal muscle is one of the organs whose function largely depends on a balanced glucose supply and for this reason it was selected for evaluation. In abdominal tissue, we observed a general decrease in glycogen content after all treatments (Fig. 3). Insulin-treated shrimps showed less glycogen contents comparing with other treatments, while their glucose hemolymph increased 5 h post-injection. The reduction of glycogen contents in the abdominal muscle may be a consequence of stress evoked by handling the animals. This stress condition causes abdominal contractions, which in turn causes energy depletion. The increase of glycogen content in the hepatopancreas 3 h post-injection was not enough to cover the glucose demand and to re-establish glycogen content of the abdominal muscle for the next 2 h. In contrast with our findings, Sanders (1983b) described an increase of glucose conversion to glycogen in lobster abdominal muscle in a dose depending manner when insulin was applied in the culture medium, suggesting the presence of insulin-like receptors, which could stimulate glycogenesis. Additionally, Richardson et al. (1997) found significant effects with rhIGF-I over glucose uptake to glycogen in crayfish muscle 24 h post-injection compared with the control value at the same time point.

Since osmoregulatory tissues such as the crustacean gills are well known to be metabolically highly active, we also examined the effect of insulin-like peptides on glucose metabolism in the gills of *P. vannamei*. Glycogen contents registered in gills of insulin-injected shrimps were higher than that of PBS-injected controls 1, 3 and 5 h post-treatment (Fig. 4). Similarly, Kucharski et al. (2002) found a significant increase of glucose uptake to glycogen in posterior gills of the crab *Chasmagnathus granulata* when incubated in the presence of insulin. They also described insulin-binding sites in gills of the same crab species by measuring the radioactivity uptake of ^{125}I-insulin (Kucharski et al., 1997) in anterior and posterior gills. As a result, they suggested that insulin action on carbohydrate metabolism in posterior gills might be important for adaptation in estuarine habitat as reported in the crabs *Ericheir sinensis* (Welcomme and Devos, 1991) and *Carcinus maenas* (Welcomme and Devos, 1994).

In vertebrates, insulin and IGF-I are key players in the regulation of anabolic and metabolic process. Nevertheless, the significance of insulin-like peptides in crustaceans is not yet clear. Although, a polypeptide similar to insulin-like peptide in crustacean has not been identified so far, this study demonstrated an *in vivo* effect of insulin. This cross-reactivity of insulin strongly suggests that insulin and their receptors might be conserved in crustaceans, as it is known in other invertebrates.

Acknowledgments

This study was sponsored by the Belgian government in collaboration with the Flemish University Council (VLIR). The authors thank G. Cardenas, M. Pacheco for assistance in the bioassay performance and Y. Paredes, F. Echeverria for technical assistance. The authors are also grateful to Dr. Elke Clynen for critical review of manuscript.

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