General and Comparative Endocrinology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

Insulin-like receptors and carbohydrate metabolism in gills of the euryhaline crab *Neohelice granulata*: Effects of osmotic stress

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ARTICLE INFO

Article history:
Received 19 October 2017
Revised 11 March 2018
Accepted 12 March 2018
Available online xxxx

Keywords:
Neohelice granulata
Crab
Insulin-like receptor
Osmotic stress
Metabolism

ABSTRACT

The present study determined the effect of osmotic stress on the insulin-like receptor binding characteristics and on glucose metabolism in the anterior (AG) and posterior (PG) gills of the crab *Neohelice granulata*. Bovine insulin increased the capacity of the PG cell membrane to phosphorylate exogenous substrate poly (Glu:Tyr 4:1) and the glucose uptake in the control crab group. The crabs were submitted to three periods of hyperosmotic (HR) and hyposmotic (HO) stress, for 24, 72 and 144 h, to investigate the insulin-like receptor phosphorylation capacity of gills. Acclimation to HO for 24 h or HR for 144 h of stress inhibited the effects of insulin in the PG, decreasing the capacity of insulin to phosphorylate exogenous substrate poly (Glu:Tyr 4:1) and decreasing the glucose uptake. Hyperosmotic stress for the same period of 144 h significantly affected ^{125}I-insulin binding in the AG and PG. However, HO stress for 24 h significantly reduced ^{125}I-insulin-specific uptake only in the PG. Therefore, osmotic stress induces alterations in the gill insulin-like receptors that decrease insulin binding in the PG. These findings indicate that osmotic stress induced a pattern of insulin resistance in the PG. The free-glucose concentration in the PG decreased during acclimation to 144 h of HR stress and 24 h of HO stress. This decrease in the cell free-glucose concentration was not accompanied by a significant change in hemolymph glucose levels. In AG from the control group, neither the capacity of bovine insulin to phosphorylate exogenous substrate poly (Glu:Tyr 4:1) nor the glucose uptake changed; however, genistein decreased tyrosine-kinase activity, confirming that this receptor belongs to the tyrosine-kinase family. Acclimation to HO (24 h) or HR (144 h) stress decreased tyrosine-kinase activity in the AG. This study provided new information on the mechanisms involved in the osmoregulation process in crustaceans, demonstrating for the first time in an estuarine crab that osmotic challenge inhibited insulin-like signaling and the effect of insulin on glucose uptake in the PG.

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1. Introduction

Molecules of the insulin family, including insulin, insulin-related growth factors (IGF-I and II) and insulin-like peptides of invertebrates, among others, are structurally homologous and have a common ancestral origin (Chan and Steiner, 2000; Smith et al., 1996). Insulin-like peptides (ILPs) have been described in invertebrates ranging from unicellular eukaryotes such as *Tetrahymena pyriformis* (Csaba and Kovács, 2000; Leick et al., 2001) to arthropods such as *Drosophila melanogaster* (Brogiolo et al., 2001; Vanden Broeck, 2001). ILPs have been identified in different organs of crustaceans, in particular the androgenic gland (Chung et al., 2011; Okuno et al., 1999; Ventura et al., 2015) and the hepatopancreas and gonads (Chandler et al., 2015; Christie et al., 2016; Chung, 2014; Gallardo et al., 2003; Huang et al., 2014; Li et al., 2015; Sanders, 1983a).

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https://doi.org/10.1016/j.ygcen.2018.03.017
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Please cite this article in press as: Trapp, M., et al. Insulin-like receptors and carbohydrate metabolism in gills of the euryhaline crab *Neohelice granulata*: Effects of osmotic stress. Gen. Comp. Endocrinol. (2018), https://doi.org/10.1016/j.ygcen.2018.03.017

The first step in the biological activity of insulin is binding to its receptor on the cell plasma membrane. Homologues of the insulin/IGF receptor show several structural similarities to the insulin receptor of vertebrates, especially the β-subunit tyrosine-kinase region (Aizen et al., 2016; Jonas et al., 1996; Skorokhod et al., 1999; Yamaguchi et al., 1995). The essential signal-transducing properties of the insulin receptor were preserved from invertebrates to mammals, despite the differences in the amino-acid sequence of their receptors (Claeys et al., 2002; Pertseva and Shpakov, 2002).

Chuang and Wang (1994) identified insulin-like receptors in muscle from the shrimp *Penaeus japonicus*. Stimulation of these receptors by mammalian insulin was followed by phosphorylation of the insulin receptor β-subunit. Aizen et al. (2016) showed in the spiny lobster *Sagmariasus verreauxi*, that the tyrosine-kinase insulin-like receptor was phosphorylated after binding with insulin-like from the androgenic gland (IAG) of crustaceans and with human insulin. Sharabi et al. (2016) identified a gene encoding an insulin-like receptor in the prawn *Macrobrachium rosenbergii*; this gene (*Mr-IR*) was expressed in the androgenic gland and in male and female gonads. In crustaceans, IAGs are involved with growth regulation, cell differentiation, intermediary metabolism and reproduction (Ventura et al., 2011).

Previous studies showed the presence of insulin-like receptors in the gills of the euryhaline crab *Neohelice granulata* (Kucharski et al., 1997). These authors also demonstrated the tyrosine-kinase activity of the insulin-like receptor by its capacity to autophosphorylate and to phosphorylate the exogenous substrate poly (Glu:Tyr 4:1) in the presence of bovine insulin (Kucharski et al., 1999). Metabolic functions of bovine insulin in the posterior gills of *N. granulata* were demonstrated by the increase in glucose uptake and glycogen synthesis when this organ was incubated in the presence of this hormone (Kucharski et al., 2002).

The crab *N. granulata* is a strong hyper-osmoregulator in saline concentrations below that of seawater and a hypo-osmoregulator in saline concentrations higher than seawater. Physiological studies performed on adult *N. granulata* showed that these crabs are euryhaline, tolerating exposure to low (2–5‰) or high salinities (40‰) for at least two weeks (Novo et al., 2005). For these reasons, this crab is considered to be an ideal model for physiological, biochemical and morphological studies on the challenge of salinity changes (Bianchini et al., 2008; 99 Luquet et al., 2005; Spivak, 2010). Crustacean gills are an important interface between the internal and external environments, since the gills are in direct contact with water of varying salinity levels inside the branchial chambers (Freire et al., 2008). Besides their role in respiratory gas exchange, crustacean gills are an important site of osmoregulation, calcium homeostasis, ammonia excretion and pH regulation (Freire et al., 2008; Henry et al., 2012). The anterior gills in *N. granulata* are involved with respiratory and excretory functions, and the posterior gills are the primary site of ion transport and osmoregulation, with high expression and activities of Na^+/K^+ ATPase and carbonic anhydrase (Castilho et al., 2001; Genovese et al., 2000; Luquet et al., 2002).

Previous studies showed that the anterior and posterior gills of *N. granulata* were the organs with the highest content of specific binding insulin-like receptors (Kucharski et al., 1997). In this habitat, the gills of *N. granulata* are in direct contact with an environment in which constant osmotic variations occur. The aims of the present study were to determine the effect of hyposmotic and hyperosmotic stresses on the first steps of insulin signaling and the effect on glucose metabolism in the anterior and posterior gills of *N. granulata*. We investigated the binding properties and the tyrosine-kinase activity of the insulin-like receptors, and the response of carbohydrate metabolism in anterior and posterior gills of *N. granulata* exposed to hyposmotic and hyperosmotic stresses.

2. Materials and methods

2.1. Animals

Male *N. granulata* crabs in the intermolt stage (Drach and Tchernigovtzeff, 1967), weighing 15 ± 2 g, were collected in summer months from Tramandaí Lagoon in the state of Rio Grande do Sul, Brazil (29°58′S, 050°08′W). All the crabs used in this study were collected with permission from the Instituto Brasileiro de Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA).

2.2. Experimental procedure

In the laboratory, the crabs were maintained for 15 days in aquaria containing running aerated water at a salinity of 20‰, temperature 25 ± 1 °C, and natural photoperiod. The animals were fed raw beef *ad libitum* once a day (50 g per 25 crabs) in late afternoon. After a 15-d acclimation period, the control group was maintained in aquaria at a salinity of 20‰ under the same conditions as in the acclimation period. The other crabs were transferred to aquaria containing running aerated water at a salinity of 34‰ (hyperosmotic group; HR) or 0‰ (hyposmotic group; HO), temperature 25 ± 1 °C and natural photoperiod, where they remained for 24, 72 or 144 h (Schein et al., 2005; Chittó et al., 2009; Martins et al., 2011) with food (raw beef) supplied *ad libitum*. The crabs remained submerged during the entire experimental period.

2.3. Gill membrane suspension preparation

Gill membranes were prepared according to Kucharski et al. (1999). For each gill-membrane preparation, 10–15 crabs were used. The animals were anesthetized by chilling on ice for 10 min. The anterior (AG) and posterior (PG) gills were carefully removed, weighed, and rinsed in ice-cold buffer (1:10 w/v) containing 0.3 M sucrose, 10 mM HEPES, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 13 mM EDTA (pH 7.4). The gills were homogenized in ice-cold buffer using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 5000*g* for 30 min at 4 °C and the supernatant was then removed and centrifuged at 30,000*g* for 20 min at 4 °C. The resulting pellet, resuspended with a Dounce-type homogenizer (Kontes Glass Co., Vineland, NJ) in Tris-HCl buffer (50 mmol/l, pH 7.4), represented the gill-membrane suspensions. The protein concentration of the gill-membrane suspensions was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA, Sigma) as a standard. The samples were stored at −80 °C until use.

2.4. Substrate phosphorylation

Phosphorylation of the substrate was measured according to Kucharski et al. (1999). Samples of gill-membrane suspensions (50 μg of protein) were pre-incubated for 30 min at 25 °C with 500 μM of genistein (a potent competitive tyrosine-kinase inhibitor; Sigma) plus 200 nM of bovine insulin, or with bovine insulin alone (without genistein). The control group was pre-incubated with 200 nM BSA alone. Samples were then incubated with 0.5 μCi of [γ^{32}P]-ATP (300 mCi/nmol; Amersham PerkinElmer) plus 5 μM unlabeled ATP (Sigma) in a total volume of 50 μL of 25 mM HEPES buffer, containing 0.05% Triton X-100, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 2 mM sodium orthovanadate, for 10 min at 25 °C. Afterwards, 1 mM poly (Glu:Tyr 4:1) (Sigma) was added and the samples were incubated for 1 h at 25 °C. The reaction was stopped by applying the samples to phosphocellulose paper (Whatman) (Kucharski et al., 1999). The paper was rinsed three times with 75 mM H_3PO_4 and a final rinse with ethanol. Incorporation of ^{32}P

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