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13-*Cis*-Retinoic Acid-Induced Hyperglycemia in the Fresh Water Edible Crab, *Oziothelphusa Senex Senex* is mediated by Triggering Release of Hyperglycemic Hormone from Eyestalks

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Abstract

The present study was aimed to investigate the effect of 13-cis-retinoic acid (13-CRA) on hemolymph glucose levels in the fresh water edible crab, *Oziothelphusa senex* senex. Injection of 13-CRA significantly increased hemolymph glucose levels in a dose-dependent manner in intact crabs. Bilateral eyestalk ablation (ESX) resulted in significant decrease in hemolymph glucose levels. Injection of 13-CRA in to ESX crabs did not cause any significant changes in hemolymph glucose level as compared to ESX crabs suggesting that the effect of 13-CRA could be on the neuroendocrine system in the eyestalks increasing secretion of hyperglycemic hormone. To test this hypothesis, eyestalks were collected from control and 13-CRA injected crabs, and tested for hyperglycemic effect and also for the hyperglycemic hormone levels. The levels of hyperglycemic hormone and the hyperglycemic effect were significantly low in the eyestalks collected from 13-CRA injected crabs when compared with eyestalks from control crabs. From results, it is hypothesized that 13-CRA-induced hyperglycemia in the crab, *O. senex*, is mediated by triggering the release of hyperglycemic hormone from the eyestalk.

Keywords: 13-*cis*-retinoic acid; Eyestalk ablation; Hyperglycemia; Hemolymph glucose, *Oziothelphusa senex senex.*

Introduction

In crustaceans, glucose homeostasis is primarily under the control of an eyestalk hormone, namely crustacean hyperglycemic hormone (CHH). Crustacean hyperglycemic hormone as a diabetogenic factor was first reported by Abramowitz et al. [1], within eyestalks of decapod crustaceans. Since then, the chemical nature, mode of action, and the target tissues of CHH have been extensively studied in several crustaceans [2-5]. The action of CHH in inducing hyperglycemia is mainly through mobilization of glucose from the tissue carbohydrate pools [6]. It has been reported that CHH stimulates glycogenolysis by activating glycogen phosphorylase in both muscle and hepatopancreas [2,7].

Retinoic acids (RA) including 9-*cis*-retinoic acid (9-CRA) and 13-*cis*-retinoic acid (13-CRA) and all-*trans*-retinoic acid (ATRA) are the metabolites of the vitamin A. Retinoids render their biological activity by binding to nuclear receptors in vertebrates. *cis*-retinoic acids like 9-CRA and 13-CRA interacts with both retinoic acid receptors (RARα,β,γ) and retinoid X receptor (RXR), whereas ATRA mainly interacts with the RARs [8]. It was reported earlier that administration of *cis*-retinoic acid increases insulin release in cultured RINm5F cells [9, 10]. The antidiabetic effects of retinoids in human skeletal muscle [11] and diabetic rodents [12] are accepted to be the mediated through the RXR/RAR heterodimer, and RXR homodimer.

In several crustaceans endogenous retinoic acid has been discovered [13] though it is not recognized as a functional hormone. Although there are sporadic reports on the identification of retinoic acid in crustaceans [14], there is little information available on the role of retinoic acid in the regulation of physiology. Recently, we have reported hyperglycemia in the freshwater crab *Oziothelphusa senex senex* after 9-CRA administration [15]. In as much as (a) RA is identified in the crustacean eyestalks and in circulation [13], (b) RXR was discovered in the eyestalks of *O. senex*, and (c) the eyestalks are the major site of CHH secretion [2], the present study was undertaken to examine the possibility that 13-CRA has any role in regulating hemolymph glucose level in the crab *O. senex senex* and if so, to determine whether it has any effect on CHH in the eyestalks.

Materials and Methods

Collection and maintenance of animals

Intact, intermolt (Stage C_4) adult male crabs, *Oziothelphusa senex senex* Fabricius, with a body weight of 30 ± 3 g and carapace width of 36 ± 3 mm were collected from the rice fields and irrigation canals around Tirupati (13°36'N, 79°25'E), Andhra Pradesh, India. The animals were housed 6-8 per glass aquaria (length: width: height=60:30:30 cm) with 40 L sand-filtered tap water (Salinity: 0.5 ppt) and transferred to fresh water every day. They were acclimatized to the laboratory conditions (temperature 28 ± 1°C and12:12 h; light: dark cycle) for 7 days before use. The crabs were fed with sheep meat *ad libitum* once daily. Feeding was stopped one day before the commencement of the experiment to avoid changes due to prandial activity.

Test chemical

13-*cis*-retinoic acid (13-CRA; chemical purity ≥ 98% HPLC) was purchased from Sigma Chemical Company (St. Louis, MO, USA). 13-CRA was dissolved in acetone and then one aliquot of this solution was mixed with nine aliquots of crustacean saline (0.2 M NaCl, 5.4 mM

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KCl, 2.6 mM $MgCl_2$, 13.5 mM $CaCl_2$, 5.6 mM maleate, 10.8 mM Tris; pH 7.4) [16] to produce the final dose for injection.

Experimental Design

Three experiments were conducted. Experiment 1 was conducted to determine the dose of 13-CRA that induces maximum hyperglycemia in intact adult male crabs. Experiment 2 was performed to determine the time-course of action of 13-CRA in inducing hyperglycemia. Experiments 3, 4 and 5 were conducted to establish whether eyestalk hyperglycaemic hormone is involved in 13-CRA-induced hyperglycemia.

In experiment 1, intact adult male crabs were injected with different doses of 13-CRA through the base of the chelae with a micro-syringe (Hamilton make) in 10 µl volume. Control crabs were injected with 10 µl crustacean saline. Hemolymph was collected from the crabs 2 h after injection and analysed for sugar levels.

In experiment 2, intact adult male crabs were injected with 25 µg retinoic acid/g live mass. This dose was selected based on the results of experiment 1 (see below). Hemolymph was withdrawn from injected crabs at different time points (30, 60, 120, and 360 min) and used for glucose quantification as described in experiment 1.

In experiment 3, both eyestalks were removed (in order to deprive the eyestalk hormones) from the crabs by cutting off the stalks at their bases at the arthrodial membrane without prior ligation, but with cautery of the wound after operation. We routinely achieve more than 95% survival of the crabs following the operation. Twenty four hours after eyestalk ablation, eyestalkless crabs (ESX) were then injected with 25 µg retinoic acid/g live mass. Hemolymph was collected from ESX crabs, 2 h after 13-CRA injection and used for glucose quantification. The dose of 13-CRA and time-point of hemolymph collection were selected based on the results of experiment 1 and 2 (see below).

In experiment 4, forty eight intact adult male crabs were divided into six equal groups. Crabs in group I served as control and the animals in group II served as saline injected controls. Animals in groups III and IV were injected with either saline (25 µl) or 25 µg retinoic acid/g live mass respectively. Two hours after injection, both eyestalks were collected from crabs in groups III and IV and eyestalk extracts were prepared by homogenizing the eyestalk neural tissue in crab physiological saline and then centrifuging at 10,000 × g for 10 min at 4°C. The supernatant was used for injections. Crabs in groups V and VI were injected with 25 µl of extract (two eyestalk equivalents per crab) prepared using eyestalks collected from crabs in groups III and IV respectively. Hemolymph was collected from control, saline injected control and eyestalk extract injected crabs 2 h after injection and glucose levels were determined.

In experiment 5, crabs were injected with either saline or 25 µg retinoic acid/g live mass. Two hours after injection, eyestalks were collected from saline injected and 13-CRA injected crabs and sinus glands were isolated. Following HPLC of sinus gland sample [17], collected fractions were assayed for CHH. CHH levels were measured using ELISA. The details of ELISA were described elsewhere [15,18]. The polyclonal antibodies for *O. senex* CHH raised in our laboratory were for the ELISA.

Hemolymph glucose determination

A 10 µl hemolymph sample was collected from the arthrodial membrane of coxa of third pair of walking leg using a micro-syringe and mixed with 250 µl of distilled water and then stored at -80°C. Glucose concentration was measured enzymatically using the glucose oxidase assay kit (Sigma Chemical Co. St. Louis, MO, USA). The assay protocol essentially followed the one provided by the manufacturer.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's test using SPSS (Student version 7.5, SPSS Inc, Chertsey, UK). Differences were considered to be significant when $p<0.05$. All data are reported as mean ± S.D.

Results

Effect of 13-*cis*-retinoic acid on hemolymph glucose levels of intact crabs

Injection of 13CRA into intact crabs resulted in significant hyperglycemia in a dose-dependent manner, whereas injection of physiological saline did not cause any significant effect on hemolymph glucose levels (Figure 1). At doses between 10 µg/g live mass and 25 µg/g live mass, the effect was statistically significant and dose-dependent. Doses lower than 10 µg/g live mass, however, retinoic acid did not elicit any hyperglycemic response and doses greater than 25 µg/g live mass exhibited a saturated response in inducing hyperglycemia. In the subsequent experiments, 25 µg/g live mass was selected as injection dose.

A time course action of 13CRA-induced hyperglycemia is presented in Figure 2. Hemolymph glucose levels increased significantly ($p<0.01$) within 30 min after 13-CRA injection and reached a highest peak in 2 h; thereafter, a decline in the hemolymph glucose levels were observed. The hemolymph glucose level was almost normal at 6 h post-injection.

Effect of eyestalk ablation and injection of 13-CRA on hemolymph glucose level in eyestalk-ablated crabs

Bilateral eyestalk ablation resulted in a significant decrease (-49.63%) in hemolymph glucose level in the crab *O. senex* (Figure 3). Injection of 13-CRA (25 µg/g live mass) into ESX crabs resulted in no significant change in the levels of hemolymph glucose when compared with ESX crabs (Figure 3).

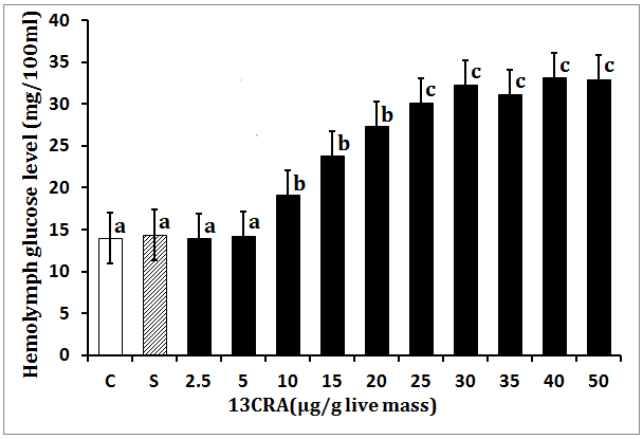

Figure 1: Dose dependent effects of 13CRA on the hemolymph glucose levels in intact crabs. Hemolymph was collected from animals for glucose quantification before (open bar; C: Control) and 2 h after injection with saline (striped bar; S) or 13CRA. Each bar represents a mean ± SD of 8 individuals. Bars with different superscript differ significantly from each other at $p<0.05$

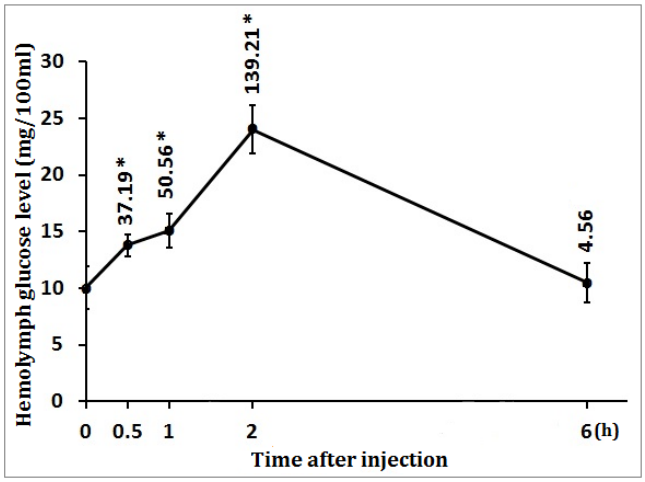

Figure 2: Time course action of 13-CRA-induced hyperglycemia in intact crabs. Hemolymph was collected from intact crabs after injection of retinoic acid (25 µg/g live mass), at the time points indicated for glucose quantification. Each point represents a mean ± S.D. of 8 individuals. Values in parenthesis represent percent change from control (0 h). *p<0.05.

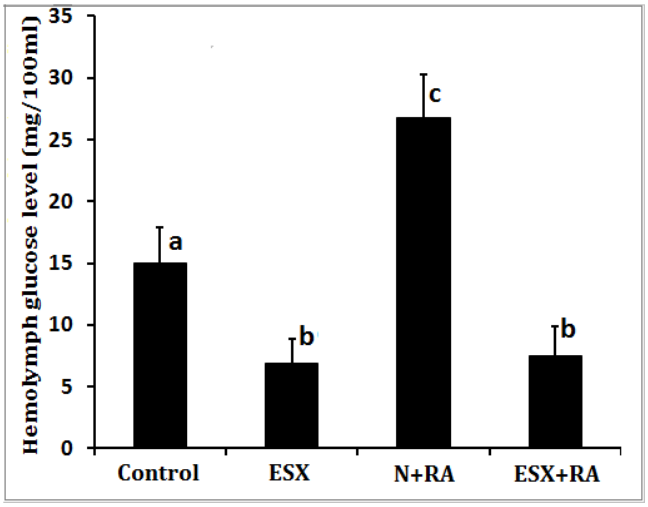

Figure 3: Effect of eyestalk ablation (ESX) and injection of 13-CRA in to normal and ablated crabs on hemolymph glucose levels in *O. senex*. Each bar represents a mean ± S.D. of 8 individuals. Bars with different superscripts differ significantly from each other at p<0.05.

Effect of 13-CRA on hyperglycemic activity and CHH content in the eyestalks

Injection of eyestalk extract of intact crabs produced significant hyperglycemia (83.05% above control) (Table 1). Injection of either saline (25 µl) or extracts of eyestalks collected from 13-CRA injected crabs did not cause any significant change in hemolymph glucose concentration when compared with uninjected crabs (Table 1).

Eyestalks of control crabs contained 76.43 ± 8.93 fmol CHH-immunoreactive peptide per sinus gland. Eyestalks of crabs injected with 13-CRA (25 µg/g live mass) contained significantly less (-62.98%) CHH-immunoreactive peptide than in the eyestalks of control crabs (Table 2).

Treatment	Hemolymph glucose level (mg/100 ml)
Control	15.75 ± 1.86
Saline injected	16.23 ± 1.69 (3.05)
Eyestalk extract from control injected	28.86* ± 2.35 (83.05)
Eyestalk extract from 13CRA injected	18.45 ± 2.46 (17.14)

Values are mean ± S.D of 8 crabs.

Values in parentheses are % change from control. *p<0.05.

Table 1: Effect of 13-CRA on hemolymph glucose concentrations in crabs injected with saline or various eyestalk extracts.

Treatment	CHH peptide level (fmol/sinus gland)
Control	76.43 ± 8.93
13-CRA injected	28.29* ± 7.86 (-62.98)

Values are mean ± SD n=5 for each group.

Values in parentheses are % change from control. *p<0.05.

Table 2: Effect of 13-CRA on levels of CHH peptides in eyestalks.

Discussion

In the crab, *O. senex*, bilateral eyestalk ablation resulted in significant decrease in the hemolymph glucose levels as compared to intact crabs. Injection of 13-CRA significantly elevated hemolymph glucose levels in intact crabs in a dose-dependent manner. An elevation in glucose concentration in the hemolymph of *O. senex* following 13-CRA injection is apparently mediated by the eyestalk CHH hormone, since an increase in the hemolymph glucose concentration following 13-CRA injection was absent after eyestalks were ablated. Zou and Bonvilliain [19] and Reddy and Sainath [15] also reported hyperglycemia in the crabs *U. pugilator* and *O. senex* respectively after 9-CRA administration. Both 13-CRA and 9-CRA most likely rendered their hyperglycemic action through stimulating the release of CHH from the eyestalk neuroendocrine cells to the circulation, since hyperglycemia was observed only in intact crabs but not in eyestalk-ablated crabs. From the results it can be hypothesized that CRA triggers the release of CHH from the sinus gland of the eyestalks. This hypothesis was further supported by the fact that hyperglycemic activity of the eyestalk extracts of the 13CRA injected crabs was less than that of the control crabs. Direct supporting evidence for this hypothesis is provided by the present studies, in which it was shown that the levels of CHH-immuno-reactive peptides were significantly reduced in the eyestalks of the crabs received 13CRA when compared to the CHH levels in the eyestalks of control crabs. These results strongly suggest that hyperglycemia caused by 13-CRA in intact crabs was due to the triggering release of CHH in the crab.

It is well known that alterations in the circulatory levels of CHH occurred during different stress conditions, including cold shock [20], parasite infection [21], exposure to pesticides [22], and heavy metals [23]. It is also known that elevated circulating titres of CHH were reported to occur following exposure to several environmental stressors [24,25] in intact but not in eyestalk ablated crabs, suggesting stress-induced hyperglycemia is CHH mediated response [26-29].

The role of RA in the regulation of glucose metabolism in vertebrates is well established [14]. Retinoids have been implicated in both stimulation of insulin secretion and expression of the glucose transporter 2 gene [10]. Further, retinoids are believed to exert their effects through the retinoic acid receptor/PPARγ heterodimer [11,

12] or retinoic acid receptor (RAR) homodimer [9]. However, such mechanisms in crustaceans are yet to be investigated.

In crustaceans, endogenous RA [13] and a nuclear RAR homologue have been detected [30, 31]. The RAR isolated from the crab has been found to bear a close similarity to vertebrate RARs in the ligand-binding domain. To date, RXR was identified from the ovaries of *C. pugilator, G. lateralis, M. japonicas, F. chinensis, C. maenas, Scylla serrata* and *Daphnia magna* [32-38]. Recently, RXR has been also identified in the eyestalks of *Callinectes sapidus* [39] *O. senex* [40] and *M. nipponence* [41]. Reduction in mRNA levels for RXR and for vitellogenin in the crab, *C. maenas* after treatment with RXR dsRNA [36] and fluctuations in expression of RXR mRNA in ovaries during reproduction [30] suggests RA acts as reproductive hormone. RAs in crustaceans are also identified as morphogens involved in the limb bud regeneration and morphogenesis [13,14]. RXR has also been identified for which terpenoids can serve as ligands [42]. It was also established that RXR binds with ecdysteroid receptor (EcR) and form a heterodimer [36,42]. This RXR-EcR complex may bind with RA or ecdysteroid (EcD) or methyl farnesoate (MF) and form a heterotrimeric complex (RXR-EcR-RA/EcD/MF) thereby regulate a range of physiological facets in crustaceans. The potential of retinoids in regulating various physiological aspects in crustaceans should now be open for analysis.

Conclusion

On the basis of the results obtained in this study it can be concluded that injection of 13-CRA resulted in significant hyperglycemia in intact crabs, but not in eyestalk ablated crabs suggesting that 13-CRA induced hyperglycemia is mediated through eyestalk hyperglycemic hormone. The levels of hyperglycemic hormone and the hyperglycemic effect was significantly lower in the eyestalks collected from 13-CRA injected crabs when compared with eyestalks from control crabs, strongly suggesting that retinoic acid act, at least in part, by triggering the secretion of hyperglycemic hormone from the eyestalk.

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