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Insulin receptor-mediated nutritional signalling regulates juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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ABSTRACT

Female reproductive processes, which comprise, amongst others, the synthesis of yolk proteins and the endocrine mechanisms which regulate this synthesis, need a considerable amount of energy and resources. The role of communicating that the required nutritional status has been attained is carried out by nutritional signalling pathways and, in particular, by the insulin receptor (InR) pathway. In the present study, using the German cockroach, *Blattella germanica*, as a model, we analysed the role of InR in different processes, but mainly those related to juvenile hormone (JH) synthesis and vitellogenin production. We first cloned the InR cDNA from *B. germanica* (BgInR) and then determined that its expression levels were constant in corpora allata and fat body during the first female gonadotrophic cycle. Results showed that the observed increase in BgInR mRNA in fat body from starved compared to fed females was abolished in those females treated with systemic RNAi *in vivo* against the transcription factor BgFoxO. RNAi-mediated BgInR knockdown during the final two nymphal stages produced significant delays in the moults, together with smaller adult females which could not spread the fore- and hindwings properly. In addition, BgInR knockdown led to a severe inhibition of juvenile hormone synthesis in adult female corpora allata, with a concomitant reduction of mRNA levels corresponding to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase and methyl farnesoate epoxidase. BgInR RNAi treatment also reduced fat body vitellogenin mRNA and oocyte growth. Our results show that BgInR knockdown produces similar phenotypes to those obtained in starved females in terms of corpora allata activity and vitellogenin synthesis, and indicate that the InR pathway mediates the activation of JH biosynthesis and vitellogenin production elicited by nutrition signalling.

1. Introduction

Nutrition and reproduction are essential processes for all organisms and have clear interconnections. To be able to reproduce, organisms need to achieve a nutritional status that ensures the viability of the progenitors and that of their progeny. How do the cells and tissues of an organism detect its nutritional status and regulate the processes that will lead to reproduction? The answer is far from simple because many factors (metabolic, endocrine, genetic or environmental) are known to be involved.

The responsibility of communicating the nutritional status of the individual lies mainly with the TOR (target of rapamycin) and insulin receptor (InR) pathways. Both pathways, interconnected at certain points by regulatory factors, are capable of determining the organism's nutritional status and subsequently modulating the activity of a series of effectors that can activate or inhibit different processes depending on factors such as the tissue, the developmental or reproductive timing, etc. Some of the processes regulated by the activity of these pathways are as important as growth, cellular proliferation, metabolism, ageing, reproduction and cancer (Baker and Thummel, 2007; Hansen et al., 2004; Maestro et al., 2009; Oldham and Hafen, 2003).

The German cockroach *Blattella germanica* is a basal hemimetabolous insect which presents an anautogenous reproductive strategy. This means that, as is the case for bloodsucking mosquitoes, females do not trigger reproductive processes until after they have fed (Maestro et al., 2009; Osorio et al., 1997). The gonadotrophic hormone in *B. germanica*, as in most insect species, is the juvenile hormone (JH) synthesised in the corpora allata (CA). JH

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activates vitellogenin production in the fat body and its incorporation into the growing oocytes as a storage protein for embryo development. Previous studies on this cockroach species indicated that nutritional signals that activate JH and vitellogenin production in adult females are mediated, at least partially, by the TOR pathway (Maestro et al., 2009).

The InR pathway is an evolutionary conserved mechanism, present in all metazoan, which detects and responds to changes in nutrient levels (Baker and Thummel, 2007; Oldham and Hafen, 2003; Wu and Brown, 2006). In the fruit fly, *Drosophila melanogaster*, the inhibition of InR signalling phenocopies starvation at a cellular and organismal level (Britton et al., 2002). Insects have a single InR, with the exception of some hymenopteran which have two (de Azevedo and Hartfelder, 2008; Lu and Pietrantonio, 2011). In contrast to the presence of a single receptor, a variable number of insulin-like peptides (ILPs) can be found in different insect species, for example, eight in *D. melanogaster* (Colombani et al., 2012; Garelli et al., 2012), four in the red flour beetle, *Tribolium castaneum* (Li et al., 2008), and up to thirty-eight in the silkworm, *Bombyx mori* (Aslam et al., 2011). Both the expression and/or release of ILPs are nutritionally regulated in different insect models (Geminard et al., 2009; Ikeya et al., 2002; Masumura et al., 2000; Sheng et al., 2011). In addition, the genetic ablation of brain neurosecretory cells that produce ILPs mimics the phenotype of starved flies (Ikeya et al., 2002; Rulifson et al., 2002). Furthermore, culture media conditioned with cells transfected with *D. melanogaster* ILP genes are able to activate autophosphorylation of the fly InR (Rulifson et al., 2002). It is then clear that the production and release of neurosecretory peptides (ILPs) in response to appropriate nutritional levels is capable of activating the InR and its signalling pathway.

The main transcriptional effector of the InR pathway is the protein FoxO (Barthel et al., 2005). Activation of the InR pathway for example, in the case of high nutritional conditions, phosphorylates FoxO and maintains it inactive within the cytoplasm, whereas starvation promotes the transport of FoxO into the nucleus so that it may perform its transcriptional activities (Baker and Thummel, 2007). We have previously demonstrated that FoxO in *B. germanica* plays an inhibitory role on JH and vitellogenin production in starved females (Suren-Castillo et al., 2012).

In the present work we aim to understand the role of the InR in communicating the organism's nutritional status to different processes, mainly JH and vitellogenin production, and at what levels its regulatory functions are carried out.

2. Material and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared on dry dog food (Panlab 125C3) and water, in the dark at 30 ± 1 °C and 60–70% relative humidity. Virgin females were used for the study of gene expression levels during the first gonadotrophic cycle. For the starvation experiments, subjects received only water after the imaginal moult or after the induction of the second gonadotrophic cycle. Dissections of the different tissues were carried out on carbon dioxide-anesthetized specimens. After dissection, tissues for mRNA levels analysis were immediately frozen in liquid nitrogen and stored at −80 °C. Fat body tissue that had adhered to the abdominal sternites was dissected out, except in the case of *in vitro* incubations, where fat body together with the abdominal sternites and epidermis was used.

2.2. Cloning of BgInR

Degenerate primers based on conserved regions of insect and chordate InR sequences were used to obtain a *B. germanica* homologue cDNA fragment by RT-PCR. The first PCR amplification was carried out using a cDNA template generated by reverse transcription of RNA extracted from UM-BGE-1 cells (derived from early embryos of *B. germanica*). The primer sequences are presented in Supplementary Data, Table 1. We amplified a 399 bp fragment, which was subcloned into the pSTBlue-1 vector (Novagen) and then sequenced. This was followed by 3′-RACE and several 5′-RACEs (5′- and 3′-RACE System Version 2.0; Invitrogen) using different specific primers to complete the sequence.

2.3. Phylogenetic analysis

We used sequences from the following insects: *Acyrthosiphon pisum* (GenBank™ Accession Number: XP_001952079), *Aedes aegypti* (AAB17094), *Anopheles gambiae* (XP_320130), *B. mori* (NP_001037011), *D. melanogaster* (AAC47458), *Nasonia vitripennis* (XP_001606180), *Pediculus humanus corporis* (XP_002430961) and *T. castaneum* (EFA11583); the tick *Ixodes scapularis* (XP_002416224); the nematode *Caenorhabditis elegans* (Daf-2: AAC47715); the amphioxus *Branchiostoma lanceolatum* (AAB50848); and the vertebrates *Homo sapiens* InR (AAA59452), *H. sapiens* IGF1R (AAI13611), *Mus musculus* InR (AAA39318) and *M. musculus* IGF1R (NP_034643). The tree was rooted in the divergence between invertebrates and chordates. Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana, 2000). The resulting alignment was analyzed with the program PHYML 3.0 (Guindon and Gascuel, 2003), based on the maximum-likelihood principle. Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates.

2.4. RNA extraction, cDNA synthesis and real-time PCR analyses

The CA and fat body expression levels of the different genes studied were analyzed using real-time PCR. cDNA was synthesized from total RNA as described previously (Maestro and Belles, 2006). 0.5 μg of total RNA was used in the case of fat bodies, whereas in the case of CA, the whole RNA from one pair of glands was used. The absence of genomic contamination was confirmed using a control without reverse transcription. cDNA levels were quantified using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad) as described previously (Maestro et al., 2010). Primer sequences to amplify BgInR are reported in Supplementary Data, Table 1. Primers used to amplify HMG-CoA synthase-1 and -2, HMGCoA reductase, methyl farnesoate epoxidase (CYP15A1), vitellogenin (BgVg) and BgActin 5C (used as a reference) have been already reported (Maestro et al., 2010; Suren-Castillo et al., 2012). The total reaction volume was 20 μl. All reactions were run in duplicate or triplicate. The schedule used to amplify the reaction was the following: (i) 95 °C for 3 min; (ii) 95 °C for 10 s; (iii) 60 °C for 1 min; and (iv) repeat steps (i) and (ii) for 50 cycles. Real-time data was collected through the iQ5 optical system software v. 2.0 (BioRad).

2.5. RNA interference

Systemic RNAi *in vivo* in females of *B. germanica* was performed as described previously (Maestro et al., 2009). Two different fragments, a 326-bp dsRNA fragment (dsInR) encompassing part of the protein tyrosine kinase domain of BgInR, and a 349-bp fragment (dsInR-II) encompassing most of the fibronectin type-III domain (spanning positions 3403 to 3728 and 2714 to 3063, respectively, of the BgInR cDNA), were used to generate two different dsRNA (Fig. 1A). A heterologous 307-bp fragment from the polyhedrin of