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Original Research Paper

NUCLEAR RECEPTORS, A PARADIGM OF IMPORTANCE FOR UNDERSTANDING HORMONE ACTION IN INVERTEBRATES: AN OVERVIEW ON CRUSTACEANS

Engineering

KEYWORDS: : Nuclear receptor; crustaceans; hormone action; endocrine control; insects; steroid hormone; ecdysteroids; HRE

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ABSTRACT

Nuclear receptors (NRs), comprising a distinct 'cohort' of (protein) molecules, belonging to the Nuclear Receptor superfamily, are suggested to play key role in mediating between the hormone molecule and the target tissue. NRs are known to bind with smaller molecules – the ligands -such as steroids, retinoids, vitamins, terpenoids and farnesol metabolites; the hormone-receptor complex, then moves towards the hormone response element (HRE) situated in the target gene, to effect hormone action. However, notwithstanding the considerable literature available with respect to vertebrates, our understanding on the exact role of receptor proteins in signalling and eliciting the response at molecular level is too fragmentary in invertebrates (excepting perhaps in insects). Significantly, invertebrates in general are suggested to rely on NR for eliciting the hormone response. However, we have only scanty information on the exact mechanism involved in hormone action with respect to non-insect invertebrates. At this juncture, we review the status of our knowledge on the structural and functional aspects of NRs of crustaceans, a phylogenetic ally of insects, and which are important organisms both from academic standpoints and applied research.

INTRODUCTION

Hormone receptors are known to mediate between the signalling molecule (the hormone) and the target gene in question, to initiate transcriptional activity. Depending on the chemistry of the ligand (the hormone), the receptors could vary in their structure and functionality.

Most of the peptide (protein) hormones, for instance, rely on the receptors housed in the cell membrane (referred to as the membrane receptors, MR). The (protein) hormone molecule, as it binds with the MR, initiates a cascade of chemical events (also referred to as the signal transduction pathways), leading to transcriptional activities. The nuclear receptors (NRs), on the other hand, comprise a totally different 'cohort' of (protein) molecules, belonging to the Nuclear Receptor Superfamily. Situated in the cytoplasm or the nucleus, unlike MRs, NRs are known to bind with smaller molecules – the ligands -such as steroids, retinoids, vitamins, terpenoids and farnesol metabolites; the hormone-receptor complex subsequently moves towards the hormone response element (HRE) situated in the target gene, to effect hormone action (O'Malley and Tsai, 1992; Kumar et al., 2011; Zheng and Murphy, 2016 for review).

Genesis of information on nuclear receptors in invertebrates:

Until early 1990's, our understanding on the hormonal mechanisms in invertebrates has primarily been based on the chemistry of the hormone molecule and the fluctuation in the hormone profiles in relation to various physiological activities. In insects for instance, the levels of ecdysteroids (the steroid hormone), were reported to fluctuate in relation to the stages of development. In crustaceans, a phylogenic ally of insects, ecdysteroids were shown to fluctuate in tune with the stages of moulting; as the individuals attain premoult, the ecdysteroids registered a perceptible increase from its intermoult levels, and subsequently declined after the exuviation of the old exoskeleton (Adiyodi, 1988 for review; Chang, 1993; Suganthi and Anilkumar, 1999; Sudha and Anilkumar, 2007; Chang and Mykles, 2011 for review; Shyamal et al., 2014).

These stage – dependent fluctuations in ecdysteroid levels have encouraged the scientists to propose ecdysteroids as the growth hormone in insects, crustaceans and several other invertebrate groups. However, the exact trajectory of events, starting from hormonal fluctuations, leading to the execution of hormone action at molecular level, was not known in invertebrates until Koelle et al. (1991) demonstrated for the first time, the importance of hormone receptor genes and its expression in facilitating hormone action in *Drosophila*. Koelle and colleagues have not only sequenced the ecdysteroid receptor gene (*EcR*), but they could as well demonstrate an enhanced gene expression towards early part of metamorphosis, clearly indicating the involvement of the receptor genes in hormone action. From then on, NRs had been gaining attention from (invertebrate) endocrinologists. Resultantly, the investigators have unravelled not only the involvement of the ecdysteroid receptor gene (*EcR*) in moulting, but also of several other receptor genes related to various physiological activities including reproduction.

Likewise, the mode of hormone action in crustaceans was not known until the investigations of Durica and Hopkins (1996) revealed the existence of NRs for ecdysteroids (*EcR*) and retinoids (*RXR*) in the fiddler crab, *Uca pugilator*. Sequence information on the various domains of crustacean NRs and its expression, paralleled with various physiological stages became available subsequently, thanks to the investigations of several researchers (Chung et al., 1998a,b; Mykles, 2011 for review). These investigations have not only paved the way for a clearer understanding on the mode of hormone action of several invertebrates, but it as well made it obvious the indispensability of studies on receptors as an important paradigm in endocrinology.

Nuclear receptor: functional domains

NRs, grouped into a very extensive superfamily, are comprised of ligand –activated transcription factors. The high degree of conservation between all NRs at the structural level (primary and tertiary) suggests for a common evolutionary origin from an ancestral NR whose origin is not exactly known. The NR superfamily contains not only receptors for known ligands but also a large number of the so-called 'orphan' receptors for which ligands do not exist or have not yet been identified. Liganded NRs include receptors for hydrophobic molecules such as steroid hormones (estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, ecdysone and oxysterols), retinoic acids, thyroid hormones, fatty acids, leukotrienes and prostagladins. The structural diversity of the ligands contrasts with the conservation and mode of action of their receptors.

These (liganded) receptors and the large number of orphan receptors have generated much speculation on the origin and evolution of the superfamily. The phylogeny of the NR superfamily, using either the C or the E domain (please see below for details on C and E domains), separately or together, has been studied by several researchers (Laudet et al., 1997; Thornton, J.W. and DeSalle, 2000; Bridgham et al. 2010 for reviews). These studies suggest that the NRs were evolved by duplication of a unique common ancestor that contained both the C

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and E domains. The phylogenetic relationships between subfamilies have been tested and confirmed by high bootstrap values in distance and parsimony analysis, so as to have an evolution – based nomenclature for the members of the superfamily (Nuclear Receptors Nomenclature Committee, 1999).

The structural design of the nuclear receptor is entrained with its functionality. Each nuclear receptor molecule is demarcated structurally into different segments (functional domains) designated to accomplish specific function leading to hormone action (Fig 1).

A typical nuclear receptor consists of a variable NH2 terminal region (A/B domain), a conserved DNA – binding domain (DBD or C domain), a conserved ligand binding domain (LBD or E domain), and a less conserved linker or hinge domain (D domain) that bridges between the DBD and the LBD (to allow free rotation of the DBD); some receptors also contain a COOH – terminal region (F domain) of unknown function. The classical ligand-receptor pathway (also referred to as the genomic pathway) can be summarized in four steps: 1) hormone diffuses through cellular membranes and binds to the receptor; 2) receptor dimerization and activation; 3) ligand – receptor complexes translocate into nucleus; 4) liganded receptor directly binds to "hormone response elements (HRE)" on the DNA to regulate gene expression or initiate transcriptional activity (David et al., 2002). A/B domain is the most variable, both in size and sequence, and in many cases, contains a hyper-variable AF-1 domain, with autonomous transcriptional activation function. Interestingly, multiple receptor isoforms generated from a single gene by alternative splicing is known to exist in A/B domain (Aranda and Pascual, 2001 for review; Weigal and Moore, 2007).



Figure 1: Schematic representation of the organization of a nuclear receptor molecule; A/B – F are the domains. DB, DNA binding domains; LBD, ligand binding domain DBD is the most conserved domain of the nuclear receptor and it confers the ability to recognize specific target sequences in the DNA (HRE) and initiate transcriptional activity at the gene level. This domain contains nine cysteines, as well as other residues that are conserved across the nuclear receptor superfamily and required for high-affinity DNA binding. DBD comprises two 'zinc fingers' that span 60-70 amino acids and a COOH – terminal extension region.

In each zinc finger, four of the invariable cysteines coordinate tetrahedrically one zinc ion, and both zinc finger modules fold together to form a compact, interdependent structure as shown by nuclear magnetic resonance and crystallographic studies (Schwabe et al., 1993; Robinson-Rechavi et al., 2003). Amino acids required for discrimination of core DNA recognition motif are present at the base of the first Zn finger in a region called the 'P' box, and the other residues of the second zinc finger that form the so-called 'D box' are involved in dimerization. (Figure 2).

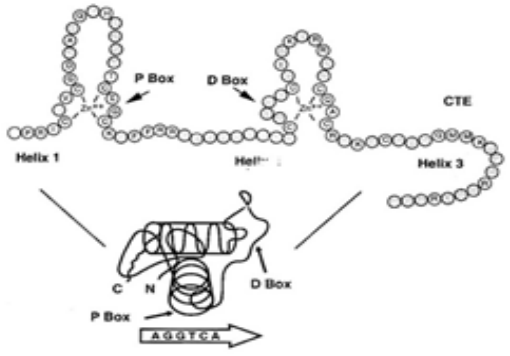

Figure 2: Diagrammatic representation of the structural design of DBD, along with the Zn fingers, P box and D box Ligand Binding Domain (LBD), as the term implies, is the region that facilitates binding of the receptor with the ligand (hormone), the primary step in eliciting the hormone action. However, this domain is also considered to be a multifunctional domain that mediates homo- and hetero-dimerization, interaction with heat-shock proteins, and also, hormone reversible transcriptional repression. The LBDs also contain two well-conserved regions: a "signature motif" and the COOH-terminal AF-2 motif responsible for ligand-dependent transcriptional activation (Aranda and Pauscal, 2001; Robinson – Rechavi et al., 2003; Weigel and Moore, 2007) (Figure 3).

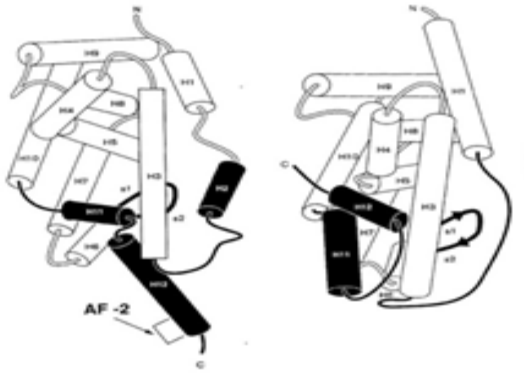

Figure 3: Schematic representation of LBD of a nuclear receptor showing the AF-2 motif To perform their extremely diverse functions, NRs are known to interact directly with the regulatory regions of target genes, called the response elements (also referred to as Hormone Response Elements, HREs), located in the regulatory sequences normally present in the 5'- flanking region of the target gene. Although the HREs are often found relatively close to the core promoter, they are also seen present in enhancer regions, several kilobases upstream of the transcriptional initiation site; in several of the instances, the ligand – receptor complexes recognize the specific sequence in question (PuGGTCA, Pu for purine for example). In general, HRE is shown to be present in two copies which can be oriented as palindromes (head to head), direct repeats or inverted palindromes (tail to tail) (Mangelsdorf et al., 1995; Huang et al., 2010). The analysis of a large number of naturally occurring as well as synthetic HREs revealed that a sequence of 6 bp constitutes the core recognition motif. The sequence AGAACA, for instance, is preferentially recognized by steroid class III receptors, whereas AGG/TTCA serves as recognition motif for the remaining receptors of the superfamily (Aranda and Pauscal 2001; Huang et al., 2010).

It has further been observed that in general, the receptor – ligand complex binds with the HRE as a combination of two units (dimers): the pairing can occur with identical molecules (homodimers) or with molecules belonging to different classes (heterodimer); in few instances though, binding can occur between a single molecule of receptor – ligand complex and the DNA (HRE) (Giguerre, 1999). Steroid receptors are known to bind almost exclusively as homodimers to the HRE.

Most of the non-steroid receptor complexes, on the other hand, bind to the HREs preferentially as heterodimers. The retinoid X receptor (RXR), for instance, is shown to be a potential (hetero)dimer partner for several of the steroid receptors (including ecdysteroid receptor). More than that, heterodimerization is shown to significantly increase the efficiency of DNA binding and transcriptional activity of the receptor in question (Manglesdorf and Evans, 1995; Amanda and Pascual, 2001).

Studies on NRs have substantially improved our understanding on the regulatory mechanisms of growth and reproduction in invertebrates

The ecdysteroid hormones act as growth promoters in several invertebrates. The function of ecdysteroids is known to be mediated

through the ecdysteroid receptor, a protein (EcR), being encoded by the ecdysteroid receptor gene (*EcR*). Researchers have reached this contention through the fluctuations shown by the *EcR* expression in a stage-dependant manner related to growth. Insects became the first model organism wherein the *EcR* was detected and the expression was studied.

Subsequently, the gene (*EcR*) was detected and sequenced in other invertebrate groups as well. Crustaceans became the other major group wherein the occurrence and expression of *EcR* were studied. Interestingly, at the beginning of the 21st century, in insects, it has also been shown that the ecdysteroids involve not only in growth, but in reproduction as well. Carney and Bender (2000) in their pioneering work, have demonstrated that ecdysteroid receptor is a requisite for successful vitellogenesis in *Drosophila*. During subsequent years, researchers have unravelled the role of *EcR* in insect reproduction. In the mosquito *Aedesaegypti*, for example, the *broad* (*br*) gene is shown to be involved in 20E regulatory hierarchy controlling vitellogenesis (Chen et al., 2004).

Investigations have also led to the identification of retinoid hormone receptors [encoded by the ultraspiracle gene (*USP*) in insects and the retinoid X receptor (*RXR*) in crustaceans, a homolog of vertebrate *RXR*] that dimerize with the EcR to accomplish the hormone action. These results have encouraged the carcinologists to hypothesize that a comparable situation might occur in crustaceans as well. Pertinently enough, 20 hydroxy-ecdysone was shown to stimulate the vitellogenin gene (*PmVg1*) expression in *Penaeus monodon* (Tiu et al., 2006; Nagaraju, 2011), and steroids from vertebrates were shown to stimulate vitellogenesisin crustaceans (Lafont et al., 2005; Brown et al., 2009; Coccia et al., 2010; Subramoniam and Kirubagaran, 2011). A series of experiments conducted in our laboratory of late, have shown fluctuating levels of*EcR*expression in the ovary and hepatopancreas of the brachyuran crab *Metopograpsus messor* (*MmEcR*) in parallel with ecdysteroid levels; the expression was significantly more in breeding females than those of the non-breeding ones, implicating a possible involvement of ecdysteroid receptors (and ecdysteroids) in reproduction. The results also reveal the ovary as a putative target for ecdysteroid hormone (Shyamal et al., 2014; 2015).

Interestingly, the experiments performed on *Drosophila* and *Tribolium* have helped us understand about a putative receptor (the 'Met' receptor) for the terpenoid hormone (JH), analog of MF, the gonadotropic hormone in crustaceans (Yamanaka et al., 2013; Dubrovsky and Bernardo, 2014). This discovery is of seminal importance to invertebrate endocrinology inasmuch as, till date, any receptor that binds with a gonadotropic hormone has been unknown in the entire group. Subsequent experiments have shown that in insects, Met heterodimerizes with steroid receptor coactivator (SRC), which in turn may function as a co-activator for ecdysone signalling.

Met was found to interact with a crustacean homolog of SRC in presence of JH III. Interestingly, the Met/SRC interaction was also found to be 10 times more sensitive to MF than to JH III. Further, of late, a Met homolog was identified in cladoceran crustaceans such as *Daphnia pulex* and *D. magna*, suggesting that MF (the JH analogue) could be a potential ligand for Met in crustaceans. This is further supported by the finding of a Met ortholog in the fiddler crab, *Uca pugilator* (Shyamal et al., 2016).

However, admittedly, more studies are to be furthered so as to better understand the exact mechanism of the sequence of events, beginning from the ecdysteroid hormone, ending up with vitellogenesis in several of the invertebrates especially, the crustaceans.

CONCLUDING REMARKS

The importance of pursuing the role of ecdysteroids in crustacean reproduction in a more systematic way is being increasingly felt (Nagaraju, 2011; Sudha et al., 2012).

Future studies involving (steroid) hormone–receptor–HRE cascade should help us address this question very effectively. Pertinently enough, the results of the Ribonuclease Protection Assay (RPA) demonstrating the occurrence of mRNA encoding the ecdysteroid receptor (UpEcR) in the ovary of the fiddler crab, *Ucapugilator* (Durica et al., 2002; Sudha and Anilkumar, 2007*),* and the expression studies conducted by Shyamal et al. (2014, 2015), as shown above, seem to strongly designate the crustacean ovary as a potential target for ecdysteroids Nevertheless, the exact cascade of events bridging between the ecdysteroids and vitellogenin synthesis is still unclear. Further, through a recent, meticulously designed experiment, Das and Durica (2013) have successfully demonstrated the use of RNAi for the knockdown of the ecdysteroid receptor gene (*EcR*) in *U. pugilator*, for disrupting the mRNA levels, leading to growth arrest. Obviously, future experiments, targeting the nuclear receptors, could offer us valuable clues to demonstrate precisely the sequence of events involved in the regulation of moulting and reproduction in invertebrates in general, but crustaceans in particular.

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