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Phenotypic and molecular analysis of the effect of 20-hydroxyecdysone on the human filarial parasite *Brugia malayi*

Amruta S. Mhashilkar [a], Swamy R. Adapa [a], Rays H.Y. Jiang [a], Steven A. Williams [b], Weam Zaky [b], Barton E. Slatko [c], Ashley N. Luck [c], Andrew R. Moorhead [d], Thomas R. Unnasch [a,*]

[a] Department of Global Health, College of Public Health, University of South Florida, Tampa, FL 33612, USA
[b] Department of Biological Sciences, 100 Green Street, Ford Hall, Smith College, Northampton, MA 01063, USA
[c] Genome Biology Division, Molecular Parasitology Group, New England Biolabs, 240 County Road, Ipswich, MA 01938, USA
[d] Department of Infectious Diseases, University of Georgia, College of Veterinary Medicine, 501 D. W. Brooks Drive, Athens, GA 30602, USA

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ABSTRACT

A homologue of the ecdysone receptor has been identified and shown to be responsive to 20-hydroxyecdysone in *Brugia malayi*. However, the role of this master regulator of insect development has not been delineated in filarial nematodes. Gravid adult female *B. malayi* cultured in the presence of 20-hydroxyecdysone produced significantly more microfilariae and abortive immature progeny than control worms, implicating the ecdysone receptor in regulation of embryogenesis and microfilarial development. Transcriptome analyses identified 30 genes whose expression was significantly up-regulated in 20-hydroxyecdysone-treated parasites compared with untreated controls. Of these, 18% were identified to be regulating transcription. A comparative proteomic analysis revealed 932 proteins to be present in greater amounts in extracts of 20-hydroxyecdysone-treated adult females than in extracts prepared from worms cultured in the absence of the hormone. Of the proteins exhibiting a greater than two-fold difference in the 20-hydroxyecdysone-treated versus untreated parasite extracts, 16% were involved in transcriptional regulation. RNA interference (RNAi) phenotype analysis of *Caenorhabditis elegans* orthologs revealed that phenotypes involved in developmental processes associated with embryogenesis were significantly over-represented in the transcripts and proteins that were up-regulated by exposure to 20-hydroxyecdysone. Taken together, the transcriptomic, proteomic and phenotypic data suggest that the filarial ecdysone receptor may play a role analogous to that in insects, where it serves as a regulator of egg development.

1. Introduction

Human filarial parasites cause diseases that inflict significant morbidity upon a large proportion of the poorest people on the planet (World Health Organization (WHO), 2010). Lymphatic filariasis (caused by infection with *Brugia malayi*, *Brugia timori* or *Wuchereria bancrofti*) and onchocerciasis (caused by infection with *Onchocerca volvulus*) together result in the loss of 5.7 million disability adjusted life years (Mathers et al., 2007). As a result, these diseases have been identified by the international community as two of the five Neglected Tropical Diseases (NTDs) worldwide, and both have been targeted for elimination by the international community in the London Declaration on Neglected Tropical Diseases (Turner et al., 2014).

Elimination programs targeting both onchocerciasis and filariasis have been implemented at the national and international levels. All rely primarily upon a strategy of mass drug distribution to interrupt transmission and thereby eventually locally eliminate the parasite (Cupp et al., 2011). However, these programs rely upon a small arsenal of drugs that must be given over a long period of time (i.e., years). This leaves the programs vulnerable to failure in the face of developing resistance. Furthermore, the prolonged treatment courses necessary for effective elimination present substantial logistical difficulties resulting from the need to maintain high drug coverage rates over a long period of time (i.e., years). The current drug regimens used by these programs face limitations in deployment in many areas. For example, diethylcarbamazine (DEC), a drug commonly used to treat lymphatic filariasis, produces severe ocular and systemic complications when given to

* Corresponding author at: Department of Global Health, 3720 Spectrum Blvd, Suite 304, Tampa, FL 33612, USA. Tel.: +1 813 974 0507; fax: +1 813 974 0992.
E-mail address: tunnasch@health.usf.edu (T.R. Unnasch).

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individuals infected with *O. volvulus* (Awadzi, 2003). This precludes the use of DEC in much of Africa, where lymphatic filariasis and onchocerciasis are co-endemic. Similarly, treatment of onchocerciasis using ivermectin is complicated in areas that are co-endemic for the eye-worm *Loa loa*, as severe adverse events have been documented to occur in individuals treated with ivermectin that are heavily infected with *L. loa* (Twum-Danso, 2003). For these reasons, there is an urgent need to develop alternative therapeutic interventions to augment the efforts of the elimination programs.

Ecdysteroids have long been known to play a central role in controlling the development of various invertebrates. They have been best characterised in insects. These hormones exhibit their effects through the activity of ecdysteroid receptors, which act as master transcriptional regulators (Koelle et al., 1991; Baehrecke, 1996). In insects, juvenile hormone and ecdysone regulate both egg development and the molting cycle. As juvenile hormone levels decrease, there is a surge in ecdysone levels leading to molting (Riddiford, 1993). This effect is mediated through a heterodimer of the ecdysone receptor (EcR) and the retinoid X receptor (RXR), two members of the nuclear hormone receptor family of transcriptional regulators (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993). The fact that molting and the receptors controlling this process are not found in vertebrates makes this process an attractive potential chemotherapeutic target.

A homolog of the EcR has been identified and shown to be active in *B. malayi* (Tzertzinis et al., 2010). However, its physiological role in controlling the developmental processes in this parasite remains unclear (Mendis et al., 1983; Tzertzinis et al., 2010). In an attempt to decipher the physiological role of the *B. malayi* ecdysone receptor (*Bma*EcR), we have conducted transcriptomic, proteomic and phenotypic studies of the effect on 20-hydroxyecdysone (20E) on gravid adult female *B. malayi* worms.

2. Materials and methods

2.1. Phenotypic studies of the effect of 20E on fecund adult female worms

Gravid adult female parasites were obtained from the Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, USA. A total of five worms per well were cultured in a 6-well plate using 3 ml of CF-RPMI media (RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml of streptomycin, 100 μg/ml of penicillin, 0.25 μg/ml of amphotericin B, and 10% heat-inactivated FBS). Experiments were designed to consist of two replicates of parasites treated with 20E and two replicate control cultures. When the parasites were received, they were allowed to acclimatise and monitored for any decrease in motility for 24 h (day 0). The 20E was added to the media of the two experimental wells at a concentration of 10 μM on day 1. Control wells received ethanol (the vehicle of 20E). Media (either containing or lacking 20E) were changed every 24 h, and the parasites cultured for an additional 3 days (days 2–4). Three aliquots of 20 μl of media were removed from each biological replicate every 24 h and the life stages released into media were counted, resulting in three technical replicates for each biological replicate for each time point. The life stages observed were counted and classified as microfilariae, pre-microfilariae or eggs/embryos. The experiment was repeated five times over a period of 1 year, with parasites isolated from different infected animals.

2.2. RNA extraction

Fecund adult females, cultured as described in Section 2.1, were used to prepare RNA for the transcriptomic analysis. Total RNA was isolated from the two biological replicates of five worms each (two wells each of treated and untreated parasites) after 2 days culture with and without 20E. The worms were flash frozen in liquid nitrogen. RNA was extracted from the worms using Trizol LS (Invitrogen, Carlsbad, CA, USA) (Griffiths et al., 2009; Choi et al., 2011). The biological replicates of the 20E-treated and untreated samples were lysed individually using TissueLyser II (Qiagen, Valencia, CA, USA) followed by chloroform extraction, isopropanol precipitation and elution in 0.1 × TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The samples were treated with DNase I (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The RNAs were subjected to drop dialysis using 45 nm Millipore membranes (EMD Millipore, Billerica, MA, USA) against 0.1 × TE buffer at 4 °C for 2–4 h and the RNA was then collected from the membranes. Purity of the samples was assessed using a NanoDrop apparatus (Thermo Scientific, Waltham, MA, USA). The quantity of RNA was determined using a Qubit apparatus (Thermo Fisher, Carlsbad, CA, USA). The purified RNA was stored at −80 °C.

2.3. RNA library preparation

The NEBNext poly(A) mRNA Magnetic Isolation Module (New England Biolabs Inc., Ipswich, MA, USA; # E7490) was used to isolate intact poly(A)+ RNA from each previously isolated total RNA preparation. The eluted RNA was used for first and second strand cDNA synthesis using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA; # E7530). The double stranded cDNA was size selected and the fractions between 250 and 550 bp in size were isolated using 1.8× Agencourt AMPure XP beads. End repair and dA tailing of cDNA library was performed, immediately followed by adaptor ligation. NEBNext Multiplex Oligos for illumina were used for adaptor ligation. The cDNA libraries were PCR amplified and purified. Quality control was performed using a Bioanalyzer (Agilent, Santa Clara, CA, USA) to analyze the quality and size selection of the cDNA. The samples were again subjected to Qubit to quantify the yield of cDNA.

2.4. Transcriptome sequencing (RNA-seq)

RNA-seq was performed on an Illumina MiSeq at the Core facility, Smith College, Northampton, MA, USA as part of the FR3 Repository, following the manufacturer's protocol. In brief, the cDNA library (at a concentration of 4 nM) was denatured using 0.2 N sodium hydroxide. The library was diluted to 20 pM in Illuminia hybridisation buffer, and the template strands hybridised to the adaptors attached to the flowcell surface as previously described (Choi et al., 2011). The library was diluted to 9 pM with Illumina HT1 buffer. The PhiX control was used as an internal standard. The PhiX standards were denatured and diluted using the same protocol as the sample library. The libraries were heated at 96 °C for 2 min followed by immediate cooling. Samples were loaded into an Illumina cartridge and single-end reads produced. The raw reads from the RNA-seq experiment are available in the National Center for Biotechnology Information (NCBI) short read archive accession **SRP064921**.

2.5. Data analysis

The Tuxedo suite of programs was used to process and analyze the data (Trapnell et al., 2012). Bowtie2 was used to build indices of the *B. malayi* reference genome from Wormbase (v. WS245) (Langmead et al., 2009). RNA-seq reads from each sample were aligned to the *B. malayi* genome using TopHat (v. 1.4.1) (Trapnell and Salzberg, 2009). A maximum of one mismatch per read was allowed. The mapped reads from TopHat were used to assemble