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Direct effects of hypoxia and nitric oxide on ecdysone secretion by insect prothoracic glands

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ABSTRACT

Insect molting and metamorphosis are controlled by the molt stimulating hormone ecdysone. A recent study suggests that reduced tissue oxygenation correlates with the size-sensing mechanism responsible for triggering molting. When reared in hypoxia, larvae of *Manduca sexta* and *Drosophila melanogaster* initiate molting at lower weights than do larvae reared in normoxia. Furthermore, in *Drosophila*, the signaling gas nitric oxide (NO) appears to be required for normal developmental timing. As observed in *Drosophila*, NO signaling targets the nuclear hormone receptor beta fushi tarazu transcription factor 1 (βFTZ-F1) through activation of *Drosophila* hormone receptor 3 (DHR3), two key regulators of ecdysone production and metamorphic tissue progression. We set out to directly examine the effects of hypoxia and NO on ecdysone secretion using prothoracic glands from feeding fifth (last) larval stage *M. sexta*. Our results indicate that in vitro treatment of prothoracic glands with hypoxia (2% oxygen) or the NO donor DETA-NONOate significantly inhibit ecdysone secretion. Protein markers of glandular activity were also in keeping with an initial inhibition, measured a decrease in phosphorylated ERK (extracellular signal regulated kinase) and an increase in non-phosphorylated 4EBP (eukaryotic initiation factor 4E binding protein). Additionally, gene expression levels of *Manduca* hormone receptor 3 (*mhr3*), β*ftz-f1*, nitric oxide synthase (*nos*), and the PTTH receptor *torso*, were quantified using real-time PCR. NO treatment increased *mhr3* expression and decreased *nos* expression. Hypoxia increased *mhr3* transcription after 2 hr, but decreased transcription after 12 hr, with no effect on *nos* expression. Both NO and hypoxia had small effects on β*ftz-f1* expression, yet strongly increased *torso* transcription. Our results demonstrate that, in isolated prothoracic glands, hypoxia and NO signaling directly inhibit ecdysteroid secretion, but at the same time alter aspects of prothoracic gland function that may enhance secretory response.

1. Introduction

The initiation of insect molting and metamorphosis have been correlated with a size threshold known as the critical weight (Nijhout and Williams, 1974; Nijhout, 1994). The mechanism by which insects determine the critical weight remains under active investigation (Callier and Nijhout, 2013a). A key hormone in the regulation of molting is the neuropeptide prothoracicotropic hormone (PTTH), which binds the PTTH receptor Torso and activates pathways in the prothoracic glands (PG) involved in the production of ecdysteroid molting hormones (Rewitz et al., 2009). Ecdysteroids then coordinate the orderly expression of genes necessary for growth and development.

Recently, gaseous signaling molecules have been linked to the regulation of insect development. When *Manduca sexta* larvae are reared under hypoxic conditions to simulate growth-induced hypoxia, they initiate molting at a lower weight than normoxic controls (Callier and Nijhout, 2011). A similar phenomenon was recently seen in *Drosophila melanogaster* (Callier et al., 2013b). These results suggest that a reduction in oxygen may serve as the insect size-sensing mechanism that corresponds with an observable critical weight. Other recent studies in *Drosophila* have focused on the involvement of nitric oxide gas (NO) on PG function. NO is a product of nitric oxide synthase (NOS) and is a

Abbreviations: AMP, adenosine monophosphate; βFTZ-F1, beta-fushi tarazu transcription factor 1; DHR3, *Drosophila* hormone receptor 3; E75, ecdysone-inducible gene 75; 4EBP, eukaryotic translation initiation factor 4E-binding protein; ERK, extracellular signal-regulated kinase; HRE, hypoxia response elements; HIF-1α, hypoxia inducible factor 1 alpha; LPS/IFNγ, lipopolysaccharide/interferon gamma; MAPK, mitogen-activated protein kinase; mTOR1, mammalian target of rapamycin 1; NO, nitric oxide; NOS, nitric oxide synthase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B; PG, prothoracic glands; RSK, p90 ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase.

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diffusible second messenger that influences a variety of cellular functions in vertebrates and invertebrates (Alderton et al., 2001; Davies, 2000; Koch et al., 1994; Yamanaka and O'Connor, 2011). In insects, NO has been implicated in the regulation of fluid production, cell proliferation, synapse formation, and innate immunity (Davies, 2000; Yamanaka and O'Connor, 2011). In *Drosophila*, the PG express high levels of NOS, and knockdown of NOS in the PG prolongs larval feeding, increases lipid storage, and blocks metamorphosis (Caceres et al., 2011). The pathway by which NO regulates the PG appears to be through βFTZ-F1, the insect homolog of the vertebrate steroidogenic factor 1, which regulates vertebrate steroidogenic enzyme transcription (Asahina et al., 2000; von Hofsten and Olsson, 2005; Yamanaka and O'Connor, 2011). The model whereby NO has been postulated to regulate steroid secretion in *Drosophila* is schematized in Fig. 1. Specifically, NO has been suggested to directly bind and inhibit the *Drosophila* nuclear transcription factor E75, a negative regulator of its heterodimer partner *Drosophila* hormone receptor 3 (DHR3) (Reinking et al., 2005). Uninhibited DHR3 then enhances the transcription of *βftz-f1* (Caceres et al., 2011). In *Drosophila*, βFTZ-F1 controls the expression of ecdysone synthetic enzymes, hence serving as a key competence factor for metamorphosis (Broadus et al., 1999; King-Jones and Thummel, 2005; Yamanaka and O'Connor, 2011).

The above studies (tissue oxygenation in *Manduca* and NO signaling in *Drosophila*) bring to light two phenomena that may play critical roles in coordinating a size-sensing mechanism with a molt signaling pathway. Linking these observations, oxygen limitation has been observed in other studies to not only control body size in insects (Kaiser et al., 2007), but also to influence NO signaling (Dijkers and O'Farrell, 2009; Ducsay and Myers, 2011; Gess et al., 1997; Wingrove and O'Farrell, 1999). It is possible that a size-related reduction in oxygen activates nitric oxide synthase in the PG, in turn enhancing ecdysone secretion. These observations could help to explain early molting in hypoxia-reared *M. sexta* and delayed molting in NOS-deficient *Drosophila*. In the present study, we tested the hypotheses that hypoxia and NO stimulate ecdysone secretion, using the PG of *M. sexta*, a well-established model for the study of insect steroidogenesis (Smith and Rybczynski, 2011). We measured in vitro levels of ecdysone secretion after direct stimulation with NO or hypoxia, and evaluated changes in the transcription of MHR3, βFTZ-F1, and the PTTH receptor Torso. Our results indicate that in the PG of *M. sexta*, NO and hypoxia inhibit, rather than directly stimulate, secretion. Nonetheless, stimulatory effects on transcription suggested a priming effect on glandular response.

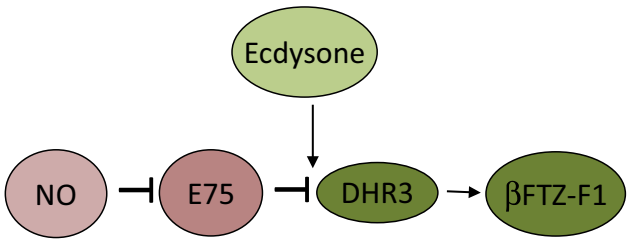

Fig. 1. Model for regulation of prothoracic gland activity by nitric oxide (NO). NO is proposed to bind and inhibit the ecdysone-inducible nuclear receptor E75, preventing E75 from blocking DHR3 (*Drosophila* hormone response element 3). DHR3 can then activate βFtz-F1 (beta fushi tarazu transcription factor 1). βFtz-F1, among other actions, regulates the transcription of steroidogenic genes. Modified from Caceres et al. (2011); Yamanaka and O'Connor (2011).

2. Materials and methods

2.1. Animals and prothoracic glands

Manduca sexta used in this study were from a strain obtained from Carolina Biological Supply (Burlington, NC). Larvae were reared on standard artificial laboratory diet (Bell and Joachim, 1976) at 25 °C on a long-day 16 hr:8 hr light:dark cycle. Larvae were transferred to fresh diet at the time of ecdysis to the fifth (final) larval instar (day 0), and were used for experiments on day 2 of the fifth instar (4.5–5.3 g). Larvae were anesthetized in water for 10 min and their prothoracic glands were removed under lepidopteran saline (Riddiford, 1978). Larvae were age/weight matched and prothoracic gland pairs were separated to ensure control and treated samples contained a single gland from each organism.

2.2. Nitric oxide/hypoxia incubation

Manduca sexta larval prothoracic glands were incubated for 2 hr or 12 hr in Grace's insect medium (2 glands/100 μl droplet) at room temperature. Glands were tested as matched pairs: one gland from a given animal was subjected to the experimental treatment, and the contralateral gland subjected to the control treatment. *Manduca* prothoracic glands are arranged as a natural monolayer of cells no greater than 100 μm thick (Hanton et al., 1993), a size that suffices for diffusion (see for example, the 200 μm diffusion limit observed in recent study of mammalian cells by Grimes et al. (2014)). Hence the glands were not incubated with trachea in an air-filled condition. Similar in vitro protocols are routinely used for assays of ecdysone secretion by insect prothoracic glands, for incubation periods ranging in length from 2 to 24 hr (*Bombyx mori* (Gu et al., 2009; Mizoguchi and Kataoka, 2005), *Pseudaletia separata* (Komiya et al., 2001), *Rhodnius prolixus* (Vafopoulou and Steel, 1998), *M. sexta* (Bollenbacher et al., 1984; Walsh and Smith, 2011; Watson et al., 1987)).

In the present study, glands were incubated under one of the following conditions: normoxic (21% oxygen), hypoxic (2% oxygen:98% nitrogen), or normoxic in the presence of DETA-NONOate sodium salt (Sigma–Aldrich, St. Louis, MO). Hypoxic conditions were generated by placing samples in an airtight modular incubator (Billups-Rothenberg, Del Mar, CA). The chamber was flushed with 2% oxygen:98% nitrogen for 10 min. The chamber was sealed for 2 hr or 12 hr after it reached the designated oxygen level. The percentage of oxygen in the airspace of the incubator was monitored with a ToxiRAE II oxygen sensor (Rae Systems, San Jose, CA) at the start and end of each experiment, and the percentage of oxygen was found to remain constant for the duration of the experiment.

For Western blots, prothoracic glands (4 glands/sample) were heated at 90 °C for 3 min in 25 μl 2X SDS sample buffer and subsequently stored at −20 °C. For RNA extractions, prothoracic glands (12 glands/sample) were flash frozen, and stored at −80 °C. For measurements of ecdysone secretion, incubation media were collected in 0.5 ml centrifuge tubes and stored at −20 °C for radioimmunoassay.

2.3. Viability assay

Trypan blue cell viability assays were conducted after 12 hr treatment with either DETA-NONOate, 2% oxygen:98% nitrogen, or Grace's medium alone. Prothoracic glands were incubated directly in 0.4% trypan blue (Beckman Coulter, Brea, CA) for 2 and 5 min. Non-viable controls were generated by exposing prothoracic glands to ultraviolet light for 5 min post incubation. The presence of trypan blue reagent within cells denotes non-viability.