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Functional divergence of dafachronic acid pathways in the control of *C. elegans* development and lifespan

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ABSTRACT

Steroid hormone and insulin/insulin-like growth factor signaling (IIS) pathways control development and lifespan in the nematode *Caenorhabditis elegans* by regulating the activity of the nuclear receptor DAF-12 and the FoxO transcription factor DAF-16, respectively. The DAF-12 ligands Δ^4- and Δ^7-dafachronic acid (DA) promote bypass of the dauer diapause and proper gonadal migration during larval development; in adults, DAs influence lifespan. Whether Δ^4- and Δ^7-DA have unique biological functions is not known. We identified the 3-β-hydroxysteroid dehydrogenase (3βHSD) family member HSD-1, which participates in Δ^4-DA biosynthesis, as an inhibitor of DAF-16/FoxO activity. Whereas IIS promotes the cytoplasmic sequestration of DAF-16/FoxO, HSD-1 inhibits nuclear DAF-16/FoxO activity without affecting DAF-16/FoxO subcellular localization. Thus, HSD-1 and IIS inhibit DAF-16/FoxO activity *via* distinct and complementary mechanisms. In adults, HSD-1 was required for full lifespan extension in IIS mutants, indicating that HSD-1 interactions with IIS are context-dependent. In contrast to the Δ^7-DA biosynthetic enzyme DAF-36, HSD-1 is dispensable for proper gonadal migration and lifespan extension induced by germline ablation. These findings provide insights into the molecular interface between DA and IIS pathways and suggest that Δ^4- and Δ^7-DA pathways have unique as well as overlapping biological functions in the control of development and lifespan.

Introduction

In replete environments, *Caenorhabditis elegans* larvae undergo four molts during reproductive development. When faced with unfavorable conditions, they enter diapause, arresting in an alternative third larval stage known as dauer. Dauers are adapted for survival and dispersal and resume development to adulthood once ambient conditions improve (Riddle, 1988).

The elucidation of signal transduction pathways that regulate dauer arrest has provided a glimpse into how organisms integrate environmental cues with developmental programs. Genetic analysis has defined an intricate signaling network that controls the transition to diapause (Fielenbach and Antebi, 2008; Hu, 2007). A pathway regulated by the DAF-11 guanylyl cyclase (Birnby et al., 2000) acts in sensory neurons to promote reproductive development by potentiating the expression of insulin-like (Li et al., 2003; Pierce et al., 2001) and transforming growth factor-beta (TGFβ)-like (Murakami et al., 2001; Ren et al., 1996; Schackwitz et al., 1996) ligands. These ligands regulate gene expression by activating conserved insulin-like and TGFβ-like signal transduction pathways in target tissues.

A hormone biosynthetic pathway also regulates dauer arrest by synthesizing dafachronic acids (DAs), which are steroid ligands for the nuclear receptor DAF-12 (Motola et al., 2006). The cytochrome P450 DAF-9 (Gerisch et al., 2001; Jia et al., 2002) acts on distinct precursors to generate two DAs, Δ^4- and Δ^7-DA (Motola et al., 2006). Precursors for Δ^4- and Δ^7-DA are thought to be synthesized by the 3-β-hydroxysteroid dehydrogenase (3βHSD) family member HSD-1 (Patel et al., 2008) and the Rieske oxygenase DAF-36 (Rottiers et al., 2006), respectively (Fig. 1). DA binding to DAF-12 promotes reproductive development, whereas unliganded DAF-12 promotes dauer arrest (Motola et al., 2006). Although synthetic Δ^7-DA is more potent than synthetic Δ^4-DA in dauer rescue bioassays (Sharma et al., 2009), exogenous Δ^4-DA rescues *daf-36* mutant phenotypes (Gerisch et al., 2007; Rottiers et al., 2006), indicating that Δ^4-DA can compensate for a reduction in Δ^7-DA *in vivo*. Whether Δ^4-DA and Δ^7-DA have distinct biological functions is not known.

daf-12 mutations suppress dauer arrest in *daf-11* and *daf-7/TGFβ* pathway mutants (Thomas et al., 1993), indicating that DAF-12 is required for dauer arrest in these mutants and suggesting that DAF-12 acts downstream of DAF-11 and DAF-7/TGFβ pathways. In contrast,

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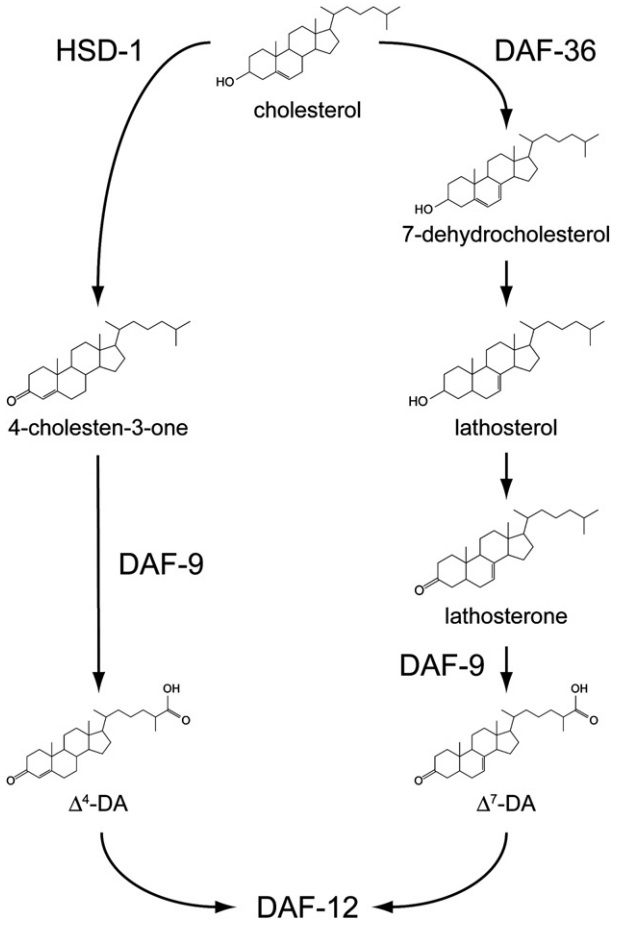

Fig. 1. Hypothetical model of dafachronic acid (DA) biosynthetic pathways. Adapted from Rottiers et al. (2006) and Patel et al. (2008).

the relationship between the DA pathway and the insulin-like pathway appears to be complex. Whereas reduced insulin-like signaling results in constitutive dauer arrest, *daf-12* mutations cause a synthetic non-dauer larval arrest phenotype in the context of reduced insulin-like signaling (Larsen et al., 1995; Vowels and Thomas, 1992). Furthermore, exogenous Δ^4-DA fully rescues dauer arrest in *daf-9* and *daf-7/TGFβ* mutants but causes non-dauer larval arrest in some strains harboring mutations in the insulin receptor (InsR) ortholog DAF-2 (Motola et al., 2006). The molecular basis for these interactions between DA and insulin-like signaling remains poorly understood.

Upon engagement by insulin-like ligands, DAF-2/InsR promotes reproductive development by activating a conserved phosphoinositide 3-kinase (PI3K)/Akt pathway (Morris et al., 1996; Paradis et al., 1999; Paradis and Ruvkun, 1998). Dauer arrest caused by reductions in DAF-2/InsR signaling is fully suppressed by mutations in the FoxO transcription factor DAF-16 (Gottlieb and Ruvkun, 1994; Vowels and Thomas, 1992), indicating that DAF-16/FoxO is the major target of DAF-2/InsR pathway signaling and that DAF-2/InsR antagonizes DAF-16/FoxO. DAF-2/InsR activation of PI3K/Akt signaling results in DAF-16/FoxO phosphorylation by Akt and its subsequent cytoplasmic sequestration *via* binding to 14-3-3 proteins (Berdichevsky et al., 2006; Hertweck et al., 2004; Lee et al., 2001; Li et al., 2007; Lin et al., 2001).

Although the inhibition of FoxO transcription factors by Akt in both *C. elegans* and mammals is well established, multiple lines of evidence support the existence of a second pathway that acts in parallel to PI3K/Akt signaling to inhibit FoxO. DAF-16/FoxO that is constitutively nuclear by virtue of either mutation of its consensus Akt/PKB phosphorylation sites or inactivation of either AKT-1 or the 14-3-3 protein FTT-2 is not fully active (Berdichevsky et al., 2006; Hertweck et al., 2004; Lin et al., 2001; Zhang et al., 2008), suggesting that a pathway acting in parallel to AKT-1 inhibits the activity of nuclear DAF-16/FoxO. To identify components of this pathway, we performed a genetic screen for enhancers of the dauer arrest phenotype seen in *akt-1* null mutants (*eak* mutants) (Hu et al., 2006; Zhang et al., 2008). Here we report the cloning and characterization of *eak-2*. Surprisingly, *eak-2* is allelic to *hsd-1* (Patel et al., 2008). This finding provided us with an opportunity to examine the interface between *C. elegans* insulin-like and DA pathways in dauer regulation and lifespan control.

Materials and methods

Strains

The following strains were used: N2 Bristol (wild-type), *hsd-1 (mg345)*, *hsd-1(mg433)* (Patel et al., 2008), *daf-9(k182)* (Gerisch et al., 2001), *daf-36(k114)* (Rottiers et al., 2006), *glp-1(e2141)* (Priess et al., 1987), *daf-16(mgDf47)* (Ogg et al., 1997), *daf-12(rh61rh411)* (Antebi et al., 2000), *akt-1(mg306)* (Hu et al., 2006), *eak-3(mg344)* (Zhang et al., 2008), *daf-2(e1370)* (Kimura et al., 1997), *sqt-1(sc13) age-1(hx546)* (Morris et al., 1996), *pdk-1(sa709)* (Paradis et al., 1999), *eak-4(mg348)* (Hu et al., 2006), and *sdf-9(mg337)* (Hu et al., 2006). DAF-16::GFP localization was assayed using strain TJ356 (*zIs356)* IV, which contains an integrated C-terminal translational fusion of the DAF-16A isoform in-frame to GFP (Henderson and Johnson, 2001). Strains CF1330 and CF1371 harbor extrachromosomal arrays encoding wild-type GFP::DAF-16/FoxO and a constitutively nuclear GFP::DAF-16/FoxO mutant lacking all AKT phosphorylation sites, respectively (Lin et al., 2001).

eak-2 mutant isolation, mapping, and sequencing

Isolation, mapping, and sequencing of *eak-2* alleles were performed as described for *eak-3* (Zhang et al., 2008).

hsd-1 cDNA isolation

hsd-1 cDNA isolation was performed as described for *eak-3* (Zhang et al., 2008), except that total RNA was isolated from fluorescent XXX cells that had been FACS-purified from cultured embryonic cells containing an integrated *sdf-9*p::RFP transgene (Hu et al., 2006).

hsd-1p::GFP construction and analysis

The *hsd-1* promoter (corresponding to nucleotides 24,173–24,413 of YAC Y6B3B, as annotated by the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) was amplified from genomic DNA, a fragment containing GFP and the *unc-54* 3′ UTR was amplified using pPD95.75 as a template, and the two PCR products were fused using overlap extension PCR to create *hsd-1*p::GFP. *hsd-1*p::GFP and the transformation marker plasmid pRF4 were coinjected at concentrations of 40 ng/μl and 60 ng/μl respectively into animals containing an integrated *sdf-9*p::RFP transgene. Transgenic animals were generated, animals were visualized using an Olympus BX61 upright microscope, and fluorescence was analyzed using SlideBook 4.1 digital microscopy software (Intelligent Imaging Innovations, Inc., Denver, CO, USA) as described (Hu et al., 2006).

Gonadal migration assays

Egg lays were performed on standard NGM plates at 20 °C, and L4 larvae were scored for gonadal migration defects (Mig phenotype) using a Nikon SMZ800 stereomicroscope. Mig phenotypes were confirmed by visualization on an Olympus BX61 upright microscope at 200× magnification.