Conversion of 3-oxo steroids into ecdysteroids triggers molting and expression of 20E-inducible genes in *Drosophila melanogaster*

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ABSTRACT

Ecdysteroids, steroid hormones in insects, coordinate major developmental transitions. During postembryonic development, ecdysone is biosynthesized from dietary cholesterol in the prothoracic gland (PG). Despite extensive studies, the initial conversion process, the so-called "Black Box", has not been characterized. A cytochrome P450 enzyme, Spookier (Spok), is speculated as a rate limiting enzyme in the Black Box during larval-pupal transitions in *Drosophila melanogaster*. RNAi mediated knockdown of *spok* expression in the PG results in arrest of molting. Because the developmental arrest can be rescued by application of an appropriate intermediate, we examined potential activities of candidate intermediates in the RNAi-treated larvae. We found that two 3-oxo steroids, cholesta-4,7-diene-3,6-dione-14α-ol (Δ^4-diketol) and 5β [H]cholesta-7-ene-3,6-dione-14α-ol (diketol), triggered molting of the RNAi-treated larvae. We also detected an enhancement of the amounts of ecdysteroids in the RNAi-treated larvae by feeding the Δ^4-diketol or diketol, indicating that the dietary 3-oxo steroids were incorporated and converted into ecdysteroids *in vivo*. Furthermore, 20-hydroxyecdysone inducible genes were induced in the RNAi-treated larvae by feeding the Δ^4-diketol or diketol. These results indicate that Δ^4-diketol and diketol are components of the ecdysteroid biosynthetic pathway and lie downstream of a step catalyzed by Spok.

1. Introduction

In insects, developmental transitions including molting and metamorphosis are triggered by pulses of steroid hormones. The most common hormone is the ecdysteroid, 20-hydroxyecdysone (20E), which is derived from ecdysone (E). E is biosynthesized from dietary cholesterol in the prothoracic gland (PG), released into hemolymph and converted into 20E in the peripheral tissues. The biosynthetic pathway has been extensively studied, but the critical steps including a rate limiting step, the so-called "Black Box", have not been elucidated [1].

The first step in the biosynthetic pathway in the PG is the conversion of cholesterol to 7-dehydrocholesterol (7dC) [2–4]. The steps from 7dC to the diketol help to build the ecdysteroid skeleton, the structure of which is characterized by a *cis* junction of rings A and B, a 7-ene-6-one chromophore, and a *trans* junction of rings C and D (Fig. 1A). However, no intermediates in these Black

Box reactions have been isolated or detected from arthropods, including insects, because of their instability and/or small concentration in the PG. From tracer experiments using radiolabeled compounds, the 3-oxo steroids, cholesta-4,7-diene-3,6-dione-14α-ol (Δ^4-diketol) and its reduced derivative, 5β [H]cholesta-7-ene-3,6-dione-14α-ol (diketol), have been considered as the first ecdysteroid-like precursors after the subsequent oxidative modifications of 7dC [5–8]. The Δ^4-diketol is converted into ecdysteroids, including 3-dehydroecdysone, in the crustacean Y-organ which corresponds to the PG of insects [6]. The diketol is efficiently converted into 3-dehydroecdysone and E in the PG of *Locusta migratoria* [8]. However, neither of these 3-oxo steroids have been detected in insects and no biological activity of metabolite(s) derived from them has been reported. In *Drosophila* and several other higher flies, the diketol undergoes reduction at C-3 to form the ketodiol [1]. The three terminal hydroxylation reactions at C-25, C-22 and C-2 of the ketodiol in the PG lead to E [9]. The synthesized E is released into hemolymph and then hydroxylated at C-20, which leads to the final product 20E in the peripheral tissues [10].

In the last decade, ecdysteroid biosynthetic enzymes have been characterized in *Drosophila melanogaster*. The first step from cholesterol to 7dC is catalyzed by a Rieske oxygenase, Neverland [11,12]. The sequential terminal oxygenations from the ketodiol to 20E are catalyzed by several cytochrome P450 enzymes, Phan-
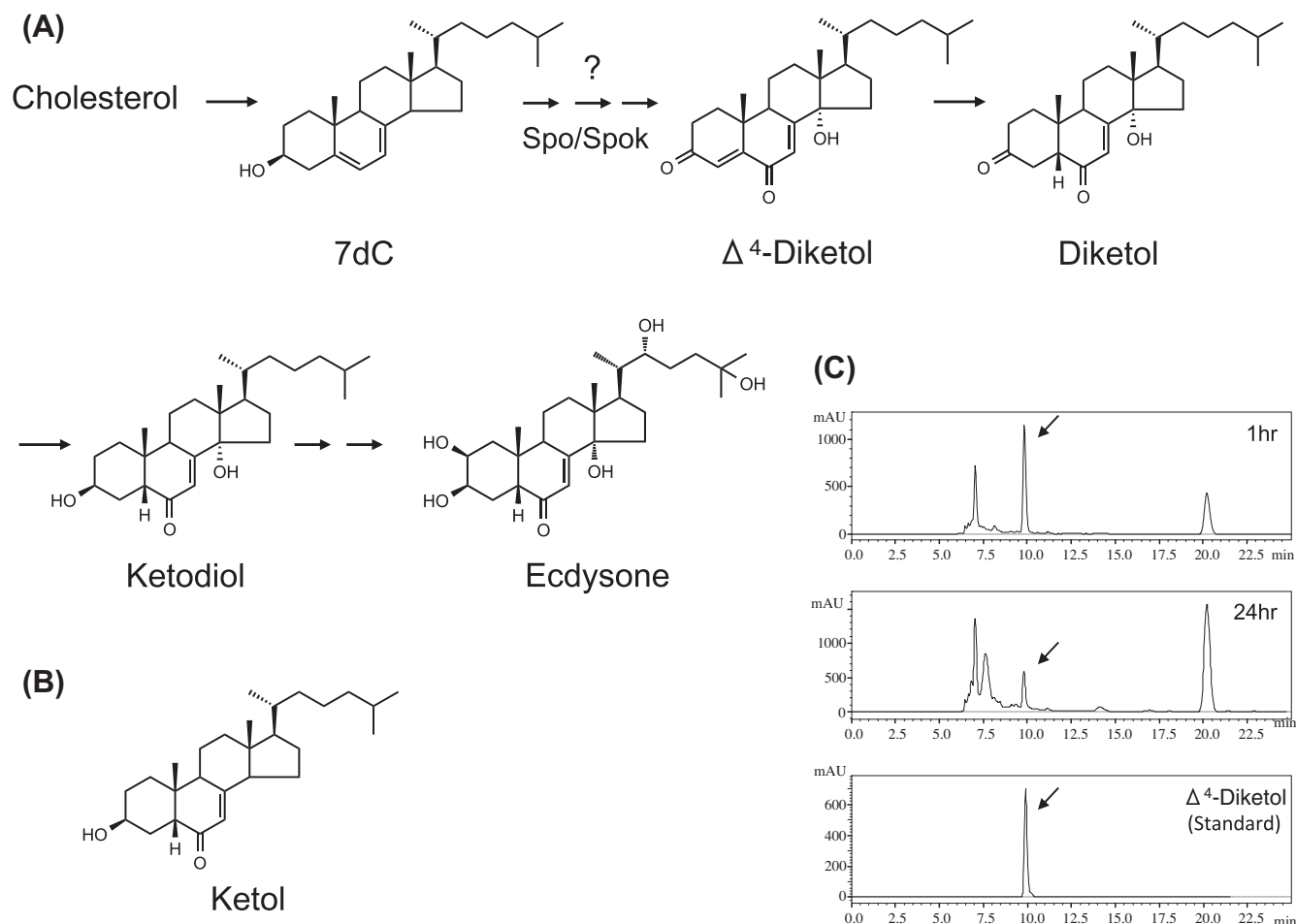
Fig. 1. Structure, biosynthesis and chemical analyses of ecdysteroid intermediates. (A) The ecdysteroid biosynthetic pathway. This study focuses on points downstream of a step catalyzed by Spok. (B) Structure of a 14-deoxyecdysteroid, ketol. (C) RP-HPLC analyses of the stability of the Δ^4-diketol. The Δ^4-diketol remaining after incubation with yeast paste for 1 or 24 h was detected. UV absorption at 274 nm was monitored.

tom, Disembodied, Shadow and Shade [10,13–15]. All of these genes encoding P450 enzymes have been identified from embryonic lethal mutants and named the Halloween genes. Of the Halloween genes, *spook* and *spookier* (*spok*) have been hypothesized to code for P450 enzymes which catalyze one of the Black Box reactions [16]. Recently, Cyp6t3 has been reported as an ecdysteroid biosynthetic enzyme that also acts on a step in the Black Box [17]. RNAi-mediated knockdown of expression of a gene which codes for an ecdysteroid biosynthetic enzyme results in arrest of molting, the phenotype of which can be rescued by feeding E or appropriate intermediate(s) to the larvae [11,16–18]. Therefore, we assume that feeding-rescue experiments for larvae in which *spok* is knocked down in the PG (hereafter called *spok*-RNAi larvae) could be applicable to clarify the intermediates in the Black Box. We focus on intermediates downstream of a step catalyzed by Spok in the Black Box and show here that application of candidate 3-oxo intermediates, the Δ^4-diketol and diketol, triggers molting of *spok*-RNAi larvae. We also detect an enhancement of ecdysteroids, E and 20E, in the *spok*-RNAi larvae fed with these 3-oxo steroids, indicating that the dietary Δ^4-diketol or diketol is converted into ecdysteroids *in vivo*. Finally, we show that induction of transcripts of 20E-inducible transcription factors occurs in Δ^4-diketol- or diketol-fed *spok*-RNAi larvae. These results reinforce that the Δ^4-diketol and diketol are components of the ecdysteroid biosynthetic pathway and lie downstream of a step catalyzed by Spok.

2. Material and methods

2.1. Drosophila strains

UAS-*spok*-IR; UAS-*spok*-IR and *phm-Gal4/TM3, sb, GFP* (gifts from M.B. O'Connor) were crossed to generate *spok*-RNAi animals [16]. Flies were cultured on a standard cornmeal/yeast extract/dextrose medium.

2.2. Chemicals

The Δ^4-diketol, ketol and ketodiol were synthesized from 7dC as described previously [6,19–21]. The diketol was synthesized from the ketodiol by chromic acid oxidation in acetone. E was purchased from Sigma. Each compound was purified by reverse-phase HPLC before experiments.

2.3. Ecdysteroid feeding experiments

Eggs were collected and placed on 2% agar-0.005% butyl 4-hydroxybenzoate in 35 mm diameter petri dish with yeast paste containing steroid or none. For preparation of yeast paste, 50 mg of dry yeast was thoroughly mixed with 90 μl of water and 10 μl of 10 mM steroid in ethanol or else only solvent. The petri dish was covered with aluminum foil to avoid light because some

intermediates are unstable under light. Non-GFP-containing (*phm > spok-IR*) first larval instar (L1) larvae were picked after hatching. The animals were reared at 29 °C in the experiments.

2.4. HPLC analysis

Yeast paste samples containing the Δ^4-diketol or diketol was placed at 29 °C under dark conditions for either 1 or 24 h. Each paste sample was extracted with ethanol and the eluate was applied to a reverse-phase column (0.5 g of Cosmosil 140C18-OPN, Nacalai Tesque, Inc., Kyoto, Japan), which was then eluted with 5 ml of water and subsequently with 5 ml of methanol. The eluate with methanol was chromatographed on a reverse-phase HPLC column (YMC-Pack ODS-AQ-323, 10 × 250 mm, YMC Co., Ltd., Kyoto, Japan) at a flow rate of 2.0 ml/min with methanol.

2.5. Ecdysteroid titer measurements

L1 larvae were collected 18–24 h after hatching and preserved in ethanol at −80 °C prior to processing. Whole bodies of 100 larvae were homogenized in 0.5 ml ethanol by hand with a plastic pestle, vortexed vigorously and centrifuged at a maximum speed. After decantation, the residues were repeatedly extracted with ethanol (0.5 ml × 3). The solvents were pooled and evaporated under low pressure. The extract was applied to a reverse-phase column (0.5 g of Cosmosil 140C18-OPN), eluted with 5 ml of water and subsequently with 5 ml of methanol. The eluate with methanol was chromatographed on a reverse-phase HPLC column (Nova-Pak C18 4 μm, 3.9 × 150 mm, Waters, Milford, MA, USA) at a flow rate of 1.0 ml/min with 30% methanol for 30 min, followed by a gradient elution to 100% methanol from 30 to 65 min. The eluates were collected in a range of Rt = 15.5–16.5 and 38.5–39.5 min, which corresponded to eluted fractions of 20E and E, respectively. These eluates were evaporated under low pressure, suspended in 50 μl of enzyme immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin [BSA] in 0.1 M phosphate buffer) and subjected to enzyme-linked immunosorbent assay (ELISA). ELISA was performed using ACE Enzyme Immunoassay system (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's

protocol. Absorbance was measured at 450 nm using a microplate reader Benchmark (Bio-Rad Laboratories, Hercules, CA, USA). The standard curves were obtained from 20E.

2.6. Quantitative RT-PCR

Ten L1 larvae were collected in 3 h intervals 15, 18 or 21 h after hatching. All were then pooled, homogenized in TRIzol Reagent (GIBCO-BRL) and total RNA was extracted from the total 30 staged larval homogenate and purified using RNeasy (Qiagen, Crawley, UK). Reverse transcription for each 0.5 μg total RNA sample was performed using PrimeScript RT reagent Kit (Takara, Shiga, Japan). Quantitative RT-PCR was performed on LightCycler (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq (Takara, Shiga, Japan). Samples were normalized with RpL23. Three independent biological samples were collected for each experiment. The following forward (f) and reverse (r) primer pairs were used: E74A, f (5′-GCCCTTTATCGACGATGCAC-3′) and r (5′-GCTCCATT-CAGTTCGTTGCC-3′); E74B, f (5′-CTGAGTTGCAGGAGGATGGG-3′) and r (5′-CTAGTGACTCGGGGACTTTGG-3′); E75A, f (5′-ACGGATAT-CAGCAGGCCAATC-3′) and r (5′-GAATGCACGCCGTAATGGAAAC-3′); βFtz-F1, f (5′- GGTGGGCATGAAGCTAGAGG-3′) and r (5′- CCAACG-CAATTGCTATGGGG-3′); rpL23, f (5′-GCTCAGGAAGAAGGTCATGC-3′) and r (5′-GGCTATAGAGCTTGCATTGGA-3′).

3. Results

3.1. Candidate ecdysteroid precursors triggered molting of spok-RNAi first instar larvae

Spok is thought to be the rate-limiting enzyme in the Black Box of the ecdysteroid biosynthetic pathway during larval-pupal transitions in *D. melanogaster*, as recent study has shown that only Spo, paralog of Spok, of the Halloween ecdysteroid biosynthetic enzymes is phosphorylated in the PG cells of the moth, *Manduca sexta*, following stimulation by the neuropeptide known as prothoracicotropic hormone (PTTH) [22]. Because intermediates downstream of the conversion step catalyzed by Spok are expected to be able to overcome the molting arrest of *spok*-RNAi larva [16],
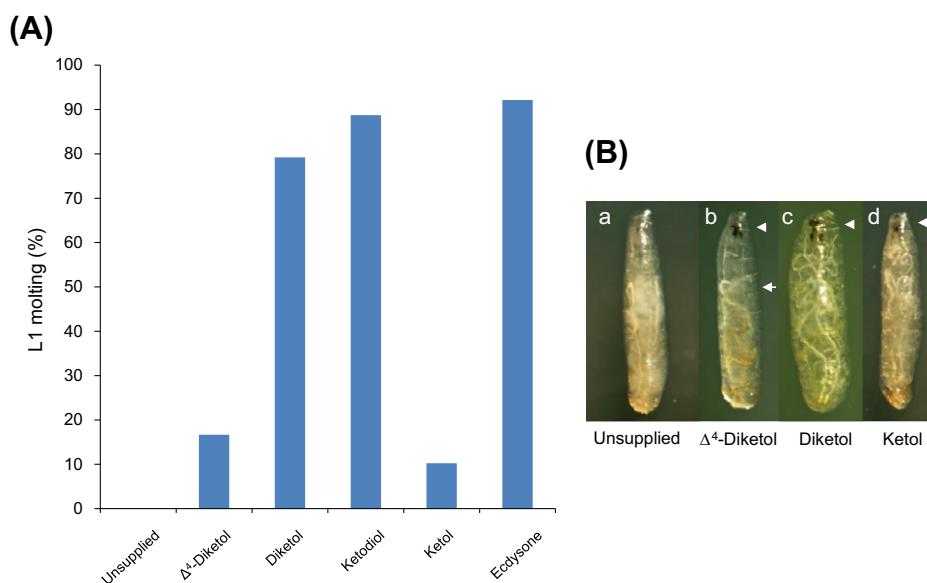
(A)

(B)

Fig. 2. Molting activity of candidate ecdysteroid intermediates in *spok*-RNAi larvae. (A) Percentage molting of L1 larvae raised on food with or without steroid. (B) *Spok*-RNAi larvae fed with or without a candidate ecdysteroid intermediate. a: Unsupplied *spok*-RNAi larva died at L1 stage. b: *Spok*-RNAi larva fed with the Δ^4-diketol died during molting from L1 to L2. Arrowhead indicates double L1 and L2 mouth hooks. Arrow indicates the boundary between new and old cuticles. c: *Spok*-RNAi larvae fed with the diketol died during molting from L2 to L3. Arrowhead indicates double L2 and L3 mouth hooks. d: *Spok*-RNAi larvae fed with the ketol died during molting from L2 to L3. Arrowhead indicates double L2 and L3 mouth hooks.

Table 1
Lethal phase of *spok*-RNAi animals by feeding steroids.

Steroid	Number of dead animals at each stage					
	Lethal stage					
	L1	L1/L2	L2	L2/L3	L3	Pupa
Unsupplied	89 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Δ^4-Diketol	110 (83)	22 (17)	0 (0)	0 (0)	0 (0)	0 (0)
Diketol	16 (21)	0 (0)	53 (69)	2 (3)	6 (8)	0 (0)
Ketodiol	8 (11)	0 (0)	50 (70)	7 (10)	5 (7)	1 (1)
Ketol	70 (90)	2 (3)	5 (6)	1 (1)	0 (0)	0 (0)
Ecdysone	4 (8)	0 (0)	7 (14)	1 (2)	33 (65)	6 (12)

Each number in parentheses refers to the percentage of animals which died at each stage. L1/L2 and L2/L3 refer to larvae that died while molting from L1 to L2 and from L2 to L3, respectively.

we investigated candidate precursor-mediated reversal of this developmental arrest. We tested two 3-oxo-intermediates, the diketol and Δ^4-diketol, and also a 14-deoxyecdysteroid, the ketol, that has been detected in the vitellogenic ovaries of *L. migratoria* [23] (Fig. 1A and B). All three steroids triggered molting, although their activities were very different (Fig. 2A). When the Δ^4-diketol was applied to *spok*-RNAi larvae, approximately 17% of them molted from the L1 to L2 stage. The resulting larvae had second larval mouth hooks and new cuticles on their upper body, but old cuticles remained on their lower body, indicating that they died during molting from L1 to L2 (Fig. 2B). As the instability of the Δ^4-diketol has been reported [6], the remaining amount of the Δ^4-diketol in yeast paste was quantified immediately after incubation for 1hr or 24hrs at 29 °C under dark conditions. Approximately, 94% or 56% of the original Δ^4-diketol was recovered from yeast paste after incubation for 1 or 24 h, respectively (Fig. 1C), indicating that the incomplete rescue of molting from L1 to L2 is not due to degradation of the Δ^4-diketol. The diketol rescued

approximate 80% of the L1 arrest of *spok*-RNAi larvae at a level similar to that observed for the ketodiol and ecdysone (Fig. 2A). Approximately 8% of diketol-fed larvae underwent development to the L3 stage, similar to that in ketodiol-fed larvae (Table 1). The ketol is not converted into E but rather into 14-deoxy-ecdysone and so has not been characterized as an intermediate in the ecdysteroid biosynthetic pathway [24]. Nevertheless, approximately 10% of ketol-fed larvae molted from the L1 to L2 stage and most of them developed to L2 or L2/L3 stage (Fig. 2A and B and Table 1). The results suggest that 14-deoxy-ecdysone synthesized from the ketol in the PG and its derivative, 14-deoxy-20E, have competence to trigger molting.

3.2. 3-Oxo steroids, the Δ^4-diketol and diketol, were converted into ecdysteroids

To confirm the conversion of 3-oxo-steroids into ecdysteroids in *spok*-RNAi larvae, we measured ecdysteroid concentrations in L1 larvae using a HPLC-ELISA combination (Fig. 3B). Both E and 20E were only marginally detected in larvae not fed steroid (referred as unsupplied), which is consistent with the arrest of molting observed following knockdown of *spok* expression. However, significant amounts of ecdysteroids were detected in Δ^4-diketol-fed larvae, i.e. the total amounts of E and 20E of Δ^4-diketol-fed larvae were similar to that of GFP-containing larvae (referred as control GFP), while in diketol-fed larvae, the amounts of both E and 20E were higher than those of control GFP larvae.

3.3. 3-Oxo steroids, the Δ^4-diketol and diketol, activated transcription of 20E-inducible genes

Binding of 20E to the ecdysone receptor (EcR) initiates complex genetic cascades which direct developmental transitions, including
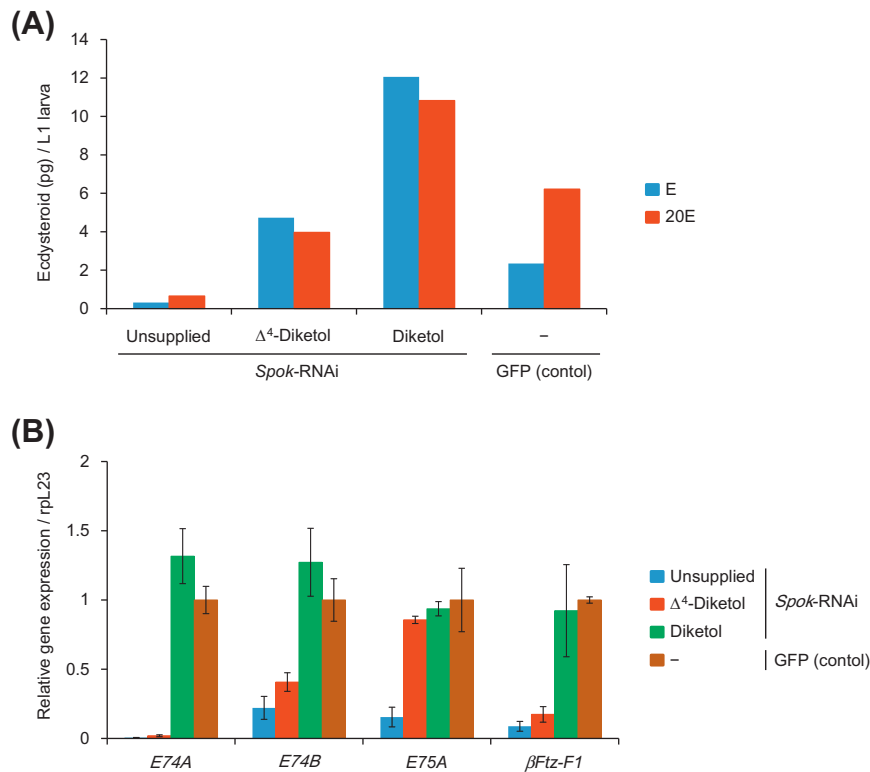

Fig. 3. Profiles of ecdysteroid titers and expression levels of 20E-inducible genes. (A) Profiles of ecdysteroid titers in middle-late stages of L1 larvae. The titers are depicted as picogram (pg) of E or 20E/larva. (B) Quantitative RT-PCR analysis of the transcriptional levels of the 20E-inducible transcriptional factors in middle-late stages of L1 larvae (mean ± SD, n = 3). The gene expression level of GFP control animals is represented as 1 on the vertical axis.

the molting process [25]. Thus, if the small amounts of 20E found in larvae fed the Δ^4-diketol were responsible for the developmental rescue of 17% of *spok*-RNAi animals, i.e. the shedding of the old cuticle, then we expected to observe partial activation of EcR signaling. To confirm if this is the case, we measured expression levels of the 20E-inducible transcription factors, E74A, E74B, E75A and βFtz-F1 [26] (Fig. 3). While E74A was not induced in either unsupplied or Δ^4-diketol- fed *spok*-RNAi larvae, in accordance with the property that the E74A transcript is induced by only high 20E concentrations [27], a small induction of both E74B and βFtz-F1 expression was observed in unsupplied and Δ^4-diketol-fed *spok*-RNAi larvae (marginally higher in the latter). In contrast to E74A, expression of E74B is induced by a low titer of 20E [27], suggesting that a trace amount of 20E is synthesized even in unsupplied *spok*-RNAi larvae, i.e. RNAi knockdown does not completely inhibit the transcription of *spok*. Interestingly, expression of E75A was induced in Δ^4-diketol-fed *spok*-RNAi larvae at a level similar to that in control GFP larvae, whereas little induction was observed in unsupplied *spok*-RNAi larvae. In diketol-fed *spok*-RNAi larvae, expression of all transcription factors tested was induced at levels similar to those in control GFP larvae, which is consistent with the significant molting activity of the diketol.

4. Discussion

Extensive studies over several decades have so far failed to completely elucidate the chemistry involved in ecdysteroid biosynthesis, but it has long been thought that the oxidation of the steroidal 3β-OH function to the 3-ketone occurs early in this process, i.e. 3-oxo-7dC may be the first step in these unknown Black Box oxidations [28]. Immediately subsequent intermediates eventually leading to the secretion of 3-dehydroecdysone likely remain oxidized at C-3. We addressed the involvement of some of these hypothetical downstream 3-oxo intermediates by a novel *in vivo* approach. In this report, we show that both the Δ^4-diketol and diketol not only trigger the molting of *spok*-RNAi L1 larvae, but also result in the elevation of E and 20E titers and increased expression of some 20E-responsive genes in late L1 larvae, all strong indications that the Δ^4-diketol and diketol are intermediates in the biosynthesis of ecdysone. However, the degree to which these compounds elicited these effects differed greatly. Indeed, only 17% of L1 larvae showed molting behavior following feeding of the Δ^4-diketol, while most developed to the L2 stage after diketol or ketodiol ingestion (as with the GFP-control). In contrast, ecdysteroid titers of Δ^4-diketol fed larvae, similar to those of GFP-control animals, were nearly 40% of those fed the diketol. However, only E75A expression was similarly elevated by both compounds (also in GFP-control) relative to unsupplied *spok*-RNAi larvae.

What could be the explanation for the low rescue of *spok*-RNAi larvae result in light of the elevated ecdysteroid titers and specific gene expression following Δ^4-diketol administration? First, it must be emphasized that only the rescue experiments resulted in unambiguous results, i.e. larvae either did or did not molt out of the first instar. In contrast, titer and gene expression determinations employed a selected population of L1 larvae, 18–24 h old for titer and 15–24 h old for expression. These larvae were not screened for normal (rescued) development, i.e. no more than 17% of the selected larvae were likely respondents to the Δ^4-diketol feeding regimen. Yet after diketol feeding, almost all were so developmentally affected. In addition, at the end of the first instar (24 h in length), the peaks of whole body ecdysteroid titer and elevated gene expression last for only a short period before falling to baseline levels. As such, they can be misrepresented in such a select population, either too low or perhaps even too high, and so may not relate mathematically to the rescue results. The significant elevations, relative to

unsupplied larvae, observed in ecdysteroid titer and 20E-inducible gene expression following both Δ^4-diketol and diketol administration support the premise that the Δ^4-diketol is an endogenous precursor of the diketol. More important is the rescue of *spok*-RNAi L1 larvae following oral Δ^4-diketol administration, relative to unsupplied controls, similar to rescue following diketol, ketodiol, ketol or ecdysone feeding. The observation that the Δ^4-diketol-mediated rescue was low relative to what might be expected based on observed ecdysteroid titers and specific gene expression levels in similarly-treated animals is not important.

It is interesting, however, and undoubtedly relates to the complex pharmacokinetics underlying the experiment. Based on their near-equal reverse-phase lipophilicities, it is likely that the Δ^4-diketol, diketol and ketodiol are equally-well absorbed from the gut into the larval whole body/circulation. However, that may not be true for their subsequent entry into the PG, because uptake by the PG of ecdysteroid-like molecules, like the secretion from the PG of ecdysone and 3-dehydroecdysone, may be an active or facilitated process that would depend on an intact ecdysteroid structure. As the Δ^4-diketol lacks the distinctive 5β (H)-*cis* A/B-ring junction, its entry into the PG could be limited to the less efficient process of simple passive diffusion. Even if this is not the case and all these molecules have equal access into the PG cytoplasm, then a kinetic barrier may exist in the subsequent PG reactions of the Δ^4-diketol. There, its conversion into the diketol via the 5β-reductase is thought to occur in the ER, similar to up-stream (Spookier) and downstream (Phamtom) P450 enzymes operating in the ecdysteroid synthetic pathway. Any problem with Δ^4-diketol equilibrating into the ER and/or being reduced to the diketol would negatively affect its ability to rescue the *spok*-RNAi larvae.

All 20E-inducible genes tested were induced by feeding the diketol to *spok*-RNAi larvae to levels similar to that of control GFP larvae, indicating that the measurement of the expression of these genes is a reliable way to evaluate whether or not a candidate intermediate can be converted into 20E *in vivo*. An intriguing finding is that application of the Δ^4-diketol to *spok*-RNAi larvae induced expression of E75A to levels similar to that of control GFP larvae. In contrast, induction of E74A, E74B and βFtz-F1 expression was at the levels observed in unsupplied *spok*-RNAi larvae. Taken together with the partial rescue of molting by Δ^4-diketol application, E75A is the most important factor to initiate molting behavior, but not enough to complete the molting process. Further study by the application of ecdysteroid intermediates to ecdysteroid-defective *Drosophila* strains could unveil molecular mechanisms, including ecdysteroid biosynthesis, transport and signal transduction required for molting.

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