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Endogenous xanthurenic acid as a regulator of the crustacean molt cycle

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Abstract -- By means of *in vitro* assay, it has been demonstrated that xanthurenic acid, a metabolite of 3-hydroxy-L-kynurenine which is contained in crustacean eyestalks (X-organ), acts as an inhibitor of ecdysteroidogenesis in Y-organs. Xanthurenic acid directly prevents an oxygenase (probably cytochrome P-450 mediated) from acting on ecdysone precursor(s). Injection with physiological doses of 3-hydroxy-L-kynurenine in saline delayed the onset of induced molts and prolonged the duration of molt cycles of eyestalk-ablated crayfish. Thus, the long-postulated molt inhibitory action of the eyestalk can at least in part be ascribed to xanthurenic acid or its precursor 3-hydroxy-L-kynurenine.

INTRODUCTION

Growth in crustaceans involves periodic shedding (ecdysis) and re-formation of the exoskeleton. The combined events, including those before and after ecdysis, are defined as a molt. The major physiological events at molting are degradation of the old exoskeleton, synthesis of the new exoskeleton, formation of gastroliths in some species and atrophy of somatic muscle in the chelae that is replaced after ecdysis. According to current views (ref. 1, 2), regulation of molting is under the influence of at least two hormones: the molt promoting hormone, 20-OH-ecdysone, and a putative molt-inhibiting hormone, "MIH". However, the precise mode of action by which these hormones interact is still unclear. The "MIH" *in vivo* assay is biological in nature and yet indirect, in that secondary morphological responses elicited by an increase in 20-OH-ecdysone titer are observed. On the other hand, the *in vitro* assay is capable of demonstrating the direct repression of ecdysteroidogenic Y-organs by the eyestalk(ES)-derived "MIH" produced in neurosecretory X-organs.

RESULTS AND DISCUSSION

An *in vitro* assay has been developed in our study (*Callinectes sapidus* and other crab species) (ref. 3), that utilizes the homogenate of Y-organs and adhering tissues (Y-organ-complex: YOC in 0.17 M KH_2PO_4/ $NaHCO_3$) to measure the ecdysone output in the presence and absence (control) of ES extract after 20 h incubation at 37°. The rapidity and reproducibility of the assay were improved by using an aliquot of the supernatant of the YOC-homogenate (7,700 x g) as control and the remainder as test solution. The supernatant contained sufficient amounts of the endogenous substrate and enzymes to synthesize ecdysteroids, which were quantitatively analyzed with HPLC, ERC-ODS-1161, MeOH : CH_3CN : H_2O, 28 : 10 : 62, 247 nm, detection limit ~3 ng. Repression was demonstrated to be dose-dependent within the same species (ED_{50} = ~0.04 ES eq.), and species-nonspecific with respect to the source of ES and YOC donors, i.e., ES and YOC from various crabs and crayfish (*Procambarus clarkii*) could be cross-reacted. Furthermore, YOCs of winter-collected animals were far more sensitive to "MIH" than those from summer-collected animals.

Isolation of "MIH" from ES extract following the assay described above led to the identification of 3-OH-L-kynurenine (3-OH-K) (ref. 3). Unless otherwise noted, 3-OH-K denotes the L-enantiomer (see Fig. 3). The activity in this isolated natural compound was as potent as in the authentic L-enantiomer. It has been also found that its metabolite, xanthurenic acid (XA), exhibits a quicker or stronger response than 3-OH-K in repressing the YOC ecdysteroidogenesis under similar conditions. However, incubation with the same dosage of synthetic DL-3-OH-K leads to no suppression of ecdysteroid biosynthesis, thus indicating that dosage deficiency is the cause of this negative response. Indeed L-enantiospecific transformation of 3-OH-K into XA was demonstrated by using a crude enzyme preparation obtained from the crab body fluid: a 1-h incubation at 37°C led to conversion of L-, DL- and D-3-OH-K in the ratio of 1 : 0.5 : 0, respectively. Although both ~28 ng 3-OH-K and ~128 ng XA per X-organ have been detected (means of 8 donors selected at random, HPLC detection at 240 nm, Asahipak GS 320, aq. $AcONH_4$ → 20% CH_3CN/0.17 M $AcONH_4$), it is conceivable that 3-OH-K is released into the hemolymph from the eyestalks and becomes bound to putative receptors at the target Y-organ, where 3-OH-K is enzymatically transformed into XA.

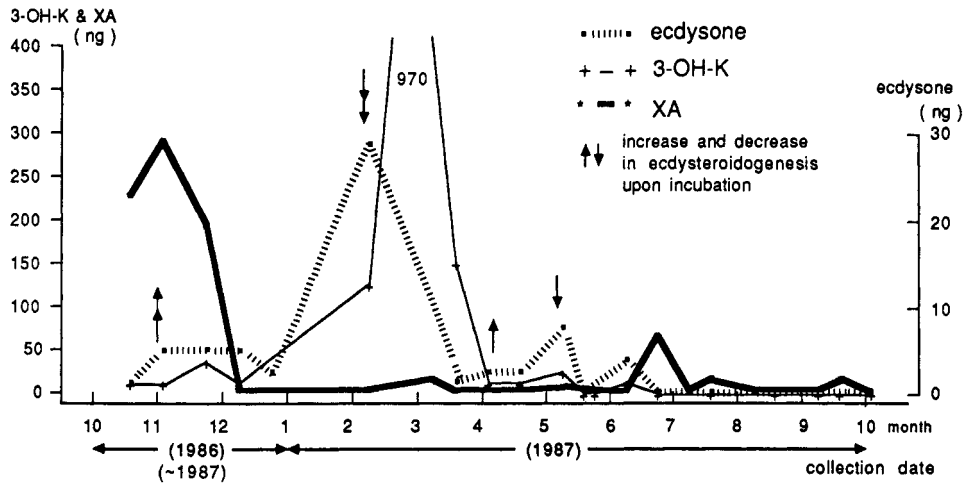

Fig. 1 Ecdysone, 3-OH-K and XA titers / YOC *(Charybdis japonica)*

The above-mentioned interpretation was supported by the following results based on the seasonal profiles which were obtained by plotting the mean values for 6 randomly-selected animals *versus* the collection date.
(i) The seasonal 3-OH-K titer in the ***ES*** showed two maxima during the year, one in March/April and the other in September/October, whereas the XA titer showed little variation. One peak in March/April was reflected in an increase of 3-OH-K titer in the ***YOC*** (Fig. 1). Another peak in September/October was presumably converted into metabolites other than XA, such as xanthommatine. As the result, 3-OH-K titers did not rise appreciably in the ***YOC***. The ratio of XA/3-OH-K titers corresponding to an increase of aminotransferase activity showed two peaks in November/December and in June/July.
(ii) The maximum in the XA titer in the ***YOC*** [Fig. 1] appeared simultaneously with the increase of aminotransferase activity in November/December, as seen in the ***ESs***.
(iii) The ecdysone content reached a peak in February/March and showed a phase-lag with the XA titer (Fig. 1).
(iv) While the level of XA per YOC was high in November/December (~300 ng), ecdysone production was greatly enhanced upon incubation (Fig.1, double-headed arrow); this indicated that during this period, the enzyme involved in ecdysteroidogenesis *in vivo* was suppressed by XA and activated upon incubation. Following this stage, the high ecdysone level (~30 ng) was greatly reduced by incubation as a result of ecdysone metabolism and suppression of ecdysteroidogenesis by the XA derived from 3-OH-K in this period. An alternative interpretation is sequestration of the ecdysteroidogenic enzyme during this period.
(v) In contrast, the low levels of both 3-OH-K and XA in the ***hemolymph*** remained practically unchanged throughout the year.

In order to check the validity of *in vitro* assay results, *in vivo* assays utilizing crayfish (10 animals) were performed (Fig. 2). Injection with physiological doses of 3-OH-K in saline delayed the onset of induced molts by 1~3 days and prolonged the duration of molt cycles of ES-ablated crayfish by ~12 days (*vs.* ~9 days for the control). This is due to inhibition of ecdysone synthesis and resultant lowering in the concentration of the circulating molting hormone; it is thus a mimic of the long-postulated inhibitory action of "MIH". To determine on which days the Y-organs would be most responsive to activation by ES-ablation, *in vivo* time courses of ecdysteroid titers in the hemolymph were examined. The level of 20-OH-ecdysone at days 8-12 after operation was enhanced (~40 ng *vs.* ~3ng of the basal level/1 ml hemolymph, the mean of 10 animals), suggesting that these animals may already have been activated for ecdysis in accordance with the above-mentioned morphological observations (Fig. 2). XA in buffer/saline also delayed the onset of molting but failed in prolonging the molt cycle (owing to death of experimental animals).

The mode of action was investigated on the basis of a working hypothesis that ecdysteroidogenesis is mediated by cytochrome P-450. Incubation of XA with a model oxygenase, oxidized cytochrome C, yielded a typical binding signal ESR spectra (Fe^{3+}- O^{-}: g=1.88, 2.12, 2.30) which could not be reversed by the action of NADPH; the substrate binding was also indicated by a shift of the Soret peak from 410 to 415 nm. It has also been demonstrated by difference UV spectroscopy showing a peak at 445 nm that XA directly acts on hepatic phenobarbital-induced cytochrome P-450 (rat) but not on a constitutive hepatic microsomal P-450 (rat). The irreversible binding probably reflects changes in the concentration of the enzyme present; this is because cultured Y-organs did not increase their rate of ecdysone secretion when sinus gland-conditioned medium was replaced by fresh saline (ref. 4). Under these conditions the low secretory rates of ecdysone continued for an additional 4 days of incubation, while addition of lipoproteins from crab serum gave only inconclusive results with respect

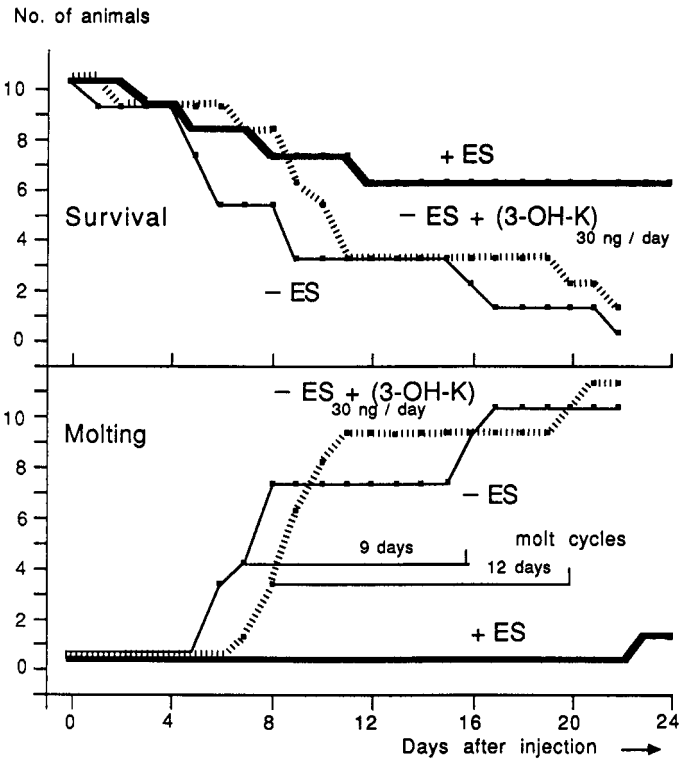

Fig. 2 Effect of 3-OH-K injection on crayfish molting *(Procambarus clarkii)*

to the increase in ecdysone secretion (ref. 4). In another series of experiments we attempted to detect cytochrome P-450 in the crab Y-organ. Although the involvement of this biocatalyst in ecdysteroidogenesis is still uncertain due to the apparently low levels of P-450, presence of cytochromes in the supernatant of the YOC homogenate was observed at critical stages of the molt cycle by monitoring the KCN-binding difference spectra around 450 nm.

We conclude that the inhibitory effect of XA (3-OH-K) in Y-organs is species-nonspecific among crabs and crayfish *in vitro* as well as *in vivo*; in addition, the inhibition mechanism probably involves a ligand exchange which competes with oxygen for the site of iron porphyrin within the enzyme to prevent electron transport. XA (3-OH-K) does not suppress the 20-hydroxylation of ecdysone at the target tissues (Fig. 2, 3). Thus, the physiological role of XA as an Ecdysone Biosynthesis Inhibitor ("EBI") has been demonstrated for the first time.

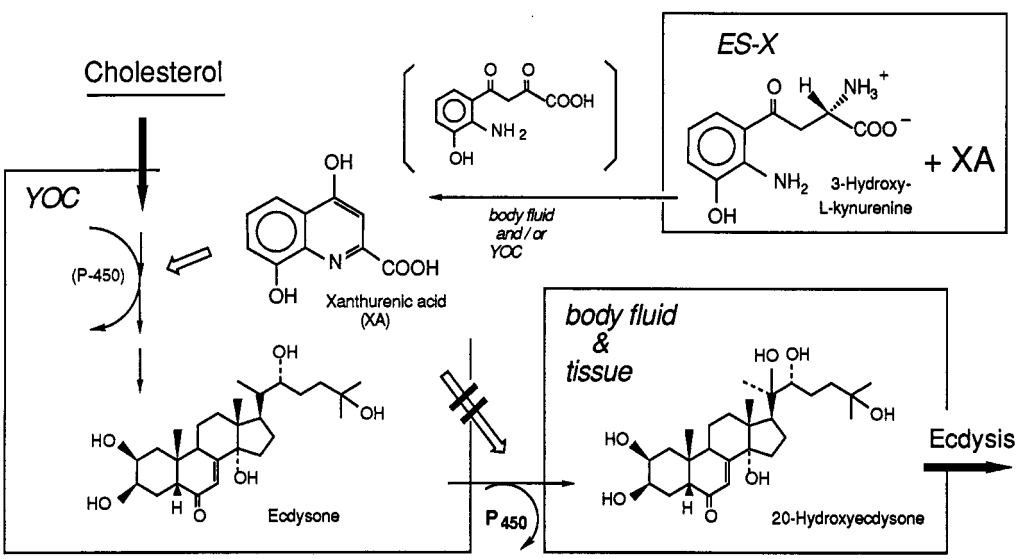

Fig. 3 Flow chart of chemicals involved in molting of crustaceans

However, the potency in the extract of 1 ES equivalent cannot be accounted for even by the combined 3-OH-K and XA present. There may be an important functional relationship between XA (3-OH-K) and novel peptides recently reported as putative MIHs (ref. 5, 6). It is worth noting that 5-hydroxytryptophan (10^{-7}M) and 5-hydroxytryptamine (10^{-6}~10^{-10}M) provide excitatory input to "MIH"-containing neurosecretory cells (ref. 7), indicating feedback metabolic regulation for tryptophan-mediated 3-OH-K (XA) biosynthesis. Since ES-ablation may interfere with the XA-mediated suppression of ecdysteroidogenesis in Y-organs but may also activate 20-hydroxylation in the peripheral tissues of the Y-organs, the negative-control of the ecdysis process is probably mediated by more than a single mechanism, in which inhibitory effects on ecdysone/ 20-hydroxyecdysone production and negative induction of enzymes are at least involved.

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