Published in final edited form as:

Comp Biochem Physiol A Mol Integr Physiol. 2010 January ; 155(1): 49–55. doi:10.1016/j.cbpa.2009.09.016.

Methyl farnesoate synthesis in the lobster mandibular organ: The roles of HMG-CoA reductase and farnesoic acid-O-methyltransferase

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Abstract

Eyestalk ablation (ESA) increases crustacean production of methyl farnesoate (MF), a juvenile hormone-like compound, but the biochemical steps involved are not completely understood. We measured the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and farnesoic acid-O-methyl transferase (FAOMeT), an early step and the last step in MF synthesis. ESA elevated hemolymph levels of MF in male lobsters. Enzyme activity suggested that increased MF production on day one was due largely to elevated HMGR activity while changes in FAOMeT activity closely paralleled changes in MF levels on day 14. Transcript levels for HMGR and FAOMeT changed little on day one, but both increased substantially on day 14. We treated ESA males with a partially purified mandibular organ inhibiting hormone (MOIH) and observed a significant decline in MF levels, FAOMeT activity, and FAOMeT-mRNA levels after 5 hours. However, no effect was observed on HMGR activity or its mRNA indicating that they must be regulated by a separate sinus gland peptide. We confirmed that lobster HMGR was not a phosphoprotein and was not regulated by reversible phosphorylation, an important mechanism for regulating other HMGRs. Nevertheless, molecular modeling indicated that the catalytic mechanisms of lobster and mammalian HMGR were similar.

Keywords

3-hydroxy-3-methylglutaryl-coenzyme A reductase; farnesoic acid O-methyltransferase; methyl farnesoate; mandibular organ; mandibular organ-inhibiting hormone; phosphorylation; molecular modeling

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INTRODUCTION

Crustacean endocrine systems produce several major hormones. One important class of hormones is the ecdysteroids, steroid hormones secreted by the prothoracic gland. Among the roles of the ecdysteroids is stimulating the synthesis of a new exoskeleton at molting (Skinner, 1985; Hopkins, 2009). A second important hormone is methyl farnesoate (MF), a sesquiterpenoid produced by the mandibular organ (MO). MF is related to the insect juvenile hormones and appears to have several roles in crustaceans, including the stimulation of reproduction and molting (Borst et al. 1987; Laufer et al. 1987, Nagaraju, 2007). Other important hormones include the neuropeptides found in the sinus gland (SG) of the eyestalk. These peptides regulate a variety of different aspects of crustacean physiology, including the prothoracic glands and the MO (Webster, 1998).

Central to the production of MF and other isoprenoids is the enzyme HMGR (3-hydroxy-3-methylglutaryl-coenzyme A reductase; EC 1.1.1.34). In many species, HMGR is the rate-limiting step in the production of mevalonate, and the levels of mevalonate control the production of important isoprenoids and other products derived from them, such as cholesterol in mammals (Friesen and Rodwell, 2004) and juvenile hormone (Bellés et al. 2005). Thus, the regulation of HMGR activity has been studied carefully in many species.

In mammals, HMGR regulation is complex and keeps the end products of different metabolic pathways from accumulating to unwanted levels (Goldstein and Brown, 1997). Regulatory mechanisms involve changing the quantity of HMGR by regulating both the transcription rate of the HMGR-mRNA (Hua et al. 1995) and degradation rate of HMGR protein (Ravid et al. 2000; Moriyama et al. 1998). Alternately, HMGR can be inactivated by reversible phosphorylation of Ser871 near its C-terminus (Omkumar and Rodwell, 1994). Several drugs (e.g., statins) that competitively inhibit HMGR have been developed and these have found significant use in lowering cholesterol production in humans (Goldstein and Brown, 1997).

Arthropods also have a mevalonate pathway for producing juvenile hormones and other products (Bellés et al. 2005). Early studies (Feyerreisen and Farnsworth, 1987) showed that HMGR is not central feature in regulating JH production, which has been generally supported by later studies (Bellés et al., 2005). However, a recent study (Belgacem and Martin, 2007) showed that HMGR may be the target in the CA that the insulin pathway used to regulate sexual dimorphism in *Drosophila melanogaster*.

In crustaceans, HMGR activity is found in several tissues, but the highest levels are found in the MO where it is used to synthesize mevalonate as a precursor for methyl farnesoate (MF). Similar to mammalian HMGR, lobster HMGR is regulated by small MW compounds (e.g. mevalonate and statins). However, cholesterol does not affect its activity. HMGR is also hormonally regulated by eyestalk peptides. Eyestalk ablation (ESA) causes an increase in HMGR activity and treatment of ESA-animals with an eyestalk extract causes a transient decrease in HMGR activity (Li et al. 2003).

In this paper, we compare the relative importance of HMGR, which produces the initial substrate for isoprenoid synthesis, with farnesoic acid O-methyl transferase (FAOMeT), which is the last step in MF synthesis. Because of its large size, the lobster MO is an excellent model to study the molecular regulation of HMGR for the synthesis of isoprenoids. Our results show that both enzymes increase in activity after ESA, but with different time courses. HMGR rises quickly (within one day) after ESA while FAOMeT rises later (after 6 days). Because they rise in response to ESA, both enzymes are regulated by eyestalk factors, presumably a SG derived peptide. Since the partially purified MOIH that we prepared only regulates FAOMeT, HMGR must be controlled by a separate SG peptide.

Material and Methods

Animals

Male lobsters (*Homarus americanus*; carapace length = 9.0 cm $\pm$ 0.3, SEM) were kept in artificial seawater at 13 °C. Some animals were eyestalk ablated (ESA) by severing each eyestalk at its base 1, 3, 6, or 14 days before use. On the indicated day, hemolymph samples were collected to measure MF levels. The MOs were then removed; the left MOs were divided in half, homogenized in appropriate buffers, and used to measure HMGR and FAOMeT activity. The right MOs were used to measure mRNA levels of HMGR.

Chemicals and reagents

(3*R*, 5*S*)-[5-³H]mevalonic acid (1.4 TBq/mmol), (3*R*, 5*S*)-3-hydroxy-[3-¹⁴C]-methylglutarylcoenzyme A (1.9 GBq/mmol), and [*methyl*-³H]-S-adenosylmethionine (SAM, 2.6 TBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA, USA).

Preparation of SG extract and partial purification of MOIH

Fresh lobster heads were obtained from Mr. William Hollier, Legal Seafoods, Boston, MA and the eyestalks removed and quick frozen in liquid nitrogen. After briefly thawing the frozen eyestalks in cold *Homarus* saline (Li et al., 2003), the sinus gland (SG, a neurohemal organ in the eyestalk that stores neuropeptides) was dissected and stored at -80 °C. SG extract was prepared by homogenizing 100 SG in 2 mL of 2 M cold acetic acid and centrifuging twice at 14,000 g for 10 min at 4 °C (Li et al. 2003). Partially-purified MOIH was produced from the SG extract in two steps. First, the extract was separated with a Poros® R2/10 reversed phase column (10 μ m, 4.6 mm $\times$ 100mm, Applied Biosystems, Foster City, CA, USA). Active fractions were collected and purified with a B10 Pore C18 column (5 μ m, 4.6 mm $\times$ 250 mm, Supelco, Bellefonte, PA, USA). MOIH was eluted from both columns with a gradient of 18-48% AcN in ddH₂O containing 0.1 % trifluoroacetic acid. The active fractions were determined by bioassay (Borst et al. 2001) and stored at 4 °C after the addition of 0.1 % methylthioethanol (Schooley et al. 1990).

HMGR assay and FAOMeT assay

The left MO from each lobster was divided along its anterior/posterior axis so that each half contained equal amounts of the fan-folded region, the area with the highest rate of MF synthesis (Borst et al. 1994). One half was homogenized in HMGR buffer (Li et al. 2003) at 4 °C and the other half in FAOMeT buffer (Holford et al. 2004). The homogenates were centrifuged at 12,000 g for 15 min at 4 °C and the supernatants used to measure HMGR (Li et al. 2003) and FAOMeT (Holford et al. 2004) activity.

Measurement of mRNA levels by quantitative PCR (qPCR)

The lobster MO contains two forms of HMGR. The two cDNAs for these proteins have identical 3'-ends and are alternative spliced products of a single gene (Li et al. 2004). We quantified the levels of HMGR-mRNA level by qPCR using two gene specific primers (H5F, a forward primer for HMGR: 5'-AATGACCAGAGCACCGAGTGTC; and H3R, a reverse primer of HMGR: 5'-CGTCCTGCAATTCCCACTTG). These amplify a 162-bp cDNA fragment that is common to both transcripts. The mRNA for FAOMeT was measured using two gene specific primers (F1F, a 5' forward primer for FAOMeT: 5'-CCAACACGGATTTCATCATGGTC; and F2R, a 3' reverse primer for FAOMeT: 5' TTT CAT CGG TGG TTG GGA AG). These amplify a 147 bp cDNA fragment.

Total RNA was isolated from the right MO using Tri Reagent (Sigma). First strand cDNA was synthesized from one μg of total RNA in 20 μL of reaction mixture including reverse transcriptase M-MLV and random primers (Promega, Madison, WI, USA). The qPCR reaction (20 μL) contained 10 μL of SYBR® Green PCR Master Mix (Applied Biosystems) and first strand cDNA equal to 0.1 μg of total RNA. Twenty pmoles of each primer (H5F and H3R) were added to amplify HMGR-mRNA. A similar amount of the F1F and F2R primers were added to other wells to amplify FAOMeT-mRNA. qPCR was run for 40 cycles of 95 °C (15 s) and 60 °C (1 min) using a GeneAmp 5700 (Applied Biosystems). Each sample was run in triplicate. PCR products of randomly analyzed samples contained a single band of the expected size when separated on a 2 % agarose gel. We created cDNA standards for HMGR and FAOMeT from their recombinant plasmids. The standards were purified by agarose gel electrophoresis and used to estimate relative mRNA levels.

Measurement of hemolymph levels of MF and the effects of MOIH in vivo

The MF levels of hemolymph samples (2 mL) were determined by normal phase HPLC (Borst and Tsukimura, 1991). Partially purified MOIH was resuspended in *Homarus* saline containing 0.1% BSA and 1 mM EDTA (Li et al. 2003) and injected (200 μL) into 14 d ESA lobsters. Other 14 d ESA animals were treated with saline only as a control. Immediately prior to (T0) and 5 h after (T5) the injection, ~2.5 mL of hemolymph was collected from each lobster. The *in vivo* activity of the partially purified MOIH is expressed by % change in MF levels 5 h after treatment ($[\text{MF}]_{\text{T5}} / [\text{MF}]_{\text{T0}} \times 100$). To calculate the effect of MOIH treatment on the enzyme activity and mRNA levels, the values in MOIH treated males were compared to data obtained from 14 d ESA lobsters that were untreated or saline-treated.

Western blot analysis

Western blots were also used to determine the phosphorylation state of recombinant lobster HMGR1 (rec-HMGR1) and purified native HMGR1 (Li et al. 2004). Kits for detecting phosphoserine and phosphothreonine residues were purchased from Invitrogen (Carlsbad, CA, USA) and used according to the enclosed protocols. As a positive control, we used recombinant CTP:phosphocholine cytidyltransferase from *Drosophila melanogaster*, shown previously to be phosphorylated on both serine and threonine (Helmink and Friesen 2004).

Molecular modeling of HMGR1

To determine the 3-dimensional structure of lobster HMGR, we analyzed its primary structure using four threading algorithms (SwissModel, 3D-PSSM, FUGUE, and 123D). These were used to predict the secondary structure and three-dimensional structure of lobster HMGR1 without a template. The generated PDB files were visualized using Swiss-PDBViewer, RasWin and GRAPH2.

Statistical analyses

The data were analyzed using Instat Software (GraphPad; San Diego, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test to determine significance.

RESULTS

ESA effects on enzyme and mRNA levels

In previous studies, this lab demonstrated that ESA increased hemolymph level of MF and MF synthesis by the lobster MO (Borst et al. 1994). Similar changes in MF levels were observed after ESA in this experiment. MF levels in intact males were 2.0 ng/mL. As shown in Figure 1A, MF levels climbed significantly after ESA to 540% of the initial MF levels on day 1, 700%

on day 6, and 1200% on day 14. This was paralleled by an increase in MO weight (Figure 1B), which rose to 170% of the initial weight after 14 days.

To determine the relative roles of HMGR and FAOMeT in MF synthesis, we measured their activity in the male MO after eyestalk ablation. Changes in MF levels were reflected in the combined increases of HMGR and FAOMeT activities. HMGR activity rose quickly after eyestalk removal, rising to 260 % of the initial value on days 1 and 3, dropping modestly to 200 % on day 6, and then increasing to 370 % on day 14 (Fig. 2A). FAOMeT activity increased slightly to 150% of the initial level on days 1 and 3, then increased robustly to 500 % and 1500 % of the initial value on day 6 and 14, respectively (Fig. 2B).

We also measured mRNA levels for HMGR and FAOMeT in the MO at the same time points using quantitative PCR (qPCR). As shown in Figure 3, the levels of HMGR-mRNA did not change much one and three days after ESA, showed a slight increase (150%) on day 6 and was significantly elevated 270 %) on day 14 ($P < 0.05$, ANOVA). The levels of FAOMeT mRNAs also didn't change one and three days after ESA, and then were significantly elevated (300 %) on days 6 and 14 ($P < 0.05$, ANOVA).

HMGR and FAOMeT activity in the MOs of eyestalk ablated lobsters can be transiently decreased by treating the animals with a SG extract (Borst et al. 2001; Li et al. 2003). We partially-purified such an extract by separating SG peptides with reversed phase HPLC. We isolated a partially-purified MOIH fraction that decreased MF levels of eyestalk-ablated male lobsters *in vivo*. As shown in Figure 4, MOIH treatment (0.05 SG equivalents/animal) significantly decreased hemolymph levels of MF to 27 % of the initial levels in these animals. Concordant with this result, MOIH treatment decreased FAOMeT activity to 14% and FAOMeT-mRNA to 31 % of the levels measured in MOs from saline-injected controls. In contrast, MOIH had no effect on HMGR activity or HMGR-mRNA levels in the MO.

The lobster HMGR is not a phosphoprotein

Purified native lobster HMGR, recombinant HMGR1, and recombinant *Drosophila* CTP:phosphocholine cytidyltransferase (CPCT) were separated on SDS-PAGE gels. The CPCT was included as a positive control for phosphoproteins. As seen in Fig. 5, CPCT (lane 2) is immunoreactive in Western blots using anti-phosphoserine (lane 5) and anti-phosphothreonine (lane 8) antibodies. Purified preparations of native HMGR and rec-HMGR1 (lanes 3 and 4) were not immunoreactive to either anti-phosphoserine (lanes 6 and 7) or anti-phosphothreonine (lanes 9 and 10) antibodies. These results indicated that rec-HMGR1 and native HMGR do not have phosphorylated forms.

Molecular modeling of lobster HMGR1

Molecular modeling using four web-based threading protein algorithms revealed two reliable templates to predict the secondary and three-dimensional structure of lobster HMGR1. The first template was the complex of the catalytic portion of human HMGR with fluvastatin (PDB ID 1hwi) (Istvan et al. 2000; Istvan and Deisenhofer, 2001). Human HMGR had an E-value of 2.72e-05 and an identity 65 % to lobster HMGR1, and a template length of 393 amino acids. The second template was the complex of *Psm*-HMGR with HMG-CoA and NAD⁺ (PDB ID 1qax) (Lawrence et al. 1995; Tabernero et al. 1999, 2003). *Psm*-HMGR had an E-value 0.00558 and an identity 19% to lobster HMGR1, and a template length of 425 amino acids. Since lobster HMGR1 was more similar to human HMGR than to *Psm*-HMGR, human HMGR was chosen as the template for predicting the structure of lobster HMGR as described below.

Similar to human HMGR, lobster HMGR contains three domains: an N-terminal "N-domain", a large "L-domain" and a small "S-domain". The N-domain (residues 82-150) is the smallest

of the three domains and contains five α -helices (green in Fig. 6 and Fig. 7). Residues 151-208 contain one α -helix and three β -strands (red in Fig. 6 and green in Fig. 7), forming the first portion of the L-domain. The S-domain (residues 209-312) is inserted into the L-domain and contains three α -helices and four β -strands (yellow in Fig. 6 and Fig. 7). Residues 313-491 form the second portion of the L-domain and contain ten α -helices and three β -strands (red in Fig. 6 and orange-red in Fig. 7). Thus, the L-domain (residues 151-208 and 313-491) contains eleven α -helices and six β -strands. The 27-residue α -helix (L α 10) forms the central structural element and is surrounded by the other elements of the protein.

DISCUSSION

The lobster MO is a good model for studying the regulation of a crustacean HMGR because of it has high levels of this enzyme (Li et al. 2003; 2004). ESA caused an increase in the size of the MO but this change was only obvious in the chronic phase of the treatment (days 6 and 14). During the acute phase of ESA (days 1 and 3), there was no change in MO weight in spite of the substantial increase in hemolymph levels of MF (to ~550 % of the initial level). Clearly, MO size is not strictly related to the output of MF by this tissue.

ESA caused an increase in hemolymph levels of MF which were mirrored in elevated levels of both HMGR and FAOMeT activity. During the acute phase of ESA, the MF levels rose substantially (to 550 % of the initial level). HMGR activity rose substantially (to ~260 % of the initial level) during this period while FAOMeT activity rose only modestly (~150 % of the initial level). These data suggest that HMGR activity is primarily responsible for the increased hemolymph levels of MF during the acute phase after ESA. Hemolymph levels of MF continued to rise in chronically ablated individuals, reaching even higher levels on day 14 (~1200 % of initial levels). HMGR activity rose only modestly after the acute phase (to ~370 % of the initial levels in day 14 animals). In contrast, FAOMeT activity showed a large increase (to ~1500 % of initial levels) in chronically ablated animals. These data support the view that FAOMeT is important for the increased hemolymph levels of MF in chronically ablated animals. It is, of course, simplistic to equate the *in vivo* levels of MF synthesis to the *in vitro* activities of these two enzymes alone. ESA may affect the activity of other enzymes in this pathway as well. In *Bombyx mori*, it was recently shown that transcripts for all of the enzymes involved in JH synthesis changed as JH synthesis changed (Kinjoh et al. 2007). In the lobster, we only have probes for two enzymes in the pathway. The rise in MF levels after ESA was reflected in the increased activities of both enzymes, though the changes in their activities depended on the length (acute or chronic) of ablation. We suspect that these differences are physiologically important in short-term and long-term changes in MF production.

In mammals, HMGR activity can be regulated by HMGR kinase, an AMP-activated protein kinase which phosphorylates a serine near the active site (Clarke and Hardie, 1990). However, in an earlier study we showed that lobster HMGR lacks the conserved serine which is found in most class I HMGRs (Li et al. 2004). Furthermore, we showed that treatment with lambda phosphatase did not increase HMGR activity and incubation of lobster MO homogenates with ATP to allow endogenous kinases to phosphorylate the enzyme did not decrease its activity (Li et al. 2004). In this study we investigated whether any other serine or threonine sites in lobster HMGR were phosphorylated. Western blots of native HMGR or recombinant HMGR1 were tested with anti-phosphoserine and anti-phosphothreonine antibodies, and neither stained the lobster protein. Hence, reversible phosphorylation is not the mechanism for the short-term regulation of lobster HMGR activity after ESA. Obviously, these data do not rule out other regulatory mechanisms, such as acetylation.

Enzyme activity can also be modified by changing the rate of protein degradation. In mammals, protein degradation mediated by the interaction of Insig-1 with the N-terminal sterol sensing

domain, is an essential mechanism for regulating HMGR activity (Rawson, 2003). Since lobster HMGR does not contain an N-terminal sterol-sensing domain, degradation by this mechanism can't occur. Likewise, lobster HMGR lacks a membrane domain at the N-terminus which can be removed by a membrane-bound cysteine protease (Moriyama et al. 1998). Lobster HMGR does contain a putative PEST site plus an unusual extension of ~100 amino acids at its C-terminus. These could be part of a mechanism that regulates lobster HMGR half-life and may be important to increase lobster HMGR levels after ESA. Clarifying the function of this region will require further experiments.

Another mechanism used to regulate HMGR levels is to change the levels of its mRNA (Nakanishi et al. 1988). We found that the acute (3 days after ESA) changes in MF levels (~550 % of initial levels) were not reflected in a change in HMGR- or FAOMeT-mRNA levels (94 % and 103 % of initial levels respectively). In contrast, chronic (14 days after ESA) changes in hemolymph levels of MF were larger (1200 % of initial levels) and were paralleled by increases in both HMGR- and FAOMeT-mRNA (300 and 270 % of initial levels, respectively). The effect of chronic ESA on these enzymes is more consistent with the results observed in *Samia cynthia ricini* (Sheng et al. 2008). While the rise in lobster HMGR activity after chronic ESA is similar to the increase in HMGR-mRNA levels, there is considerable difference between the increased activity and mRNA levels of FAOMeT.

Although lobster HMGR has several unusual structural features, it is still a class I HMGR. Molecular modeling indicates that the catalytic mechanism of lobster HMGR is similar to that of human HMGR (Istvan et al. 2000; Istvan and Deisenhofer, 2001). This hypothesis is supported by the high identity and similarity of the primary sequences of lobster HMGR and human HMGR. Both enzymes have the same three domains (N-, L-, and S-domains), secondary structure, and three-dimensional structure. In each domain, the number and organization of α -helices and β -strands are the same.

The residues of human HMGR involved in substrate binding (CoA, bold and pink; NADPH, bold and gray; HMG, bold and turquoise in Fig. 6) were conserved in lobster HMGR. Considering the similarity of sequence and structure between human HMGR and lobster HMGR, we propose that lobster HMGR binds its substrates in the same way as human HMGR. Likewise, statins are potent competitive inhibitors of HMGRs, and contain a HMG moiety and rigid hydrophobic groups linked to the HMG moiety. As reported previously, the K_I values of the native lobster HMGR and rec-HMGR1 for lovastatin are 0.45 and 1.3 nM, respectively (Li et al. 2003,2004). These values are similar to those observed in human HMGR. Concordant to observation, we noted that all the amino acid residues in the HMG-binding pocket in the lobster enzyme were similar to those in human HMGR. Thus, it seems likely that statins inhibit lobster HMGR by binding to the HMG-binding site and sterically block the substrate from binding to the enzyme's active site. Taken together, our analysis strongly supports the hypothesis that lobster HMGR and human HMGR monomers share common three-dimensional structures and catalytic mechanisms. Finally, HMGR enzymes in several species form active multimeric complexes. The residues responsible for forming these complexes are conserved across species. Thus, the residues responsible for dimer formation in human HMGR and *Psm*-HMGR were well conserved in lobster HMGR. The key dimerization element (ENVIGX₃I/LP) is located in the N-terminal residues (L β 1) of the L-domain. The second dimerization element is located in L α 6 and L α 7 of the L-domain of HMGR (bold in Fig. 6). While it appears that dimeric HMGR is catalytically active, in mammals the hydrophobic nature of the protein surfaces makes tetrameric HMGR more stable than dimeric HMGR. Human HMGR contains three residues which link two dimers together to form the final tetramer structure. These residues were also conserved in lobster HMGR1: buried salt bridges between residues R264 and E400 and hydrogen bonds between residue E318 and E318 from neighboring monomers establish four anchor points of the saddle (bold and dark yellow in Fig.

6). We showed in a previous study (Li et al. 2004) that when native lobster HMGR was separated by gel filtration the HMGR activity eluted in a major peak (~500 kDa) and a minor peak (~120 kDa). Likewise, the enzyme activity of rec-HMGR1 eluted from the gel filtration column with similar sized peaks (Li and Borst, unpublished). These results strongly support the suggestion that lobster HMGR subunits form multimeric structures.

The regulation of the MO appears to be complex. However, one source of regulating factors obviously is from the eyestalk SG, since removal of the eyestalk causes MF production by the MO to increase. Peptides identified as potential MOIHs were isolated from two crab species a decade ago (Wainwright et al., 1996; Liu et al., 1997). In both species, these peptides are closely related to CHH. Both peptides were isolated by testing MOs incubated *in vitro* with high levels of peptide. Neither peptide is very active in regulating MF production *in vivo*, though one of the MOIH peptides appears to affect glucose levels in *Uca pugilator* (Liu et al., 1997). The cellular and molecular mechanisms of these peptides are not well understood (Wainwright et al., 1998). Recently, a novel MOIH from the crab *Carcinus pagurus* was partially characterized (Borst et al., 2002). Since then, we have partially purified a similar peptide from the lobster eyestalk. These novel MOIH peptides do not cross-react with antisera to CHH or the MOIH described by Wainwright and colleagues (1998) in *Cancer pagurus*. They work at low levels *in vivo* but have little effect on MO tissue *in vitro*. Thus, we assume that these novel MOIH peptides must act upon the MO indirectly.

MOIHs are thought to inhibit MF synthesis in the MO by decreasing the activities of enzymes involved in MF biosynthesis, such as FAOMeT and HMGR (Wainwright et al. 1998; Li et al. 2003). Our experimental data indicated that the lobster MOIH we isolated tightly regulated FAOMeT but had no effect on HMGR. Since SG extracts can decrease lobster HMGR levels (Li et al. 2003), there must be an additional factor in the extract that regulates HMGR activity. Isolation of this compound and the identification of its signal transduction pathway may explain the different characteristics of the MOIH peptides that have been isolated to date.

Acknowledgments

This work was supported by NIH R15 HD37953-01 and NSF IBN 0240903 to DWB. We express our appreciation to Mr. William Hollier at Legal Seafoods in Boston for the generous donation of lobster heads for harvesting eyestalks.

Abbreviations used

HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MO, mandibular organ; MF, methyl farnesoate; ESA, eyestalk-ablated; SG, sinus gland; MOIH, mandibular organ inhibiting hormone; FAOMeT, farnesoic acid O-methyltransferase.

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Figure 1. ESA causes an increase in hemolymph levels of MF in male lobsters

Day 0 animals were intact or controls (n = 4). The other animals had been ESA for one day (n = 5), three days (n = 4), six days (n = 5), and 14 days (n = 9). A) MF levels after ESA. B) Mandibular organ (MO) weight (bars) and MF levels (gray line, values from 1A). Data show the mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$; ANOVA).

Figure 2. ESA increases HMGR and FAOMeT activity of the MO

One MO from each animals analyzed in Figure 1 was analyzed for HMGR and FAOMeT activity. **(A)** HMGR activity in the MO increased to 250% of the initial (control) value one day after ESA and then slowly rose to 370% of the initial levels 14 days after ESA. **(B)** FAOMeT activity in the MO increased modestly to 150% of the initial level one and three days after ESA, and then rose significantly six (500%) and 14 (1500%) days after ESA. Data show the mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$; ANOVA).

Figure 3. ESA increases mRNA levels

The MOs from the animals in Figure 1 were used to measure the levels of HMGR and FAOMeT-mRNA. A) mRNA levels for HMGR were unchanged from control values (day 0) on days 1 and 3 after ESA, increased modestly by day 6 (150%) and were significantly elevated (270%) by day 14. B) mRNA levels for FAOMeT were unchanged days 1 and 3 after ESA, then increased (300%) on days 6 and 15. Data show the mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$; ANOVA).

Figure 4. The effects of MOIH on MF production in ESA lobsters

Injection of a partially purified MOIH (0.05 SG equivalents in *Homarus* saline) in animals ESA for 14 days caused a significant decrease in MF levels after 5 hours. A sharp decline in FAOMeT activity and its mRNA levels was also observed after the injection. However, MOIH treatment had no significant effect on either HMGR activity or the level of its mRNA. The mean $\pm$ SEM for each group (N = 5) is shown (** = $P < 0.01$, * = $P < 0.05$, t-test).

Figure 5. Lobster HMGR is not a phosphoprotein

An immunoblot of lobster HMGR using anti-phosphoamino acid antibodies is shown. Lanes 1-4: Coomassie-stained SDS-PAGE. Lanes 5-7: Western blot probed with an anti-phosphoserine antibody. Lanes 8-10: Western blot probed with an anti-phosphothreonine antibody. Lane 1, standard proteins (kDa); lanes 2, 5, and 8, *D. melanogaster* CTP:phosphocholine cytidyltransferase isoform 2; lanes 3, 6, and 9, lobster HMGR from MO; lanes 4, 7, and 10, recombinant lobster HMGR1 expressed in Sf9 insect cells.

Figure 6. Structure-based sequence alignment of lobster HMGR1 and human HMGR
 HMGR1_PSS: potential secondary structure of lobster HMGR1; HMGR1_Seq: sequence of lobster HMGR1; -----: sequence alignment between HMGR1 and ihwi, the PDB ID for human HMGR (Istvan et al. 2000; Istvan and Deisenhofer, 2001); ihwi_Seq: sequence of ihwi; ihwi_SS: secondary structure of ihwi; CORE: comparison score of the secondary structures between HMGR1 and ihwi. Lobster HMGR1 contains three domains: the N-domain is green, the L-domain is red, and the S-domain is yellow. Solid arrows indicate β -strands and outlined rectangles indicate α -helices. Residues involved in dimerization are bold, and residues involved in tetramerization are bold and marked by dark yellow. Residues involved in binding with CoA, NADPH, and HMGR are all bold, marked by pink, gray, and turquoise, respectively. The phosphorylation site of ihwi is bold and blue. Predicted by 3D-PSSM and visualized by RasWin.

Figure 7. The three-dimensional structure of lobster HMGR1

Ribbon diagram of the HMGR1 monomer structure shows it contains three domains (N, L, and S). In this figure, the N-domain is green, the first portion of L-domain is also green, the second portion of L-domain is orange to yellow, and the S-domain is yellow. Predicted by SwissModel and visualized by RasWin.