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Arrest of transcription following anoxic exposure in a marine mollusc

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Abstract The intertidal marine snail, *Littorina littorea*, is an anoxia tolerant species that endures long-term oxygen deprivation using a suite of compensatory metabolic adaptations that includes overall metabolic rate depression. Nuclear run-off assays were used to quantify the relative rates of mRNA transcription in nuclear extracts from hepatopancreas of aerobic and anoxic snails. Total [^{32}P]-UTP incorporation into RNA by nuclei from 48 h anoxic snails ranged from 42 to 50% of that observed for nuclei from normoxic snails. When this data is transformed with respect to incubation time, the rate of [^{32}P]-UTP incorporation by nuclei from 48 h anoxic snails showed a decrease of 68% as compared with the normoxic level. Examination of selected expressed sequence tags also showed an overall decrease in mRNA transcription levels in samples derived from anoxic nuclei as compared with normoxic nuclei. Control of ribosomal translation was also examined by assessing the levels of the eukaryotic initiation factors eIF-2α and eIF-4E and the eukaryotic elongation factor-1γ (eEF-1γ). Levels of eIF-4E and eEF-1γ did not change between aerobic and anoxic states, but the amount of phosphorylated inactive eIF-2α rose strongly under anoxic conditions indicating that control of this factor is key to suppressing protein translation in anoxic snails. Since gene transcription is an ATP expensive process in cells, suppression of transcription to minimum levels provides substantial energy savings for the hepatopancreas, and the organism as a whole, under anoxic conditions.

Keywords *Littorina littorea* · Snail · Transcription · Metabolic suppression · Anoxia · Mollusc

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Introduction

A variety of marine organisms, especially those that occupy the intertidal zone, experience intermittent periods of aerial exposure as a routine occurrence and show adaptive responses at cellular, molecular, and systemic levels that prevent injury due to hypoxia/anoxia when O_2 uptake by gills is interrupted [1]. Indeed, many intertidal invertebrates can endure days or weeks of severe hypoxia and/or anoxia. Studies to date have placed heavy emphasis on the adaptations of intermediary metabolism and enzyme regulation that accompany the aerobic–anaerobic transition and allow intertidal organisms to sustain anaerobic life but little is yet known about the molecular biology underlying these processes.

One of the critical adaptations that supports long-term anaerobiosis is the ability to strongly reduce metabolic rate to a level that can be sustained over the long-term by fermentative pathways of ATP generation [2]. By coordinating and prioritizing the suppression of a wide variety of ATP-expensive cellular processes, the metabolic rate of marine molluscs under anoxia is often reduced to <10% of the resting aerobic rate, and this greatly extends the time that fixed tissue reserves of fermentable fuels (carbohydrates, selected amino acids) can sustain life [3–5]. Other key biochemical adaptations of anoxia tolerant molluscs

include the maintenance of high reserves of fermentable fuels in all tissues and the use of alternative pathways of fermentative metabolism to reduce the acid load normally associated with anaerobic glycolysis and increase the yield of ATP per molecule of glucose catabolized [3, 5]. Marine molluscs also adjust pathways of macromolecular synthesis to selectively synthesize specific mRNA transcripts and proteins in response to anoxia and do this against a background of overall strong suppression of translational activity [1, 6]. The present study examines aspects of gene transcriptional control in response to anoxia in the marine intertidal snail, *Littorina littorea*, to determine whether anoxia-responsive metabolic rate depression includes a reduction in the overall rates of mRNA synthesis.

Materials and methods

Animals

Marine periwinkles, *L. littorea*, were acclimated at 10°C in aerated tanks of artificial seawater without feeding for 1 week prior to experimentation. To prepare for anoxia exposure, a small amount of seawater (~3 cm depth) was placed in the bottom of glass incubation jars to maintain high humidity; gas bubblers were then inserted into the water and each jar was gassed with nitrogen gas (N_2) for 30 min. After this time, snails were quickly transferred into the jars, which were then sealed except for the N_2 port line. Gassing was continued for a further 20 min and then the gas line was removed and jars were fully sealed. Jars were transferred to a 10°C incubator and the timing of anoxia exposure was begun with snails sampled after 12 and 48 h. Control animals were held at 10°C in aerated jars and were sampled after the time course. Prior to sampling, the N_2 bubbler was reinserted into jars containing anoxic snails. Individual snails were quickly removed from the jar, the shell was removed, and the hepatopancreas quickly dissected out. Freshly dissected tissue was used for nuclear isolation.

Nuclear isolation

Nuclear isolations were performed as described previously [7]. All chemicals and glassware were treated with DEPC (diethyl pyrocarbonate) or were RNAse-free. Samples of hepatopancreas (5 animals, ~2.5 g) were homogenized using a Potter–Elvejhem homogenizer in ice-cold homogenization buffer (5 ml) consisting of 250 mM sucrose, 50 mM HEPES, pH 7.5, 25 mM KCl, 1 mM EGTA, and 1 mM EDTA. The homogenate was filtered through two layers of cheesecloth and 4 ml of eluant was placed on top of a 10 ml sucrose cushion (2.0 M sucrose, 10% v/v glycerol, 50 mM HEPES, pH 7.5, 25 mM KCl, 1 mM EGTA, 1 mM EDTA). The cushion was centrifuged at 13,000*g* for 30 min at 4°C to separate nuclei from other organelles and cell debris. The cushion was decanted and nuclei were resuspended in a glycerol storage buffer (200 µl) consisting of 40% v/v glycerol, 75 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.5 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM EDTA, and 0.125 mM phenylmethylsulphonyl fluoride. A 10 µL aliquot of nuclei was removed, stained with 0.01% w/v methylene blue, and used to confirm nuclear integrity by light microscopy.

Transcriptional assays

Aliquots of nuclei equivalent to 20 µg of protein from aerobic (normoxic control) and anoxic snails were incubated for 0–20 min at 26°C in a final volume of 800 µl containing 0.1 M Tris-HCl, pH 7.4, 200 mM NaCl, 4 mM $MgCl_2$, 4 mM $MnCl_2$, 1.2 mM dithiothreitol, 0.4 mM EDTA, 1.25 mM GTP, 1.25 mM CTP, 1.25 mM ATP, 500 µCi [α-^{32}P]-UTP (Amersham), 10 mM creatine phosphate, 2 µg of creatine phosphokinase, 20 units/ml RNasin, 10 µl of heparin stock (1/10 dilution), and 20% v/v glycerol. Deoxyribonuclease (8 units) was then added and incubation proceeded for an additional 15 min at 37°C to terminate the reaction. Total RNA was isolated from nuclei using Trizol reagent (Invitrogen) and resuspended in 200 µl of DEPC-treated water. Aliquots (20 µl) from each sample of RNA were passed through G-50 Sephadex columns (0.5 ml) and the incorporation of [α-^{32}P]-UTP was quantified using a Packard 1900CA scintillation counter.

Dot blot preparation and hybridization

Differential screening of a cDNA library made from hepatopancreas of anoxic snails (as described in [8]) revealed a selection of clones representing a variety of expressed sequence tags (ESTs) from *L. littorea*. Inserts were isolated from plasmids by digestion with appropriate restriction enzymes and separated via electrophoresis on a 1% TAE agarose gel. The insert bands were isolated from the gel slices and blotted in aliquots of 1 µl onto a piece of Hybond 0.45 µm nylon membrane (Amersham) that had been presoaked in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Blots were air-dried and baked in a BioRad Model 583 gel dryer for 2 h at 80°C. Pre-hybridization of dot blots was carried out at 44°C with 1 ml of modified Church's buffer (0.25 M Na_2HPO_4, 0.25 M NaH_2PO_4, pH 7.5, 1 mM EDTA, 7 % w/v SDS) in a hybridization oven for a minimum of 30 min. Radiolabeled RNA transcripts from the run-off assays were boiled for 5 min, snap-cooled on ice, then added to the hybridization solution. Hybridization was conducted for 16 h at 44°C, after which blots

were washed in 6× SSC at room temperature for 15 min. Membranes were exposed to Kodak XAR5 film at –80°C for an appropriate time period and developed manually using Kodak chemicals (Rochester, NY). Densitometric analysis of the developed autoradiograms was performed with the aid of computer imaging software. A Scan Jet3C scanner (Hewlett-Packard, Palo Alto, CA) was used in conjunction with Deskscan II v2.2 software (Hewlett-Packard) to produce high-resolution computer generated images, which were then analyzed with ImagequaNT v4.2 (Molecular Dynamics).

Western blotting

Hepatopancreas samples (~0.5 g each) were gently homogenized with a ground glass homogenizer in 2 ml of homogenization buffer (25 mM Tris, pH 7.6, 25 mM NaCl, 100 mM sucrose, 1% w/v SDS) and centrifuged at high speed to remove debris. Soluble protein concentrations were measured using the Coomassie blue dye-binding method and the BioRad commercial reagent (BioRad, Hercules, CA) with an MR 5000 Microplate Reader (Dynatech Laboratories, Chantilly, VA). Aliquots containing 20 μg of protein were separated on 12% SDS-polyacrylamide gels. Protein was wet-transferred to PVDF membrane using a trans-blot apparatus (BioRad) and transfer buffer (25 mM Tris, 192 mM glycine, 10% v/v methanol, pH 8.5). The membrane was placed in a petri dish and blocked for 2 h at room temperature using MTBST (1% w/v powdered skim milk, 150 mM NaCl, 50 mM Tris, 0.05% v/v Tween 20). Membranes were incubated with primary antibody (1:1,000) in 10 ml of MTBST overnight at 4°C. Primary antibodies to eIF-2α, phospho-eIF-2α (Ser51), and eIF-4E were purchased from Cell Signaling Technology. eEF-1γ antibody was a gift of Dr. J. Dijk (Sylvius Laboratory, Leiden University, The Netherlands). Secondary rabbit IgG HRP-conjugated antibody (1:1,000) (Cell Signaling Technology) was used to detect bound primary antibody and visualized using enhanced chemiluminescence (Renaissance, Perkin Elmer Life Sciences, Inc.). Blots were exposed to X-ray film for an appropriate length of time, film was developed, and subsequent densitometric analysis was performed using ImagequaNT v4.2 (Molecular Dynamics).

Results

RNA synthesis and activity

Nuclear run-off assays were used to measure the relative rate of mRNA transcription. Nuclei isolated from hepatopancreas of normoxic *L. littorea* were used to determine the optimal incubation period to quantify the rate of incorporation of [^{32}P]-UTP into nascent mRNA transcripts. Initiation of new mRNA transcripts is negligible [9], so this system measures the elongation of mRNA transcripts that are being actively transcribed. Linear incorporation of [^{32}P]-UTP into transcripts was observed up to 20 min incubation time (Fig. 1). The level of [^{32}P]-UTP incorporation was then determined for hepatopancreas nuclear extracts from 48 h anoxic and aerobic control snails at three different incubation times (10, 15, and 20 min). [^{32}P]-UTP incorporation into RNA by anoxic nuclei was significantly lower than incorporation by normoxic nuclei within this timeframe, with mean levels ranging from 42–50% of the aerobic values (Fig. 2A). Transforming the data with respect to time yielded an overall rate of [^{32}P]-UTP incorporation into RNA transcripts. The rate of [^{32}P]-UTP incorporation using nuclei obtained from 48 h anoxic snails was determined to be 3.4 ± 0.5 cpm × 10^3/μg nuclear protein/h compared with 10.6 ± 0.8 cpm × 10^3/μg nuclear protein/h for normoxic controls, a reduction under anoxia of 68% (Fig. 2B).

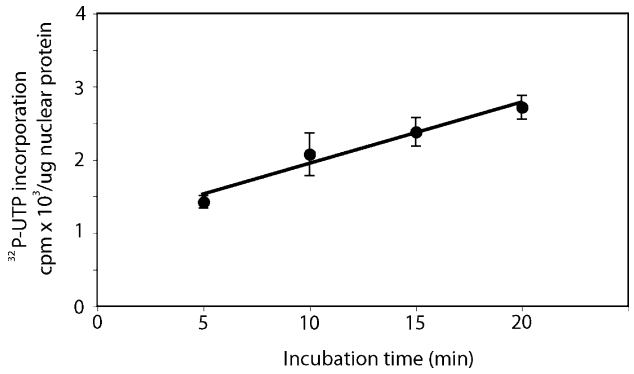

Fig. 1 Determination of the optimal incubation period to quantify the incorporation of [^{32}P]-UTP into nascent mRNA transcripts. Nuclei were isolated from hepatopancreas of aerobic snails (normoxic control) and levels of incorporation of [^{32}P]-UTP were measured at intervals up to 20 min incubation time. Data are means ± SEM, $n = 6$ independent samples for each time point

Expression patterns of selected mRNA transcripts

A variety of expressed sequence tags (ESTs) were obtained through the initial screening of a cDNA library constructed using hepatopancreas from anoxia-exposed *L. littorea*, as described in Larade et al. [8]. Representative clones were dotted onto a membrane and reverse-screened using the radiolabeled nuclear mRNA isolated for the transcription assays described above. Normoxic and anoxic samples were compared to determine if transcriptional levels of selected genes changed relative to the internal control gene α-tubulin. Previously examined genes (KVN and RPL26) [7, 8] were used as positive controls and ferritin [10] was

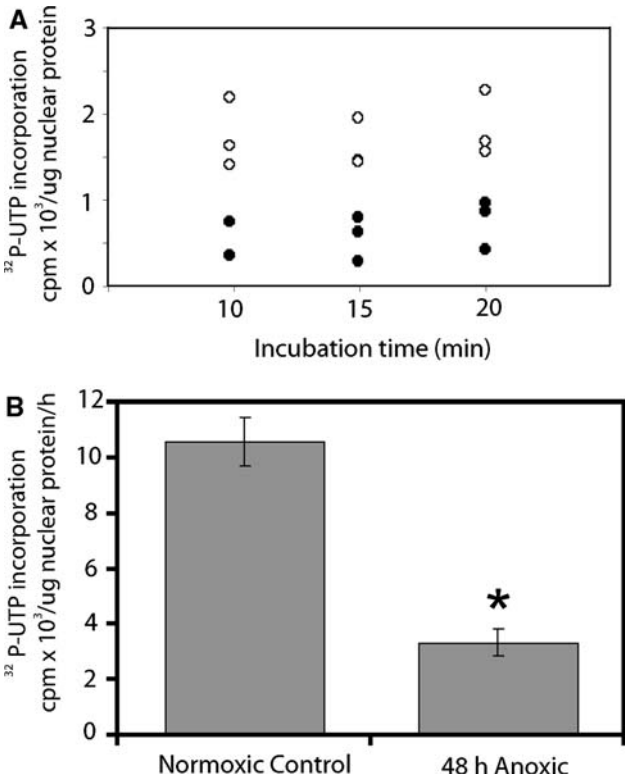

Fig. 2 (**A**) Determination of [^{32}P]-UTP incorporation into mRNA in hepatopancreas nuclei from 48 h anoxic (filled circles) and aerobic (open circles) snails. Nuclei from each condition were incubated for 10, 15, or 20 min, followed by reaction termination and mRNA isolation. Radiolabel incorporation was determined by scintillation counting. Data show $n = 3$ samples for each condition for each time point. (**B**) The data from A was transformed with respect to time to yield an overall rate of [^{32}P]-UTP incorporation into RNA transcripts. Data are means ± SEM, $n = 9$ for each condition. *Significantly different from aerobic control as determined by the Student's t-test, $P < 0.001$

used as a neutral control. As predicted from previous studies, mRNA transcript levels for KVN and RPL26 were elevated in nuclei from hepatopancreas of anoxic snails as compared with aerobic controls, whereas ferritin heavy chain transcript levels were unchanged between the two states (Fig. 3A, left panel). We then assessed the transcription of three ESTs that were identified from cDNA library screening as not differentially regulated during anoxic exposure. Figure 3B shows that the mRNA levels of these three ESTs (determined from Northern blots) were not altered under anoxia when evaluated in whole tissue extracts relative to their corresponding rRNA band intensities that control for loading differences. However, in all cases, these representative genes showed reduced transcript production in vitro in samples from nuclei of anoxic, compared with aerobic, snails (Fig. 3A, right panel). This strongly indicates that anaerobiosis involves an overall suppression of nuclear gene transcription as compared with the normoxic state.

Protein synthesis

Western blotting was used to determine the total content and phosphorylation status of selected ribosomal initiation and elongation factors in *L. littorea* hepatopancreas in response to anoxia exposure (Fig. 4). The relationships between the different transcription factors are outlined in Fig. 4A, with the specific factors assessed highlighted in gray. The eukaryotic initiation factor 2 (eIF-2) carries the initiating methionine residue for peptide synthesis and phosphorylation of its alpha subunit is key to turning off eIF-2 function. Immunoblotting showed that total eIF-2α content of hepatopancreas did not change after 12 h of anoxia exposure but the amount of phosphorylated eIF-2α increased dramatically by 14-fold under anoxic conditions (Fig. 4B). The total content of another translation initiation factor, eIF-4E, also remained unchanged under anoxic conditions (Fig. 4C, left panel) but, unfortunately, the phospho-specific antibody for this protein could not successfully detect the correct protein in snail hepatopancreas extracts (results not shown). The eukaryotic elongation factor-1 is another major translational control point. Immunoblotting was also applied to measure levels of the gamma subunit (eEF-1γ) in snail hepatopancreas, but no change in the content of this protein was detected between aerobic and anoxic states (Fig. 4C, right panel).

Discussion

Many marine invertebrates have excellent anoxia tolerance [1, 11]. For example, periwinkle snails, *L. littorea*, readily endure natural periods of oxygen deprivation in the intertidal zone and survive long-term anoxia exposure in a laboratory setting. When oxygen is limiting, these snails employ a suite of compensatory mechanisms and enter a hypometabolic state, minimizing ATP use by nonessential processes. Pathways of macromolecule biosynthesis are highly sensitive to ATP supply and eukaryotic systems have shown a strong dependence on ATP concentration for both RNA and protein synthesis [12, 13]. The present data demonstrate a profound suppression of nuclear transcription in *L. littorea* hepatopancreas after 48 h of anoxic exposure, with the rate of elongation of nascent mRNAs dropping to one-third of the normoxic rate (Fig. 2B). As demonstrated previously by our lab, *L. littorea* also reduce the overall rate of protein synthesis under anoxia; the rate of protein synthesis by hepatopancreas fell by 50% within 30 min of anoxia exposure and this level of translational suppression was maintained over several days of anoxia exposure [14]. Hence, both transcriptional and translational suppression are coordinated energy-saving responses to anoxia exposure in this species.

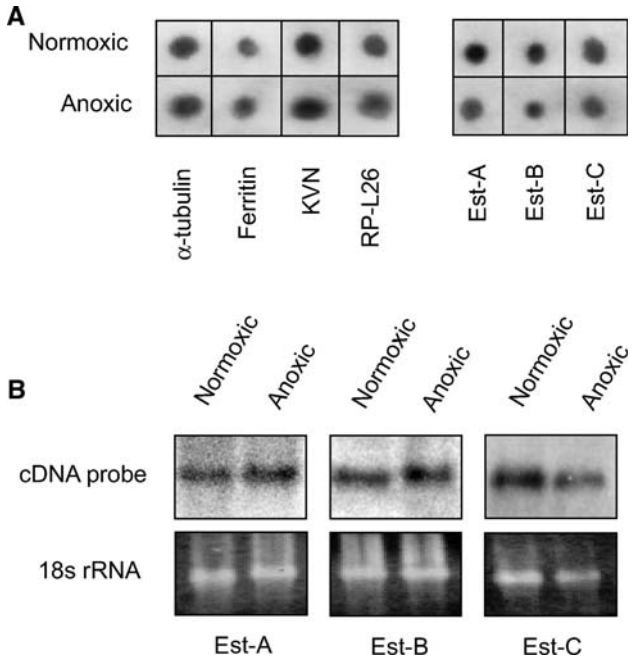

Fig. 3 (**A**) Nuclear run-off assay using nuclei prepared from the hepatopancreas of normoxic and 48 h anoxic *L. littoria*. Transcripts labeled with [^{32}P]-UTP were hybridized to nylon membranes carrying immobilized inserts coding for the clones indicated. (**B**) Expression of ESTs in normoxic (aerobic) and anoxic (48 h) hepatopancreas of *L. littorea* as shown by Northern blots. Total RNA was resolved on a 1.5% formaldehyde gel, stained with ethidium bromide, and visualized under UV light (bottom panel) to confirm equal loading. RNA was blotted onto nitrocellulose and hybridized at 44°C to [^{32}P]-labeled probes produced from each cDNA clone (top panel)

Dot blots using inserts representing selected ESTs were used to illustrate the extent of labeling of individual transcript types after incubation of nuclei isolated from normoxic versus 48 h anoxic snails. ESTs that did not show increased expression during anoxia, as determined via Northern blotting, had lower levels of [^{32}P]-dUTP incorporation into existing nuclear transcripts (Fig. 3B) indicating that transcription of these genes is reduced under anoxic conditions. This is not surprising, given the sharp decrease in global transcription, with suppression of transcription expected for genes that may not be critical for survival under anoxic conditions. Transcription of specific mRNAs is significant for the reason that these genes may play an important role during oxygen lack or during the re-oxygenation period, whereas transcription of other genes that may not be required for anoxia survival are effectively ''turned off'' as a means to conserve energy. Consequently, ''economical'' use of limited energy resources is achieved by focusing ATP use on the synthesis of a few selected transcripts of putative importance to anoxia survival. As expected, KVN and RPL26 transcripts were increased

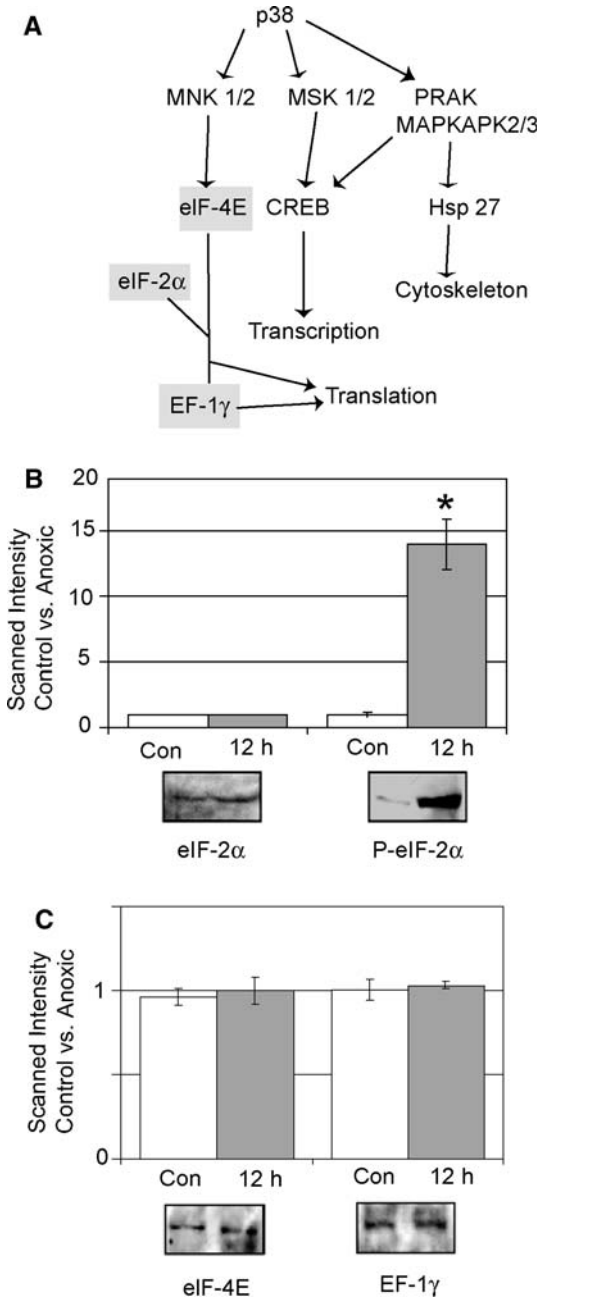

Fig. 4 (**A**) Map of selected proteins involved in signal transduction and regulation of ribosomal translation. Interacting factors are joined by straight lines, with arrows leading from activator (e.g., kinase) to target protein (e.g., transcription factor). Proteins and factors analyzed via Western blotting in the present study are highlighted by a shaded box. (**B**) Western blotting of protein samples (20 μg) from hepatopancreas of aerobic versus 12 h anoxic snails. Total and phosphorylated protein content was determined for eIF-2α (top panel) and total protein content was measured for eIF-4E (bottom left) and eEF-1γ (bottom right). Representative blots are shown and histograms present the scanned intensities of aerobic control (Con) and 12 h anoxic samples as means + SEM, $n = 3$ independent samples. *Significantly different from aerobic control as determined by the Student's t-test, $P < 0.001$

relative to α-tubulin in nuclei from anoxic snails, which is in agreement with the increased transcription and expression observed in our previous studies [7, 8].

Additional experiments were performed to follow-up previous results from this lab that examined the status of the translational machinery in hepatopancreas of anoxic snails [14]. Ribosomal translation is controlled by the activity states of multiple initiation and elongation factors; in turn, the activities of these factors are frequently regulated by reversible protein phosphorylation [15]. Translation initiation begins with delivery of the initiating methionine residue to the assembling ribosome by eIF-2α, a process that is inhibited by phosphorylation of its alpha subunit. Eukaryotic initiation factor 4 then brings the m7G-capped mRNAs to the assembling ribosome and is regulated by reversible phosphorylation control of the 4E binding protein (4E-BP1). Western blotting demonstrated that the total content of eIF-2α protein remained unchanged in *L. littorea* hepatopancreas after 12 h anoxia exposure. However, the amount of phosphorylated eIF-2α, which was barely detectable in aerobic control hepatopancreas, increased by 14-fold after 12 h of anoxia exposure (Fig. 3). This result demonstrates that protein synthesis would be strongly suppressed under anoxia due to inhibition of the delivery of the initiating methionine residues to ribosomes. We also examined the translation initiation factor eIF-4E, which binds the 7-methyl guanosine residues at the 5′ end of mRNA (known as the cap). eIF-4E is phosphorylated by the kinase MNK-1 [16], which is activated through phosphorylation by p38 MAPK [17, 18], a kinase that we have previously shown to be activated in during anoxia in *L. littorea* [19]. The levels of translation factor eIF-4E were examined to determine if there was a change following 12 h anoxia exposure. Total content remained unchanged (Fig. 4C, left panel), but the eIF-4E phospho-specific antibody could not successfully detect the correct protein in hepatopancreas extracts (results not shown), so it is not known whether the phosphorylation state of this protein also changed under anoxia. The eukaryotic elongation factor-1γ (eEF-1γ) plays an important role in peptide elongation on the ribosome; this factor is responsible for linking eEF-1β and eEF-1δ during GDP: GTP exchange in the translation process. Levels of this protein were also assessed by Western blotting but no change in protein content was detected between aerobic and anoxic states (Fig. 3). It is possible that phosphorylation of eIF-2α alone is sufficient to suppress protein translation in anoxic snails, although phosphorylation of other factors involved in translation cannot be ruled out at this time.

The examination of mRNA expression by *L. littorea* during anoxia exposure is interesting for a number of reasons. As mentioned above, the cost of producing mRNA and protein for a cell is high, particularly under anoxia when ATP availability is limited. Hence, suppression of transcription and translation to minimum levels can provide substantial energy savings for the hepatopancreas, and the organism as a whole, under anoxic conditions. However, the generation of specific transcripts under anoxia is significant because such genes may play an important role either during the period of oxygen lack or during the re-oxygenation period afterwards. Consequently, an ''economical'' use of limited energy resources to synthesize selected transcripts can be justified.

A number of adaptations allow the marine snail *L. littorea* to cope with anoxia exposure, including a net suppression of metabolic rate. The present data show that suppression of the energy-consuming processes of transcription and translation accompanies anoxia exposure to make a significant contribution to overall metabolic suppression. However, neither process comes to a complete halt under anoxia, thereby allowing the sustained low expression of some constitutive genes that are necessary in order to maintain homeostasis as well as the selected strong up-regulation of a few specific genes that play essential roles in anoxia survival.

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