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Activation of the carbohydrate response element binding protein (ChREBP) in response to anoxia in the turtle *Trachemys scripta elegans*

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ABSTRACT

Background: ChREBP (carbohydrate response element binding protein) is a glucose-responsive transcription factor that is known to be an important regulator of glycolytic and lipogenic genes in response to glucose. We hypothesized that activation of ChREBP could be relevant to anoxia survival by the anoxia-tolerant turtle, *Trachemys scripta elegans*.
Methods: Expression of ChREBP in response to 5 and 20 h of anoxia was examined using RT-PCR and Western immunoblotting. In addition, subcellular localization and DNA-binding activity of ChREBP protein were assessed and transcript levels of liver pyruvate kinase (LPK), a downstream gene under ChREBP control were quantified using RT-PCR.
Results: ChREBP was anoxia-responsive in kidney and liver, with transcript levels increasing by 1.2–1.8 fold in response to anoxia and protein levels increasing by 1.8–1.9 fold. Enhanced nuclear presence under anoxia was also observed in both tissues by 2.2–2.8 fold. A 4.2 fold increase in DNA binding activity of ChREBP was also observed in liver in response to 5 h of anoxia. In addition, transcript levels of LPK increased by 2.1 fold in response to 5 h of anoxia in the liver.
Conclusions: The results suggest that activation of ChREBP in response to anoxia might be a crucial factor for anoxia survival in turtle liver by contributing to elevated glycolytic flux in the initial phases of oxygen limitation.
General significance: This study provides the first demonstration of activation of ChREBP in response to anoxia in a natural model of anoxia tolerance, further improving our understanding of the molecular nature of anoxia tolerance.

1. Introduction

Availability of molecular oxygen is indispensible to many complex life forms on Earth due to its role as the final electron acceptor in oxidative phosphorylation. In vertebrates, oxygen deprivation quickly leads to inhibition of oxidative phosphorylation and a subsequent interruption of mitochondrial ATP production, resulting in disruption of many ATP-utilizing processes in the cell. Many animals experience situations of oxygen deprivation that occur either due to variations in environmental oxygen levels or to behaviors that interrupt oxygen supply and have evolved adaptations to cope with short or long term oxygen deprivation [1]. Among vertebrates, some freshwater turtles such as red-eared sliders (*Trachemys scripta elegans*) and painted turtles (*Chrysemys picta*) have highly developed anoxia tolerance and can survive without oxygen for several months when submerged in cold water (as occurs naturally in the winter) [2]. Several key mechanisms that contribute to anoxia tolerance have been identified and include profound metabolic rate depression, the ability to avoid acidosis through storage or buffering of lactic acid, and large tissue stores of glycogen to fuel anaerobic glycolysis [3–7]. In addition, various enzymatic and gene expression processes have been studied [7,8] but more remains to be learned about the molecular mechanisms that contribute to natural anoxia tolerance.

Re-adjustment of metabolism is important to optimize metabolic efficiency during anaerobiosis. Under aerobic conditions ATP can be generated by oxidizing multiple fuel sources, including carbohydrates, lipids and amino acids whereas under anoxia, anaerobic glycolysis typically becomes the sole method for ATP production. In addition, studies have shown that metabolic priorities are reorganized under anoxia, providing both global suppression of metabolic functions and a reprioritization of ATP use by different specific cell functions [9]. Reversible protein phosphorylation of key enzymes and functional proteins is a crucial mechanism mediating this metabolic reorganization [6]. However much still remains to be explored about the transcriptional responses that underlie metabolic reorganization in anoxia. Mechanisms that have to date been shown to contribute to anoxia tolerance include FoxO transcription factor regulation of downstream gene targets [10], controlled suppression of the cell division by selective adjustment of

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cell cycle proteins [11], and control of the p53 tumor suppressor protein [12].

The carbohydrate response element binding protein (ChREBP) is a glucose-responsive transcription factor that is predominately expressed in the liver, kidney and adipose tissue of mammals [13]. In recent years, ChREBP has emerged as an important regulator of glycolytic and lipogenic genes in response to glucose [14–18]. It does so by binding to glucose-responsive DNA elements, termed carbohydrate response elements (ChRE), in the promoter regions of selected genes [19–21].

ChREBP contains several phosphorylation sites for cAMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK) and is likely to be regulated by these kinases [22]. According to the present model, PKA targets the serine 196 residue on ChREBP, located near the nuclear localization sequence. Under basal conditions, ChREBP is phosphorylated on Ser-196, resulting in cytoplasmic localization. By contrast, high glucose conditions prompt the activation of protein phosphatase 2A (PP2A) by xylulose-5-phosphate (X5P), an intermediate of the pentose phosphate pathway, which then dephosphorylates ChREBP on Ser-196, allowing it to localize to the nucleus [23]. Once in the nucleus, a second dephosphorylation on Thr-666 enhances the DNA-binding activity of ChREBP, allowing it to bind to the ChRE in the promoter regions of glycolytic and lipogenic genes [22]. In addition to this 'classical' model for ChREBP regulation, it has recently been described that a constitutively active form of ChREBP, termed ChREBP-β, is localized in the nucleus [24]. In addition, it has been shown that ChREBP can also be regulated by other post-translational modifications including acetylation [25] and O-linked GlcNAcylation [26,27], suggesting that regulation of ChREBP is more complicated than previously thought.

One major glycolytic target gene of ChREBP is the liver isozyme of pyruvate kinase (LPK). Pyruvate kinase is a regulatory enzyme that contributes to the control of flux through glycolysis together with phosphofructokinase and hexokinase (or glycogen phosphorylase depending on the carbon source) [28]. Inhibitory control over PK is also required to promote gluconeogenesis in the liver when substrates such as lactate and alanine are high. PK catalyzes the formation of pyruvate and ATP from phosphoenolpyruvate (PEP) and ADP. The enzyme has four isozymes (L, R, M1 and M2) that are expressed in different tissues [29]. LPK is expressed predominately in the liver, but also in kidney, small intestine and pancreatic beta cells [29,30]. PK activity can be regulated in response to various hormones, nutrients and stresses by changes in its expression levels (protein synthesis and degradation), post-translational modifications (mainly reversible phosphorylation), and allosteric effectors (e.g. fructose-1,6-bisphosphate activation, L-alanine inhibition) [31].

Because of the pivotal role of glycolysis as the central and/or sole pathway of energy production under anoxia, and because of the importance of ChREBP in the regulation of glycolytic enzyme transcription, we examined the response of this transcription factor to anoxia in turtle tissues. The present study analyzes ChREBP transcript and protein expression, nuclear localization, and DNA-binding activity, as well as the transcript levels of its target gene LPK, in *T. s. elegans* in response to anoxia. Our results suggest a probable involvement of this transcription factor in early adaptation to anoxia in turtle tissues.

2. Materials and methods

2.1. Animals

Adult red eared slider turtles, *T. s. elegans*, were purchased from Carolina Biological. The turtles were originally placed in a holding tank containing dechlorinated water at ~11 °C for several days and then the temperature was lowered to ~4 °C over 1 week. Animals were acclimated at 4 °C ± 1 °C for 2 weeks before use. Aerobic control turtles were sampled from this condition, euthanized by decapitation, and then tissue samples were quickly dissected and immediately frozen in liquid nitrogen. To impose anoxia, other turtles were moved into containers filled with dechlorinated water at 4 °C that had been previously bubbled with 100% nitrogen gas for at least 6 h beforehand. After the turtles were placed in a tank, a wire mesh was fitted ~10 cm below the water line so that the turtles could not surface during the anoxic episode; a low level of nitrogen bubbling continued throughout. Animals were exposed to 5 or 20 h of anoxic submergence and then tissues were rapidly sampled. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee.

2.2. RNA extraction and cDNA synthesis

All solutions and materials were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved prior to use. Total RNA was isolated from liver and kidney of normoxic, 5 h and 20 h anoxic turtles using Trizol™ (Invitrogen). Briefly, 100 mg of tissue was homogenized in 1 mL Trizol using a Polytron homogenizer followed by the addition of 200 μL of chloroform and centrifugation at 10,000 ×*g* for 15 min at 4 °C. The upper aqueous phase (containing the RNA) was removed to a fresh tube and precipitated by the addition of 500 μL isopropanol followed by incubation for 10 min at room temperature (RT). The samples were then centrifuged again at 10,000 ×*g* for 15 min at 4 °C. The total RNA pellet was washed with 1 mL of 70% ethanol and centrifuged again as above. The resulting pellet was air-dried for 10–15 min and resuspended in 50 μL of DEPC-treated water. The quality of RNA was judged based on the ratio of absorbances at 260 and 280 nm as well as gel electrophoresis on a 1% agarose gel stained with ethidium bromide to check for the integrity of 18S and 28S ribosomal RNA bands.

To synthesize cDNA, 4 μg aliquots of RNA were diluted with DEPC water to a 10 μL final volume. Briefly, 1 μL of 200 ng/μL *Oligo dT* (5′-TTTTTTTTTTTTTTTTTTTTTV-3′) primer was added to each RNA sample and incubated at 65 °C for 5 min. The mixture was then chilled rapidly on ice and 4 μL 5× first strand buffer, 2 μL 100 mM dithiothreitol (DTT), 1 μL dNTP mixture (10 mM each) and 1 μL reverse transcriptase enzyme M-MLV (all reagents from Invitrogen) were added for a total volume of 19 μL. The mixture was incubated at 42 °C for 1 h and chilled to 4 °C. The resulting cDNA was diluted (10^{-1} to 10^{-3}) and used as a template for PCR.

2.3. Primers

The *ChREBP* forward and reverse primers were designed from a consensus sequence of mammalian and chicken *ChREBP* using the Primer Designer program, version 3.0 (Scientific and Educational Software). The nucleotide sequence of the forward *ChREBP* primer was 5′-CACA GCGGWCACTTCATGGT-3′ (W = A or T) whereas the reverse primer sequence was 5′-GGTCYGGCTGGATCATGTCA-3′ (Y = C or T). The *LPK* primers were designed based on a partial mRNA sequence for *LPK* available from NCBI (accession#: AJ243137). The nucleotide sequence of the forward *LPK* primer was 5′-GGCGAGAACATGGAGGTGGA-3 whereas the reverse primer sequence was 5′-CCACGAGCCACCATTATTCC-3′. Primers for the control gene *α-tubulin* were forward (5′-AAGGAAGATGCTGCCA ATAA-3′) and reverse (5′-GGTCACATTTCACCATCTG-3′). Primers were purchased from Sigma Genosys.

2.4. RT-PCR

The PCR program was as follows: 7 min at 94 °C followed by 36 cycles of 1 min at 94 °C, 1 min at 54 °C and 1.5 min at 72 °C. The final step was 72 °C for 10 min. PCR products were separated on 1% agarose gels, stained with ethidium bromide, visualized using the ChemiGenius imaging system (Syngene, Frederick, MD, USA) under UV light, and quantified using the GeneTools program. Bands from the most dilute cDNA sample that gave visible product were used for quantification to make sure that the products had not reached

amplification saturation. PCR products were sequenced by StemCore Laboratories (Ottawa, ON). The sequences were verified as encoding *ChREBP* or *LPK* using the program BLASTN (http://www.ncbi.nlm.nih.gov/blast) at the NIH. The partial sequence for *ChREBP* was shown to have 100% identity with the published sequence for the western painted turtle, *Chrysemys picta bellli* (NCBI accession: XM_005294339.2)

2.5. Protein extracts and Western blotting

Protein extracts were prepared from tissue samples of aerobic, 5 h and 20 h anoxic turtles, as previously described [32]. Aliquots containing 30 μg of protein were loaded into lanes of 10% SDS polyacrylamide gels, electrophoresed, and electroblotted onto polyvinylidene difluoride (PVDF) membranes, also as described previously. After the transfer of proteins, membranes were blocked for 10 min in Tris-buffered saline containing Tween-20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.1% v/v Tween-20) with 2.5% non-fat dried milk added. The blots were then probed overnight with a polyclonal antibody for ChREBP (Santa Cruz Biotechnology; diluted 1:1000 in TBST). After incubation, blots were washed several times with TBST and then incubated at room temperature for 45 min with secondary antibody, HRP-linked donkey anti-goat IgG (Santa Cruz Biotechnology; diluted 1:2000 in TBST). Immunoblots were developed using enhanced chemiluminescence (ECL) reagents.

2.6. Preparation of nuclear extracts

Nuclear extracts were prepared using a slight modification of the method described by Dignam et al. [33]. Briefly, 0.5 g of frozen powdered tissue samples were homogenized using a Dounce homogenizer in 1 mL of homogenization buffer (10 mM HEPES, 10 mM KCl, 10 mM EDTA, 1 mM DTT, pH 7.9). The DTT, as well as 10 μL of Protease Inhibitor Cocktail (Sigma) were added just prior to homogenization. Samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C and the supernatant (cytoplasmic extract) was removed. The pellet was resuspended in 150 μL of extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 10% v/v glycerol, 1 mM DTT); again, the DTT as well as 1.5 μL of Protease Inhibitor Cocktail (Sigma) were added just prior to adding buffer to the pellet. Tubes containing the samples were put on ice horizontally on a rocking platform for 1 h. Samples were then centrifuged at 10,000 ×*g* for 10 min at 4 °C. The supernatant (nuclear extract) was collected. Soluble protein contents of extracts were quantified and then samples were standardized to a common protein concentration using extraction buffer and prepared for Western immunoblotting [32]. The integrity of the isolated nuclei was confirmed by immunoblotting of cytoplasmic and nuclear fractions and probing with a histone H3 antibody (Cell Signaling; diluted 1:1000 in TBST).

2.7. DNA-binding assay

Nuclear extracts from all tissues were prepared as above, with the exception that following quantification, the protein concentration of the extracts was adjusted to 8 μg/μL by the addition of a calculated small volume of extraction buffer. Aliquots containing equal amounts of protein from each sample were then used to assess the amount of binding by ChREBP to its response element using a DNA-binding assay kit from Cayman Chemical (Ann Arbor, MI). Binding assays were performed according to the manufacturer's protocol. Briefly, ChREBP binding (16 μg of protein) to its DNA-element (provided by the kit) was carried out for 1.5 h at room temperature with mild agitation on a rocking platform. The plates were then washed with wash buffer to remove unbound transcription factors. ChREBP-specific primary antibody diluted 1:100 v/v in antibody buffer was then added to each well and incubated for 1 h at room temperature without agitation. The wells were then washed three times with washing buffer and the secondary antibody (HRP-linked anti-rabbit antibody diluted 1:100 v/v in antibody buffer) was then added to each well and incubated for 1 h at room temperature without agitation. The wells were then washed five times with washing buffer and the manufacturer's developing solution was then added. After developing for 45 min in the dark, the stop solution was added and color development was quantified by absorbance readings at 450 nm. Absorbance values (minus blanks) are proportional to the amount of DNA-binding over a wide range.

2.8. Data analysis

Bands on agarose or SDS-PAGE gels were scanned using a ChemiGenius Bio-Imaging system and densitometric analysis was performed using the associated GeneTools software (Syngene). RT-PCR bands for all genes were normalized against bands for *α-tubulin* amplified from the same cDNA sample. To control for loading irregularities in Western blots, the band intensity of immunoreactive material in each lane was standardized against the combined density of a group of Coomassie stained protein bands that were constant between control and experimental conditions and that were not located close to the protein band of interest, as previously described [10,32,34]. To standardize DNA-binding results, the mean OD from the negative controls was subtracted from the OD of each sample. Mean normalized band densities ± SEM were then calculated for control and anoxic samples. Significant differences among groups containing three conditions were assessed using analysis of variance followed by the Dunnett's post-hoc test. Significant differences between groups containing two conditions were determined using the Student's *t*-test. Statistical difference was accepted if $P < 0.05$.

3. Results

3.1. Expression of ChREBP in response to anoxia

Using RT-PCR and the primers derived from the consensus sequence of ChREBP from other vertebrates, a 640 bp PCR product was retrieved from total RNA prepared from *T. s. elegans* liver and submitted to GenBank (GenBank ID: KF984204). The product was confirmed as encoding a portion of ChREBP using BLAST. Amino acid translation of the nucleotide sequence showed it to have a 100% similarity to ChREBP from painted turtle (*C. picta bellii*, NCBI accession: XP_005294396.2), 83.5% similarity to ChREBP from chicken (*Gallus gallus*, NCBI accession: NP_001104311.1) and 79.7% similarity to ChREBP from mouse (*Mus musculus,* NCBI accession: NP_067430.2). Alignment of the amino acid sequences showed that the PKA phosphorylation site, Ser-196, was preserved in both turtle sequences (data not shown), suggesting a comparable mode of regulation to that in mammals.

Transcript levels of *chrebp* in kidney and liver from normoxic, 5 h anoxic and 20 h anoxic *T. s. elegans* were assessed using RT-PCR (Fig. 1A). A constitutively expressed gene, *α-tubulin,* was also amplified from the same samples and *chrebp* transcript levels were normalized against the tubulin transcript level in each sample. Transcript levels of *chrebp* increased slightly in the kidney by 1.16 ± 0.03 fold as compared with controls in response to 5 h anoxia and remained at 1.19 ± 0.03 fold higher after 20 h anoxic submergence (both $P < 0.05$ as compared with controls). In the liver, *chrebp* transcripts increased by 1.89 ± 0.06 fold compared with controls in response to 5 h of anoxia but returned to normoxic levels by 20 h anoxia.

ChREBP protein levels were assessed via Western blotting in kidney and liver extracts from normoxic, 5 h anoxic, and 20 h anoxic *T. s. elegans* (Fig. 1B). The polyclonal antibody crossreacted with a band of 95 kDa corresponding to the known molecular mass for ChREBP from other vertebrates. In both tissues, ChREBP protein levels increased significantly after 5 h anoxia but had returned to control values by 20 h. After 5 h anoxia exposure ChREBP protein values were 1.92 ± 0.21 fold higher

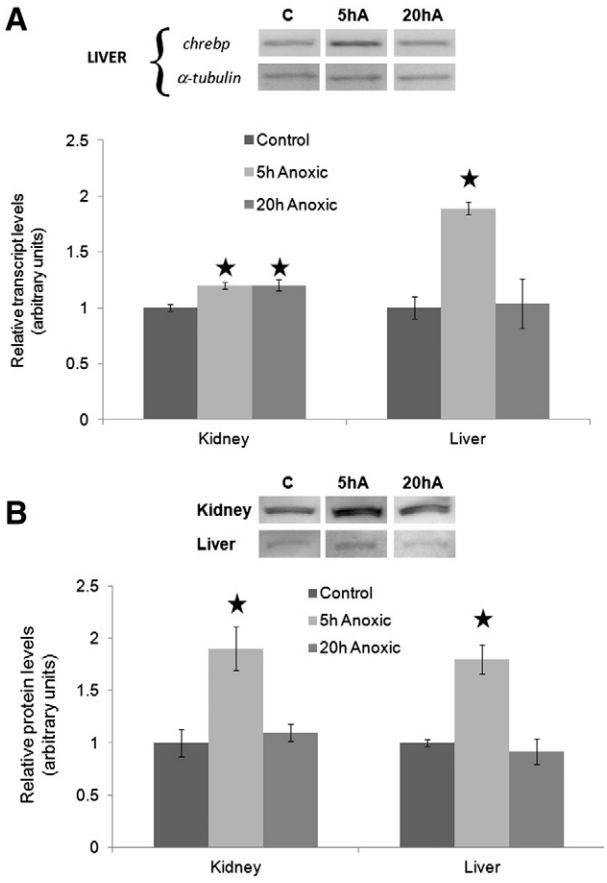

Fig. 1. Effect of 5 or 20 h of anoxic submergence on ChREBP gene and protein expression in *T. s. elegans* kidney and liver. (A) Changes in *chrebp* transcript levels in response to 5 or 20 h anoxia exposure, as determined by RT-PCR. Bands show representative *chrebp* amplicons for liver with corresponding *α-tubulin* bands amplified from the same samples. The histogram shows standardized transcript levels under control and anoxic conditions for liver and kidney; bands were standardized against the corresponding *α-tubulin* bands amplified from the same sample. (B) Changes in ChREBP protein levels in response to anoxia, as determined by Western immunoblotting. Representative immunoblot bands are shown above the histogram. The histogram shows normalized protein levels under control and anoxic conditions. Data are means ± SEM, $n = 3$–5 independent trials. * Significantly different from the corresponding control, $P < 0.05$.

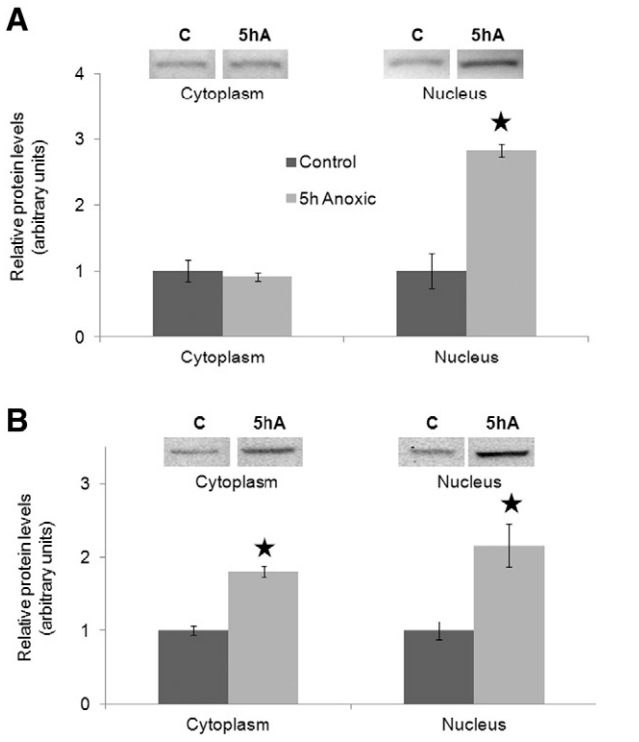

Fig. 2. Effect of 5 h anoxic submergence on subcellular distribution of ChREBP. Changes in ChREBP protein levels in kidney (A) and liver (B) cytoplasm and nucleus were determined by Western immunoblotting. Representative immunoblot bands are shown above the histogram. Data are means ± SEM, $n = 3$–4 independent trials. * Significantly different from the corresponding control, $P < 0.05$.

than the normoxic level in kidney ($P < 0.05$) and 1.83 ± 0.14 fold higher than controls in liver ($P < 0.05$).

3.2. Subcellular distribution of ChREBP

The subcellular distribution of ChREBP in kidney and liver of normoxic and 5 h anoxic turtles was assessed using Western blotting. Fig. 2A shows the relative protein levels of ChREBP in the cytoplasm and nucleus of kidney during normoxia and anoxia, whereas Fig. 2B shows the comparable data for liver. Cytoplasmic levels of ChREBP in kidney remained unchanged between normoxic and anoxic conditions, but the nuclear ChREBP content increased significantly during anoxia to 2.82 ± 0.10 fold higher than controls ($P < 0.05$). This increase indicates translocation of ChREBP to the nucleus under anoxic conditions. Liver ChREBP was elevated in both compartments in response to 5 h anoxia; cytoplasmic and nuclear ChREBP levels rose by 1.81 ± 0.07 and 2.16 ± 0.30 fold, respectively ($P < 0.05$).

3.3. DNA binding activity of ChREBP

Glucose-mediated activation of ChREBP is known to result in both nuclear translocation and enhanced DNA-binding activity, leading to induction of the transcription of downstream genes [22,23]. Relative changes in ChREBP DNA binding activity between normoxic and 5 h anoxic states were assessed in turtle kidney and liver (Fig. 3). Kidney showed no significant change in the DNA binding activity of ChREBP between the two states. By contrast, the liver showed a significant increase in the DNA binding activity of ChREBP during anoxia, rising by 4.63 ± 0.39 fold as compared with the control ($P < 0.05$).

3.4. cDNA cloning and mRNA levels of LPK

The primers designed for *LPK* were based on a *T. s. elegans* LPK sequence obtained from GeneBank (accession number: AJ243137). A 415 bp PCR product was retrieved from the total RNA prepared from turtle liver and was confirmed as *LPK* by BLAST. Transcript levels of *LPK* in livers from normoxic and anoxic turtles were assessed using RT-PCR (Fig. 4). *LPK* transcripts in liver rose to 2.14 ± 0.03 fold higher

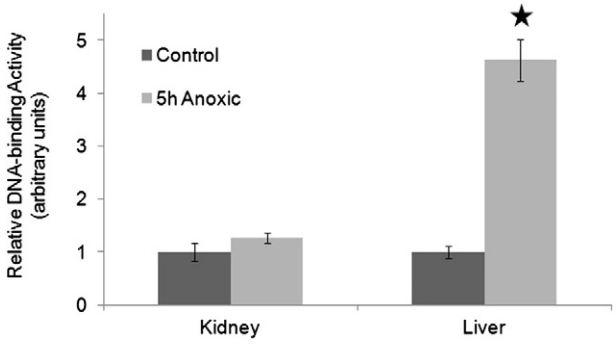

Fig. 3. Effect of 5 h anoxic submergence on the DNA-binding activity of ChREBP in nuclei isolated from kidney and liver as determined by a DNA-binding assay. Data are means ± SEM, $n = 3$–4 independent trials for each tissue. * Significantly different from the corresponding control, $P < 0.05$.

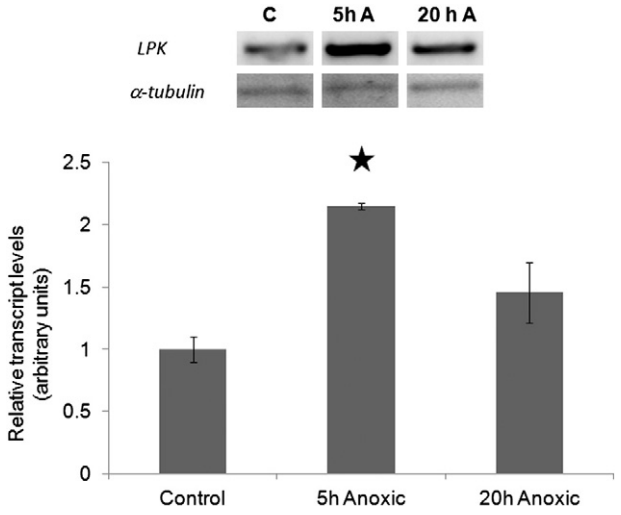

Fig. 4. Effect of 5 or 20 h anoxic submergence on *LPK* transcript expression in *T. s. elegans* liver as determined by RT-PCR. Bands show representative *lpk* amplicons with corresponding *α-tubulin* for control and anoxic conditions. The histogram shows normalized transcript levels under control and anoxic conditions; bands were normalized against the corresponding *α-tubulin* bands amplified from the same sample. Data are means ± SEM, $n = 3$–4 independent trials. * Significantly different from the corresponding control, $P < 0.05$.

than control values ($P < 0.05$) after 5 h anoxia but returned to normoxic values by 20 h.

4. Discussion

Several important processes occur when animals are subjected to oxygen limitation. Inability to proceed with oxidative phosphorylation renders anaerobic glycolysis the chief energy producer in the cell, creating a need for a higher glycolytic flux to compensate for energy demands. This can be obtained by changing the properties of the regulatory enzymes of glycolysis, as well as up-regulating the amounts of these enzymes, often mediated by the Hypoxia Inducible Factor (HIF). In anoxia-intolerant animals (such as mammals) elevated levels of glycolysis cannot be sustained for long because of the inefficiency of glycolysis, insufficiency of substrate stores (glucose, glycogen), and acidosis associated with the accumulation of lactate as the end product [1]. Turtles have solved these problems through several mechanisms, including large glycogen stores in the liver that fuel both liver glycolysis and provide glucose for export to other organs, buffering of acid build-up via Ca^{+2} and Mg^{2+} carbonates released from the shell, storage and buffering of lactate in the shell, and very strong metabolic rate depression that can lower energy demands during anoxia to just 10–20% of the corresponding aerobic rate at the same body temperature [6,35]. However, little is known to date about metabolic adjustments for anoxia survival at the gene level. The present study investigated ChREBP, a pivotal transcription factor that regulates carbohydrate metabolism, and its potential role in anoxia tolerance.

ChREBP has important roles in regulating enzymes of glycolysis and lipogenesis. To date its function has been primarily investigated in the liver, due to this organ's key role in carbohydrate metabolism. However, this transcription factor is also expressed in other tissues (including kidney), and is of potential importance in them. The classical model of ChREBP regulation is that under basal conditions it is found in the cytoplasm in a phosphorylated state but undergoes nuclear translocation and activation in response to elevated glucose levels [22,23].

Both transcript and protein levels of ChREBP increased in response to 5 h anoxia exposure (Fig. 1) in liver and kidney of turtles, suggesting that regulation at the level of gene expression could be a relevant mechanism of ChREBP activation during anoxia. Strong evidence of ChREBP activation under anoxia was also observed in liver including a 2.2 fold increase in nuclear ChREBP levels (Fig. 2), a 4.6 fold increase in ChREBP DNA-binding activity (Fig. 3), and a 2.1 fold increase in transcript levels of *LPK* in liver, a known target gene of ChREBP (Fig. 4). Kidney also showed elevated amounts of ChREBP in the nuclear fraction from 5 h anoxic turtles but DNA binding activity did not change. Notably, in both tissues, most anoxia-responsive changes were seen only in 5 h anoxic-exposed animals, and had reverted to control values after the longer term 20 h anoxia exposure. Although *LPK* gene expression probably also responds to other transcription factors, the correlation seen in turtle liver between ChREBP activation and the up-regulation of *LPK* strongly suggests that *LPK* expression is regulated in a ChREBP-dependent manner in response to anoxia. Brooks and Storey [36] reported a 20% increase in PK maximal activity in the livers of turtles subjected to 5 h anoxic submergence. This increase in activity could be potentially attributed to an increase in LPK protein levels resulting from ChREBP-mediated up-regulation of LPK gene expression. However, it should also be noted that the measured increase in PK activity could also be derived from a posttranslational mechanism such as enzyme phosphorylation [31]. PK is also subjected to allosteric regulation by small molecules such as alanine or $F1,6P_2$. Kelly and Storey [37] showed that $F1,6P_2$ levels rise quickly in response to anoxic submergence, which indicates glycolytic activation and the potential for PK activation in vivo by rising $F1,6P_2$.

Interestingly, other data from the Brooks and Storey study actually suggest that glycolysis is inhibited in turtle liver in response to anoxia. For example, changes in the kinetic constants for liver PFK under anoxia are consistent with a less active form [36]. However, it is difficult to draw a direct comparison here to this previous study, since the thermal conditions of the anoxia exposure were different: in the present study 4 °C was used, while 18 °C was used in the previous study. Since the metabolic rate of turtles is higher at higher temperatures, it is expected that 5 h of anoxic submergence at 18 °C would represent a much more advanced state of oxygen deprivation (and metabolic reorganization) compared to 4 °C. For example, in a study of the effect of temperature on lactate and glycogen in anoxic turtles, Warren and Jackson [35] showed that plasma glucose levels was 8-fold higher for 24 h anoxic turtles held at 15 °C compared to those held at 5 °C. Furthermore, another study from our group that characterized fructose-1,6-bisphosphate aldolase under normoxic and anoxic conditions at a lower temperature showed that enzyme to be more active under anoxia in liver, suggesting an upregulation of glycolysis. Therefore, it appears that changes in the regulation of glycolysis in response to anoxia in turtle liver are very temperature-dependent.

The response of turtles to oxygen deprivation during submergence occurs in two stages. In the initial stage, conditions become increasingly hypoxic as remaining oxygen in tissues is used up, ATP levels fall, and animals compensate by increasing glycolysis to meet organ demands for ATP. However, when oxygen levels drop below a critical value, a second phase is entered where conservation strategies are initiated to promote long term survival without oxygen. In this phase, metabolic rate is strongly suppressed to lower the demand for ATP by cellular processes, metabolic functions are re-prioritized to minimize nonessential cellular processes, and the resulting energy conservation allows a partial recovery of ATP levels [37]. Therefore, a possible explanation for the ChREBP activation pattern observed in liver in the present study is that enhanced expression of genes under ChREBP control (e.g. LPK) is needed within the early hours of anoxic submergence to support the hypoxia transition stage. The up-regulation of LPK seen after 5 h of anoxic submergence could be crucial to enhancing the glycolytic rate in liver cells as they become more and more hypoxic. However, after 20 h of anoxic submergence, turtles will have entered the conservation phase and significantly lowered metabolic rate in response to the continuing decline or complete lack of oxygen in tissues, resulting in lower energy demands and reducing glycolytic rate to a level that can be sustained over the long term to allow survival for days or weeks of continuing anoxia

submergence. Hence, continuing activation of the ChREBP pathway becomes unnecessary.

Interestingly, while kidney ChREBP showed increased protein expression and nuclear presence, no significant increase in DNA-binding activity by ChREBP was observed (Fig. 1-3). The differences in ChREBP responses seen in kidney versus liver might potentially be attributed to the functional differences between the two tissues. While the liver does significantly decrease its metabolic rate in response to anoxia, it still continues to function as a central regulator of metabolism, whereas kidney function is greatly curtailed [38]. Therefore, it is possible that the energy demands of kidney are lower under anoxia than those of the liver. Warren and Jackson [35] showed that tissue glycogen content in the turtle liver is much higher than in other tissues and that glycogen is depleted significantly slower in the liver compared to other tissues. In addition, glucose concentrations have increased more significantly in the liver after 7 days of anoxia at 5 °C compared to other tissues, likely due to glycogenolysis. Since enhanced glucose content is the primary factor responsible for activation of ChREBP, this could potentially explain why complete activation of this transcription factor was observed in the liver, but not in kidney. Alternatively, it is also possible that mechanisms other than ChREBP-based up-regulation of genes are employed for glycolytic activation in the kidney. For example, reversible phosphorylation has been shown to be an important control mechanism of glycolytic enzymes in response to anoxia in turtles, and is likely to also play a role in the kidney.

In summary, a strong activation of the transcription factor ChREBP was observed in turtle liver in response to short-term anoxic submergence. This activation was concomitant with enhanced levels of *LPK* transcripts, suggesting that activation of ChREBP in response to anoxia might be contributing to increasing glycolytic enzyme levels to facilitate enhanced glycolytic flux, thereby allowing hepatic cells to retain energy homeostasis during the hypoxic transition stage.

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