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Effects of acute hypoxia on nutrient metabolism and physiological function in turbot, *Scophthalmus maximus*

Qiang Ma · Houguo Xu · Yuliang Wei · Mengqing Liang

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Abstract Acute hypoxia is a common stress in aquaculture, and causes energy deficiency, oxidative damage and death in fish. Many studies have confirmed that acute hypoxia activated *hif1α* expression, anaerobic glycolysis and antioxidant system in fish, but the effects of acute hypoxia on lipid and protein metabolism, organelle damage, and the functions of *hif2α* and *hif3α* in economic fishes have not been well evaluated. In the present study, turbot was exposed to acute hypoxia (2.0 ± 0.5 mg/L) for 6 h, 12 h, and 24 h, respectively. Then, the contents of hemoglobin (HB), metabolite, gene expressions of *hifα* isoforms, energy homeostasis, endoplasmic reticulum (ER) stress, and apoptosis were measured. The results suggested that turbot is intolerant to acute hypoxia and the asphyxiation point is about 1.5 mg/L. Acute hypoxia induced *perk*-mediated ER stress, and increased lipid peroxidation and liver injury in turbot. The blood HB level and liver *vegfab* expression were increased under hypoxia, which enhances oxygen transport. At hypoxia stress, *hif3α*, anaerobic glycolysis-related genes expression, and lactate content were increased in the liver, and glycogen was broken down to ensure ATP supply. Meanwhile, *hif2α*, lipid synthesis-related genes expression, and TG content were increased in the liver, but the lipid catabolism and protein synthesis were suppressed during hypoxia, which reduced the oxygen consumption and ROS generation. Our results systematically illustrate the metabolic and physiological changes under acute hypoxia in turbot, and provide important guidance to improve hypoxia tolerance in fish.

Keywords Hypoxia · Metabolism · Glucose · Lipid · Protein · Oxidative damage

Q. Ma · H. Xu · Y. Wei · M. Liang (✉)
Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 106 Nanjing Road, Qingdao 266071, China
e-mail: liangmq@ysfri.ac.cn

H. Xu · Y. Wei · M. Liang
Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, 1 Wenhai Road, Qingdao 266237, China

Introduction

Oxygen (O_2) is necessary for living organisms. During oxidative phosphorylation, O_2 receives electrons from NADH and $FADH_2$ in the respiratory chain, which generate ATP for growth and development of organism (Saraste 1999). Terrestrial mammals take in O_2 through their lungs from the air, and fish take in O_2 through their gills from the water. However, the water dissolved oxygen (DO) content is constantly changing and is influenced by extreme weather, eutrophication, circadian rhythms, high-density culture and power interruption and so on (Karim et al. 2002; Keeling et al. 2010; Phan-Van et al. 2008; Wu 2002). Therefore, fish are more susceptible to hypoxia

stress than most terrestrial animals. Under hypoxia, the efficiency of electron transport of the respiratory chain decrease, which would hinder ATP synthesis and generate harmful reactive oxygen species (ROS) (Goda and Kanai 2012; Majmundar et al. 2010). Studies showed that hypoxia could affect the behavior, feed intake, digestion, growth, fecundity, immunity, antioxidant capacity and metabolic pattern of aquatic animals (Wu et al. 2003; Zhu et al. 2013). In particular, acute hypoxia would lead to mass death of cultured fishes within a short time and cause severe economic losses (Ma et al. 2021), so it is crucial to explore the adaptation mechanism of fish to acute hypoxia.

Hypoxia-inducible factors (Hifs) are the main regulators in cellular adaptation to hypoxia and are evolutionarily conserved from *Caenorhabditis elegans* to human beings (Xiao 2015). In mammals and fish, Hifs are composed of an α subunit (Hifα) that is easily degraded in oxygen and a β subunit (Hifβ/Arnt) that is stably expressed in oxygen (Semenza Gregg 2012) . In hypoxia condition, cumulative Hifα transports to nucleus and dimerizes with Hifβ, then binds to hypoxia-response elements (HRE) and activates expression of target genes, such as glycolysis, erythropoiesis, and angiogenesis (Koukourakis et al. 2001; Wang et al. 1995). There are three Hifαs isoforms in mammals and non-cyprinid fishes, including Hif1α, Hif2α (Epas1), and Hif3α (Hif1al). Studies have proved that Hif1α and Hif2α play a major role under hypoxia, and Hif3α is a negative regulator of Hif1α and Hif2α by competitive binding of Hifβ to inhibit the expression of target genes in mammals (Yuichi Makino et al. 2001). At hypoxia condition, accumulated Hif1α directly activates pyruvate dehydrogenase kinase (PDK), then PDK phosphorylates and inactivates pyruvate dehydrogenase complex (PDH), which prevents pyruvate to enter tricarboxylic acid cycle (TCA cycle) (Gudi et al. 1995; Kim et al. 2006). In Largemouth bass (*Micropterus salmoides*), acute hypoxia (1.2 ± 0.2 mg/L) significantly increased the expression of *hif1α* and *ldha* genes in the liver, glucose and lactate contents in the blood, but decreased the liver glycogen content (Yang et al. 2019). In Indian major carp, *hif1α* expression in the gill, superoxide dismutase (SOD), glutathione peroxidase (GPX) enzymes activities, and reduced glutathione (GSH) level in the gill and liver were increased significantly during acute hypoxia (0.5 mg/L for 72 h) (Varghese et al. 2018), and the similar results were found in Nile tilapia (*Oreochromis niloticus*) (Li et al. 2018). Surprisingly, unlike mammalian Hif3α, zebrafish Hif3α had a similar role as Hif1α and enhanced transcription of downstream genes (Zhang et al. 2012, 2014). So far, most fish hypoxia studies all focused on the Hif1α protein, glucose metabolism and antioxidant system, but the effects of acute hypoxia on organelle damages, lipid and protein metabolism changes, and the functions of Hif2α and Hif3α in economic fishes are not clear.

Turbot is a cultured marine fish with high economic value, rapid growth rate and high meat yield. Industrial aquaculture with high-density is the main culturing model for turbot and the annual production of turbot in China is around 50,000 tons (Jia and Lei 2019). Turbot is a typical benthic flatfish and is susceptible to hypoxia stress because of interruption of electricity and air pump, and the DO level should be kept above 6 mg/L to maintain normal life activity during the cultivation process (Jia and Lei 2019). However, the effects of acute hypoxia on nutrient metabolism and physiological health have not been well evaluated in turbot. In the present study, turbot was exposed to hypoxic water (2.0 ± 0.5 mg/L) for 6 h, 12 h, and 24 h, respectively. Then, the contents of hemoglobin (HB) and metabolite, antioxidant parameters, gene expressions related to *hifα* isoforms, energy homeostasis, endoplasmic reticulum (ER) stress, and apoptosis were assayed in the blood, liver, or muscle. The results indicated that acute hypoxia enhanced oxygen transport, anaerobic glycolysis, lipid synthesis, and liver injury, but inhibited lipid catabolism and protein synthesis in turbot. Our study systematically demonstrates the metabolic and physiological changes under acute hypoxia in turbot, which could be helpful to understand the hypoxia adaption and improve hypoxia tolerance in fish.

Materials and methods

Experimental fish and diet

Experimental juvenile turbots (six-month-old) with an average initial body weight of 76.6 ± 0.5 g were bought from Huanghai Aquaculture Co. Ltd. (Haiyang, China). All fish were reared in an indoor

Table 1 Formulation of the experimental diets

Dietary ingredient (%)	
Fishmeal	41
Soybean protein concentrate	25
Wheat meal	21
Fish oil	6
Soybean lecithin	1.5
Vitamin premix[a]	0.4
Mineral premix[b]	0.8
Choline chloride	0.5
Butylated hydroxytoluene	0.02
Dimethyl-beta-propiothetin	0.1
$Ca(H_2PO_4)_2$	1.5
Vitamin C	0.5
Carboxymethyl cellulose	1.68
Total	100
Nutrients compositions:	
Moisture (%)	9.31
Crude fat (%)	10.62
Crude protein (%)	49.46
Ash (%)	8.65

[a]Vitamin premix

[b]Mineral premix, designed for marine fish, were purchased from Qingdao Master Biotech Co., Ltd., Qingdao, China

flow-through seawater system and fed with a same diet for 4 weeks before acute hypoxia experiment. The diet was made following the standard procedures in our laboratory and the dietary compositions are listed in Table 1. Fishmeal and soy protein concentrate is the main protein sources and fish oil is the main lipid source. Fish were fed twice per day (at 8:00 and 20:00) and the feeding rate was set as 4% of body weight. Water dissolved oxygen, temperature, salinity, pH, and total ammonia nitrogen were kept at 6.5 ± 0.5 mg/L, 20.5 ± 1 °C, 26.5 ± 2.5‰, 7.5 ± 0.5, and < 0.02 mg/L, respectively.

Acute hypoxia challenge test and fish sampling

In order to obtain the asphyxiant point of turbot, sixteen fish were selected for acute hypoxia challenge test. Turbots were transferred to a customized hypoxic equipment and the water DO values were continuously decreased from 7.0 mg/L to 0.8 mg/L as time went on and the experiment was repeated three times at 20.5 °C (Fig. 1a). The number of dead fish were recorded every 6 h and a fish was regarded dead when the gill stopped moving for at least 1 h. Then, 48 fish were randomly distributed into four treatment groups (12 tanks, 3 tanks

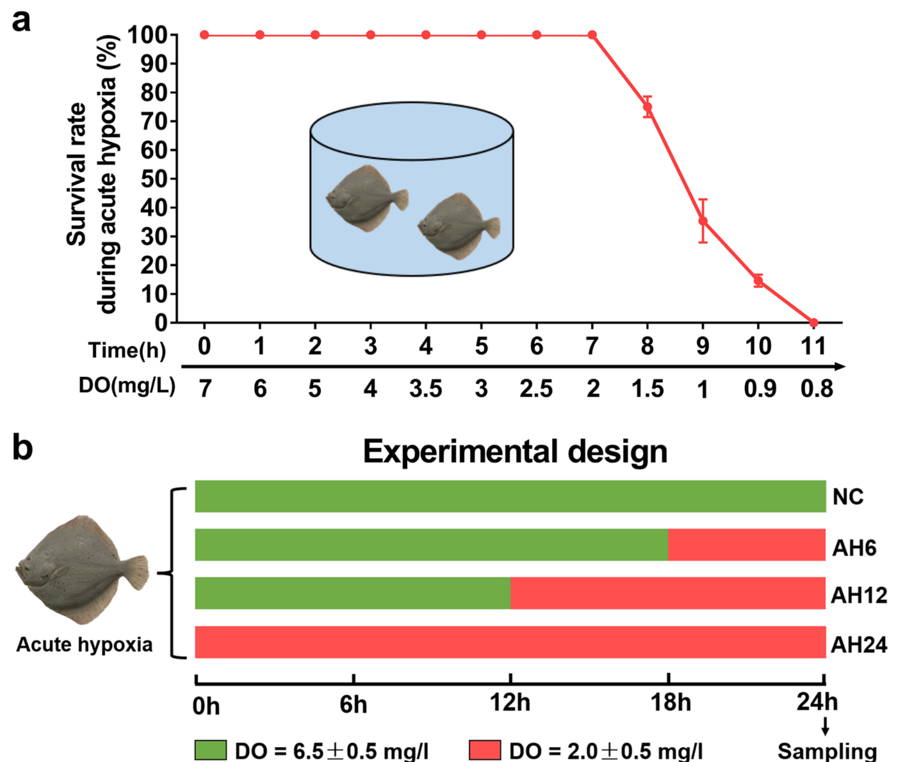

Fig. 1 Effect of acute hypoxia on survival rate of turbot. **a** Survival rate of turbot during acute hypoxia ($n = 16$ for 3 replicates). **b** Turbots were exposure to hypoxic water (2.0 ± 0.5 mg/L) for 0, 6, 12, or 24 h and sampled simultaneously

per treatment group, 4 fish per tank): normoxia control group (NC), acute hypoxia 6 h (AH6), acute hypoxia 12 h (AH12), and acute hypoxia 24 h (AH24). The DO level of NC group was maintained at 6.5 ± 0.5 mg/L for 24 h, but DO levels of AH6, AH12, and AH24 groups were reduced to 2.0 ± 0.5 mg/L in 0.5 h, and were maintained at 2.0 ± 0.5 mg/L for 5.5, 11.5, and 23.5 h, respectively. After 0–24 h acute hypoxia (2.0 ± 0.5 mg/L), 2 fish per tank (6 fish per treatment group) were anesthetized with MS-222 (30 mg/L), and the serum, liver, and muscle were collected and stored at − 80 ℃ for further analysis.

Proximate analysis of feed constituents

Diet compositions were measured by the standard methods of Association of Official Analytical Chemists. Feed samples were weighed and dried at 105 ℃ for 24 h to calculate moisture and dry matter contents. Crude protein content was detected by the Kjeldahl method ($N \times 6.25$) using UDK142 automatic distillation unit (VELP, Usmate, MB, Italy). Crude lipid content was detected by petroleum ether extraction using the Soxhlet method (Foss Tecator, Hoganas, Sweden). The feed was weighed and put into a muffle furnace at 550 ℃ for 4 h to calculate ash content.

Biochemical parameters assays

The contents of glucose, pyruvate, lactate, glycogen, triglyceride (TG), free fatty acid (FFA), total amino acid (TAA), total protein (TP), malondialdehyde (MDA), and the activities of glutamic propylic transaminase (GPT), glutamic oxaolacetic transaminase (GOT), and SOD in the serum, liver, or muscle were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). Insulin content of serum was measured using fish insulin enzyme-linked immune sorbent assay (ELISA) kit (Shanghai Hengyuan Biological Technology Co., Ltd., China). All measurement steps refer to relevant kit protocol and the results were read by a microplate reader (Tecan infinite M200, Switzerland). HB content of blood was detected by using a portable HB monitor (electrochemistry method, Taiwan BeneCheck™, China).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from liver or muscle by using RNAiso Plus kit (Takara, Japan). The quality and quantity of total RNA were detected by Titertek-Berthold Colibri spectrometer (Colibri, Germany). The absorbance ratios of 260/280 nm of the RNA samples were from 1.9 to 2.0, and showed high purity of the RNA samples for future analysis. First-strand cDNA was synthesized using Evo M-MLV RT Mix kit with gDNA Clean (Accurate biotechnology, China) by 96 universal gradient PCR (PEQSTAR, Germany) following the manufacturer's instructions. The primers of reference genes (*rpl19* and *β-actin*) and target genes (Table 2) for quantitative real-time PCR were designed in NCBI and were synthesized by Tsingke Biotechnology Co., Ltd. The reaction system of quantitative real-time PCR was 10 µL and include 5 µL of 2×SYBR Mixture, 1 µL cDNA (20 ng), 0.5 µL of qPCR primers (10 µM), and 3.5 µL nuclease-free water. The program of qPCR was performed in 12×8 well plates on a Roche LightCycler 96 system (Roche, Switzerland) and included 95 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The melting curves were generated to ensure the specificity of amplified products at the end of PCR. Amplification efficiency of primers was between 90 and 110%, and the correlation coefficient was over 0.9 for each gene. The amplification efficiency was calculated according to the following equation $E = 10^{(-1/\text{Slope})} - 1$ (Ma et al. 2021) and the expression of target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

All data are shown as means ± standard error of means (SEM) and were analyzed by using the SPSS Statistics 21.0 software (IBM Company, USA). All results were tested for normality and homogeneity of variances by using Shapiro–Wilk and Levene's tests. One-way ANOVA was applied to determine significant differences among treatments, followed by a Duncan's multiple comparison test and significant differences were set at $p < 0.05$. Bar graphs were made by GraphPad prism 8.0 software.

Results

Effect of acute hypoxia on survival rate of turbot

As shown in Fig. 1a, acute hypoxia challenge was carried out to determine the asphyxiation point of turbot. Turbot began to die when water DO fell below

Table 2 Primers used for the analysis of genes expressions

Gene name	Sequences (5′ to 3′) forward and reverse	Product length	GenBank no
gck	CTTCCGTGACAGGTTCCACA	117	XM_035639201.1
(glucokinase)	CAGGCCACCGCTGAGATAAG		
pk	TGGCGACGCGATTCTACAC	175	XM_035623784.1
(pyruvate kinase L/R)	GGCGTTGGATGAAAGAGTCC		
ldha	TCCAAGGTGGTGGTGGTTAC	129	XM_035643663.1
(lactate dehydrogenase A4)	GCAGTTGGGGCTGTACTTGA		
pdha1a	GGCCAATATCTCCAACGTGC	80	XM_035625777.1
(pyruvate dehydrogenase E1 subunit alpha 1a)	CAGCATATGTGCGTGCTGAC		
cs	CGGCAAAACCAACATAGGGC	126	XM_035644242.1
(citrate synthase)	CTGTAGCCACGGAAACGGAT		
ogdha	GGGTTCGAGTTGGGTTTTGC	81	XM_035641152.1
(oxoglutarate dehydrogenase a)	CGTGTTGTGGAAGTCTCCGA		
hif1α	GACTACTACCGGGCACAAGG	82	XM_035610073.1
(hypoxia inducible factor 1 subunit alpha a)	CTCAATGTTGGAGGGGTGCT		
hif2α/epas1b	AGCAGACGGAGACCTTGTTCA	185	XM_035605652.1
(endothelial PAS domain protein 1b)	ATCCGACAAACCGAAGTCGAGG		
hif3α/hif1αl	ATGCATGTTCGAAACCCAGC	136	XM_035623708.1
(hypoxia inducible factor 1 subunit alpha, like)	ACGAACGACAGCTCGACAAA		
vegfab	CGACAAAACAATTCACGACGC	82	XM_035617034.1
(vascular endothelial growth factor Ab)	GTCTTGCACGAACAAACGCT		
atpase	GAGAGGGCAGCGTCAGTATC	155	XM_035618637.1
(ATPase H + transporting V1 subunit Ab)	GAGCCAGTTCACAGACGGAA		
ampk2/prkaa2	AAGGCAGGGTAAAGATCGGC	171	XM_035648540.1
(protein kinase AMP-activated alpha 2)	TCTCGCGTTTGATCTTCCCC		
fasn	CAACTCGTTCGGATTTGGCG	109	XM_035612875.1
(fatty acid synthase)	GGCATTCAGAACCCTGGGAA		
srebf1	GCTTCCAATCAACCGCATCG	163	XM_035615397.1
(sterol regulatory element binding transcription factor 1)	GAGCTTGGCCTCAGTACCAG		
atgl/pnpla2	ATGTCGCGAAAGAGGCAAGA	85	XM_035643755.1
(patatin-like phospholipase domain containing 2)	CGCAGCATATGACGCACAAT		
cpt1b	GTATGTTCCGAGACGGACGG	87	XM_035642881.1
(carnitine palmitoyltransferase 1B)	CACTTGTGTCCTCCATGGCT		
mtor	GGTTGGGAGCAGACAGGAAT	94	XM_035644866.1
(mechanistic target of rapamycin kinase)	TGCTGGAAGAAGAACGTGGG		
glud1a	TCCCCATCAAGAGAGACGGC	165	XM_035605065.1
(glutamate dehydrogenase 1a)	CCACAGCACACTTGTAGGTCA		
gcn2/eif2ak4	TCAGCCCAGAAAAGGTGTCC	125	XM_035609728.1
(eukaryotic translation initiation factor 2 alpha kinase 4)	GTCCACCGCCAGAATCTCAA		
uab1	CATCCGCCAGCTACTTCACA	99	XM_035644760.1
(ubiquitin-like modifier activating enzyme 1)	AATTCTAGGGGGTGGGGACA		
nox4	GCTGGACAAGAGCAAGACCT	115	XM_035623825.1
(NADPH oxidase 4)	CGAGTAGCTCACGCTGAAGT		
gpx1a	CTCGCGTTATTCTGCCAAGG	118	XM_035631441.1

Table 2 (continued)

Gene name	Sequences (5′ to 3′) forward and reverse	Product length	GenBank no
(glutathione peroxidase 1a)	CCCTGGACGGACATACTTCAG		
bip/hspa5/grp78	CTCCGACAACCAGCCTACTG	96	XM_035608312.1
(heat shock protein 5)	CCAGTCAGGTCGAAGGTTCC		
ire2	ATGGCCCATCTTGTGTCGAG	90	XM_035636207.1
(endoplasmic reticulum to nucleus signaling 2)	CGAGCCGTAGTTCTGACTCC		
xbp1	GGAAAAGACGACGGGCCTAC	141	XM_035639024.1
(X-box binding protein 1)	GACTCAGAAGACCCGATCCC		
perk/eif2ak3	GCTGTATCCCTTCAGGACGC	178	XM_035610116.1
(eukaryotic translation initiation factor 2-alpha kinase 3)	GAGGGGCATACCCGTGATGT		
atf4	CCCCAAGGTCAAATCGGTGT	149	XM_035636250.1
(activating transcription factor 4a)	TCTTCTCCAGCTCATCGCAC		
atf6	ACTACGCACCTCATCACACC	82	XM_035646916.1
(activating transcription factor 6)	CGGAAGGACACCACGTAGAA		
chop/ddit3	AGTGCCTTGTCCTCTTTCGC	100	XM_035632938.1
(DNA-damage-inducible transcript 3)	TCCTTTCGTTCTCGTGCTCTC		
caspase3a	GATACAGCCTCGGTTTCCCC	98	XM_035637276.1
(apoptosis-related cysteine peptidase a)	GTCCGTCCCATTTCGCTGAT		
rpl19	TGGATCCCAACGAGACCAAC	97	XM_035614206.1
(ribosomal protein L19)	GTGACAGGCTTGCGAATGAT		
β-actin	CTTCCCTTCTATCGTCGGTC	91	XM_035614479.1
(actin beta 2)	GCTCTGGGCTTCATCACCTA		

2 mg/L, and survival rate was 75% at 1.5 mg/L for 30 min. When water DO was dropped to 0.8 mg/L, the fish all died. Turbot is intolerant to acute hypoxia and the asphyxiation point of turbot is about 1.5 mg/L at 20.5 °C. To study adaptation of turbot to acute hypoxia stress, turbots were exposed to hypoxic water (2.0 ± 0.5 mg/L) for 0, 6, 12, or 24 h, respectively (Fig. 1b), and metabolism- and health-related indicators were measured.

Effects of acute hypoxia on glucose metabolism

Compared with the NC group, the glucose and insulin contents in the serum were increased with the extension of hypoxic time, and the significant difference was found in the AH24 group ($p < 0.05$, Fig. 2a, b). The serum pyruvate and lactate contents were significantly increased in the AH6 group ($p < 0.05$) and then recovered to the control level in the AH12 and AH24 group (Fig. 2c, d). Accordingly, turbot exposed to hypoxia significantly decreased glycogen content in the liver ($p < 0.05$, Fig. 2f) but did not affect the muscle glycogen content ($p < 0.05$, Fig. 2i). Meanwhile, turbot exposed to hypoxia for 12 h significantly increased the blood HB and liver lactate contents ($p < 0.05$, Fig. 2e, h). Moreover, compared with NC group, acute hypoxia did not change pyruvate content in the liver and muscle, and lactate content in the muscle ($p > 0.05$, Fig. 2g, j, k). All these suggest that acute hypoxia enhanced oxygen transport capacity, glycogen breakdown and lactate production in the liver.

Effects of acute hypoxia on expression of *hifα* isoforms and glucose metabolism-related genes

As shown in Fig. 3, compared with normoxia control (NC) group, the expression of glycolysis-related genes such as glucokinase (*gck*), pyruvate kinase L/R (*pk*), and lactate dehydrogenase A4 (*ldha*) in the liver were increased after acute hypoxia exposure, and the similar results were also found at *pk* and *ldha* expressions in the muscle ($p < 0.05$, Fig. 3a). Acute hypoxia increased the mRNA expressions of TCA cycle-related genes such as pyruvate dehydrogenase E1

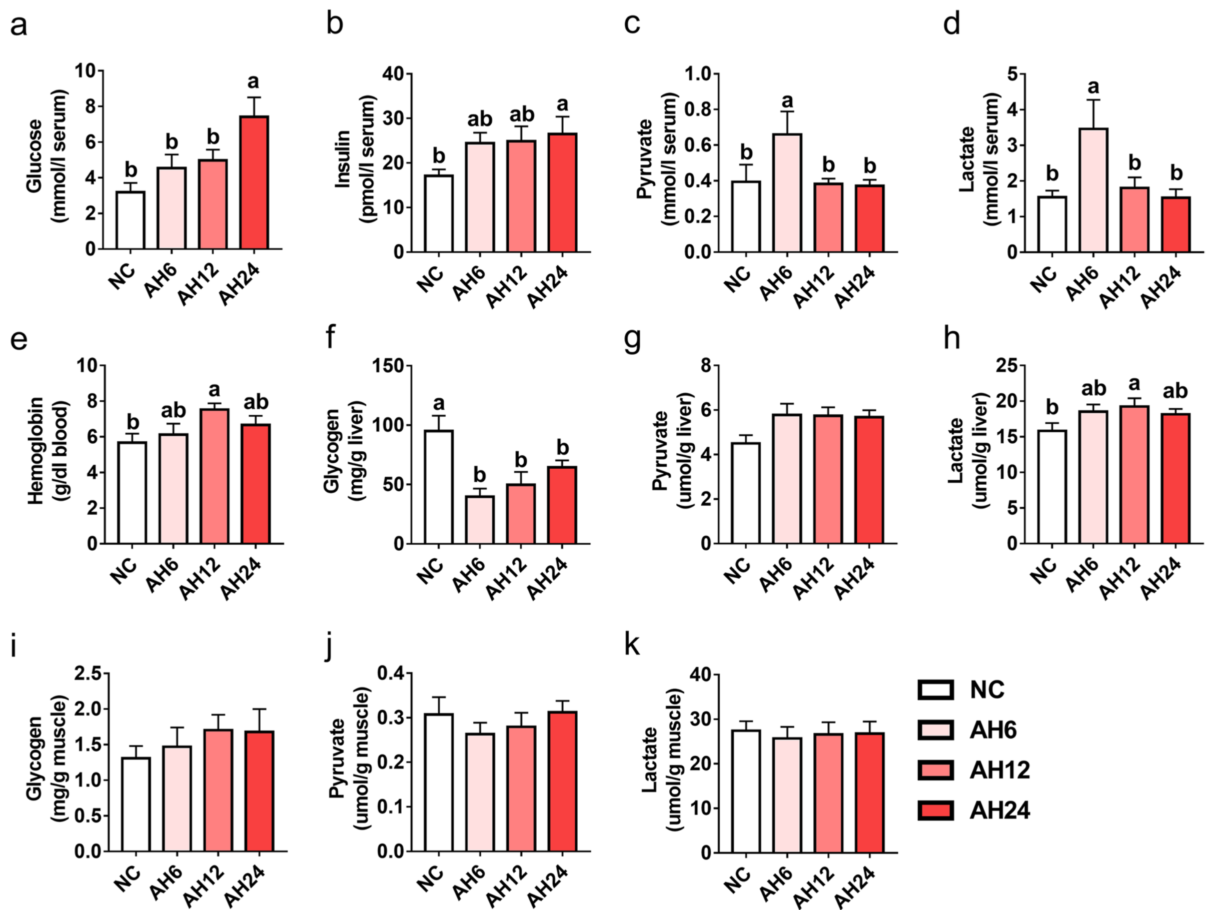

Fig. 2 Effects of acute hypoxia on glucose metabolism. **a**–**d** Glucose, insulin, pyruvate and lactate contents in the serum. **e** Hemoglobin in the blood. **f**–**h** Glycogen, pyruvate and lactate contents in the liver. **i**–**k** Glycogen, pyruvate, and lactate contents in the muscle. All values are means ± SEM ($n=6$)

subunit alpha 1a (*pdha1a*), citrate synthase (*cs*), and oxoglutarate dehydrogenase a (*ogdha*) in the muscle ($p<0.05$), but did not change these gene expressions in the liver ($p>0.05$, Fig. 3b).

Turbot exposed to hypoxia for 12 h significantly increased endothelial PAS domain protein 1b (*hif2α/epas1b*) expression ($p<0.05$, Fig. 3c), but did not affect hypoxia inducible factor 1 subunit alpha a (*hif1α*) expression in the liver. Hypoxia inducible factor 1 subunit alpha like (*hif3α/hif1αl*) and vascular endothelial growth factor Ab (*vegfab*) genes expression were significantly increased in the both liver and muscle after acute hypoxia exposure ($p<0.05$, Fig. 3c, d). Meanwhile, 6 h hypoxia significantly enhanced the mRNA expressions of *hif1α* in the muscle ($p<0.05$, Fig. 3d) and protein kinase AMP-activated alpha 2 (*ampk2/prkaa2*) in the liver ($p<0.05$, Fig. 3e). Compared with NC group, the liver mRNA expressions of ATPase H + transporting V1 subunit Ab (*atpase*) in the AH24 group, muscle mRNA expressions of *ampk2* in the AH12 and AH24 group were increased significantly ($p<0.05$, Fig. 3e). These data indicate that acute hypoxia could promote *hifas* and glycolysis-related genes expression to supply energy.

Effects of acute hypoxia on lipid metabolism

AS seen in Fig. 4, compared with NC group, TG content in the serum and liver was increased with the prolongation of hypoxic time, and the significances were found in AH24 group in the serum ($p<0.05$,

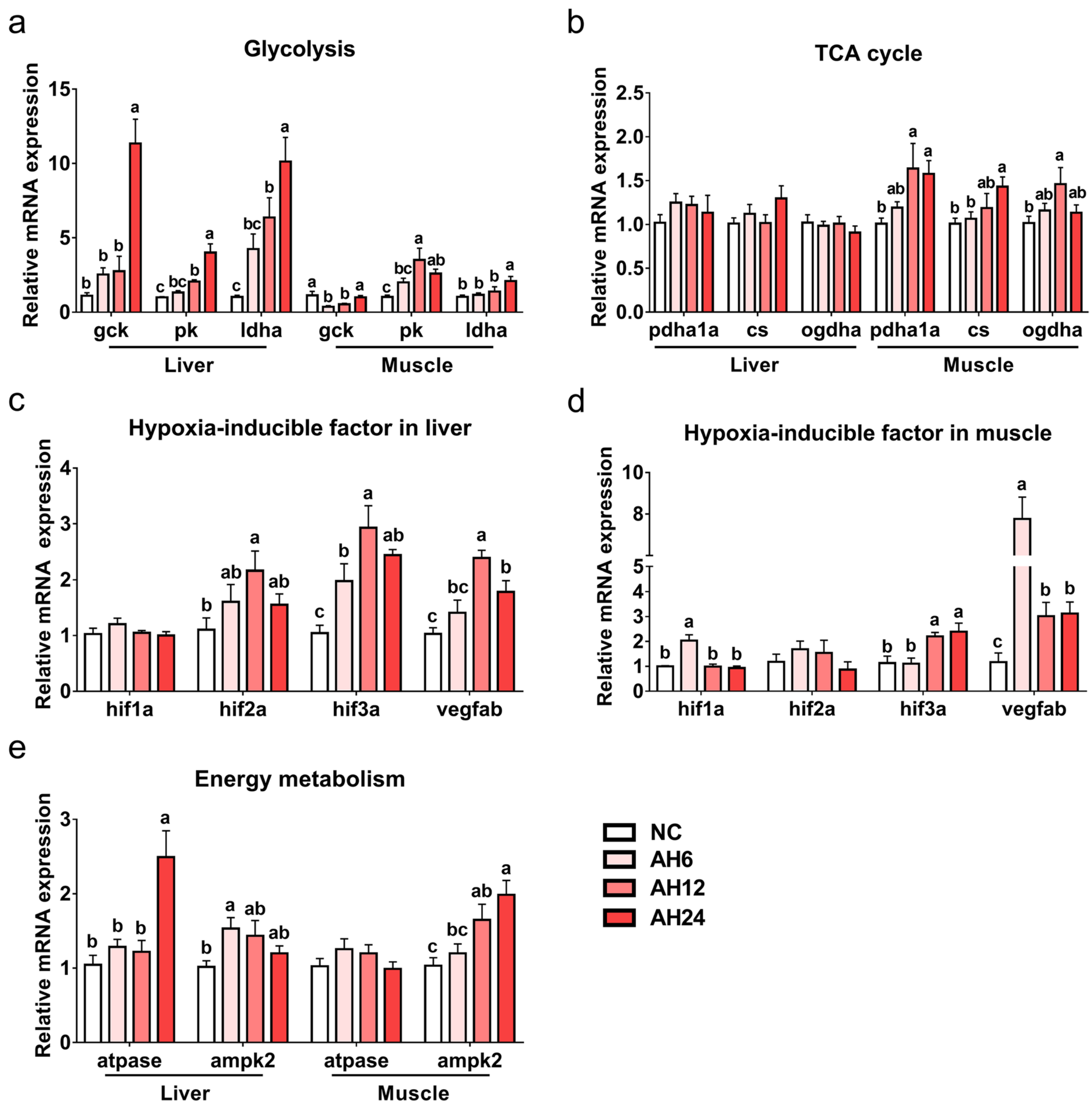

Fig. 3 Effects of acute hypoxia on expression of *hifα* isoforms and glucose metabolism-related genes. **a** *gck*, *pk*, and *ldha* genes expression in the liver and muscle. **b** *pdha1a*, *cs*, and *ogdha* genes expression in the liver and muscle. **c**, **d** *hif1α*, *hif2α*, *hif3α*, and *vegfab* genes expression in the liver and muscle. **e** *atpase* and *ampk2* genes expression in the liver and muscle. All values are means ± SEM ($n = 6$)

Fig. 4a), AH12 and AH24 group in the liver ($p < 0.05$, Fig. 4b). Simultaneously, acute hypoxia significantly reduced the FFA level in the serum ($p < 0.05$, Fig. 4c), but did not affect TG content in the muscle ($p > 0.05$, Fig. 4d). Accordingly, hypoxia for 24 h significantly increased the mRNA expressions of fatty acid synthase (*fasn*) and sterol regulatory element binding transcription factor 1 (*srebf1*) in the liver ($p < 0.05$, Fig. 4e), but these indicators were reduced in the muscle (Fig. 4e). Hypoxia for 12 and 24 h significantly decreased the mRNA expressions of patatin-like phospholipase domain containing 2 (*atgl/pnpla2*)

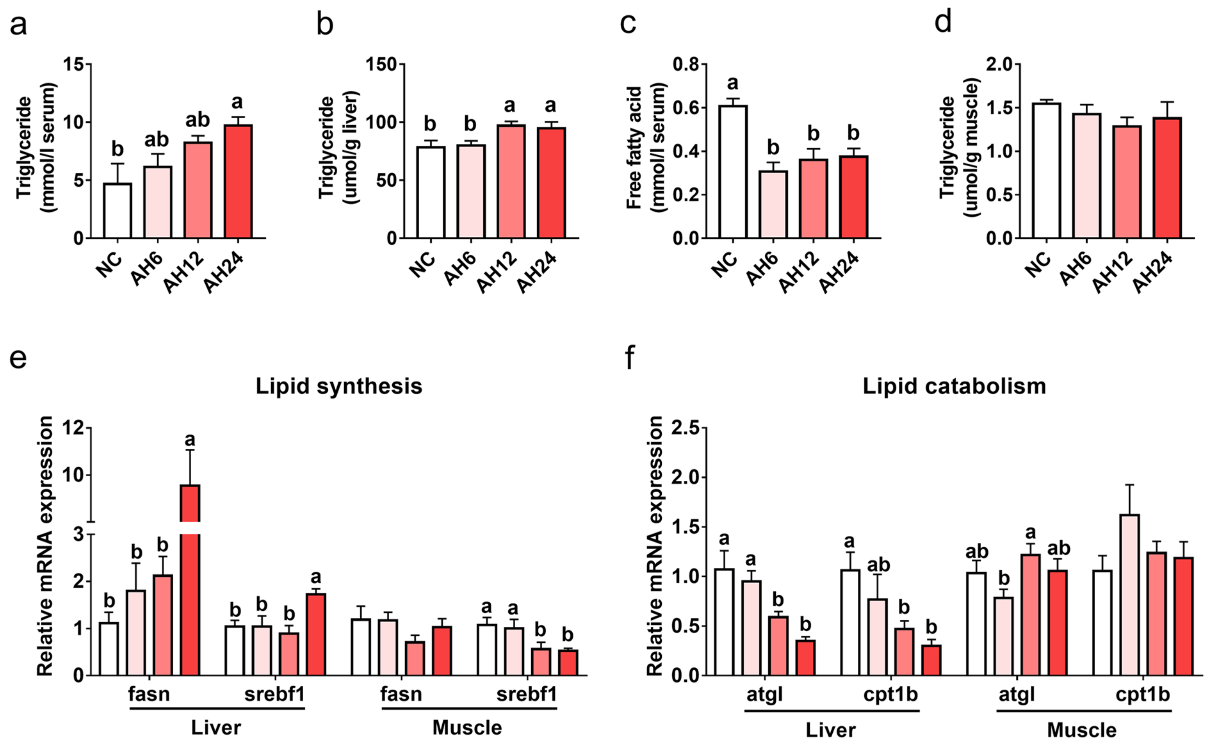

Fig. 4 Effects of acute hypoxia on lipid metabolism. **a**, **b** TG contents in the serum and liver. **c** FFA content in the serum. **d** TG content in the muscle. **e** *fasn* and *srebf1* genes expression in the liver and muscle. **f** *atgl* and *cpt1b* genes expression in the liver and muscle. All values are means ± SEM ($n = 6$)

and carnitine palmitoyltransferase 1B (*cpt1b*) in the liver than those in the NC group ($p < 0.05$, Fig. 4f), but did not affect *atgl* and *cpt1b* genes expression in the muscle ($p > 0.05$, Fig. 4f). These data suggest that acute hypoxia could enhance lipid synthesis and inhibit lipid catabolism in the liver.

Effects of acute hypoxia on protein metabolism

As the prolonging of hypoxic time, the serum total amino acids (TAA) and total soluble protein (TP) levels were elevated than the NC group, and the significances were found in the AH24 group ($p < 0.05$, Fig. 5a) and AH12 group, respectively ($p < 0.05$, Fig. 5b). However, the TAA and TP contents in the muscle were reduced after acute hypoxia, and the significances were found at the AH24 group ($p < 0.05$, Fig. 5c, d), and similar trends were also found in the liver ($p < 0.05$, Fig. 5e, f). Furthermore, turbot exposed to hypoxia for 24 h decreased the mRNA expression of mechanistic target of rapamycin kinase (*mtor*) in the both liver and muscle, and eukaryotic translation initiation factor 2 alpha kinase 4 (*gcn2/eif2ak4*) in the liver ($p < 0.05$, Fig. 5g, h). There were no significant differences in glutamate dehydrogenase 1a (*glud1a*) and ubiquitin-like modifier activating enzyme 1 (*uab1*) genes expression in the liver and muscle, and *gcn2* in the muscle between NC group and hypoxia groups ($p > 0.05$, Fig. 5g, h). All these data indicate that acute hypoxia could suppress the synthesis of protein in the muscle especially.

Effects of acute hypoxia on oxidative damage

As shown in Fig. 6, compared with the NC group, the GOT activity tended to increase during acute hypoxia in the serum ($p > 0.05$, Fig. 6a), and the GPT activity was significantly increased in the AH24 group ($p < 0.05$, Fig. 6b). MDA content in the both serum and liver was increased during acute hypoxia, and the significances were found at the AH24 group in the serum, AH6 and AH12 group in the liver ($p < 0.05$, Fig. 6c,

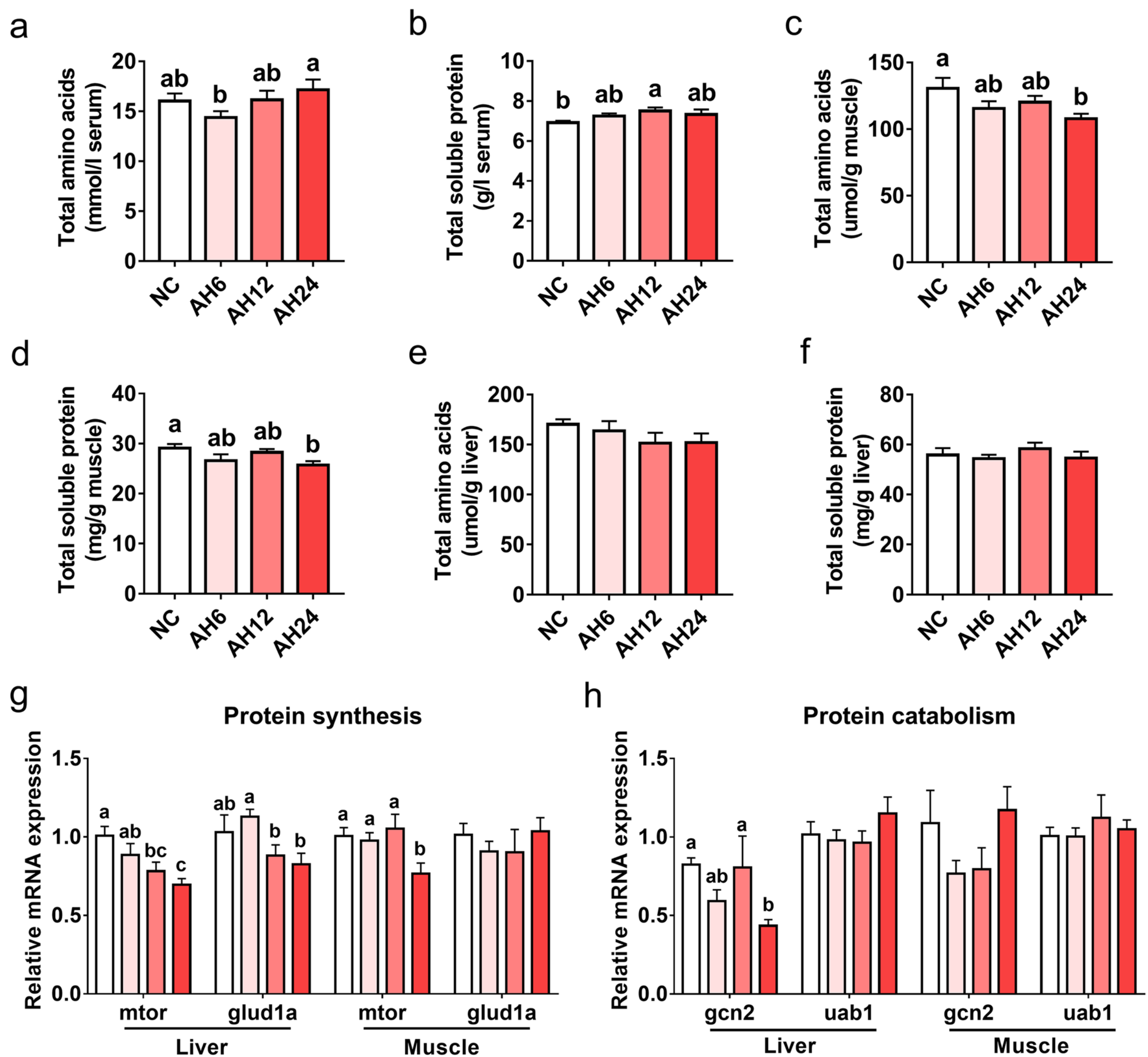

Fig. 5 Effects of acute hypoxia on protein metabolism. **a**, **b** TAA and TP contents in the serum. **c**, **d** TAA and TP contents in the muscle. **e**, **f** TAA and TP contents in the liver. **g** *mtor* and *glud1a* genes expression in the liver and muscle. **h** *gcn2* and *uab1* genes expression in the liver and muscle. All values are means ± SEM ($n = 6$)

d), but acute hypoxia didn't affect the muscle MDA content and serum SOD activity ($p > 0.05$, Fig. 6e, f). Meanwhile, acute hypoxia increased the genes expression of heat shock protein 5 (*bip/hspa5/grp78*), eukaryotic translation initiation factor 2-alpha kinase 3 (*perk/eif2ak3*), activating transcription factor 4a (*atf4*), NADPH oxidase 4 (*nox4*), and DNA-damage-inducible transcript 3 (*chop/ddit3*) in the liver ($p < 0.05$, Fig. 6g, i and j), but decreased the genes expression of *perk* and *chop* in the muscle ($p > 0.05$, Fig. 6h, j). Moreover, there were no significant differences in genes expression of endoplasmic reticulum to nucleus signaling 2 (*ire2*), X-box binding protein 1 (*xbp1*), glutathione peroxidase 1a (*gpx1a*), apoptosis-related cysteine peptidase a (*caspase3a*) in both liver and muscle, *bip*, *xbp1*, *atf4*, activating transcription factor 6 (*atf6*), and *nox4* in the muscle between the NC and hypoxia groups ($p > 0.05$, Fig. 6g–i). Overall, these data indicate that acute hypoxia could induced *perk*-mediated ER stress, injury, and apoptosis in the liver.

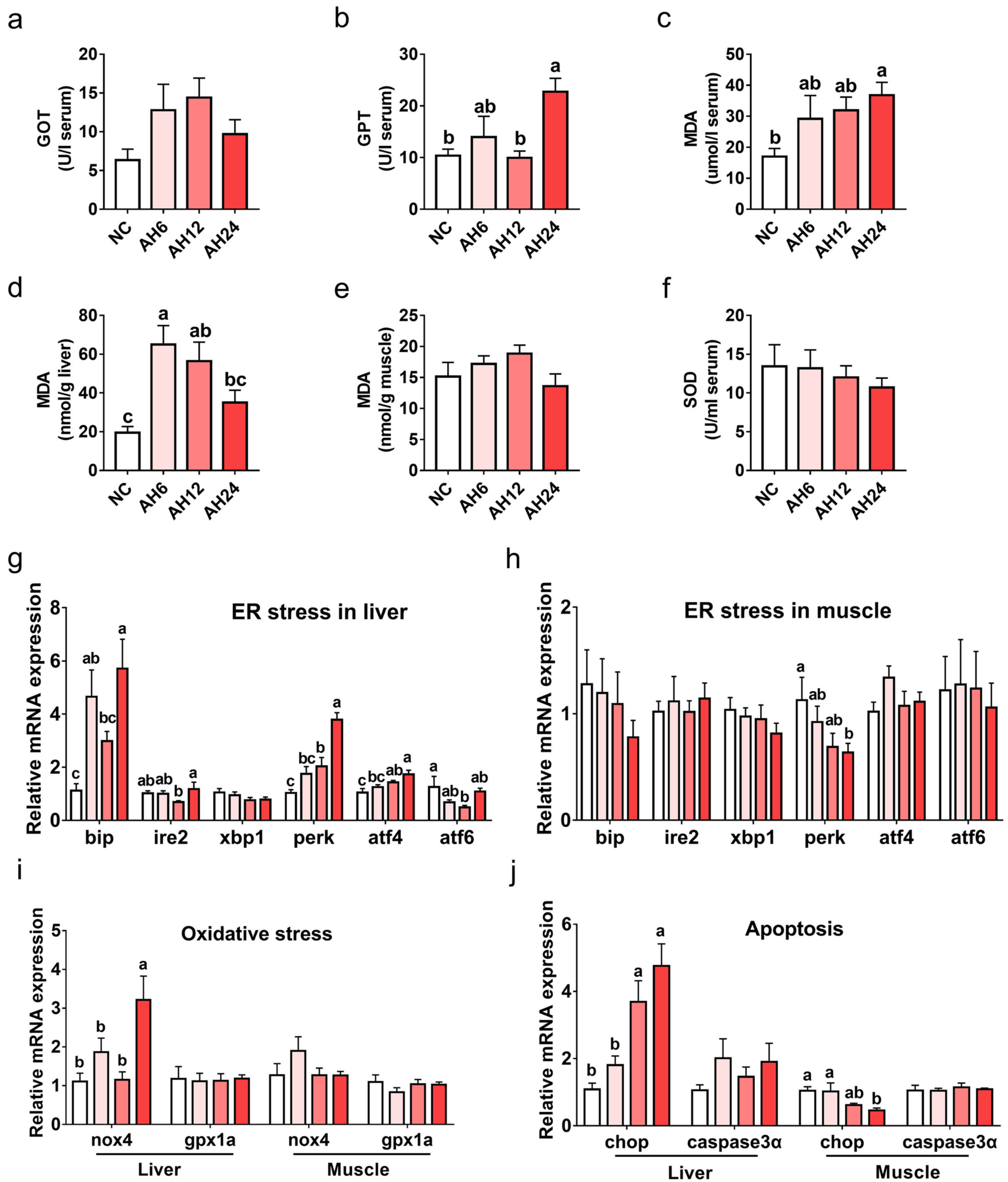

Fig. 6 Effects of acute hypoxia on oxidative damage. **a**, **b** GOT and GPT activities in the serum. **c**–**e** MDA content in the serum, liver and muscle. **f** SOD activity in the serum. **g**, **h** *bip*, *ire2*, *xbp1*, *perk*, *atf4* and *atf6* genes expression in the liver and muscle. **i** *nox4* and *gpx1a* genes expression in the liver and muscle. **j** *chop* and *caspase3a* genes expression in the liver and muscle. All values are means ± SEM ($n = 6$)

Discussion

Animals could adapt to hypoxia environment by altering their physiology and metabolism. In hypoxia condition, Nile tilapia and Atlantic silversides (*Menidia menidia*) exhibited aquatic surface respiration (ASR) behavior, and reduced metabolic rate and oxygen uptake (Dixon et al. 2017; Verheyen et al. 1994). However, in the present study, ASR behavior was not observed in turbot that is a benthic flatfish, which may cause intolerant to acute hypoxia in turbot. And during selective breeding process of turbot, the scientists got the fast growth phenotype, but may lose the tolerance to hypoxia. Moreover, crucian carp (*Carassius carassius*) and mangrove killifish (*Kryptolebias marmoratus*) could remodel the gill morphology reversibly to increase the respiratory surface area (Nilsson 2007; Sollid et al. 2003). Vascular endothelial growth factor (VEGF) and erythropoietin (EPO) play a crucial role in regulating erythropoiesis and angiogenesis (Wisniewska et al. 2020). In flounder (*Pleuronectes flesus luscus*), acute hypoxia (2.6–2.7 mg/L for 2–24 h) promoted the differentiation of juvenile red blood cells (RBCs) in the pronephros and enhanced the number of mature RBCs in the blood (Soldatov 1996). In the Nile tilapia, Indian major carp (*Cirrhinus mrigala*) and Flowerhorn fish (*Amphilophus trimaculatus* × *Amphilophus citrinelllus* × *Vieja synspilum*), the HB content and RBCs count in the blood were increased significantly after acute hypoxia (Li et al. 2018; Varghese et al. 2018; Kupittayanant and Kinchareon 2011). Similarly, in the present study, compared with NC group, the blood hemoglobin level was increased significantly after 12 h hypoxia, which enhanced the binding and transport of oxygen to resist acute hypoxia.

When mammals were exposed to hypoxia, anaerobic glycolysis was activated to generate energy and reduce oxygen consumption, which is regulated by the Hif pathway (Kim et al. 2006). In rat, acute hypoxia increased glucose uptake, the mRNA expressions of Hif1α, glucose transporter 1 (GLUT1) and monocarboxylate transporter 1 (MCT1) and lactate content in the brain (Coimbra-Costa et al. 2017; Vega et al. 2006). At high altitude hypoxic environment, the expression of Hif1α, PDK4, ATP-independent HSP27 and HSP60 genes, lactate dehydrogenase (LDH) activity, ATP and NADH contents were increased, but citric acid content was decreased in the heart, muscle or lung in Gannan yaks (*Bos grunniens*) (Wen et al. 2021). In fish, acute hypoxia (45% DO for 24 h; 1.9 mg/L for 4 h) significantly increased the gill GLUT1 expression in Atlantic cod (*Gadus morhua*) and the liver GLUT2 expression in Sea bass (*Dicentrarchus labrax*) (Hall et al. 2005; Terova et al. 2009). In largemouth bass (*Micropterus salmoides*), the liver glycogen and pyruvic acid contents, gluconeogenesis-related genes expression were decreased after acute hypoxia (3.0 ± 0.2 mg/L and 1.2 ± 0.2 mg/L), but lactic acid content, HIF, VEGF, AMPK signaling pathways, and glycolysis-related genes expression were increased in the liver (Sun et al. 2020). In Indian major carp, the mRNA expression of *hif1α* in the gill, anaerobic glycolysis-related enzyme activities, such as HK, LDH, and G6pase in the liver and muscle were increased after acute hypoxia (Varghese et al. 2018). At high-attitude area, the natives utilize more glucose to produce energy in the heart, and promote the adaptation to hypoxia environment (Holden et al. 1995). The similar results were also found in the present study. Compared with the NC group, the glucose, pyruvate, lactate, and insulin contents in the serum were increased, and glycogen content in the liver was decreased in the hypoxia groups. Meanwhile, the mRNA expressions of *hif2α*, *atpase*, *gck*, *pk*, and *ldha* in the liver, *hif1α* in the muscle, *ampk2*, *hif3α*, and *vegfab* in the both liver and muscle were enhanced after acute hypoxia exposure. These data suggest that *hifas* genes expression, glycogenolysis, and anaerobic glycolysis were activated to supply energy under acute hypoxia in turbot.

In fish hypoxia studies, there are many studies focused on *hif1α* and glucose metabolism (Xiao 2015). However, few papers explored the effects of acute hypoxia on lipid and protein metabolism, and the roles of *hif2α* and *hif3α* in fish hypoxia adaptation are not yet clear. In mice, the gene expression of *srebp1* and lipid biosynthesis were upregulated in the liver after hypoxia (Li et al. 2005). In rat cardiac myocyte, 48 h acute hypoxia inhibited CPT1 expression and PPARa/RXR binding efficiency, which lead to reduction of mitochondrial FAO rate and accumulation of intracellular neutral lipid significantly (Huss et al. 2001). Under hypoxia, glial cells could convert lactate to lipid

droplet to protect neuronal cells in mouse and the process dependent on ROS and APOE/APOD (Liu et al. 2017). Similarly, promoting lipid droplet formation could protect mouse astrocytes against ROS toxicity, but knockout FABP7 could suppress lipid synthesis and decrease the survival rate of cell (Islam et al. 2019). Especially in cancer cells, such as clear cell renal cell carcinoma and glioma cell, hypoxia inhibited lipolysis and mitochondrial FAO, and promoted lipid synthesis, and Hif2α played an important role during the process (Majmundar et al. 2010; Metallo et al. 2011; Rankin et al. 2009). In the present study, compared with the NC group, TG content in the serum and liver, and the mRNA expressions of *hif2α*, *fasn*, and *srebf1* in the liver were increased significantly after hypoxia stress. Meanwhile, the FFA level in the serum was reduced, which indicates the FFA was utilized for fat synthesis during hypoxia exposed. In largemouth bass, 8 h hypoxia also increased TG and FFA contents in the liver and inhibited fatty acid β-oxidation-related genes expression. Moreover, glycolysis was inhibited, and gluconeogenesis and lipid oxidation were increased after 12 h reoxygenation (Sun et al. 2020). When turbot was exposed to acute hypoxia, the TAA and TP contents, the mRNA expression of *mtor* was decreased in the both liver and muscle in the present study. Studies have found that acute hypoxia could cause lack of ATP, then raised AMP level would directly phosphorylates and activates Thr172 residue of AMPK to maintain energy homeostasis (Hardie et al. 2012). In mouse and Arctic char (*Salvelinus alpinus*), hypoxia activated AMPK, and suppressed mTOR expression and protein synthesis (Cassidy and Lamarre 2019; Wouters and Koritzinsky 2008). Similarly, in Amazonian cichlid (*Astronotus ocellatus*) and crucian carp (*Carassius carassius*), acute hypoxia also decreased the protein synthesis rates in the both liver and muscle (Cassidy et al. 2018; Smith et al. 1996). As we all know, the mitochondrial FAO and TCA cycle would consume large amounts of oxygen, but anaerobic glycolysis occurred in the cytoplasm would directly generate 2 ATP without any oxygen (Scott 2012). Moreover, compared with fatty acids or amino acids, glucose is more rapidly utilized for energy supply under stress conditions (Mickelson et al. 2005). These indicate that promoting anaerobic glycolysis, lipid synthesis, and inhibiting protein synthesis is a metabolic adaptation of turbot to acute hypoxia.

It is classically accepted that hypoxia could decrease the electron transport efficiency in the respiratory chain, which could cause incomplete utilization of O_2 and generate harmful reactive oxygen species (ROS) (Majmundar et al. 2010). Previous study demonstrated that 10 h hypoxia exposure increased the MDA content, SOD and GPX activities in the serum of healthy exercise trained participants (Ribon et al. 2016). Intermittent hypoxia increased Nox4 expression and ROS level in pancreatic beta cell, and the process was abolished in Hif1α$^{-/-}$ mice (Wang et al. 2020). In juvenile yellow catfish (*Pelteobagrus fulvidraco*), acute hypoxia (1.14 ± 0.04 mg/L) increased the activities of total SOD and catalase (CAT) in the brain and gill, but significantly reduced the total antioxidant capacity (T-AOC) in the liver (Wang et al. 2021). In Largemouth bass, acute hypoxia (1.2 ± 0.2 mg/L) significantly increased the number of RBCs, antioxidant enzyme activities (SOD, CAT, GPX) in the liver and muscle at the initial stage of acute hypoxia, but decreased HB concentration and antioxidant enzyme activities in the gill (Yang et al. 2017). In the present study, compared with the NC group, acute hypoxia increased the GOT and GPT activities in the serum, *nox4* and *chop* expressions in the liver, MDA content in the both serum and liver, but didn't affect the muscle MDA content and serum SOD activity. These results indicate that the effects of acute hypoxia on antioxidant system were influenced by the duration and severity of hypoxia, and different tissues and fish species. In Gibel carp (*Carassius gibelio*), addition of vitamin C or emodin in the feed all alleviated acute hypoxia-induced ER stress, inflammation, oxidative damage and apoptosis by activating Nrf2/Keap1 or AMPK/mTOR pathway in the liver, respectively (Wu et al. 2022a, b). ER stress can cause the accumulation of misfolded or unfolded proteins, then the unfolded protein response (UPR) was activated to recover ER function and maintain cell viability. UPR is regulated by three classical ER transmembrane proteins, including PERK, IRE1 and ATF6 (Hetz 2012). In the present study, acute hypoxia only activated *perk*-mediated ER stress in the liver, but didn't affect the genes expression of *ire2* and *atf6*, which suggest *perk*-mediated

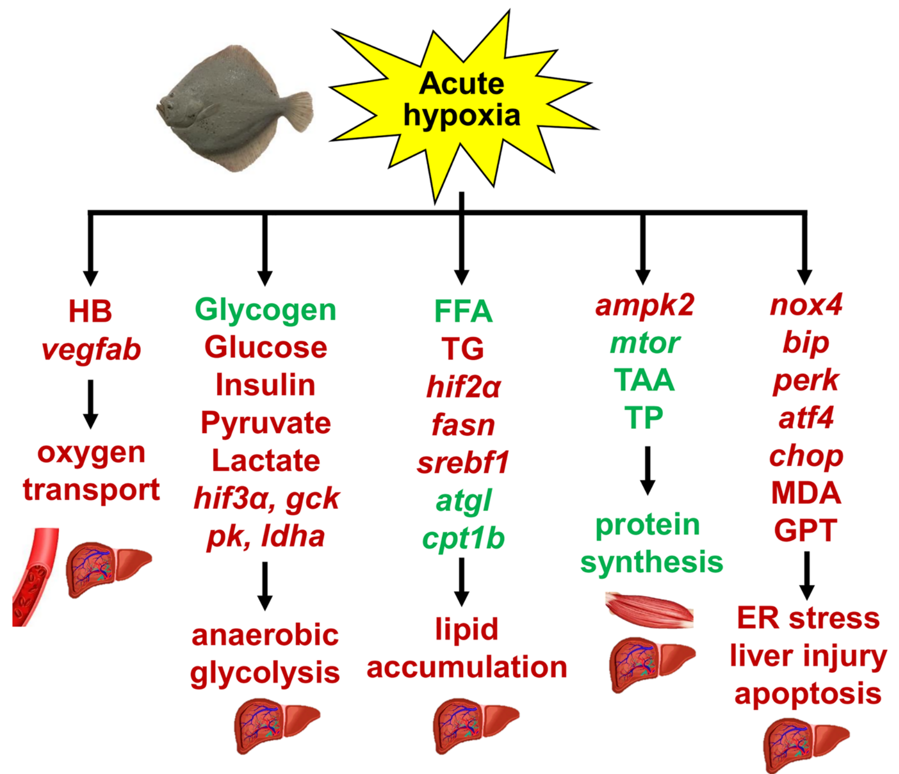

Fig. 7 The summary diagram of acute hypoxia affects nutrient metabolism and physiological function in turbot. Red font represents uptrend and green font represents downtrend

ER stress plays an important role in hypoxic adaptation in turbot. However, acute hypoxia decreased the genes expression of *perk* and *chop* in the muscle, and the mechanisms behind are waiting for further studies. In brief, the effects of acute hypoxia on nutrient metabolism and physiological function in turbot are provided in Fig. 7. On the one hand, acute hypoxia results in *perk*-mediated ER stress, liver injury, and apoptosis in turbot; on the other hand, hypoxia promotes HB level, anaerobic glycolysis, and lipid synthesis, but suppresses lipid catabolism and protein synthesis. These changes increase oxygen transport, energy supply from glucose, and reduce the oxygen consumption and ROS production, which promote the adaptation of turbot to hypoxia environment.

Conclusion

Water dissolved oxygen is an important environmental factor for aquatic animals. Our present study suggested that turbot is intolerant to acute hypoxia and the asphyxiation point is about 1.5 mg/L at 20.5 °C. Acute hypoxia induced *perk*-mediated ER stress, and increased lipid peroxidation, liver injury, and apoptosis in turbot. Under hypoxia, the hemoglobin level in the blood and *vegfab* gene expression in the liver were increased to enhance oxygen transport. Moreover, *hif3α* expression and anaerobic glycolysis were activated in the liver to ensure ATP supply. Meanwhile, *hif2α* expression and lipid synthesis were activated, but the lipid catabolism and protein synthesis were suppressed to reduce the oxygen consumption and ROS production. In short, our study proves the effects of acute hypoxia on nutrient metabolism and physiological function in turbot, and is important for understanding hypoxia adaptation in fish.

Authors' contributions QM designed the experiment and wrote the manuscript. ML revised the manuscript. HX performed the experiments. YW analyzed the data. All authors read and approved the final manuscript.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All experimental procedures and animal care were conducted under a protocol approved by Institutional Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute.

Consent to participate All authors have agreed to participate in this study.

Consent for publication All authors have read the final manuscript for publication.

Competing interests The authors declare that they have no competing interests.

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