Comparative Biochemistry and Physiology, Part A 166 (2013) 399–405

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Effects of food-deprivation and refeeding on the regulation and sources of blood glucose appearance in European seabass (*Dicentrarchus labrax* L.)

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ARTICLE INFO

Article history:
Received 25 February 2013
Received in revised form 9 July 2013
Accepted 10 July 2013
Available online 17 July 2013

Keywords:
^2H_2O
Gluconeogenesis
Glycogenolysis
Glucose 6-phosphatase
Glucokinase
NMR

ABSTRACT

Sources of blood glucose in European seabass (initial weight 218.0 ± 43.0 g; mean ± S.D., n = 18) were quantified by supplementing seawater with deuterated water (5%-^2H_2O) for 72 h and analyzing blood glucose ^{2}H-enrichments by ^{2}H NMR. Three different nutritional states were studied: continuously fed, 21-day of fast and 21-day fast followed by 3 days of refeeding. Plasma glucose levels (mM) were 10.7 ± 6.3 (fed), 4.8 ± 1.2 (fasted), and 9.3 ± 1.4 (refed) (means ± S.D., n = 6), showing poor glycemic control. For all conditions, ^{2}H-enrichment of glucose position 5 was equivalent to that of position 2 indicating that blood glucose appearance from endogenous glucose 6-phosphate (G6P) was derived by gluconeogenesis. G6P-derived glucose accounted for 65 ± 7% and 44 ± 10% of blood glucose appearance in fed and refed fish, respectively, with the unlabeled fraction assumed to be derived from dietary carbohydrate (35 ± 7% and 56 ± 10%, respectively). For 21-day fasted fish, blood glucose appearance also had significant contributions from unlabeled glucose (52 ± 16%) despite the unavailability of dietary carbohydrates. To assess the role of hepatic enzymes in glycemic control, activity and mRNA levels of hepatic glucokinase (GK) and glucose 6-phosphatase (G6Pase) were assessed. Both G6Pase activity and expression declined with fasting indicating the absence of a classical counter-regulatory stimulation of hepatic glucose production in response to declining glucose levels. GK activities were basal during fed and fasted conditions, but were strongly stimulated by refeeding. Overall, hepatic G6Pase and GK showed limited capacity in regulating glucose levels between feeding and fasting states.

1. Introduction

Glucose is an essential energy substrate for many cells and as principal regulator of its supply, the liver controls the requirements for endogenous glucose production (EGP) in order to maintain homeostasis. Despite large variation in dietary carbohydrate (CHO) availability and whole body glucose demands between species, the biochemical pathways and control mechanisms of glucose metabolism are highly conserved (Polakof et al., 2011). Therefore, the plasticity of this system determines how well a particular organism adapts to dietary CHO. For farmed carnivorous fish species such as the European seabass *Dicentrarchus labrax* L., there is current interest from both economic and ecological perspectives about their capacity to adapt from their natural high protein/low CHO diet to feedstock where a significant portion of protein is replaced by CHO (Fernández et al., 2007; Enes et al., 2009).

It is widely accepted that seabass and other carnivorous fish ineffectively utilize dietary CHO, instead they are highly dependent on gluconeogenesis from amino acids for sustaining endogenous glucose demands (Moon, 2001; Stone, 2003; Enes et al., 2009; Polakof et al., 2012). In addition, glycemia is poorly controlled in these fish compared to mammals (Polakof et al., 2011). This may in part reflect a weak coupling between plasma glucose levels and regulation of glucose production and disposal pathways. For example, in seabass the activities of hepatic gluconeogenic enzymes were not suppressed by chronic high-CHO feeding thereby contributing to postprandial hyperglycemia (Enes et al., 2006, 2008b). Meanwhile, the transition from feeding to fasting is characterized by a steep drop in plasma glucose concentrations (Gutiérrez et al., 1991; Echevarría et al., 1997; Pérez-Jiménez et al., 2007; Viegas et al., 2011; Pérez-Jiménez et al., 2012) among other tissue-dependent effects (Blasco et al.,

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http://dx.doi.org/10.1016/j.cbpa.2013.07.013

2001). Other fish species can tolerate extended periods of starvation without apparently suffering from hypoglycemia like *Brycon cephalus* (Figueiredo-Garutti et al., 2002), gilthead seabream *Sparus aurata* (Sangiao-Alvarellos et al., 2005; Polakof et al., 2006) or Senegalese sole *Solea senegalensis* (Costas et al., 2011). To date, our knowledge of fish CHO metabolism is largely informed by relating measurements of activities and gene expression levels of key enzymes in the regulation of glucose fluxes with changes in plasma glucose levels. While this approach has provided important insights into fish glucose metabolism, it does not directly unravel the metabolic processes that contribute to the appearance of blood glucose. Hence, the contribution of dietary CHO absorption vs. EGP to blood remains incompletely defined.

Deuterated water (^2H_2O) reveals the fraction of blood glucose derived from glucose 6-phosphate (G6P) via glucose 6-phosphatase (Basu et al., 2008; Nunes and Jones, 2009). In addition, the sources of G6P can be resolved into gluconeogenic and glycogenolytic contributions by measuring the ratio of enrichment in positions 5 and 2 (H5/H2) of blood glucose (Landau et al., 1996) (Fig. 1). This approach was recently applied in seabass (Viegas et al., 2011) revealing that gluconeogenesis was the main source of hepatic G6P production regardless of nutritional state. However, for fasted fish, where blood glucose appearance was expected to be completely dependent on G6P hydrolysis, the fraction of blood glucose derived from G6P was unexpectedly low, accounting for only ~70% of the total. Therefore, to confirm feeding to fasting transitions and metabolic rebound by refeeding, we aimed at assessing the available endogenous sources of hepatic G6P. For this purpose we also measured hepatic glycogen concentration as well as the activities and mRNA levels of the hepatic catalytic subunit of glucose 6-phosphatase (G6Pase, EC 3.1.3.9) and glucokinase (hexokinase IV; GK, EC 2.7.1.2). To our knowledge, mRNA levels for these enzymes have never been assessed in any studies using seabass.

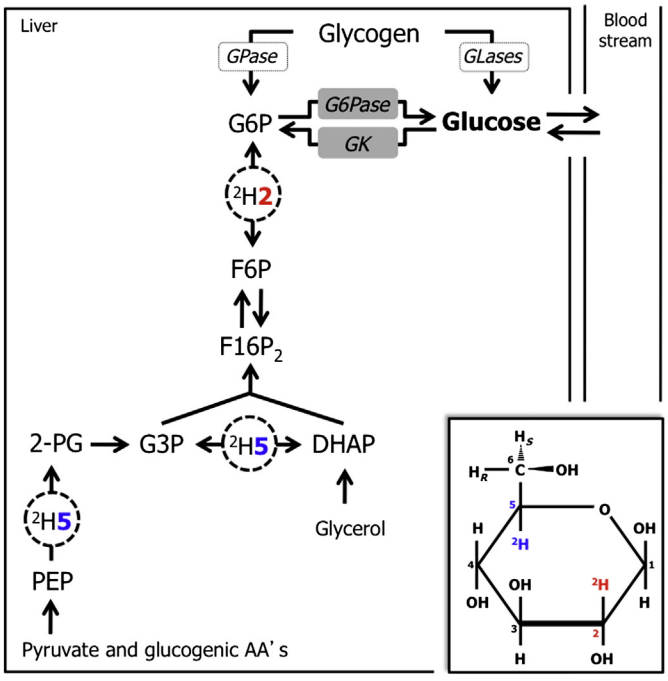

Fig. 1. Metabolic model representing gluconeogenesis and glycogenolysis in the liver. Gluconeogenic precursors are represented by pyruvate and gluconeogenic amino acids (metabolized via the anaplerotic pathways of the Krebs cycle) as well as glycerol from lipolysis. The sites for glucose enrichment in position 5 ($^2H5$) and position 2 ($^2H2$) from ^2H_2O are indicated. Note that for $^2H5$, the incorporation mechanisms include the addition of water hydrogen via enolase and formation of glyceraldehyde 3-phosphate from dihydroxyacetone phosphate via triose phosphate isomerase hence enrichment of this site is obligatory for glucose derived from gluconeogenic precursors. Glucose molecule with incorporation of ^2H in positions 2 and 5 is presented in detail. Some metabolic intermediates were omitted for clarity. Abbreviations are as follows: G6P = glucose 6-phosphate; F6P = fructose 6-phosphate; $F16P_2$ = fructose 1,6-bisphosphate; DHAP = dihydroxyacetone phosphate; G3P = glyceraldehyde 3-phosphate; 2-PG = 2-phosphoglycerate; PEP = phosphoenolpyruvate; AA's = amino acids; GK = glucokinase; G6Pase = glucose 6-phosphatase; GPase = glycogen phosphorylase (EC 2.4.1.1); GLases = glycosidases (E.C. 3.2.1) which include glucosidases, amylases and glycogen debranching enzymes that do not hydrolyze glycogen into G6P.

2. Material and methods

2.1. Fish handling and sampling

A total of 18 fish provided by a local farm (initial mean length of 28.0 ± 1.7 cm and initial mean body weight of 218.0 ± 43.0 g) were transported to the laboratory and randomly assigned to 3 different tanks (n = 6). These consisted of ~200 L polyethylene circular tanks supplied with well-aerated filtered seawater from a recirculation system set to 18 ± 1 °C temperature, 30 ± 1‰ salinity and O_2 levels above 80% saturation under natural photoperiod. These conditions were kept throughout the experiment. One of the tanks served as control and was provided with food once a day, 5 d per week, with ration of 2% mean body weight d^{-1} (Table 1) for 21 d. Fish from the remaining two tanks were fasted for 21 d. Following this period, each group was transferred to a separate 5% ^2H-enriched seawater tank for a period of 72 h. This consisted of a ~200 L tank of similar characteristics to the ones used in the previous phase. In order to insure similar water parameters, this tank was maintained with an independent closed filtering system. During this 72 h period in 5% ^2H-enriched seawater, the fed fish were kept in the same feeding protocol and one group of fasted fish was kept in the fasting protocol. The remaining fasted group started a refeeding protocol with the same ration size and diet than the fed fish. Fed and refed fish were provided with last meal 24 h before sacrifice. At this stage, fish were anesthetized in 5% ^2H-enriched seawater containing 0.1 g L^{-1} of MS-222, measured, weighed and sampled for blood from the caudal vein with heparinized syringes. Fish were sacrificed by cervical section; liver was excised, weighed, freeze-clamped in liquid N_2, ground and stored at −80 °C.

2.2. Sample treatment

From the total blood withdrawn, an aliquot was separated and kept on ice until centrifugation (3000 *g* for 10 min) to separate plasma. This aliquot was used to quantify plasma glucose (Amplex® Red Glucose Assay Kit, Invitrogen, Spain) and assess plasma ^2H-enrichment

Table 1
Ingredients and proximate composition of the diet provided to *D. labrax* (5 mm standard pellet).

Ingredients (%)	Diet
Fishmeal	37.0
Soybean meal	21.0
Corn gluten	13.5
Wheat gluten	0.3
Fish oil	13.5
Rapeseed oil	5.0
Wheat	4.5
Pea starch	2.5
Premix	0.2
Co-product of formation of L-glutamate from fermentation	2.5
Proximate composition (% dry weight)	
Crude protein	44.1
Crude fat	18.0
Starch	2.2
Ash	9.2
Phosphorous	1.3
Gross energy (kJ g^{-1} dry mass)	20