Fish Physiol Biochem (2011) 37:681–692
DOI 10.1007/s10695-011-9468-1

Influence of increased environmental water salinity on gluconeogenesis in the air-breathing walking catfish, *Clarias batrachus*

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Received: 20 August 2010 / Accepted: 22 January 2011 / Published online: 15 February 2011

Abstract The present study was aimed at determining the effect of hypertonicity due to increased environmental water salinity on gluconeogenesis in air-breathing walking catfish (*Clarias batrachus*). In situ exposure to hypertonic saline solution (150 mM NaCl) led to a significant stimulation of glucose efflux due to gluconeogenesis from the liver after 7 days with further elevation after 14 days in the presence of each of the three potential gluconeogenic substrates (lactate, pyruvate, and glutamate). This was accompanied by significant increase of activities of three key gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-biphosphatase (FBPase), and glucose 6-phosphatase (G6Pase) in liver and kidney by about twofold to threefold. Environmental hypertonicity also led to a significant elevation in the levels of PEPCK, FBPase, and G6Pase enzyme proteins in both the tissues by about 2- to 2.75-fold, accompanied by a significant elevation in the level of PEPCK mRNA by about 2- to 2.5-fold after 7 days, and further enhancement to about 3.5- to 4-fold after 14 days. Thus, the upregulation of PEPCK, FBPase. and G6Pase activities appears to be a result of transcriptional regulation of these genes. The induction of gluconeogenesis under environmental hypertonicity, which this catfish faces regularly in its natural habitat, possibly occurs as a consequence of changes in hydration status/cell volume of different cell types. This would certainly assist in maintaining glucose homeostasis, and also for a proper energy supply to support metabolic demands for ion transport and other altered metabolic processes under various environmental hypertonic stress-related insults.

Keywords Hypertonic stress · Phosphoenolpyruvate carboxykinase · Fructose 1, 6-biphosphatase · Glucose 6-phosphatase · Hydration status · Gluconeogenesis · *Clarias batrachus*

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Introduction

Glucose plays a key role in mammalian energetics but its importance as a metabolic fuel in fishes is not fully understood (for review, see Moon and Foster 1995). Even though most of the enzymes involved in glucose metabolism have been detected in fish, the regulation of carbohydrate metabolism differs in some aspects from that of mammals (Moon and Foster 1995). The regulation of hepatic glucose metabolism in teleost fishes is influenced by different stressful conditions, such as nutritional status of carbohydrates and proteins (for review, see Enes et al. 2009) and changes in

hepatocellular hydration status (Goswami and Saha 1998; Goswami et al. 2004). Gluconeogenesis has been extensively studied in the liver and kidney in various fish species (Saurez and Mommsen 1987). In some teleostean fishes, gluconeogenesis occurs at relatively higher rates (Hayashi and Ooshiro 1979; Renaud and Moon 1980) and is thought to be a key process in maintaining glucose homeostasis (Carneiro and Amaral 1982), especially in carnivorous fishes that have high protein and low carbohydrate diets (De la Higuera and Cardenas 1986).

A remarkable property of living cells is their ability to maintain a comparatively constant cell volume under different physiological conditions (for reviews, see Häussinger 1996; Perlman and Goldstein 1999). Cell volume may be challenged by a variety of factors such as the intestinal absorption of water and of various amino acids and metabolites; or by exposure to different osmotic environments especially in the case of aquatic animals. Most cells possess various volume-regulatory mechanisms [regulatory volume decrease (RVD) and regulatory volume increase (RVI)] to maintain the constancy of cell volume and also the hydration status of the cell largely by changing the permeability of various ions such as K^+, Na^+, H^+, Cl^-, and HCO_3^-, and certain organic osmolytes (Goswami and Saha 2006; Häussinger 1996). However, it has been noticed in many cell types that they remain either in a slightly swollen or in a shrunken state for the duration of the anisotonic exposure (for review, see Häussinger 1996). Irrespective of the route of RVD or RVI, increase in hepatic cell volume generally results in increased anabolism and curtailment of catabolic pathways, while the reverse is true during the decrease in hepatic cell volume (Biswas et al. 2009, 2010; Goswami and Saha 1998; Saha et al. 1992; Weiergräber and Häussinger 2000), with a few exceptions, such as in goldfish, where hypotonic exposure leads to increase in the rate of oxygen consumption (Pafundo et al. 2008).

In comparison with mammals, teleost fishes face more problems of osmotic stress primarily owing to osmolarity changes in their external environment. Reports are available concerning the cell volume-regulatory mechanisms in red blood cells (Fugelli and Thoroed 1986; Haynes and Goldstein 1993), renal cells (Kanli and Norderhus 1998), intestinal cells (Lionetto et al. 2001, 2005), and liver cells in fishes (Bianchini et al. 1988; Espelt et al. 2003). More recently, it has been demonstrated that liver cells of the air-breathing walking catfish (*Clarias batrachus*) possess efficient volume-regulatory mechanisms, but remain in partly swollen or shrunken state as long as they are exposed to anisotonicity (Goswami and Saha 2006). These changes of cell volume due to anisotonicity have been reported to cause changes in glucose, pyruvate and lactate efflux, glycogen metabolism (Goswami and Saha 1998), hexose monophosphate pathway (Saha and Goswami 2004), and also on gluconeogenesis (Goswami et al. 2004) in the perfused liver of this fish. Hallgren et al. (2003) also reported similar effects of cell volume changes at least on glycogen metabolism in the hepatocytes of three fish species.

The walking catfish (*C. batrachus*) found predominantly in tropical Southeast Asia is reported to be more resistant to various environmental challenges such as high environmental ammonia, hypoxic and desiccation stresses (for review, see Saha and Ratha 2007). Further, they are reported to be euryhaline, inhabiting fresh, and brackish waters as well as muddy marshes, thus facing wide variations of external osmolarity changes (Sen 1985); they frequently encounter the problem of osmolarity changes in the same habitat during different seasons of the year, especially in summer when the ponds and lakes dry up, thus compelling them to migrate inside the mud peat to avoid total dehydration, and during the monsoon season when the water in the same habitat gets diluted. Studies from our laboratory suggested that this fish is capable of surviving up to 200 mM NaCl under the laboratory conditions for months without having any mortality (Saha and Jyrwa, unpublished observation). Thus, looking at its enormous capacity in challenging the external osmolarity changes, the present study was undertaken to elucidate the possible effect of hypertonic stress due to increased environmental water salinity (150 mM NaCl) on gluconeogenesis.

Materials and methods

Animals

The walking catfish (*C. batrachus*, weighing 150 ± 15 g body mass) were purchased from commercial sources and acclimatized in the laboratory for

approximately 1 month at room temperature of 28 ± 2°C in plastic aquaria with 12 h: 12 h light and dark photoperiod before using for experiments. No sex differentiation of fish was done while performing these studies. Minced pork liver and rice bran (5% of the body wt) were given as food, and the water was changed on alternate days. Food was withdrawn 24 h prior to experiments.

Experimental set up

Fish of similar size were placed individually in plastic buckets containing 2 l of 150 mM NaCl solution prepared in fresh water (pH 6.99 ± 0.05) and kept for 7 and 14 days. Another group of fish was kept individually in plastic buckets containing 2 l of fresh water (pH 7.05 ± 0.06) and served as controls. Both the NaCl solution and water from each bucket were replaced with a fresh medium every day at a fixed time. On day 7 and 14, five fishes each from control and treated buckets were removed and killed immediately by decapitation after collecting blood from the caudal vein. Liver and kidney tissues were dissected out, plunged into liquid nitrogen and stored at −60°C. All analyses were completed within 2 weeks of collecting the tissues. In another set of experiments, a group of fish was treated separately with 150 mM NaCl for 7 and 14 days along with a group kept in fresh water for performing the liver perfusion experiments.

Liver perfusion technique

Fishes were anesthetized in neutralized 3-aminobenzoic acid ethyl ester (MS-222, 0.2 g l^{-1}) for 5 min before operation to perform the liver perfusion. The isolated livers were perfused via the portal vein in a non-circulating manner with hemoglobin-free medium following the method described by Saha et al. (1995). The isotonic medium (265 mOsmol l^{-1}, determined by freezing point depression method) contained 119 mM NaCl, 5 mM $NaHCO_3$, 5.4 mM KCl, 0.35 mM Na_2HPO_4, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, and 1.25 mM $CaCl_2$ and served as a basic solution for perfusion. The perfusate was gassed with O_2/CO_2 (99:1, v/v) and its pH adjusted to 7.6. Livers were perfused at a flow rate of 4–5 ml g^{-1} liver min^{-1} and at a temperature of 30°C.

To study the rates of gluconeogenic efflux in the perfused liver of both NaCl-treated and control fishes, livers were initially perfused for 40 min with isotonic medium, followed by infusion of gluconeogenic substrates (lactate, pyruvate, or glutamate) at a concentration of 5 mM (a concentration suitable for studying gluconeogenic efflux, Goswami et al. 2004) for 30 min. Effluents were collected at 2-min intervals for the determination of glucose flux from the perfused liver and the steady-state efflux of glucose, obtained between 22 and 30 min of infusion of substrates, was used to calculate the rate of gluconeogenic efflux. A steady-state continuous efflux of glucose normally occurs from the perfused liver while perfusing with isotonic medium at least for 100–120 min (Goswami and Saha 1998). Therefore, the rate of gluconeogenic efflux was calculated by subtracting the value of steady-state efflux of glucose, obtained just before infusion, from the value of steady-state efflux obtained after 20 min of infusion of gluconeogenic substrates (Goswami et al. 2004).

Estimation

For estimation of glucose in the perfusate, 10 µl of 2 M perchloric acid (PCA) was added to 1 ml of effluent collected at 2-min intervals, and the precipitated protein was removed by centrifugation. The supernatant was neutralized by adding 10 µl of 2 M NaOH before estimation of glucose. Concentrations of glucose in the effluent were measured enzymatically following the method of Bergmeyer et al. (1974).

Enzyme assay

A 10% homogenate (w v^{-1}) of each frozen tissue was prepared in a homogenizing buffer containing 50 mM Tris–HCl buffer (pH 7.4), 0.25 M sucrose, 1 mM ethylene diamine tetra-acetic acid (EDTA), 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 3 mM 2-mercaptoethanol, and a cocktail of protease inhibitor (Roche, Germany) using a motor-driven Potter–Elvehjem type glass homogenizer with a Teflon pestle. The homogenate was treated with 0.5% Triton X-100 in 1:1 ratio for 30 min, followed by mild sonication for 30 s to rupture the mitochondria since some of the gluconeogenic enzymes are localized both in cytosol and in mitochondria (Goswami et al. 2004). The homogenate was then centrifuged at 10,000 × *g* for 10 min and the supernatant used for assaying the enzymes. All steps were carried out at 4°C. It is to be

noted that there no difference of activity of any of the enzymes in frozen tissue, preserved at −60°C maximum for 2 weeks, compared to fresh tissue.

Phosphoenolpyruvate carboxykinase (PEPCK) was assayed following the method of Mommsen et al. (1985) with two-step enzymatic reactions. One ml of reaction mixture contained 50 mM Tris–HCl buffer (pH 7.4), 4.5 mM phosphoenolpyruvate, 20 mM $NaHCO_3$, 0.6 mM deoxyGDP, 0.15 mM NADH, 1 mM $MnCl_2$, 5 units of malate dehydrogenase (MDH), and 50–100 µl tissue extract. Fructose 1, 6-biphosphatase (FBPase) was also assayed following the method of Mommsen et al. (1985) with three-step enzymatic reactions. One ml of reaction mixture contained 50 mM Tris–HCl buffer, 15 mM $MgCl_2$, 0.3 mM $NADP^+$, 10 units G6PDH, 400 units phosphoglucose isomerase, 0.15 mM fructose 1,6-biphosphate, and 50 µl tissue extract. Glucose-6-phosphatase (G6Pase) was assayed following the method of Nordlie and Arion (1966). One ml of reaction mixture contained 100 mM Na-acetate buffer (pH 6.5), 40 mM glucose-6-phosphate, and 100 µl tissue extract. In both the cases the reaction was stopped by adding 0.5 ml 10% perchloric acid after a specific period of time. The inorganic phosphate, formed in the G6Pase reaction, was estimated in the supernatant spectrophotometrically at 700 nm following Fiske and Subbarow (1957) against a tissue blank, and expressed as enzyme activity. The decrease in absorbance (due to oxidation of NADH to NAD^+) in case of PEPCK, the increase in absorbance (due to reduction of $NADP^+$ to NADPH) in case of FBPase were recorded at 30-s interval at 340 nm in a UV–visible spectrophotometer (Varian, Model Cary 50) fitted with a peltier temperature-controlled device. One unit of enzyme activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 µmol of NADH h^{-1} for PEPCK, or the reduction of 1 µmol of $NADP^+$ h^{-1} for FBPase at 30°C. For G6Pase, one unit of enzyme activity was expressed as that amount of enzyme which catalyzed the formation of 1 µmol of inorganic phosphate h^{-1} at 30°C.

Western blot analysis

Tissues were homogenized in 20 mM Tris–HCl buffer (pH 7.4) containing 0.33 M sucrose, 1 mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail (Roche, Germany) and sonicated for 30 s. The homogenate was centrifuged at 10,000×*g* for 10 min at 4°C. The protein concentration in supernatants was determined by the dye binding method (Bradford 1976). Supernatants were mixed with loading buffer containing 150 mM Tris–HCl buffer (pH 8.8), 15 mM EDTA, 60 mM DTT, 1.2 M sucrose, 6% sodium dodecyl sulphate (SDS), and 0.125% bromophenol blue in the ratio of 2:1, followed by incubation at 37°C for 30 min. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH), the house keeping protein, was used as a protein loading control in each experiment. In each lane, 50 µg of protein was applied and electrophoresed in 7.5% SDS–PAGE in case of PEPCK, and 12% SDS–PAGE in case of FBPase, G6Pase, and GAPDH. In one lane, 5 µl of Rainbow molecular weight marker (GE Healthcare, USA) was also run. Proteins were electroblotted from gels onto nitrocellulose membrane in a semidry blotter (Hoefer TE 70) for 2 h in presence of transfer buffer (40 mM glycine, 48 mM Tris, 0.03% SDS, and 20% methanol). Blots were blocked overnight in 1% bovine serum albumin (BSA) prepared in PBST (10 mM Na-phosphate buffer, pH 7.4, 137 mM NaCl, 3 mM KCl, and 0.1% Tween) at 4°C. Blots were then incubated with goat polyclonal antibodies to PEPCK, FBPase, G6Pase, and GAPDH (1:2,500 dilution) for 2 h, followed by incubations with horseradish peroxidase (HRP)-conjugated anti-goat IgG (1:5,000 dilution) secondary antibody for 2 h with washing steps with PBST in between. Immunodetection was carried out using an enhanced chemiluminescence kit (PerkinElmer, USA) according to the manufacturer's directions. After exposure to X-ray film, bands were scanned with a Gel Logic 200 Imaging System (Kodak, Japan) and the band densities determined using the LabImage software.

Blood sampling and osmolarity measurement

The blood was collected with a heparinized syringe from the caudal vein and centrifuged at 10,000×*g* for 10 min at 0 ± 2°C for separating out the plasma from blood cells. The plasma osmolarity was measured with a Camlab (Model 200) osmometer using the freezing point depression method.

Measurement of water content

The water content in cells of different tissues of both control and saline water-treated fish was determined

by oven drying method following Goswami and Saha (1998).

RNA extraction and cDNA synthesis

The total RNA was isolated from liver and kidney tissues using TRI® Reagent (Sigma Chemicals, St. Louis, USA), following the manufacturer's protocol. RNA extracted as pellets from different tissues was washed in 75% ethanol, air-dried at room temperature for 10–15 min, and redissolved in RNAase free diethylpyrocarbonate-treated water. Purity of RNA was confirmed by reading absorbance at 260 and 280 nm. The ratio of background-free absorbance at 260/280 nm was always between 2.0 and 2.2. The RNA solution was then further purified using the RNAase miniprotocol for RNA cleanup (Qiagen, Germany). Purified RNA was quantified spectrophotometrically, diluted to 5 μg μl^{-1} and electrophoresed on 1% agarose gel stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from 1 μg total RNA (DNase I-treated, Invitrogen) in a total volume of 20 μl with High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA) as per the standard protocol.

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was performed on cDNAs produced from total RNA for PEPCK mRNA. Quantitative analysis of mRNA expression for FBPase and G6Pase was not possible due to non-availability of cDNA sequencing in the gene bank. qPCR was performed in the 7500 FAST RT–PCR (Applied Biosystems, USA) with Power Sybr® Green PCR Master Mix (Applied Biosytems, USA). The reaction mixture of 25 μl each contained 12.5 μl of 2× Sybr Green/ROX PCR Master Mix (Applied Biosystems, USA), 2.5 μl of cDNA, 8 pmol of each primer, and 6 μl of MilliQ H_2O. The PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 54°C for 1 min. Data were collected at 54°C. qPCR was performed in triplicate and negative controls using no cDNA were run for each gene. Melting curve analysis was used to re-confirm amplification of only a single PCR product. The level of β-actin was invariant between the control and treated fish validating its choice as an endogenous control. Fold changes of PEPCK gene in treated fish compared to untreated control was calculated using the modified delta-delta C_T method (Livak and Schmittgen 2001).

The primer pairs were chosen from the published cDNA sequences of *Clarias batrachus* PEPCK (EU797496) and β-actin (EU527190). The primers for PEPCK were as follows: forward (5′-CGGGA ACCTCACTGAAGACAA-3′) and reverse (5′-GTG AATATCGT GTTCTTTGAA-3′), and for β-actin the primers were as follows: forward (5′-CCGAGCG AGGCTACAGCTT-3′) and reverse (5′-TGAAATC GTGCGTGACATCA-3′), which were designed with the help of Primer Express software 3.0 (Applied Biosystems, USA).

Chemicals

Enzymes, co-enzymes, substrates, and oligonucleotide primers were purchased from Sigma Chemicals (St. Louis, USA). PEPCK, FBPase, G6Pase, and GAPDH goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (USA). Other chemicals were of analytical grades and obtained from local sources. MilliQ water was used in all preparations.

Statistical analyses

The data collected from different replicates were statistically analyzed and presented as mean ± SEM (n = number of animals in each set of experiment). Student's t-test followed by multiple comparisons of means by Student–Newman–Keuls Multiple Range Test was performed to evaluate differences between means where applicable. Two way ANOVA was performed to evaluate the significance of difference of activity of gluconeogenic enzymes due to time of exposure and hypertonicity and their interaction. Differences with $P < 0.05$ were regarded as statistically significant.

Results

Effect of environmental hypertonicity on glucose efflux due to gluconeogenesis from the perfused liver

Effect of environmental hypertonicity on gluconeogenic activity was studied in the liver organ of walking

catfish by perfusion technique in presence of each three potential substrates such as lactate, pyruvate, and glutamate (Goswami et al. 2004; Table 1). In control fish, a steady-state efflux of glucose due to gluconeogenesis from the perfused liver was obtained with almost no changes of efflux during the experimental periods, except for a significant increase of efflux by 34% with glutamate only after 14 days (Table 1). However, the gluconeogenic efflux increased significantly in fishes following in situ exposure to hypertonic saline environment with all the three substrates. With lactate as a substrate, the rate of gluconeogenesis by the perfused liver increased significantly by 109 and 125% after 7 and 14 days of exposure, respectively. With pyruvate, the rate increased by 73 and 82%, and with glutamate, by 57 and 89% after 7 and 14 days of exposure, respectively.

Upregulation of gluconeogenic enzymes following exposure to hypertonic saline environment

The changes of activity of the three key gluconeogenic enzymes namely PEPCK, G6Pase, and FBPase, following exposure to hypertonic saline environment (150 mM NaCl) for 7 and 14 days in liver and kidney tissues of the catfish, are depicted in Fig. 1. In control fish, no significant ($P < 0.05$) changes in the activity of gluconeogenic enzymes were observed during the experimental periods. However, the activities of all the three enzymes increased significantly in fish exposed to hypertonic saline environment with respect to controls. The PEPCK activity in liver increased maximally by 171% after 14 days, whereas in kidney it increased maximally by 106% after 7 days of exposure. The FBPase activity increased maximally in liver and kidney by 161 and 91%, respectively, after 14 days of exposure. In case of G6Pase, the enzyme activity increased maximally both in the liver and in the kidney by 158 and 91%, respectively, after 7 days with no further changes of activity after 14 days of exposure. Two way ANOVA showed a highly significant ($P < 0.001$) effect of the time of exposure in relation to hypertonicity exposure on all the three enzymes both in liver and in the kidney of treated fish compared to control fish.

As evidenced by Western blot analysis (Figs. 2 and 4), the increase of PEPCK, FBPase, and G6Pase activities in liver and kidney tissues of walking catfish, following exposure to environmental hypertonicity, was accompanied by a significant elevation in the levels of all the enzyme proteins after 7 and 14 days. In case of PEPCK, the maximal elevation by 2.5-fold was observed in liver after 14 days and by 2.05-fold in kidney after 7 days of exposure (Fig. 2). For FBPase, the enzyme protein concentration elevated maximally by 2.4- and 1.8-fold, respectively, in liver and kidney after 14 days of exposure (Fig. 3). Similarly, for G6Pase, the enzyme protein concentration also elevated maximally by 2.75- and 2.45-fold, respectively, both in liver and kidney after 7 days of exposure (Fig. 4).

Analyses of relative expression of mRNA for PEPCK by real-time qPCR technique indicated that the mRNA level elevated significantly both in liver and in kidney tissues by 2.45- and 2.18-fold after 7 days, with further elevation by 3.6- and 3.8-fold, respectively, after 14 days of exposure to hypertonic saline environment (Fig. 5).

Table 1 Changes of glucose efflux due to gluconeogenesis (μmol g^{-1} liver h^{-1}) in presence of different substrates (5 mM) from the perfused liver of *C. batrachus* following 7 and 14 days of hypertonic exposure

Substrates	Days of Exposure				
	0	7		14	
	Control	Control	Treated	Control	Treated
Lactate	19.7 ± 1.13	21.0 ± 1.15 (7)*	43.86 ± 2.11^{c} (109)**	24.0 ± 1.52 (17)*	54.0 ± 2.65^{c} (125)**
Pyruvate	14.7 ± 0.52	15.6 ± 0.77 (6)*	27.0 ± 2.65^{c} (73)**	16.8 ± 0.91 (14)*	30.60 ± 1.85^{b} (82)**
Glutamate	24.1 ± 0.90	25.2 ± 1.01 (5)*	39.6 ± 2.01^{b} (57)**	32.4 ± 2.35^{a} (34)*	61.2 ± 4.21^{b} (89)**

Values are expressed as mean ± SEM ($n = 5$)

* Percentage increase of glucose efflux due to gluconeogenesis by the 7 and 14 days control fish compared to 0 time control fish

** Percentage increase of glucose efflux due to gluconeogenesis by the hypertonically treated fish compared to respective controls

$^{a, b, c}$ Significantly different from control values at $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels, respectively (Student's *t*-test)

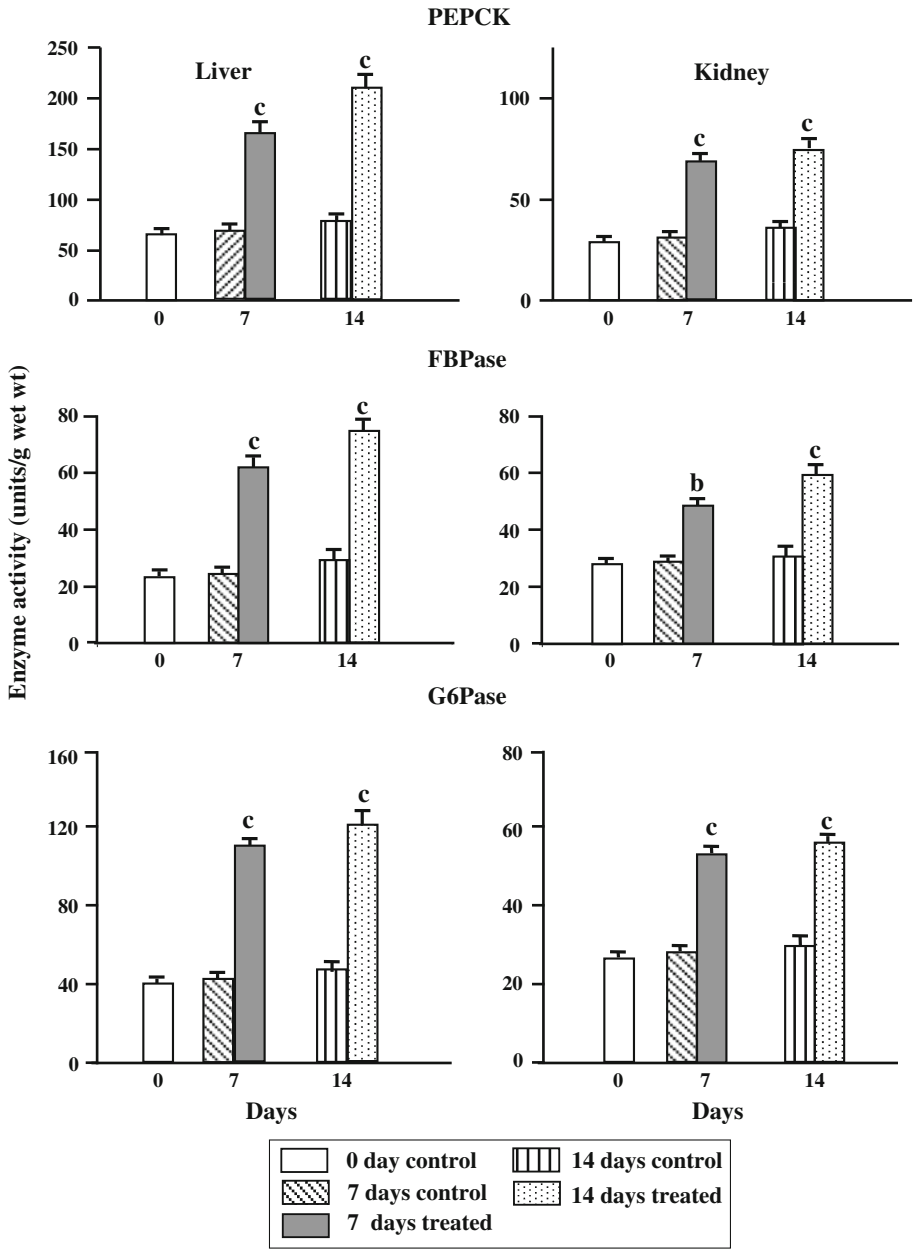

Fig. 1 Changes in activities (units g^{-1} wet wt) of different gluconeogenic enzymes in walking catfish following exposure to environmental hypertonicity. Values are plotted as mean ± SEM ($n = 5$). One unit of enzyme activity was expressed as that amount of enzyme that catalyzed the oxidation of 1 μmol of NADH h^{-1} in case of PEPCK, reduction of 1 μmol of $NADP^+$ h^{-1} in case of FBPase at 30°C, and 1 μmol of inorganic phosphate formed h^{-1} at 30°C in case of G6Pase. $^{b,c}P$ values significant at <0.01 and <0.001 levels, respectively, compared to respective controls (Student's *t*-test)

Changes of blood osmolarity and tissue water content due to environmental hypertonicity

In situ exposure of walking catfish in hypertonic environment (150 mM NaCl) led to a significant ($P < 0.05$) increase of blood osmolarity from 265 ± 4 to 320 ± 5 ($n = 5$; 21%) after 7 days and to 332 ± 6 ($n = 5$; 25%) after 14 days. This also led to a decrease of water content in liver and kidney tissues by 11 ± 1.2 and 10 ± 0.8%, respectively, after 7 days with no further changes at later stages of exposure.

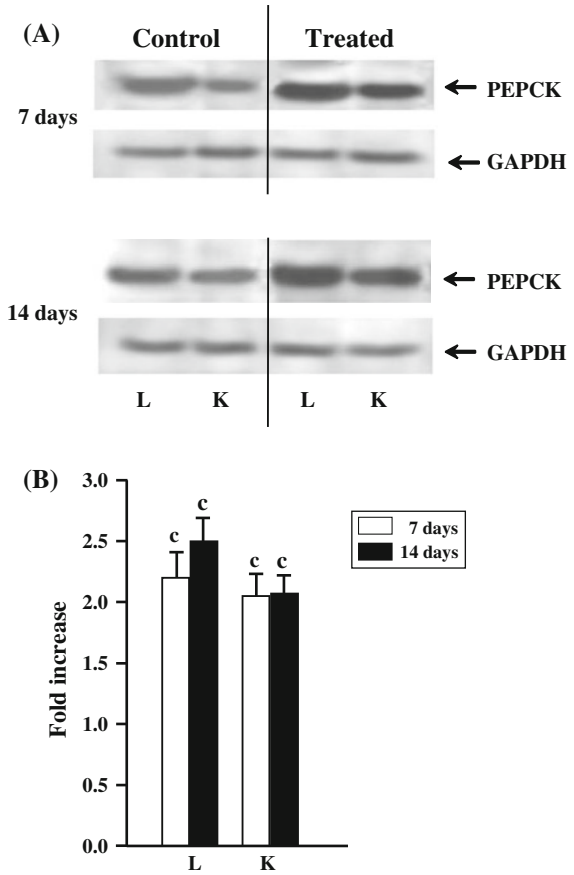

Fig. 2 Western blot analysis showing changes in the levels of expression of PEPCK enzyme protein in liver (*L*) and kidney (*K*) of walking catfish following exposure to environmental hypertonicity. **a** A representative plot of 5 individual experiments. GAPDH was taken as a protein loading control. **b** Densitometric analysis showing the fold of increase of PEPCK enzyme protein concentration in treated fish compared to respective controls. Values are plotted as mean ± SEM ($n = 5$). ^{c}P value significant at <0.001 level compared to respective controls (Student's *t*-test)

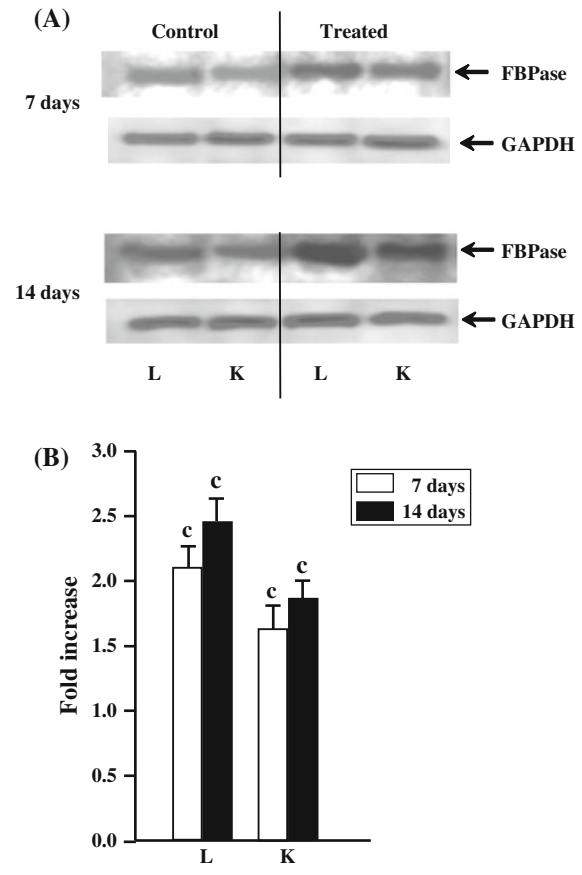

Fig. 3 Western blot analysis showing changes in the levels of expression of FBPase enzyme protein in liver (*L*) and kidney (*K*) of walking catfish following exposure to environmental hypertonicity. **a** A representative plot of 5 individual experiments. GAPDH was taken as a protein loading control. **b** Densitometric analysis showing the fold of increase of FBPase enzyme protein concentration in treated fish compared to respective controls. Values are plotted as mean ± SEM ($n = 5$). ^{c}P value significant at <0.001 level compared to respective controls (Student's *t*-test)

Discussion

The effect of various environmental factors such as temperature, hypoxia, starvation, and certain hormones on carbohydrate metabolism including gluconeogenesis in different fish species has been reported by several workers (for review, see Moon and Foster 1995). There are also reports on the influence of dietary carbohydrates on gluconeogenesis in teleosts (for review, see Enes et al. 2009); however, the reports on the influence of environmental hypertonicity in this process in teleosts are scanty. Using an intact organ model of perfused liver, it was demonstrated that the alterations of hepatic cell volume due to anisotonicity lead to changes in carbohydrate and oxidative metabolisms (Goswami and Saha 1998; Goswami et al. 2004; Saha and Goswami 2004), and also autophagic proteolysis (Biswas et al. 2009) in walking catfish.

Our results on the present investigation clearly demonstrated that the gluconeogenic activity could be stimulated by environmental hypertonicity in walking catfish by exposing the fish to hypertonic saline water (150 mM NaCl). Exposure to hypertonic saline environment for 7 and 14 days leads to

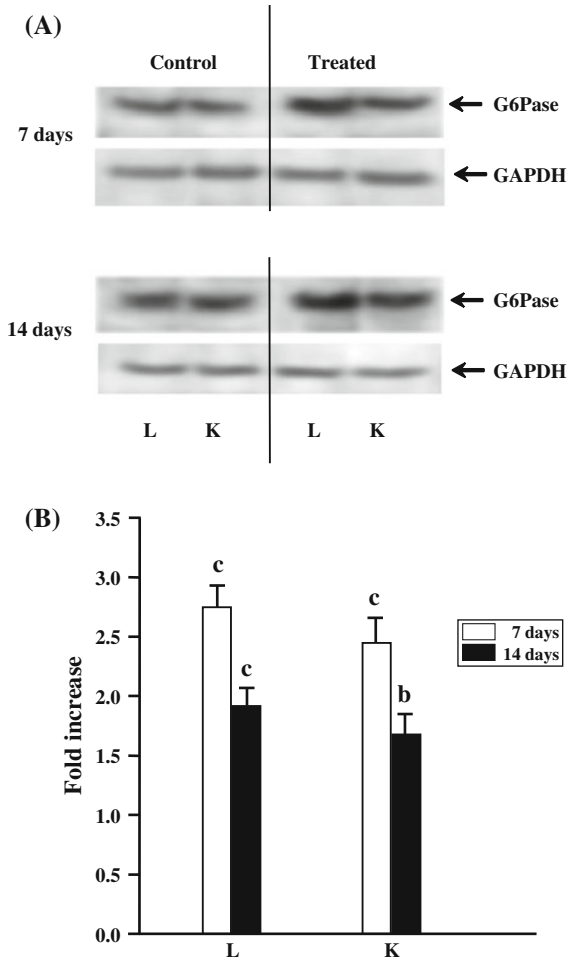

Fig. 4 Western blot analysis showing changes in the levels of expression of G6Pase enzyme protein in liver (*L*) and kidney (*K*) of walking catfish following exposure to environmental hypertonicity. **a** A representative plot of 5 individual experiments. GAPDH was taken as a protein loading control. **b** Densitometric analysis showing the fold of increase of G6Pase enzyme protein concentration in treated fish compared to respective controls. Values are plotted as mean ± SEM ($n = 5$). $^{b,c}P$ values significant at <0.01 and <0.001 levels, respectively, compared to respective controls (Student's *t*-test)

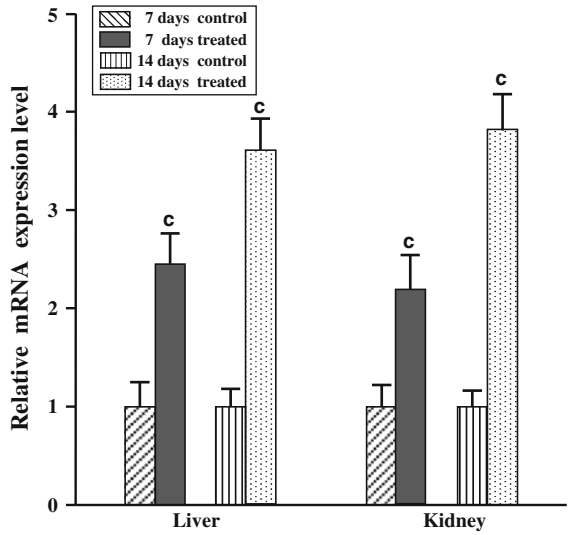

Fig. 5 qPCR analysis showing the levels of relative expression of PEPCK mRNA in liver and kidney of walking catfish following exposure to environmental hypertonicity. Values are plotted as mean ± SEM ($n = 5$). ^{c}P value significant at <0.001 level, compared to respective controls (Student's *t*-test)

significant increase of glucose efflux due to gluconeogenesis from the liver with all the three substrates that are considered to be most potential gluconeogenic substrates in this air-breathing catfish (Goswami et al. 2004). As such, the glucose production due to gluconeogenesis is reported to be quite high in this fish liver when compared to carps and certain other teleosts (Goswami et al. 2004). The glucose production by gluconeogenesis got further elevated significantly in presence of all the three substrates following after exposing the fish to hypertonic saline environment. The maximum elevation was seen with lactate, indicating that an active Cori cycle is prevailing in this walking catfish. Thus, lactate gluconeogenesis could be one of the major sources of energy in this fish under various environmental constraints including hypertonicity. Further, this catfish is predominantly carnivorous in its feeding habit and primary depends on high protein and low carbohydrate diets (Munshi and Ghosh 1994). Thus, amino acid gluconeogenesis, which is of great physiological significance in this fish (Goswami et al. 2004), also gets further stimulated following exposure to environmental hypertonicity as shown with glutamate in the present study. Stimulation of autophagic proteolysis due to hypertonicity, reported in this fish liver (Biswas et al. 2009), should favor gluconeogenesis from proteolysis-derived amino acids as a coordination of a functionally linked physiological process in response to decrease of cell volume under hypertonic stress.

Further, the increase of glucose efflux due to gluconeogenesis was accompanied by favorable changes of activities of three key gluconeogenic enzymes both in liver and in kidney. Induction of activities of gluconeogenic during exposure to

hypertonic saline environment led also to similar folds of increase of all the three enzyme protein concentrations (Figs. 2, 3, 4) and also elevation in the level of PEPCK mRNA both in liver and in kidney (Fig. 5). Thus, the induction of PEPCK, FBPase, and G6Pase activities could suggest be a result of transcriptional regulation of genes of these three enzymes under environmental hypertonic stress. Further, the two way ANOVA test indicated that the effect of environmental hypertonic exposure on three gluconeogenic enzymes in relation to the time of exposure is highly significant ($P < 0.001$) both in liver and in kidney of treated fish in relation to control fish.

The enzyme PEPCK is known to occur as two isoforms (the mitochondrial and the cytosolic) with different distribution and regulatory patterns in various groups of vertebrates (Hanson and Reshef 1997). It has been demonstrated that in animals such as chicken in which both the mitochondrial and the cytosolic forms occur (Weldon et al. 1990), only the cytosolic form is acutely regulated by diet and hormones, whereas the gene for mitochondrial PEPCK is largely constitutive in its pattern of expression (Hanson and Reshef 1997). Similarly, in rainbow trout, the PEPCK gene which exclusively codes for the mitochondrial type of PEPCK could not be regulated by dietary carbohydrates (Panserat et al. 2001). In walking catfish, however, the PEPCK and FBPase enzymes have been reported to be equally distributed both in mitochondria and in cytosol (Goswami et al. 2004). But, with our present data and with the partial sequence data of PEPCK gene from this fish (EU797496), which could not discriminate between the cytosolic and the mitochondrial isoforms, it may be difficult to conclude about which isoform was regulated at transcriptional level resulting to an increase of PEPCK enzyme activity in this fish during hypertonicity. However, compartmentalization of gluconeogenic enzymes could be of regulatory significance in this catfish as suggested in other fishes such as plaice (*Pluronectis platessa*; Moon and Johnston 1980). The subcellular localization of G6Pase is not known in this fish. Nonetheless, the increase of activity of this enzyme during hypertonicity could also be the result of transcriptional regulation of G6Pase gene leading to more abundance of this enzyme protein concentration in liver and kidney. Upregulation of PEPCK and FBPase genes at transcriptional level has been demonstrated in perfused rat liver within 3–6 h of hypertonic exposure and correlated with the hydration status of hepatic cells (Newsome et al. 1994). Exposure of walking catfish in hypertonic environment led to a significant increase of blood osmolarity, which was accompanied by a decrease of water content both in liver and in kidney tissues. Already there are reports that although the hepatocytes of walking catfish possess a very efficient volume-regulatory mechanism, the hepatocytes remain partly swollen/shrunken state in a hypo-/hypertonic environment, shown both in intact liver organ (Goswami and Saha 2006; Biswas et al. 2009) and also in isolated cells (Biswas et al. 2010). Therefore, the upregulation of PEPCK, FBPase, and G6Pase genes probably takes place at a transcriptional level, and the increasing activity of key gluconeogenic enzymes, following exposure to hypertonic environment, could be a result of decreasing of water content or cell volume in gluconeogenic tissues of this catfish. Thus, the regulation of gluconeogenesis by changing the hydration status or alterations of cell volume in different gluconeogenic tissues adds a new aspect to the complex regulation of PEPCK gene and possibly other genes of key gluconeogenic enzymes in this catfish.

A major question arises now from this study concerning the mechanisms by which environmental hypertonicity, thereby decreasing the cellular hydration status of different tissues, exerts effect on PEPCK gene transcription, and possibly also other related genes so as to enhance the gluconeogenic activity. Modulation of PEPCK mRNA level due to hypertonicity could be due to upregulation of gene transcription in addition to leading to a more stability of mRNA transcript by accumulating certain metabolites like cyclic AMP (cAMP; Hanson and Reshef 1997), thereby resulting to a more abundance of PEPCK enzyme protein. However, as a crucial enzyme in gluconeogenesis, PEPCK transcription is probably also tightly controlled by various pre-existing regulatory elements that bind to PEPCK promoter due to altered phosphorylation status induced in response to hypertonicity, as noticed in mammalian system during varied physiological stimuli, including dietary carbohydrate content, nutritional status, cAMP, and various hormones like glucocorticoids and thyroid hormone (Beale et al.

2004; Hanson and Reshef 1997). Further, the inhibition of mammalian target of rapamycin (mTOR) signaling, which might have resulted due to decrease of hydration status of tissues under hypertonicity, could be another reason of stimulation of gluconeogenesis in this air-breathing catfish (Schliess et al. 2006; Houde et al. 2010). Additionally, there could be allosteric modulation of other key gluconeogenic enzymes under hypertonic stress so as to ensure a prompt adaptation of the gluconeogenic flux leading to glucose homeostasis and energy status. However, to understand the real mechanism(s) of regulation of gluconeogenesis during osmotic stress in this air-breathing catfish, it requires a detailed investigation.

In conclusion, the present study provides evidences that environmental hypertonicity leads to an increase in the level of PEPCK mRNA, and more abundance of PEPCK, FBPase, and G6Pase proteins, accompanied by an increase of enzymatic activities of PEPCK and other key gluconeogenic enzymes, thereby leading to a stimulation of glucose efflux due to gluconeogenesis, in coordination with the increase of glucose output by stimulating glycogenolysis and inhibiting glycogenesis, as shown in perfused liver organ of walking catfish (Goswami and Saha 1998). Further, the induction of autophagic proteolysis in liver during hypertonicity (Biswas et al. 2009) would favor gluconeogenesis from proteolysis-derived amino acids in this catfish. These biochemical adaptational strategies, possibly as a consequence of changes of hydration status/cell volume of different cell types during environmental hypertonicity, would assist in maintaining glucose homeostasis and proper energy supply mainly to support metabolic demands for ion transport and other altered metabolic processes.

Acknowledgments This study was supported by the DSA programme to the Department of Zoology and the UPE-Biosciences project to the North-Eastern Hill University, Shillong by the University Grants Commission, New Delhi. The financial support as a research fellowship to LMJ from the Council of Scientific and Industrial Research (CSIR), New Delhi is gratefully acknowledged.

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