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Regulation of Lactate Dehydrogenase in Tilapia (*Oreochromis mossambicus*) Gills during Acclimation to Salinity Challenge

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Yung-Che Tseng, Jay-Ron Lee, Joshua Chia-Hsi Chang, Chien-Hsien Kuo, Shyh-Jye Lee, and Pung-Pung Hwang (2008) Regulation of lactate dehydrogenase in tilapia (*Oreochromis mossambicus*) gills during acclimation to salinity challenge. *Zoological Studies* **47**(4): 473-480. Upon salinity challenge, euryhaline teleosts immediately regulate the functions of many ion transporters and enzymes in gill mitochondrion-rich (MR) cells in order to maintain their internal homeostasis. A large amount of energy is necessary to operate these transporters and enzymes; however, the mechanism of energy metabolism in fish gills is still unclear. In the present study, tilapia (*Oreochromis mossambicus*) was used as a model animal to investigate the roles of lactate dehydrogenase (LDH) in the energy metabolism of fish gills during acclimation to seawater (SW) challenge. Results of LDH isozyme gel electrophoresis and Western blotting demonstrated that LDH1 is the major form and LDH5 the minor form expressed in tilapia gill epithelial cells. Immunocytochemical experiments indicated that both LDH1 and LDH5 were expressed in MR cells (using Na^+-K^+-ATPase as a marker). Protein expression levels of LDH1 and citrate synthase (CS) increased immediately in the 1st hour of acclimation to SW, while that of LDH5 was significantly enhanced during the 1st-3rd h. Taken together, an energy metabolism model in gill epithelial cells during SW acclimation is proposed: the tricarboxylic acid cycle in MR cells is stimulated to produce adenosine triphosphate (ATP) which initially triggers salt secretion; conversion of pyruvate to lactate is subsequently stimulated to enhance anaerobic respiration to produce additional energy.
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Key words: Lactate dehydrogenase, Energy metabolism, Tilapia gills, Salinity challenge, Osmoregulation.

Pyruvate and lactate are the end products in the process of glycolysis (Mannen et al. 1997). In mammalian brain glycogen metabolism, lactate is an anaerobic metabolite in the presence of anoxia, a hypoxic metabolite in the presence of dysoxia, and an aerobic metabolite in the presence of an adequate O_2 supply (Gladden 2004). Recently, a new lactate paradigm of metabolism was discovered in the mammalian brain, and lactate has been proposed to be another energy source (Izumi et al. 1997, Brown et al. 2004, Gladden 2004). Lactate has also been shown to support respiration, maintain ATP levels, and reduce glucose utilization in brain slices and in cultured neurons (Izumi et al. 1994, Tabernero et al. 1996, Pellerin et al. 1998).

Lactate dehydrogenase (LDH; EC 1.1.1.27) has 5 isozymes and is responsible for the conversion of pyruvate to lactate. Pyruvate is converted to acetyl-coA and then to citrate in mitochondria by citrate synthase (CS; EC 2.3.3.1), which is the 1st enzyme in the tricarboxylic acid (TCA) cycle (Roche and Reed 1974, Elcock and McCammon 1996). LDH is a tetrameric molecule

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composed of polypeptide subunits that are encoded by 2 structurally distinct genes, *ldha* and *ldhb* (Markert 1984). The association of subunits A and B is random and generates 5 isoenzymes, LDH1, -2, -3, -4, and -5. The composition of LDH1 is 4 B subunits (B4), and that of LDH5 is 4 A subunits (A4). Indeed, LDH1 is the key enzyme in charge of converting lactate to pyruvate, while LDH5 is responsible for the reverse reaction.

Euryhaline teleosts have to maintain their internal ionic and osmotic balance regardless of fluctuating salinities in the aquatic environment, and that is achieved via efficient mechanisms of active salt secretion and absorption in fish gills (Evans et al. 2005, Hwang and Lee 2007). In gill epithelium, mitochondrion-rich (MR) cells are the main ionocytes responsible for active ion transport, which is an energy-consuming process (Hwang and Lee 2007).

Acclimation of teleosts to different environmental salinities causes depletion of energy which is used to stimulate or modulate the operations of various pumps and ion transporters in gill MR cells, which are highly energy-consuming (Boeuf and Payan 2001, Chang et al. 2007, Hwang and Lee 2007, Tseng et al. 2007). Previous studies documented that changes in oxygen consumption reflect energy metabolic processes in response to variations in salinity (Morgan et al. 1991, Sangiao-Alvarellos et al. 2003). Other studies also examined the effects of fluctuating environments on changes in glycolysis metabolites and enzymes activities, including concentrations of plasma glycogen, glucose, lactate, lactate dehydrogenase, glycogen phosphorylase, pyruvate dehydrogenase, citrate synthase, and glucose 6-phosphate dehydrogenase (Perry and Walsh 1989, Nakano et al. 1998, Marshall et al. 1999, Sangiao-Alvarellos et al. 2003, Chang et al. 2007, Tseng et al. 2007). During salinity challenge, evident changes in lactate contents and LDH activities in gills of some euryhaline teleosts have been reported (Vijayan et al. 1996, Polakof et al. 2006), implying the involvement of monocarboxylate metabolites in gill energy consumption during osmoregulation. Regulating LDH for converting between lactate and pyruvate in fish gills is apparently important for energy metabolism during acclimation to salinity changes; however, very little is known about this issue.

The purpose of the present study was to examine the role of LDH in energy metabolism for osmoregulation in tilapia (*Oreochromis mossambicus*), a euryhaline teleost, by answering the following questions: (1) Do specific isozymes of LDH exist in tilapia gills?; (2) Are LDHs specifically expressed in the main gill ionocytes, MR cells?; and (3) How does environmental salinity affect the expression of LDHs in tilapia gills? The tilapia was selected as the experimental species because of its excellent adaptability to fresh water and seawater.

MATERIALS AND METHODS

Experimental animals

Tilapia (*Oreochromis mossambicus*), 40-60 g in body weight and 10-14 cm in total length, from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica were kept in a tank with a fresh water (FW) circulating system at 27-28°C under a 14: 10 h light: dark photoperiod.

Acclimation experiment

Seawater (SW) was prepared by adding an appropriate amount of artificial sea salt (Taikong, Taipei, Taiwan) to FW. Tilapia were transferred from FW to SW at 25 parts per thousand (ppt), and were sampled at 1, 3, 6, 12, 24, 48, and 168 h for short-term acclimation. After transfer, all samples were starved during the short-term acclimation experiment from FW to 25 ppt SW and then sacrificed at 11:00-12:00.

Isolation of epithelial cells from tilapia gills

Gill tissues contain blood cells, which have substantial amounts of LDHs (Nano et al. 1991, Rezzani et al. 1999). Epithelial cells have to be isolated from gill tissues in order to exclude the effects of blood cells on the experimental data. Tilapia were cautiously killed on ice, and the gills were immediately removed and transferred to phosphate-buffered saline (PBS; 0.9% NaCl in 0.1 M phosphate buffer) to remove most of the blood cells. The epithelial tissues were scraped from the gill filaments in dissociation buffer (0.5 M EDTA and 500 μl Percoll in PBS), and then gently agitated with a stirring bar on ice for 30 min. After stirring violently, cells were isolated from tissues by gently passing the tissue suspension through a nylon mesh (with a 100 μm mesh size) to remove larger tissue fragments. Remnants of the cell suspension were poured in a Percoll solution (2: 1: 1 of cell suspension: Percoll: PBS), and centrifuged

at 2000 x*g* and 4°C for 10 min. Afterwards, the cell suspension was isolated and washed with PBS several times. Isolated cells were stored at -70°C for the subsequent experiments.

Isozyme gel electrophoresis

Isozyme gel electrophoresis for LDHs followed Jean et al. (1995) with some modifications. Tilapia gills, heart, muscles, and isolated gill epithelial cells were homogenized in 1-2 volumes of extraction buffer (0.01 M Tris-HCl (pH 7.0), 1 mM EDTA, and 0.05 mM NADP) using a sonicator in an ice bath. Homogenates were centrifuged at 14,000 rpm and 4°C for 40 min, and then the supernatants were stored at -70°C until electrophoresis. A piece of filter paper was dampened with roughly 15 μl of protein extract and placed into the sample slot at the cathode end of a 12% (w/v) starch gel. Horizontal electrophoresis was performed using a TC 8.0 buffer system at 4°C for 6 h at 120 V. After electrophoresis, the starch gel was stained with staining buffer (10 ml 0.2 M Tris-HCl (pH 8.0), 25 mg NAD^+, 15 mg NBT, 1 mg PMS, 5 ml 1 M sodium-DL-lactate pH 7.0 substrate, and ddH_2O to bring the volume up to 50 ml). Stained gels were then fixed with acetic acid/methanol, photographed, and dried for storage.

Protein extraction

Isolated epithelial cells from tilapia gills were homogenized with homogenization solution (100 mM imidazole-HCl, 5 mM Na EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate; pH 7.6) at 600 rpm on ice, and were then centrifuged at 14,000 rpm and 4°C for 30 min. The supernatant was stored at -70°C. The extracted protein concentration was measured with a U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

Western blotting

Electrophoresis sample buffer (250 mM Tris-base, 2 mM Na_2EDTA, 2% sodium dodecylsulfate (SDS), and 5% dithiothreitol) was added to the protein samples (30-50 μg of total protein), and then incubated at 95°C for 10 min. Denatured samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE and transfer to polyvinylidene difluoride membranes, the membranes were incubated in 5% nonfat milk for blocking, and then washed with PBST (0.01 M phosphate, 0.9% NaCl (pH 7.5), and 0.05% Tween 20). The blotted membranes were incubated overnight with a mouse anti-human LDH1 monoclonal antibody (mAb) (Acris Antibodies, Hiddenhausen, Germany, diluted 1: 1000), a sheep anti-human LDH5 polyclonal antibody (pAb) (USBiological, Swampscott, MA, USA, diluted 1: 1000), or a mouse anti-porcine CS mAb (USBiological, diluted 1:1000) at 4°C. After washing with PBST, the blotted membranes were reacted with alkaline-phosphatase (AP)-conjugated goat anti-mouse immunoglobulin G (IgG) (Jackson Immunoresearch, Cambridgeshire, UK, diluted 1: 1000), AP-conjugated rabbit anti-sheep immunoglobulin G (IgG) (Jackson Immunoresearch, diluted 1: 1000), or AP-conjugated goat anti-mouse IgG (Jackson Immunoresearch, diluted 1: 1000), respectively. Immunoreactive proteins were visualized with a BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate kit for AP (Zymed Laboratories, San Francisco, CA, USA). Immunoblots were scanned and exported to JPEG files, and differences between the band intensities of FW and SW were compared using commercial software (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA).

Fluorescence immunocytochemistry

Fresh tilapia gills were fixed with 4% paraformaldehyde at 4°C for 3 h, then gradually immersed in PBS containing different concentrations of sucrose of 5%, 10%, and 20% at 4°C. Samples were soaked in a mixed PBS solution (OCT compound: 20% sucrose 1: 2) overnight, and were then embedded with OCT compound-embedding medium (Sakura, Tokyo, Japan) at -20°C. Cryosections at 10 μm were made with a cryostat (CM 1900, Leica, Heidelberg, Germany), and were stuck to poly-L-lysine-coated slides (Erie, Hooksett, NH, USA). Prepared slides were then rinsed in PBS, and blocked with 3% bovine serum albumin (BSA) for 30 min. Afterwards, the slides were incubated with a mouse anti-human LDH1 mAb (Acris Antibodies, Hiddenhausen, Germany, diluted 1: 300), or a sheep anti-human LDH5 pAb (USBiological, diluted 1: 300) overnight at 4°C, and then washed with PBS before being respectively incubated with anti-mouse IgG conjugated with FITC or anti-sheep IgG conjugated with FITC antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA; 1: 200 dilution) for 2 h at room temperature. For double staining, these slides were incubated

with an Na^+-K^+-ATPase α5 mAb (a marker for MR cells) (Hybridoma Bank, University of Iowa, Ames, IA, USA; diluted 1: 200), and then incubated with an anti-mouse IgG antibody conjugated with Texas red (Jackson Immunoresearch, Hiddenhausen, PA, USA; 1: 200 dilution). Images were acquired with a Zeiss Axioplan 2 fluorescence microscope (Oberkochen, Germany).

Statistical analysis

Values are presented as the mean ± standard error (S.E.) (n = 4-6). All time-course data were statistically analyzed by two-way analysis of variance (ANOVA), and pairwise multiple comparisons were made by Tukey's test ($p < 0.05$).

RESULTS

Isozyme gel electrophoresis and Western blotting of LDHs

As shown in the native gel for isozyme electrophoresis (Fig. 1), expression patterns of LDH isozymes in tilapia heart and muscle were similar to those in mammals. Tilapia heart and muscles expressed the most abundant amounts of LDH1 (the heart form of LDH) and LDH5 (the muscle form of LDH), respectively. Intact tilapia gills without isolation expressed LDH1 and LDH5, as well as a little LDH4; LDH1 was the major form. In isolated gill epithelial cells, a lot of LDH1 and very little LDH5 (detectable by Western blot) were detected, but no other isozymes were found, indicating that only LDH1 and LDH5 are expressed by gill epithelial cells. To exclude the effects of other LDH isozymes from the non-epithelial cells, isolated gill epithelial cells were used in all subsequent experiments.

Western blots with LDH1, LDH5, and CS antibodies (Fig. 2) supported the data of the isozyme gel electrophoresis as described above. Immunoreactive bands of tilapia gill epithelial cells were similar in size, at 37, 37, and 49 kDa, to LDH1, LDH5, and CS in rat tissues, respectively, providing molecular evidence for the expressions of these 3 proteins in tilapia gill epithelial cells.

Localization of LDH1, LDH5, and Na^+-K^+-ATPase in gills

Figures 3A-D show double-staining for LDH1 and Na^+-K^+-ATPase α5 proteins in tilapia gill epithelia. LDH1 protein was stained with a green signal (FITC, Fig. 3B), whereas the Na^+-K^+-ATPase α5 was shown as a red one (Texas red, Fig. 3C). Figure 3A is the differential interference contrast (DIC) image of a tilapia gill. The merged image of figure 3D shows that LDH1 was obviously co-localized with MR cells in the gill epithelia. The same expression pattern as LDH1 (Figs. 3E-H) showed that LDH5 was also localized in tilapia gill epithelia and co-localized with MR cells.

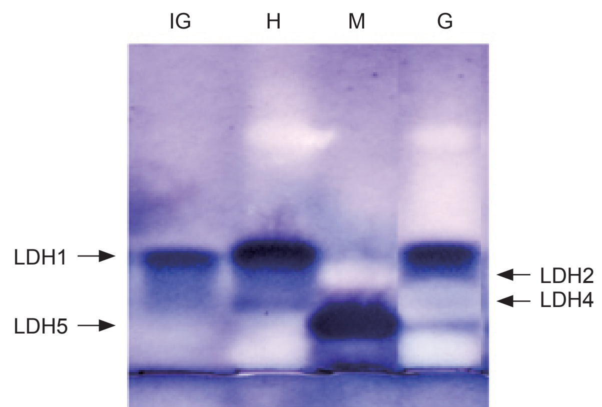

Fig. 1. Lactate dehydrogenase (LDH) isozymes in tilapia tissues. IG, isolated gill epithelial cells; H, heart; M, muscle; G, whole gill without isolation.

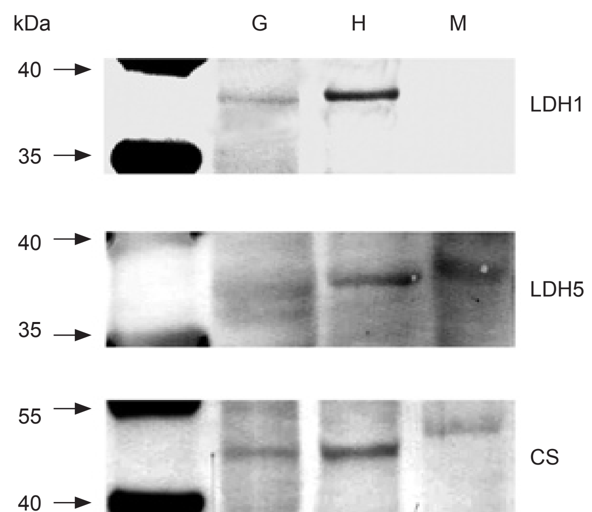

Fig. 2. Western blots of lactate dehydrogenase (LDH)1, LDH5, and citrate synthase (CS) proteins in different tissues. IG, isolated epithelial cells from tilapia gills; H, rat heart; M, rat muscle.

Changes in LDH1, LDH5, and CS protein amounts in isolated gill epithelial cells after transfer to SW

In Figure 4A, the protein amount of LDH1 obviously increased in the 1st hour after transfer to 25 ppt SW, and then decreased in the 3rd hour. Thereafter, the amount of LDH1 protein almost returned back to the level in the control group and remained constant. The amount of LDH5 protein significantly increased during 1 to 3 h after transfer to 25 ppt SW as shown in figure 4B. Then, the protein level of LDH5 almost returned back to the same level as the control group.

CS is an enzyme that catalyzes the 1st step of the Krebs cycle in the mitochondrial matrix of eukaryotic cells (Goldenthal et al. 1998). Therefore, information about the CS level and its regulation implies that the capability of mitochondrial oxidative metabolism was intact. Similar to LDH1, the amount of CS protein significantly increased 1 h after transfer to 25 ppt SW, and thereafter almost remained at the same level as the control group (Fig. 4C).

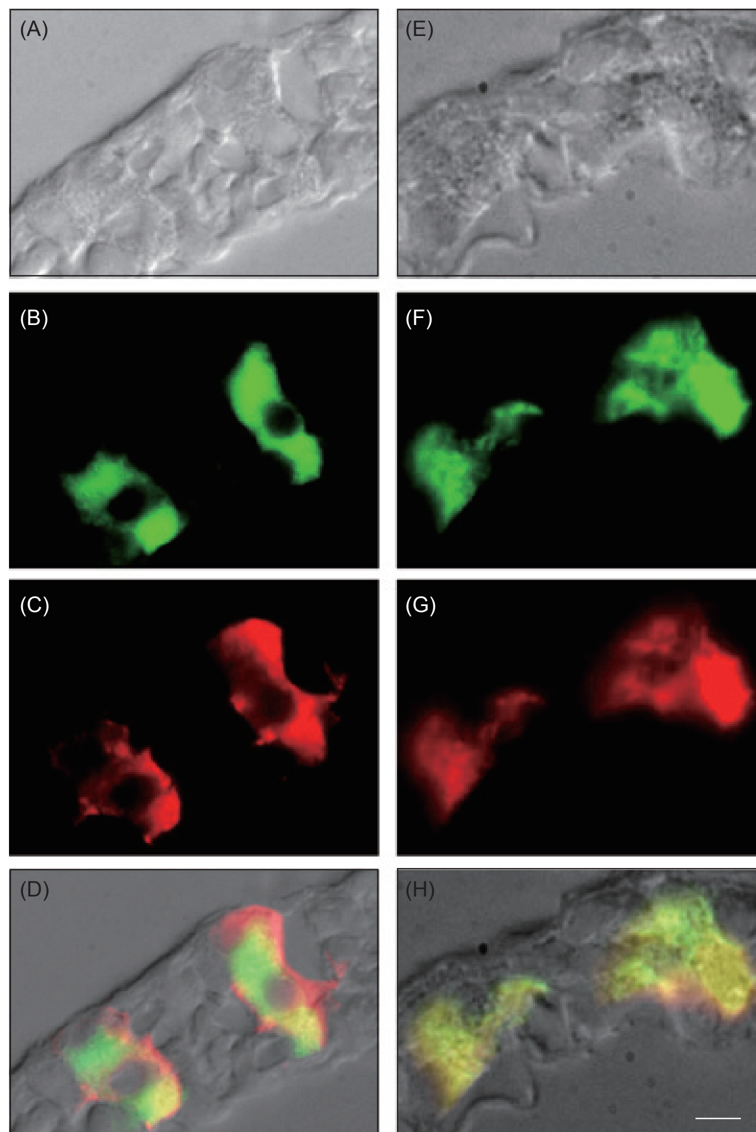

Fig. 3. Images of gill frozen sections double-labeled with lactate dehydrogenase (LDH)1, LDH5, and Na^+-K^+-ATPase antibodies. A and E, differential interference contrast (DIC) image of a gill cryosection. B and F, FITC signals of LDH1 and LDH5, respectively. C and G, Texas red signals of Na^+-K^+-ATPase. D, merged images of A, B, and C. H, merged images of E, F, and G. Scale bar: 5 μm.

DISCUSSION

The major findings of the present study were that (1) LDH1 is the major isozyme expressed in tilapia gill epithelial cells, while LDH5 is a minor form; (2) both LDH1 and LDH5 are expressed in MR cells, the main ionocytes in gill epithelium; and (3) immediately after acute exposure to 25 ppt SW, LDH1 was stimulated to convert gill lactate to pyruvate, which subsequently enters the TCA cycle to produce ATP for MR cells; thereafter, stimulation of LDH5 switched on anaerobic respiration, which converts pyruvate to lactate and produces additional ATP for ion transporter operation.

During acclimation to fluctuating salinity, the ionic and osmotic regulation mechanisms of teleosts are modulated, and these processes also induce changes in energy metabolism. Previous studies indicated that acclimation to salinity induced changes in activities of some metabolites and metabolic enzymes, including LDH (Sangiao-Alvarellos et al. 2003, Polakof et al. 2006). There are 5 LDH isozymes, and they show different expression patterns in various types of cells (Yuan et al. 1998, Koslowski et al. 2002). LDH1 is the key enzyme responsible for converting lactate to pyruvate, whereas LDH5 is responsible for the reverse reaction (Pellerin 2003). Previous studies examined LDH activities in whole gill tissues, but were unable to ascribe those activities to certain isozymes. Therefore, no information was provided by those studies as to whether lactate or pyruvate is the energy metabolite in fish gills during acclimation to salinity. Gills are composed of ionocytes, pavement cells, blood cells, muscle cells, novel glycogen-rich (GR) cells, etc. (Hwang and Lee 2007, Tseng et al. 2007). Without isolation of gill epithelial cells, one cannot study the role of LDH isozymes that are specifically expressed in gill ionocytes or other specific cell types.

Based on isozyme electrophoresis of whole gill tissues, many studies indicated that LDH1 is the major form, with LDH3 and/or LDH5 also being expressed in gills of several teleosts (Fonseca de Almeida-Val and Val 1993, Jean et al. 1995, Basaglia 2000 2002). Those observations cannot be applied to gill epithelial cells because the effects resulting from gill blood cells, which contain substantial amounts of LDHs, were not excluded in those previous studies. In the present study, isozyme electrophoresis of isolated gill epithelial cells demonstrated the expressions of LDH1, the major form, and LDH5, the minor form, in tilapia gill epithelial cells, and this was further supported by the Western blots with LDH1 and LDH5 antibodies. More importantly, the present localization experiments for the first time indicate that both LDH1 and LDH5 are expressed in MR cells, the main ionocytes in fish gill epithelium, providing convincing molecular and cellular evidence for the role of specific LDH isozymes in energy metabolism for fish osmoregulation.

Upon acute challenge with SW, emergency energy is required to carry out physiological

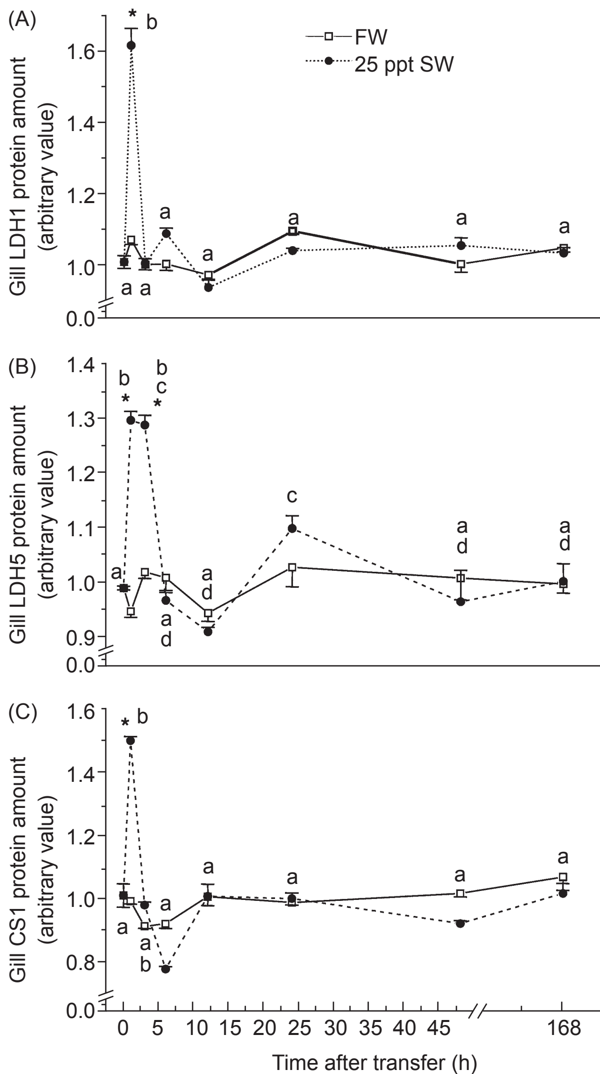

Fig. 4. Time-course changes of gill lactate dehydrogenase (LDH)1 (A), LDH5 (B), and citrate synthase (CS) (C) relative protein amounts in tilapia transferred from freshwater (FW) to 25 ppt seawater (SW). Protein amounts were measured by Western blotting. Data are presented as the mean ± S.E. (n = 4-6). * Indicates a significant difference from the respective control in FW ($p < 0.05$). Different letters indicate a significant difference ($p < 0.05$) among sampling times in fish transferred to SW.

processes to maintain the osmotic and ionic balance in fish bodily fluids (Hwang and Sun 1989, Marshall et al. 1999, Sangiao-Alvarellos et al. 2003, Lin et al. 2004, Chang et al. 2007). Sangiao-Alvarellos et al. (2003) demonstrated that glucose and lactate contents in the plasma, kidneys, and liver increased in gilthead sea bream (*Sparus aurata*) after acclimation to different salinity environments. In tilapia, both CS and Na^+-K^+ ATPase activities were increased in isolated gill epithelial cells after transfer to SW for 2 wk (Perry and Walsh 1989), indicating stimulation of aerobic respiration in mitochondria during acclimation to SW. Immediately after an acute challenge with SW, gill Na^+-K^+ ATPase activity showed a rapid and drastic increase from the 1st hour in order to recover the impaired internal ion levels and osmolalities in tilapia (Hwang et al. 1989), implying that a prompt energy supply for stimulating ion-secretion mechanisms is critically needed. Our previous studies revealed dramatic rises in gill Na^+-K^+-ATPase activity, glycogen phosphorylase (GP) messenger (m)RNA activities, and protein amounts immediately after acute exposure to 25 ppt SW (Chang et al. 2007, Tseng et al. 2007). Enhancement of gill GP activity during 1-3 h after acclimation to 25 ppt SW appears to be associated with the present results of the simultaneous stimulation of LDH1, LDH5, and CS protein expressions. These results provide cellular and molecular evidence for the stimulation of glycolysis and the TCA cycle being associated with the operation of Na^+-K^+ ATPase in gill MR cells during acute acclimation to SW. However, we cannot exclude the possibility that energy metabolites (glucose, lactate, and/or pyruvate) may also be provided by other organs including the liver, because plasma lactate and glucose increase immediately after SW transfer (Sangiao-Alvarellos et al. 2003).

Lactate has been demonstrated to be an important fuel substrate in the mammalian central nervous system (CNS) (Pellerin 2003), that develops from the neural ectoderm. Lactate and pyruvate are transported between astrocytes and neurons via specific types of monocarboxylate transporters (MCTs). The transportation type occurring in the CNS is called a “lactate shuttle” (Chih et al. 2001, Pellerin 2003, Gladden, 2004, Pellerin et al. 2005). Based on the present results, we propose a model for energy metabolism in fish gills, which is derived from the non-neural ectoderm, during acclimation to SW. The TCA cycle for aerobic respiration in gill epithelial cells is stimulated to produce an enormous amount of energy to maintain homeostasis and ion regulation in the 1st hour after transfer to SW. Subsequently in the 3rd hour, LDH5 is continually activated to enhance anaerobic respiration to produce emergent energy. Moreover, LDH5 in the epithelia was stimulated in the 3rd hour at the same time while LDH1 was not, suggesting that epithelial cells may store lactate as an energy fuel for subsequent environmental challenges.

Much remains to be further studied to support the proposed model of energy metabolism in fish gills during acclimation to SW.

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