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REGULATION OF RENAL Na, K-ATPASE ACTIVITY IN THE CLIMBING PERCH, *Anabas testudineus* (BLOCH) EXPOSED TO WATER-BORNE AMMONIA

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ABSTRACT

The osmoregulation inteleosts is regulated by the gills, kidney, and gut. Na^+/K^+-ATPase (NKA) is an index of osmoregulation expressed in animal cells to create an electrochemical gradient providing the driving force for the transport of ions in fish gills and kidneys. The present study evaluated the Na^+, K^+-ATPase activity (NKA) and expression in the kidney of the climbing perch, *Anabas testudineus,* exposed to different concentrations of water-borne ammonia (10 and 100µM ammonium sulphate) for 48 hrs and duringrecovery in clean freshwater for another 96 hrs. There was a significant change in Na^+, K^+-ATPase activity and expression in control and ammonia exposed freshwater adapted fish. The western blot demonstrated the immunoreactive bands at ~100 kDa in the kidney of freshwater adapted perch and ammonia exposed groups. NKA protein expression as well as specific activity in kidneys significantly increased in the FW anabas. The primary function of the kidney in fresh water, is to excrete excess water, while reabsorbing most of the filtered solutes. The low levels of ions in freshwater with higher Na^+, K^+-ATPase activity in kidneys of the euryhalineteleosts provides an enhancement of reabsorbing tubular NaCl destined for large volumes of urinary output. To the best of our knowledge, however, the positive correlation between distribution of NKA activity and protein abundance in kidneys of Anabas in response to water borne ammonia stress has been demonstrated here for the first time. The present study concludes that the climbing perch can tolerate fairly high levels of water-borne ammonia, resulting in compensatory modifications of both hydro mineral and metabolic processes.

KEYWORDS: Na^+K^+ATPase, Kidney, Osmoregulation, Anabas, Western Blot

The aquatic environment is particularly sensitive to the toxic effects of contaminants since a considerable amount of the chemicals used in industry, urbanization and in agriculture enters marine and other aquatic ecosystem (Osman,et al., 2009). Ammonia is the major nitrogenous excretory product that is lost from the body across the gills to the aqueous environment, in teleost fish. Fishes tolerate ammonia and are able to maintain their plasma ammonia levels within a range (Mommsen and Walsh, 1992). Freshwater fishes excrete ammonia whereas ammonia is converted into urea in sea water fish (Frick and Wright, 2002). Maintaining an internal environment with stable conditions is essential for animals to survive in a variety of habitats. The osmoregulation of teleosts is regulated by the gills, kidney and gut. Na^+K^+ATPase (NKA) is a crucial primary enzyme expressed in animal cells to create an electrochemical gradient providing the driving force for ion transport in osmoregulatory organs, including fish gills and kidneys (Yang et al., 2016). Therefore, the present study focused on the kidney NKA expression of the freshwater fish, when exposed to water borne ammonia environment.

MATERIALS AND METHODS

Experimental Animals and Environments

The experimental animal used in the present study is the Climbing perch, *Anabas testudineus*, Bloch (1792). Perch of both sexes weighing 45-50g were maintained in large tanks and fed once a day with 1.5% body weight commercial fish feed. The specimens were given prophylactic treatment by bathing twice in 0.05%potassium permanganate solution for two minute to avoid any dermal infections. Before commencement of the experiment, the fish were transferred to glass aquaria(20L) and kept for two weeks at water temperature 28±1^{o}Cand photoperiod 12hL:12h D cycle. Twenty- four fish were divided into 4 groups of six each and placed in separate glass aquaria. The fish of group I were freshwater control; group II and III were treated with10 and 100µM ammonium sulphate for 48 hr respectively. The group IV fish were first kept at 100µM ammonium sulphate for 48h and later kept for recovery in clean freshwater for another 96 h. Feeding was stopped 24h prior to sampling.

Sampling and Analyses

After the treatment, the experimental fish were anaesthetized in 2-phenoxyethanol (SRL, Mumbai) and blood was collected from the caudal vein. The fish were then sacrificed by spinal transsection and kidney tissues were excised and washed in ice-cold 0.25 M SEI buffer (*p*H 7.1) and kept at -20°C.

Electrophoretic Analyses

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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SDS-PAGE analysis was performed using discontinuous buffer system (Laemmli, 1970) on mini gels 910 cm × 8 cm). The reagents included acrylamide solution (30% stock solution contained acylamide and methylenebis acrylamide in the ratio 30:08), stacking gel buffer (0.5MTris*p*H 6.8),separating gel buffer (0.5MTris*p*H 8.8), Tris - Glycine electrode buffer (0.1%SDS, 0.05 MTris and 0.38 Mglycine, *p*H 8.3), sample treatment buffer (15%SDS, 25% glycerol, 10% β-mercaptoethanol, 0.31 MTris (*p*H 6.7), 0.1%bromophenol blue, TEMED, 10% ammonium persulphate (APS) and 10%SDS.Tissues of kidney were weighed and homogenized in buffer. The homogenized samples were centrifuged at 5000rpm. The supernatant was collected and used for SDS-PAGE and western blotting. Total protein was analyzed using 7 well gel. Each well was loaded with 20µl samples. Electrophoresis was performed under denaturing and discontinuous conditions on 10 % SDS polyacrylamide gel using a mini vertical unit (Bio-Rad, India) by the method of Laemmli (1970). Known molecular weight proteins ranging from 250- 29 KDa were used as markers. Routine electrophoresis was carried out on 3 % stacking gels (0.266 mlacrylamide, 0.5 ml stacking gel buffer, 50 µl 10% APS, 10 µl TEMED, 0.05 ml SDS and distilled water to make 2 ml) and 10% separating gels (2.35 mlacrylamide solution, 1.75 ml separating gel buffer, 170 µl 10% APS, 10 µl TEMED, 0.07 ml SDS and distilled water to make 7 ml) at a constant current of 15 mA for stacking and 100V for running gel buffer for 3 h. The electrophoretic run was terminated when the tracking dye migrated out of the gel. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The appropriate molecular weights of resolved proteins were determined by comparison with a known standard.

Western Blot Analysis for Na^+, K^+-ATPase

Na^+, K^+-ATPase antibody: The chicken Na^+, K^+-ATPase antibody (α5) developed by D. M. Fambrough (Johns Hopkins University, MD, USA) was obtained from the Developmental Hybridoma Bank maintained by the University of Iowa Department of Biology, The Johns Hopkins University, 34th & Charles Streets, Baltimore, MD 21218 under contract NO1-HD-7-3263 from the National Institute of Child Health and Human Development (NICHD). The primary antibody was purchased as cell culture supernatant ($0.9 mgml^{-1}$).

Secondary Antibody: The secondary antibody used was Polyclonal anti mouse IgGFab-specific - peroxidase produced in goat (A9917, Sigma, USA).Kidneys of *A. testudineus* were homogenized and suspended in a buffer solution (pH 7.9) containing 10 mM HEPES, 1 mM EDTA, 0.6% NonidetP-40, 150 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM orthovanadate) (Sigma Chemical Co.,St. Louis, MO, USA). Protein samples (20 µg/lane) were mixed with sample buffer, boiled for 5 min, and loaded on 12% SD Spolyacrylamide gels for electrophoresis (Laemmli,1970). Proteins were blotted on to a sheet of nitrocellulose transfer membrane (Bio-Rad Laboratories, Hercules, CA) and were blocked with 5% dry milk in PBS, 0.1% Tween 20.Membranes were then washed and incubated with Monoclonal antibody, chicken Na^+, K^+-ATPase (1: 1000),(DSHB,USA) with 3% dry milk, washed 3 x10 min with PBS, 0.1% v/v Tween 20, and then exposed to the goat antimouse IgG Fab-specific -peroxidase (Sigma,USA) Polyclonal secondary antibody (1: 3000) for 1 h at room temperature. The membrane was then developed for 5 min with peroxidase substrate (diaminobenzidine and ureahydrogenperoxide tablets, Sigma Chemicals Co., St. Louis, MO, USA) untilbands appeared. A parallel gel was stained for total protein with coomassiebrilliant blue R-250. The appropriate molecular weights of resolved proteins were determined by comparison with a kaleidoscope prestained standard(Bio-Rad Laboratories, Hercules, CA). The blots were scanned and the respective bands were quantified using a Bio-Rad GS 800 densitometer and was quantified by Bio-Rad Quantity One version 4.4 software.

Statistical Analysis

One-way analysis of variance,(ANOVA) followed by Student-Newman-Keul's test was employed to test the significant difference between the treatment groups using Instat-3 Software (GraphPad Software Inc., San Digeo, California). Significant difference between groups was accepted if $P< 0.05$ and the values are in mean ± (Standard error (SE)(n = 6).

RESULTS

Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Kidney Na^+, K^+-ATPase SDS-PAGE analysis showed that there was a significant change in renal Na^+, K^+-ATPase activity and expression in control and ammonia exposed freshwater adapted fish. It was noticed that Na^+, K^+-ATPase specific protein was high in the kidney tissue. The Western blot demonstrated the differences in expression of NKA in the kidney of freshwater adapted fish and ammonia exposed fish at

various concentrations. This is the first experiment focusing on kidney NKA expression in an aquatic air-breathing fish. NKA protein expression as well as specific activity in kidneys significantly increased in the FW anabas. Immunoblots of kidney tissue of the control group, 10 and 100mMammonia and recovery fish, all revealed a single immunoreactive band of NKA at ~100 kDa molecular mass. Electrophoretic patterns and Western blot analysis of SDS-extracted microsomal fractions from kidney of anabas are shown in Figure 1.

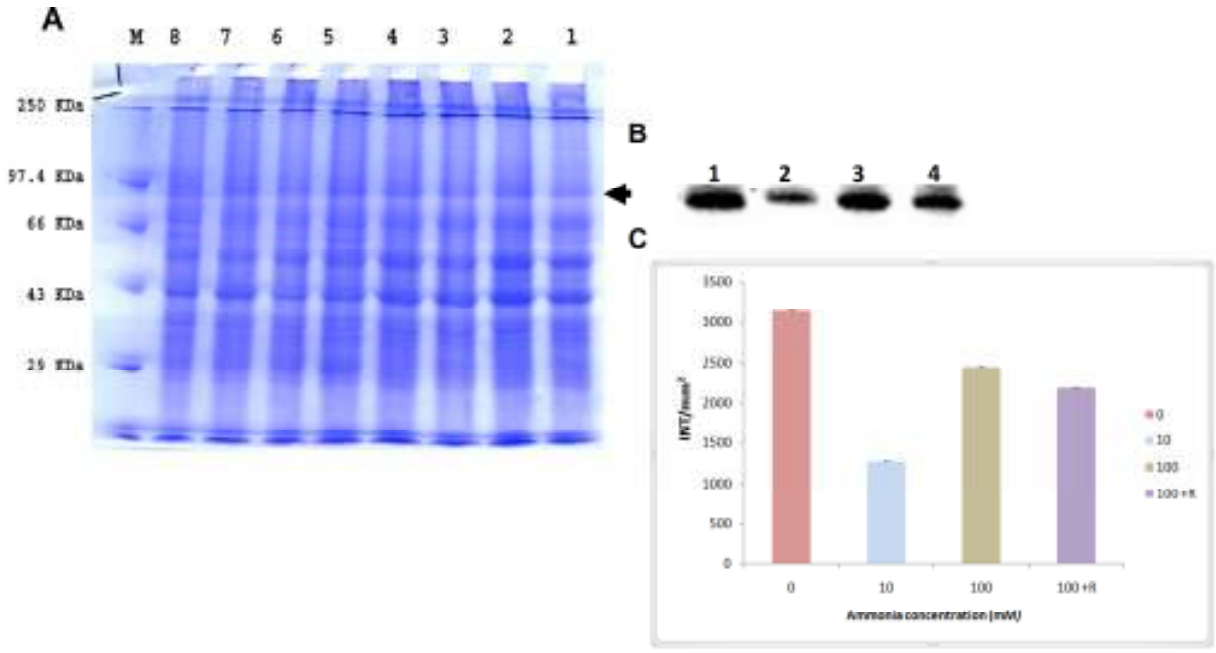

Figure 1: SDS-PAGE analysis in the kidney tissue of *A. testudineus* adapted to FW(A) The lane numbers (1–8) refer to 0, 10, 100 and 100 + R respectively in duplicates, molecular mass standards (from top to bottom) were 250, 97.4, 66, 43, and 29 kDa. Arrowheads indicate bands of interest. (B) Representative Western Blot indicates immunoreactive bands ~100 kDa in kidney of freshwater adapted perch. Lanes 1-4 refer to 0, 10, 100 & 100+R respectively (C) Densitogram showing abundant Na^+, K^+-ATPase expression in the FW control compared to ammonia treated groups.

DISCUSSION

In thc prcscnt study, immunoblotting tcchniquc revealed that kidney NKA protein of FW adapted fish proved to be higher than the ammonia exposed groups. Na^+, K^+-ATPase, (NKA) a ubiquitous membrane-bound enzyme is important not only for sustaining intracellular homeostasis, but also for providing a driving force for many transporting systems including fish gills and kidneys. NKA is a P- type ATPase consisting of an $(\alpha\beta)_2$ protein complex and the catalytic α-subunit with a molecular mass of about 100 kDa, while the smaller glycosylated β-subunit exists with amolecular mass of approximately 55 kDa.

The Na^+, K^+-ATPase (E.C.3.6.9) or Na^+ pump is a ubiquitous energy transducing ion pump which actively transport Na^+ out and K^+ into the animal cell across the plasma membrane. This membrane protein complex maintains a high electrochemical gradient of Na^+ and K^+ across the plasma membrane and comprises two sub-units, α and β (Glynn, 1993; Lingrel and Kuntzweiler,1994). The catalytic α-subunits encompass the sites of nucleotide and cationbinding and undergo conformational transitions associated with the coupling of ATP hydrolysis to the translocation of Na^+ and K^+.Changes in gill NKA expression in response to salinity changes or environmental disturbances were found in many teleosts (Marshall et al., 2002; Perry,et al., 2003; Horng,et al., 2007; Hwang and Lee, 2007; Hwang, 2009).

NKA protein expression as well as specific activity in kidneys significantly increased in the FW anabas. The primary function of the kidney in fresh

water, is to excrete excess water, while reabsorbing most of the filtered solutes. Due to the low levels of ions in FW, higher NKA activity in kidneys of the euryhalineteleosts provides one mechanism by which such an enhancement of reabsorbing tubular NaCl destined for large volumes of urinary output might be accomplished (Perry,et al., 2003). NKA α1 is predicted to be the crucial isoform for osmoregulation of teleosts. To the best of our knowledge, however, the positive correlation between distribution of NKA activity and protein abundance in kidneys of anabas in response to water borne ammonia stress has been demonstrated here for the first time.

CONCLUSION

The present study concludes that the climbing perch can tolerate fairly high levels of water-borne ammonia, resulting in compensatory modifications of both hydro mineral and metabolic processes.

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