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Cloning and expression of Na^+/K^+-ATPase and osmotic stress transcription factor 1 mRNA in black porgy, *Acanthopagrus schlegeli* during osmotic stress

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Abstract

We cloned complementary DNA (cDNA) encoding the Na^+/K^+-ATPase (NKA) and the osmotic stress transcription factor 1 (OSTF1) from the kidney and gill, respectively, of the black porgy, *Acanthopagrus schlegeli*. Black porgy NKA full-length cDNA consists of 3078 base pairs (bp) and encodes a protein of 1025 amino acids; OSTF1 partial cDNA consists of 201 bp. To investigate the osmoregulatory ability of black porgy when black porgy were transferred to freshwater (FW), we examined the expression of NKA and OSTF1 mRNA in osmoregulatory organs, i.e., gill, kidney and intestine, using quantitative polymerase chain reaction (QPCR). To determine the hypoosmotic stressor specificity of the induction of NKA and OSTF1, black porgy were exposed to 30°C water temperature for 24 h. In the gill, NKA mRNA was 4.2 times higher in FW, its expression in the kidney was 5.7 times higher in 10‰ seawater (10‰ SW) than in SW. In contrast, OSTF1 mRNA in the gill was 3.7 times higher in FW than in SW. The expression of heat shock protein 90 (HSP90) mRNA occurred not only during transfer to FW, but also in high-temperature water in all tested tissues, although the mRNA levels were not significantly different. Plasma osmolality level was decreased and cortisol level was increased when the fish were transferred from SW to FW. These results suggest that NKA and OSTF1 genes play important roles in hormonal regulation in osmoregulatory organs and that these genes are specific to hypoosmotic stress, improving the hyperosmoregulatory ability of black porgy in hypoosmotic environments.

Keywords: Black porgy; Hypoosmotic stress; Osmoregulation; Na^+/K^+-ATPase; Osmotic stress transcription factor 1

1. Introduction

In euryhaline teleosts, osmoregulation in response to changes in salinity takes place by the movement of water and ions such as Na^+ and Cl^- in the osmoregulatory tissues of the gills, kidneys and intestine (Evans, 1993). In seawater (SW), because the osmolality of the body is higher than that of the surrounding environment, a large volume of SW is absorbed by the intestine to supplement water loss due to osmolality. In addition, Na^+ and Cl^- ions are discharged via the gills and kidneys. In freshwater (FW), however, ion discharge through the gills is suppressed, and a large quantity of urine is generated in the kidneys for discharge (Moyle and Cech, 2000). Proteins and hormones such as cortisol, prolactin (PRL), growth hormone (GH), Na^+/K^+-ATPase (NKA), arginine vasotocin (AVT) and aquaporins (AQPs) are involved in osmoregulation (Pickford and Phillips, 1959; Geering, 1990; Madsen and Bern, 1992; Warne and Balment, 1995).

Of these proteins and hormones, NKA, as a sodium pump, activates K^+ inflow and Na^+ discharge (Geering, 1990). This enzyme is important not only to sustain intracellular homeostasis, but also to provide a driving force for many transport systems in a variety of osmoregulatory epithelia, including the gills of fish (McCormick, 1995). NKA is composed of a catalytic α-subunit with a molecular mass of approximately 100 kDa and a smaller glycosylated β-subunit with a molecular mass of approximately 55 kDa (Mercer, 1993). The NKA α-subunit has all of the catalytic domains necessary for enzyme function (Mercer, 1993) and has been cloned in a large number of teleosts (Schonrock et al., 1991; Cutler et al., 1995; Feng et al., 2002; Semple et al., 2002; Richards et al., 2003).

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bpNKA    1:MGLIQGKKEYKLAATSDGKEKKKAKKGKKGEKDMDELKKEVDLDDHKLTLDELHRKYGTD   60
ssNKA    1:MGLIQGKEEYKLAATSDGKE-KK-KKGKKGEKDMDELKKEVDLDDHKLTLDELHRKYGTD   58
kfNKA    1:MGLGKGKDDYKPAATSE-PGDKKSKK-EKKKMDMDELKKEVDLDDHKLTLDELHRKYGTD   58
mtNKA    1:MGLGKGKDEYKLAATSE-DGGKKDKK-AKAKKDMDDLKKEVDLDDHKLTLDELHRKYGTD   58
rtNKA    1:MGRGEGREQYELAATSEQGGKKKNAKAMKKERDMDELKKEVDLDDHKLTLDELNRKYGTD   60

bpNKA   61:LSRGLSNSRAKEILARDGPNALTPPPTTPEWVKFCRQLFGGFSMLLWIGAILCFLAYGIQ  120
ssNKA   59:LSRGLSNSRAKEILARDGPNALTPPPTTPEWVKFCRQLFGGFSMLLWIGAILCFLAYGIQ  118
kfNKA   59:LSRGLSSSRAKDILARDGPNALTPPPTTPEWVKFCKQLFGGFSMLLWIGAILCFLAYGIQ  118
mtNKA   59:LTRGLSSSRAKEILARDGPNALTPPPTTPEWVKFCKQLFGGFSMLLWIGAILCFLAYGIQ  118
rtNKA   61:LSKGLSSAKAAENLARDGPNSLTPPPTTPEWVKFCKQMFGGFSMLLWTGALLCFLAYGIQ  120

bpNKA  121:AASEDEPANDNLYLGVVLSAVVIITGCFSYYQEAKSSKIMDSFKNLVPQQALVLRDGEKK  180
ssNKA  119:AASEDEPANDNLYLGVVLSAVVIITGCFSYYQEAKSSKIMDSFKNLVPQQALVLRDGEKK  178
kfNKA  119:AASEDEPANDNLYLGIVLSAVVIITGCFSYYQEAKSSKIMESFKNLVPQQALVVRDGEKN  178
mtNKA  119:AASEDEPANDNLYLGIVLSAVVIITGCFSYYQEAKSSKIMESFKNLVPRQALGIRDGEKK  178
rtNKA  121:AAMEDEPANDNLYLGVVLSAGVIVTGCFSYYQEAKSSKIMDSFKNLVPQQALVVRDGEKM  180

bpNKA  181:SINAEEVVVGDLVEVKGGDRIPADLRIISAHGCKVDNSSLTGESEPQTRTPDFSNDNPLE  240
ssNKA  179:SINAEEVVVGDLVEVKGGDRIPADLRIISAHGCKVDNSSLTGESEPQTRTPDFSNDNPLE  238
kfNKA  179:SINAEEVVAGDLVEVKGGDRIPADLRIISSHGCKVDNSSLTGESEPQTRSPDFTNDNPLE  238
mtNKA  179:NINAEEVVLGDLVEVKGGDRIPADLRIISAHGCKVDNSSLTGESEPQTRSPDFSNENPLE  238
rtNKA  181:NINAQQVVVGDLVEVKGGDRIPADLRIISASGCKVDNSSLTGESEPQTRTPDYSNDNPLE  240

bpNKA  241:TRNIVFFSTNCVEGTARGVVINTGDHTVMGRIATLASSLEGGKTPIAVEIEHSIHIITGV  300
ssNKA  239:TRNIVFFSTNCVEGTARGIVINTGDRTVMGRIATPASSLEGGKTPIAKEIEHFIHIITGV  298
kfNKA  239:TRNIAFFSTNCIEGTARGIVINTGDRTVMGRIATLASSLDGGKTPIAKEIEHFIHIITGV  298
mtNKA  239:TRNISFFSTNCIEGTARGIVINTEDRTVMGRIATLASSLEGGKTPIAIEIEHFIHIITGV  298
rtNKA  241:TRNIAFFSTNCVEGTARGIVINTGDRTVMGRIATLASGLEVGRTPISIEIEHFIHIITGV  300

bpNKA  301:AVFLGVSFFILSLILGYGWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMAKKNCLV  360
ssNKA  299:AVFLGVSSFILSLILGYGWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMAKKDCLV  358
kfNKA  299:AVFLGASFFILSLILGYGWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMAKKNCLV  358
mtNKA  299:AVFLGVSFFILSLILGYNWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMAKKNCLV  358
rtNKA  301:AVFLGMSFFVLSLILGYSWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMAKKNCLV  360

bpNKA  361:KNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDKTSASW  420
ssNKA  359:KNLEAVETLGSSSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDKTSASW  418
kfNKA  359:KNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDRSSSTW  418
mtNKA  359:KNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDRSSATW  418
rtNKA  361:KNLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDRSSATW  420

bpNKA  421:SALARIAGLCNRAVFLAEQNNVPILKRDVAGDAAEAALLKCIELCCGSVGGMRDKYPKIS  480
ssNKA  419:SALARIAGLCNRAVFLAEQNNVPILKRDVAGDASEAALLKCIELCCGSVGGMRDKYLKIS  478
kfNKA  419:AALARVAGLCNRAVFLAEQNNVPILKRDVAGDASEAALLKCIELCCGSVKDMRDKYPKVA  478
mtNKA  419:ANLSRIAGLCNRAVFLADQSNIPILKRDVAGDASEAALLKCIELCCGSVNEMREKYPKIA  478
rtNKA  421:AALARVAGLCNRAVFLAEQNGIPILKRDVAGDASESALLKCIELCCGSVQGMRDQYTKVA  480

bpNKA  481:EIPFNSTNKYQLSIHKNATPGETKHLLVMKGAPERILDRCSTIVIQGKEQPLDDEMKDAF  540
ssNKA  479:EIPFNSTNKYQLSIHKNTTPGETKHLLVMKGAPERILDRCSTIMIQGKEQPLDDEMKDAF  538
kfNKA  479:EIPFNSTNKYQLSIHKNATPGETKHLLVMKGAPERILDRCSTIVLQGKEQPLDDEMKDSF  538
mtNKA  479:EIPFNSTNKYQLSIHKNTTPGETKHLLVMKGAPERILDRCNSIVLQGKVQALDDEMKDAF  538
rtNKA  481:EIPFNSTNKYQLSVHLNKNEGESKHLLVMKGAPERILDRCSTILIQGKEQPLDDEMKDSF  540
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Fig. 1. Comparison of amino acid sequences of black porgy, *Acanthopagrus schlegeli* NKA, Silver seabream, *Sparus sarba* NKA, killifish, *Fundulus heteroclitus* NKA, Mozambique tilapia, *Oreochromis mossambicus* NKA and rainbow trout, *Oncorhynchus mykiss* NKA optimally aligned to match identical residues, indicated by shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The NKA sequences used for alignment are black porgy NKA (bpNKA), Silver seabream NKA (ssNKA, AAT48993), killifish NKA (kfNKA, AAL18002), Mozambique tilapia NKA (mtNKA, Q9YH26) and rainbow trout NKA (rtNKA, AAQ82788).

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bpNKA  541:QNAYVELGGLGERVLGFCHFSLPDDQFPEGFAFDTEEVNFPTDNLCFVGLMSMIDPPRAA  600
ssNKA  539:QNAYVELGGLGERVLGFCHFHLPDDQFPEGFAFDTEEVNFPTENLCFVGLMSMIDPPRAA  598
kfNKA  539:QNAYVELGGLGERVLGFCHFHLPDDQFPEGFAFDADEVNFPTENLCFIGLMAMIDPPRAA  598
mtNKA  539:QNAYVELGGLGERVLGFCHYHLPDDEFPEGFAFDTDEVNFPTENLCFVGLMAMIDPPRAA  598
rtNKA  541:QNAYMELGGLGERVLGFCHFQLPDDQFAEGFQFDCEEVNFPTENLCFVGLMSMIDPPRAA  600

bpNKA  601:VPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNVPISEVNPR  660
ssNKA  599:VPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPINEVNPR  658
kfNKA  599:VPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNVPISEVNPR  658
mtNKA  599:VPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNVPVSEVNPR  658
rtNKA  601:VPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPVNEVDPR  660

bpNKA  661:DAKACVVHGGELKDMTSEQLDDILKHHTEIVFARTSPQQKLIIVEGCQRQGAIVAVTGDG  720
ssNKA  659:DAKACVVHGGDLKDLTAEQLDDILKYHTETVFARTSPQQKLIIVEGCQRQGAIVAVTGDG  718
kfNKA  659:DAKACVVHGGELKDLTSDQLDEILKHHTEIVFARTSPQQKLIIVEGCQRQGAIVAVTGDG  718
mtNKA  659:DAKACVVHGSELKDMTSEELDDLLKHHTEIVFARTSPQQKLIIVEGCQRQGAIVAVTGDG  718
rtNKA  661:DAKACVVHGGDLKDLSAEQLDDILKYHTEIVFARTSPQQKLIIVEGCQRQGAIVAVTGDG  720

bpNKA  721:VNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIAY  780
ssNKA  719:VNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIAY  778
kfNKA  719:VNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIAY  778
mtNKA  719:VNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIAY  778
rtNKA  721:VNDSPALKKADIGVAMGISGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKSIAY  780

bpNKA  781:TLTSNIPEITPFLFFIIVNIPLPLGTITILCIDLGTDMVPAISLAYEAAESDIMKRQPRN  840
ssNKA  779:TLTSNIPEITPFLLFIIANIPLPLGTVTILCIDLGTDMVPAISLAYEAAESDIMKRQPRN  838
kfNKA  779:TLTSNIPEISPFLLFIIANIPLPLGTVTILCIDLGTDMVPAISLAYEAAESDIMKRQPRN  838
mtNKA  779:TLTSNIPEISPFLLFIIANIPLPLGTVTILCIDLGTDMVPAISLAYEKAESDIMKRQPRN  838
rtNKA  781:TLTSNIPEITPFLFFIIANIPLPLGTVTILCIDLGTDMVPAISLAYEAAESDIMKRQPRN  840

bpNKA  841:PFRDKLVNERLISIAYGQIGMIQALGGFFSYFVILAENGFLPSLLVGIRLNWDDRSVNDL  900
ssNKA  839:PKTDKLVNERLISIAYGQIGMIQALAGFFTYFVILAENGFLPSTLLGIRVNWDNKYINDL  898
kfNKA  839:PKTDKLVNERLISMAYGQIGMMQATAGFFTYFVILAENGFLPMDLLGIRVLWDDKYVNDL  898
mtNKA  839:PKTDKLVNERLISIAYGQIGMMQATAGFFTYFVILAENGFLPMDLIGVRVLWDDKYVNDL  898
rtNKA  841:SKTDKLVNERLISIAYGQIGMIQALAGFFTYFVILAENGFLPSRLLGIRVDWDNKFCNDL  900

bpNKA  901:EDSYGQQWTYEQRKIVELTCHTAFFVSIVVVQWADVIICKTRRNSVFQQGMKNKILIFGL  960
ssNKA  899:EDSYGQQWTYEQRKIVEFTCHTAFFVSIVIVQWADLIICKTRRNSVFQQGMKNKILIFGL  958
kfNKA  899:EDSYGQQWTYERRKIIEFTCHTAFFSSIVIVQWADLIICKTRRNSIVQQGMKNRILIFGL  958
mtNKA  899:EDSYGQQWTYERRKIVEYSCHTAFFASIVIVQWADLIICKTRRNSIVQQGMTNRILIFGL  958
rtNKA  901:EDSYGQQWTYEQRKIVEFTCHTAFFASIVVVQWADLIICKTRRNSVFQQGMRNKILIFGL  960

bpNKA  961:FEETALAAFLSYCPGMDVALRMYPLKPTWWFCAFPYSFLIFVYDEVRKLLLRRNPGGWVE 1020
ssNKA  959:FEETALAAFLSYCPGMDVALRMYPLKPNWWFCAFPYSLLNFIYDEIRKLILRRSPGGWVE 1018
kfNKA  959:LEETALAAFLSYCPGMDVALRMYPLKPAWWFCAFPYSLLIFLYDEARRYILRRNPGGWVE 1018
mtNKA  959:FEETALAAFLSYCPGMDVALRMYPMKPLWWFCAFPYSLLIFLYDEARRYILRRNPGGWVE 1018
rtNKA  961:FEETALAAFLSYCPGMGIALRMYPLKPSWWFCAFPYSLLIFIYDEIRKLIIRRSPGGWVE 1020

bpNKA 1021:METYY                                                        1025
ssNKA 1019:RETYY                                                        1023
kfNKA 1019:LETYY                                                        1023
mtNKA 1019:KETYY                                                        1023
rtNKA 1021:RETYY                                                        1025
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Fig. 1 (*continued*).

The NKA β-subunit maintains the correct orientation of the enzyme in the membrane and stabilizes the sodium pump in the membrane (McDonough et al., 1990; Noguchi et al., 1990). However, there have not been many reports on this subject (Richards et al., 2003). Immunocytochemical studies have reported that NKA occurs mainly in the epithelia of the kidney tubules (Ura et al., 1996) and in mitochondrion-rich cells of the branchial epithelium in euryhaline teleosts (Wilson and Laurent, 2002). In euryhaline teleosts, NKA activity in the kidneys was higher in FW fish than in SW fish (Lasserre, 1971; Gallis and

Bourdichon, 1976; Venturini et al., 1992; Madsen et al., 1994), but NKA activity in the gills was generally higher in SW fish (Sakamoto et al., 2001; Marshall, 2002). This result has been described as the "diadromid paradigm" (Marshall and Bryson, 1998; Lee et al., 2000, 2003).

Osmotic stress transcription factor 1 (OSTF1) is the gene that is initially expressed under osmotic stress. In fish, OSTF1, which was reported for the first time in tilapia (Fiol and Kültz, 2005), is a transcription factor specific to osmolality that is expressed only during osmotic stress, regardless of oxidative or water-temperature stress. Recently, many studies have examined the expression of aquaporin 3 mRNA (Cutler and Cramb, 2002), as well as the ions that are regulated according to salinity levels by water transporter proteins such as NKA (Tipsmark et al., 2002), $Na^+/Cl^-/2Cl^-$ co-transporter (Tipsmark et al., 2002), urea transporter (Mistry et al., 2001), and taurine transporter (Takeuchi et al., 2000).

Black porgy (*Acanthopagrus schlegeli*) is a euryhaline teleost and moves from coastal waters near land or in estuaries to near-shore shallow areas as it transitions from larvae to juveniles. Being euryhaline, black porgy has excellent osmoregulatory ability (Kitajima and Tsukashima, 1983). Therefore, our purpose was to investigate the changes in NKA and OSTF1 mRNA expression in osmoregulatory organs when the black porgy were transferred to FW, and to determine whether these genes are specifically expressed under hypoosmotic stress. We compared the mRNA expression of these genes and of heat shock protein 90 (HSP90), which is a representative stress protein. In addition, we analyzed changes in plasma osmolality and cortisol levels to provide basic data on the osmoregulation of black porgy in a hypoosmotic environment.

2. Materials and methods

2.1. Experimental animals and transfer to FW

Black porgy (length 14.3±0.4 cm, mass 51.0±6.0 g) were collected from a commercial fish farm. Experiments were performed in 220-L circulation filter tanks containing either full-strength seawater (SW), diluted SW (10‰ SW), freshwater (FW), and SW at 30°C over 24 h (temperature stress). The transfer to FW of black porgy and rising of water temperature were performed according to the methods of Min et al. (2003) and Choi et al. (2006), respectively. During the experimental periods, except during the temperature stress experiment, the water temperature and photoperiod were maintained at 20°C and a 12L/12D cycle, respectively. No food was supplied during the experimental periods.

2.2. Sampling

Six fish from each treatment [SW, 10‰ SW, FW and 30°C water temperature] were randomly selected for blood and tissue sampling. Fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, USA) prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000 rpm, 5 min) and stored at −80°C until analysis. Fish were euthanized by spinal transection for the collection of gill, kidney and intestine tissue samples. Immediately after collection, samples were frozen in liquid nitrogen and stored at −80°C until the total RNA was extracted for analysis.

2.3. Identification of NKA cDNA

Primers for NKA were designed using highly conserved regions of Silver seabream (GenBank accession no. AY553205), tilapia (GenBank accession no. U82549), and killifish (GenBank accession no. AY057072): NKA forward primer (5'-ACT GGW GAA TCW GAG CCY CA-3') and NKA reverse primer (5'-GTC AGG GTG TAR GCR ATG GA-3'). Total RNA was extracted from the kidney using a TRIzol kit (Gibco/BRL, Grand Island, NY, USA). Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Bioneer, Seoul, Korea), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using a BS Taq Master Mix (Biosesang, Sungnam, Korea). PCR was carried out as follows: initial of denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min; followed by 5 min at 72°C for the final extension. The amplified PCR product was processed by electrophoresis in 1% agarose gels. The PCR product was purified and ligated into the pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α, and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoRI (Fermentas, USA). Based on the plasmid DNA, NKA cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.4. Identification of OSTF1 cDNA

Primers for OSTF1 were designed using a reported sequence of Mozambique tilapia OSTF1 (GenBank accession no. AY679524): OSTF1 forward primer (5'-TGC TAG CGT TGT GGC CAT T-3') and OSTF1 reverse primer (5'-ACT GGA ACT TCT CCA GCT G-3'). Total RNA extraction and RT were conducted using the same methods as for NKA. PCR was subsequently carried out as follows: one cycle of denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min; followed by 5 min at 72°C for the final extension. After PCR, the transformation and sequencing were the same as for NKA.

2.5. NKA rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the NKA RACE reaction, the total RNA was extracted from the kidney using a TRIzol kit (Gibco/BRL, Grand Island, NY, USA). Using 3 μg of total RNA as a template, 3' RACE cDNA and 5' RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5'-CTG TGA ATG CTG CGA CTA CGA T $(T)_{18}$-3') and a CapFishing™ adaptor (Seegene, Korea).

NKA-specific primers were selected from the PCR product obtained by PCR. For the 3' RACE, the 50-μL PCR reaction mixture contained 5 μL of 3' RACE cDNA, 1 μL of 10 mM 3' RACE target primer (5'-CTG TGA ATG CTG CGA CTA CGA T-3'), 1 μL of 10 mM 3' RACE gene-specific primer (5'-CGT TAC AGG AGT GGA AGA AGG TCG TCT G-3'), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out as follows: denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s; followed by 5 min at 72°C for the final extension.

For 5' RACE, the 50-μL PCR reaction mixture contained 5 μL of 5' RACE cDNA, 1 μL of 10 mM 5' RACE target primer (5'-GTC TAC CAG GCA TTC GCT TCA T-3'), 1 μL of 10 mM 5' RACE gene-specific primer (5'-AGT CAG GAG TAC GAG TCT GAG GCT CTG A-3'), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out as follows: denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s; followed by 5 min at 72°C for the final extension. The amplified PCR product was processed by electrophoresis in 1% agarose gels. The transformation and sequencing were conducted as described above.

2.6. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of NKA and OSTF1 mRNA using total RNA extracted from the kidney and gill, respectively, of black porgy. Primers for QPCR were designed with reference to the known sequences of black porgy as follows: NKA forward primer (5'-GGT GAT GGT GTG AAC GAC TC-3'), NKA reverse primer (5'-CTG GTC AGG GTA TAG GCG AT-3'), OSTF1 forward primer (5'-AGG TGG AGA TCC TCA AGG AGC AG-3'), OSTF1 reverse primer, (5'-GCG AGG TTC TTC AGC AGG TAG TTC-3'), β-actin forward primer (5'-GGA CCT GTA TGC CAA CAC TG-3') and β-actin reverse primer (5'-TGA TCT CCT TCT GCA TCC TG-3'). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data are expressed as the change with respect to the corresponding β-actin CT levels.

2.7. Semi-quantitative PCR

Semi-quantitative PCR was conducted to determine the relative expression of NKA, OSTF1, HSP90 and β-actin mRNA in tissues of black porgy. To optimize the number of cycles used for semi-quantitative PCR, the RT reaction (1 μL) from the gill, kidney, and intestine was used as the template for PCR amplification. NKA, OSTF1, HSP90, and β-actin-specific primers for RT-PCR were designed from published sequences: bpNKA forward primer (5'-ACC GGT GAA TCA GAG CCT CA-3') and bpNKA reverse primer (5'-GTC AGG GTA TAG GCG ATG GA-3'), bpOSTF1 forward primer (5'-TGC TAG CGT TGT GGC CAT T-3') and bpOSTF1 reverse primer (5'-ACT GGA ACT TCT CCA GCT G-3'), bpHSP90 forward primer (5'-AAC GAC TGG GAG GAT CAC CTG-3') and bpHSP90 reverse primer (5'-CAT GAT GCG CTC CAT GTT CGC-3'), and bpβ-actin forward primer (5'-TCG AGC ACG GTA TTG TGA CC-3') and bpβ-actin reverse primer (5'-ACG GAA CCT CTC ATT GCC GA-3'). PCR amplification was conducted using a BS Taq Master Mix (Biosesang, Korea), according to the manufacturer's instructions. PCR was carried out as follows: one cycle of denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, annealing at 55°C (OSTF1, HSP90) or 57°C (NKA) for 30 s; a total of 27 (HSP90) and/or 30 (OSTF1, NKA) cycles for 1 min at 72°C for 30 s; followed by one cycle of 5 min at 72°C for the final extension.

The β-actin mRNA was amplified in each PCR reaction as a loading control. The amplification of β-actin was carried out as follows: denaturation at 94°C for 5 min; 27 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, 72°C for 1 min for extension; followed by 5 min at 72°C for the final extension. The PCR products from different cycles of amplification were visualized on a UV-transilluminator after electrophoresis on 1% agarose gels containing ethidium bromide (0.5 μg/μL). The signal intensity was quantified using the Gel-Doc System and Gelpro 3.1 software (KBT, Incheon, Korea).

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bpOSTF1    1: ASVVAIDNKIEQAMDLVKNHLMYAVREEVEILKEQIKELAEKNNQLERENYLLKNLASPE  60
mtOSTF1  118: ASVVAIDNKIEQAMDLVKNHLMYAVREEVEILKEQIKELAEKNNQLERENYLLKNLASPE 177
hmTSC22  104: ASVVAIDNKIEQAMDLVKNHLMYAVREEVEILKEQIRELVEKNSQLERENTLLKTLASPE 163
ckTSC22  942: ASVVAIDNKIEQAMDLVKSHLMYAVREEVEVLKEQIKELIEKNSQLEQENTLLKTLASPE 1001

bpOSTF1   61: QLEKFQ                                                      66
mtOSTF1  178: QLEKFQ                                                     183
hmTSC22  164: QLEKFQ                                                     169
ckTSC22 1002: QLAQFQ                                                    1007
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Fig. 2. Comparison of amino acid sequences of black porgy, *Acanthopagrus schlegeli* OSTF1, Mozambique tilapia, *Oreochromis mossambicus* OSTF1, human, *Homo sapiens* TSC22 and chicken, *Gallus gallus* TSC22 optimally aligned to match identical residues, indicated by shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. The OSTF1 sequences used for alignment are black porgy OSTF1 (bpOSTF1), Mozambique tilapia OSTF1 (mtOSTF1, AAT84345), human TSC22 (hmTSC22, EAX02707) and chicken TSC22 (ckTSC22, NP_001012778).

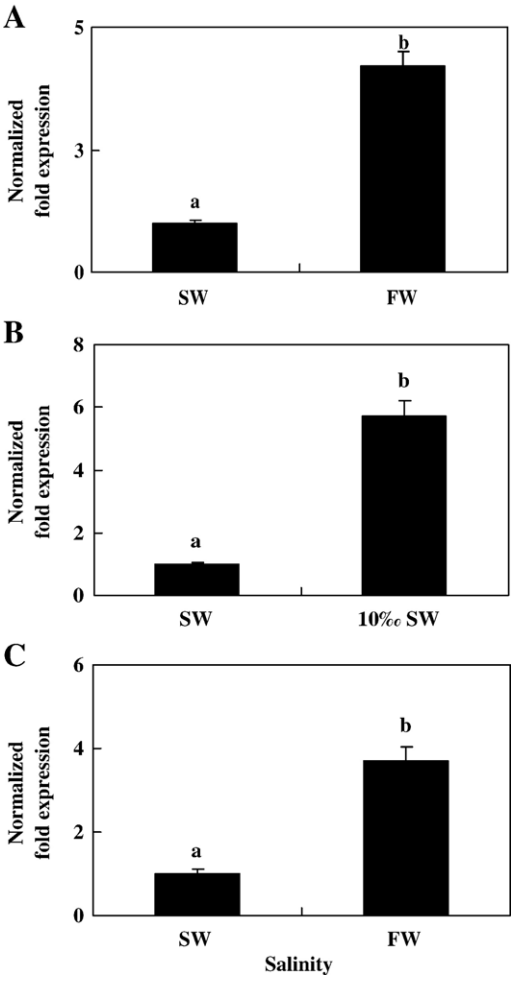

Fig. 3. Expression of NKA (A, gill; B, kidney) and OSTF1 (C, gill) mRNA levels in tissues of black porgy by quantitative PCR. One microgram of total RNA prepared from kidney and gill, respectively, was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=6$).

2.8. Plasma parameters analysis

Plasma osmolality was examined using a Vapor Pressure Osmometer (Vapro 5520, Wescor Co., Logan, UT, USA). Plasma cortisol was analyzed by radioimmunoassay (RIA) using an RIA kit (Diagnostic System Laboratories, Webster, TX, USA).

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). One way ANOVA followed by post hoc Duncan's multiple range test multiple comparisons was used to compare differences in the data ($P<0.05$).

3. Results

3.1. Identification of NKA full-length cDNA

RT-PCR was used to clone a fragment of NKA cDNA using total RNA extracted from kidney of black porgy from 10‰ SW,

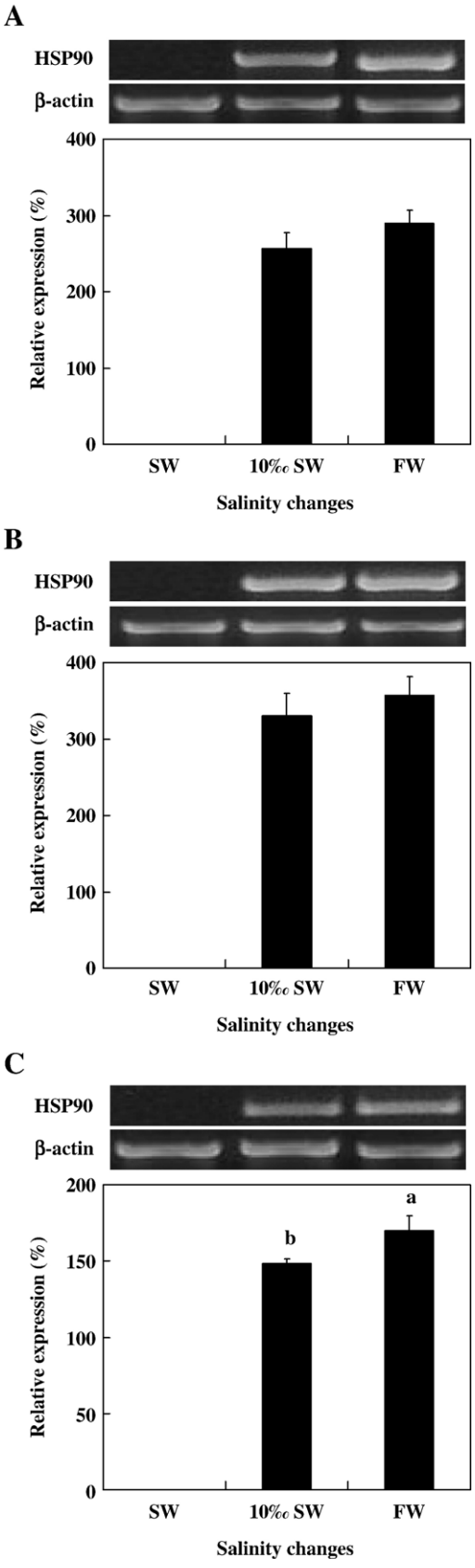

Fig. 4. Expression of HSP90 mRNA in tissues of black porgy. One microgram of total RNA prepared from gill (A), kidney (B) and intestine (C) was reverse transcribed and amplified using black porgy HSP90-specific primer. Tissue distribution of black porgy HSP90 was analyzed by RT-PCR. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=6$).

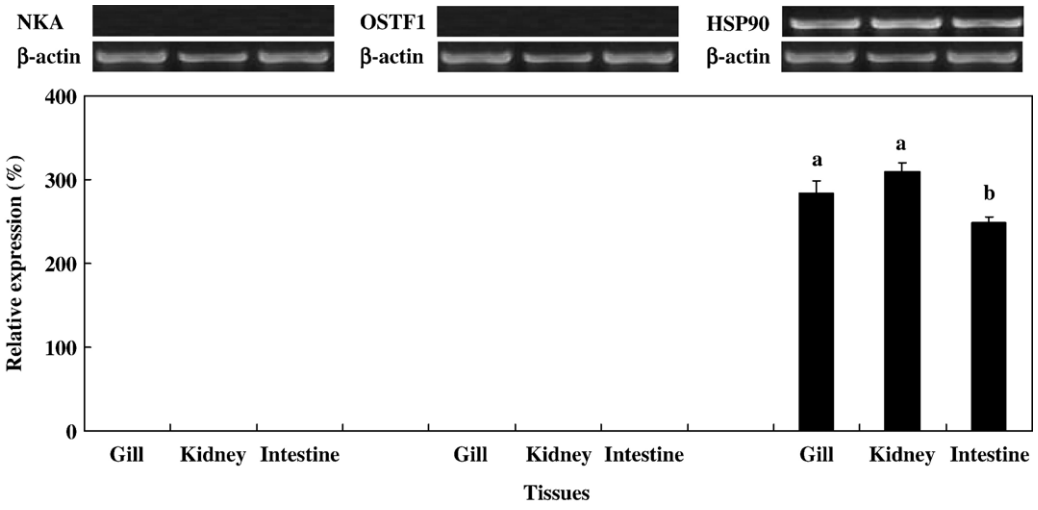

Fig. 5. Expression of NKA, OSTF1 and HSP90 mRNA in tissues of black porgy during water-temperature rising. One microgram of total RNA prepared from gill, kidney and intestine was reverse transcribed and amplified by RT-PCR. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=6$).

in which the expression was highest. A single PCR product of the expected size (1688 base pairs [bp]) was obtained. A PCR-based cloning strategy (PCR followed by 3' and 5' RACE) was used to clone a full-length cDNA encoding a putative NKA from kidney of black porgy. NKA full-length cDNA contained 3078 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 1025 amino acids (Fig. 1). The amino acid sequence of black porgy NKA was compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid identity of NKA with those of other fish species is: 94% with Silver seabream NKA (GenBank accession no. AAT48993), 90% with killifish NKA (GenBank accession no. AAL18002), 89% with Mozambique tilapia NKA (GenBank accession no. Q9YH26), and 88% with rainbow trout NKA (GenBank accession no. AAQ82788; Fig. 1).

3.2. Identification of OSTF1 partial cDNA

RT-PCR was used to clone a fragment of OSTF1 cDNA using total RNA extracted from the gill of black porgy from FW, in which the expression was highest. A single PCR product of the expected size (201 bp) was obtained. The amino acid sequence of black porgy OSTF1 was compared to those deduced from the cDNA of other species (Fig. 2). The amino acid identity of OSTF1 with those of other species is: 100% with Mozambique tilapia OSTF1 (GenBank accession no. AAT84345), 92% with human TGF-β-stimulated clone 22 (TSC22; GenBank accession no. EAX02707) and 86% with chicken TSC22 (GenBank accession no. NP_001012778; Fig. 2).

3.3. QPCR for NKA and OSTF1 mRNA expression

We measured using the QPCR the levels of NKA and OSTF1 mRNA expression in the osmoregulatory organs of black porgy when the fish were transferred to FW. The expression of NKA mRNA in the gill was 4.2 times higher in FW than in SW (Fig. 3A), and the level in the kidney was 5.7 times higher in 10‰ SW than in SW (Fig. 3B), and the expression of OSTF1 mRNA in the gill was 3.7 times higher in FW than in SW (Fig. 3C).

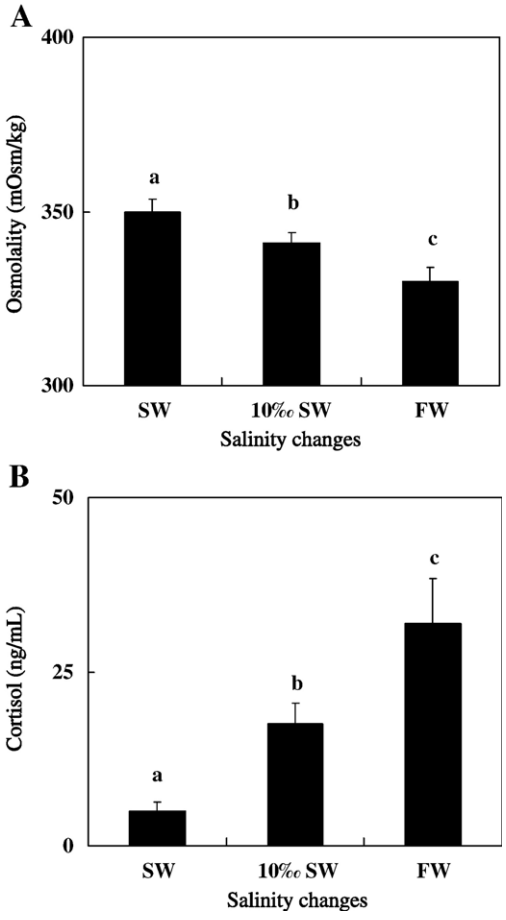

Fig. 6. Levels of plasma osmolality and cortisol in black porgy during freshwater acclimation. Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=6$).

3.4. Tissue distribution of HSP90 mRNA

The expressions of HSP90 mRNA in the gill, kidney, and intestine tissues using semi-quantitative PCR were not observed in fish from SW, but the levels were high in 10‰ SW and FW (Fig. 4).

3.5. Water-temperature rising experiment

The expressions of NKA and OSTF1 mRNA were undetectable from the high-temperature stress experiment. On the other hand, the HSP90 mRNA expression was high in fish from the temperature stress experiment (30°C water temperature; Fig. 5).

3.6. Plasma parameters

The plasma osmolality was 350.0±3.5 mOsm/kg in fish from SW, which was significantly greater than the 329.8±8.4 mOsm/kg in fish from FW (Fig. 6A). And the plasma cortisol levels were 5.3±1.3 ng/mL in SW, reaching their highest level of 32.0± 6.4 ng/mL in FW (Fig. 6B).

4. Discussion

The NKA and OSTF1 genes were cloned from the euryhaline black porgy as expressed when the fish were transferred from SW to FW. We also compared the expression of these genes in the osmoregulatory tissues, i.e., gill, kidney, and intestine, under hypoosmotic stress. To determine whether NKA and OSTF1 are specifically expressed under hypoosmotic stress, we compared their expression with that of HSP90 mRNA, which is a representative stress gene, under hypoosmotic and high water-temperature stress.

Full-length NKA cDNA was separated using the total RNA extracted from the kidney of black porgy from 10‰ SW, in which NKA mRNA expression was the highest. The NKA cDNA of black porgy encodes 1025 amino acids in an open reading frame and has a total of 3078 nucleotides. This NKA amino acid displayed high identity with the NKA α subunit and was well conserved among various species of teleost fish (Fig. 1).

The results of the QPCR of black porgy tissue indicated that NKA expression in the gill was approximately 4.2 times higher in FW than in SW (Fig. 3A), NKA expression in the kidney was 5.7 times higher in 10‰ SW than in FW (Fig. 3B). In general, the activation of NKA in the gills of fish was high in SW. In FW, NKA activation and mRNA expression in the kidney is high (Marshall and Bryson, 1998; Lee et al., 2000, 2003); these results are supported by ours. Similarly, we observed high NKA mRNA expression in the kidney. This is because the expression of NKA mRNA occurred due to the re-absorption of ions in the kidneys to prevent the loss of ions such as Na^+ and Cl^-. Accordingly, we assume that NKA activation increased. These results correspond to those in tilapia (Lee et al., 2000, 2003) and pufferfish (Lin et al., 2004), in which NKA mRNA expression and activation were higher in the kidneys than in the gills during osmotic stress. Thus, we presume that black porgy carries out hyperosmoregulation in hypoosmotic environments by suppressing ion loss through the re-absorption of ions in the kidneys and by regulating water inside the body as it takes water into the blood to create a large volume of dilute urine. The higher NKA mRNA expression in the kidneys than in the gills in FW corresponds to the diadromid paradigm (Marshall and Bryson, 1998; Lee et al., 2000, 2003).

OSTF1 partial cDNA was separated using the total RNA extracted from the gills of black porgy. The decided OSTF1 partial cDNA of black porgy consisted of 201 bp. Its amino acid sequence had 100% similarity with that of tilapia OSTF1. It also displayed high amino acid identity with the TSC22 protein of mammals such as human and chicken (Fig. 2). TSC22 is a transcription factor that is derived from transforming growth factor β (TGF-β). Its transcription is triggered by a variety of stimuli such as anticancer drugs, progesterone, and growth inhibitors and it suppresses tumorigenesis (Kester et al., 1999). In addition, because OSTF1 has a binding site for putative phosphorylation, DNA-PK, cdk, and MAP kinases, OSTF1 is thought to take part in regulating apoptosis and DNA destruction under osmotic stress (Kültz and Chakravarty, 2001). Therefore, because OSTF1 mRNA expression in black porgy was high in FW, it is possible that hypoosmotic stress affects DNA destruction and apoptosis. However, because there is little published information on this subject, further examination of OSTF1 is necessary.

OSTF1 mRNA expression as quantified by QPCR was observed only in the gill. QPCR of gill tissue showed expression levels that were approximately 3.7 times higher in FW than in SW (Fig. 3). Unlike OSTF1 expression in tilapia under hyperosmotic stress, the expression of OSTF1 mRNA in black porgy occurred under hypoosmotic stress. Thus, tilapia, which is a FW fish, and black porgy, which is a SW fish, became subject to osmotic stress under hyperosmolality and hypoosmolality, respectively. This is because OSTF1 is activated not only in response to hyperosmolality, but also in response to hypoosmotic stress. OSTF1 mRNA expression was not observed in the kidneys and intestine, possibly indicating that OSTF1 does not have an osmoregulatory function in these organs.

The mRNA expression of HSP90, a representative stress protein, increased in the gills, kidney, and intestine in hypoosmotic environment (Fig. 4), as well as during exposure to a high water temperature of 30 °C (Fig. 5). Thus, HSP90 reacts not only to hypoosmolality, but also to high water-temperature stress. This corresponds to the results of previous studies in which the expression of the stress protein HSP90 was induced by factors such as water temperature, salinity, and heavy metals (Beckmann et al., 1990) and in which HSP90 mRNA expression increased during increases in water temperature in Chinook salmon (Palmisano et al., 2000), rainbow trout (Sathiyaa et al., 2001), zebrafish (Murtha and Keller, 2003), and black porgy (Choi et al., 2006). Thus, black porgy became stressed by hypoosmolality during transfer to FW, as indicated by the expression of HSP90 mRNA.

The exposure of black porgy to heat shock (30 °C) does not induce the expression of NAK or OSTF1 mRNA in gill, kidney, or intestine tissues (Fig. 5). These data indicate that NAK and OSTF1 mRNA expression is specific to osmotic stress and does not occur during other common type of stress such as heat

shock. These results correspond to those of a study in which changes in water temperature did not affect NKA activation in the gills (Fiess et al., 2006) and of a study in which OSTF1 mRNA expression did not occur in tilapia after 2 h of exposure to a high water temperature of 36 °C (Fiol and Kültz, 2005).

Plasma cortisol is potentially a valuable hormonal index of stress. This study demonstrated that black porgy are sensitive to salinity changes as indicated by the significant increases in plasma cortisol concentrations. In the present study, FW transfer caused an immediate increase in plasma cortisol that reached its peak in 24 h (Fig. 6B). This result agrees with that of Chang et al. (2002). Such stress responses with regard to cortisol change can be explained that abrupt salinity changes cause more stress to fish than gradual changes, and after a salinity change.

In SW, fish emit ions and absorb water. In FW, they absorb ions and discharge water to maintain osmolality within the body. The plasma osmolality was lowest in FW during transfer to FW (Fig. 6A). This imbalance in osmolality occurred in black porgy exposed to a hypoosmotic environment. This result corresponds to those of previous studies (Mancera et al., 1993; Lin et al., 2004; Min et al., 2005). Therefore, as with other teleosts, black porgy maintain osmolality within the body through hyperosmoregulation while acclimating to the hypoosmotic environment of FW.

In summary, we compared differences in the expression of the NKA and OSTF1 genes that are specifically expressed in reaction to hypoosmotic stress when the black porgy were transferred to FW. Through a comparison with the expression of HSP90 mRNA, we determined that the NKA and OSTF1 genes reacted specifically during osmotic stress. By observing the expression of these genes, we deduced that black porgy carries out hyperosmoregulation in a hypoosmotic environment.

Acknowledgements

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