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Nitric oxide synthase in the gill of Atlantic salmon: colocalization with and inhibition of Na^+,K^+-ATPase

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Summary

We investigated the relationship between nitric oxide (NO) and Na^+,K^+-ATPase (NKA) in the gill of anadromous Atlantic salmon. Cells containing NO-producing enzymes were revealed by means of nitric oxide synthase (NOS) immunocytochemistry and nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) histochemistry, which can be used as an indicator of NOS activity, i.e. NO production. Antibodies against the two constitutive NOS isoforms, neuronal and endothelial NOS, both produced immunoreactivity restricted to large cells at the base and along the secondary lamellae. NADPHd-positive cells showed a corresponding distribution. Antibodies against the inducible NOS isoform only labeled small cells located deep in the filament. Using *in situ* hybridization and NKA immunoreactivity, cells expressing Na^+,K^+-ATPase α-subunit mRNA were found to have a similar distribution to the NOS cells. Double labeling for NOS immunoreactivity and NKA α-subunit mRNA revealed cellular colocalization of NKA α-subunit mRNA and nNOS protein in putative chloride cells at the base of the lamellae and interlamellar space. Along the lamellae, some NOS- or NKA-immunoreactive cells possessed a relatively lower expression of NKA α-subunit mRNA in smolts. A clear increase in NADPHd staining in the gill was demonstrated from parr to smolt. The regulatory role of NO on gill NKA activity was studied *in vitro* using sodium nitroprusside (SNP; 1 mmol l^{-1}) and PAPA-NONOate (NOC-15; 0.5 mmol l^{-1}) as NO donors. Both SNP and NOC-15 inhibited gill NKA activity by 30% when compared to controls. The study shows that NO systems are abundant in the gill of Atlantic salmon, that NO may be produced preferentially by a constitutive NOS isoform, and suggests that NO influence on gill functions is mediated via intracellular, possibly both auto/paracrine, inhibition of Na^+,K^+-ATPase activity in chloride cells. Furthermore, the increase in NADPHd in the gill during smoltification suggests a regulatory role of NO in the attenuation of the smoltification-related increase in Na^+,K^+-ATPase activity prior to entering seawater.

Key words: nitric oxide, Na^+,K^+-ATPase, osmoregulation, parr–smolt transformation, development, metamorphosis, *Salmar salmar*, fish, teleost.

Introduction

Nitric oxide (NO) is a short-lived freely diffusible radical gas that acts as an important biological signal in a variety of physiological processes, including vasodilatation, neurotransmission and recently in ion transport (Evans, 2002). A family of NO synthases (NOS) generates NO from the oxidation of L-arginine to L-citrulline. Three main NOS isoforms have been identified, two constitutive, neuronal NOS (nNOS) and endothelial NOS (eNOS), and one inducible NOS (iNOS). All three isoforms require nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, which has led to the widely used NADPH-diaphorase (NADPHd) histochemical technique to identify NOS activity, i.e. NO production. In fish, NOS and NADPHd have been identified in numerous tissues, including brain (Brüning et al., 1996; Brüning et al., 1995; Holmqvist and Ekström, 1997; Holmqvist et al., 2000, 1994; Øyan et al., 2000), retina (Östholm et al., 1994) peripheral nervous system (Green and Campbell, 1994; Li and Furness, 1993; Mauceri et al., 1999; Olsson and Holmgren, 1996), caudal neurosecretory system (Cioni et al., 2002), gut (Olsson and Holmgren, 1997), kidney (Jimenez et al., 2001) and head kidney (Gallo and Civinini, 2001).

Evidence for the role of NO in osmoregulation in fish includes localization of NOS in osmoregulatory organs and effects on ion regulatory proteins and vascular tonus. NADPHd has been localized in branchial nerves in the cod (Gibbins et al., 1995) and neuroendocrine cells in the catfish gill (Mauceri

et al., 1999). NO donors and NOS inhibitors have been shown to decrease and increase, respectively, the short-circuit current across the killifish opercular membrane (Evans, 2002). Recently, several studies have shown that NO affects Na^+,K^+-ATPase (NKA) activity in the kidney and other tissues in higher vertebrates (Liang and Knox, 1999). Tipsmark and Madsen (2003) showed for the first time that NO exerts an inhibitory effect on Na^+,K^+-ATPase activity in fish kidney and gill tissue. In addition, NO is well recognized as a vasodilator in mammals, and more recently in fish (Fritsche et al., 2000; Haraldsen et al., 2002). NOS has also been located in nerves along blood vessels in the gill (Gibbins et al., 1995), and therefore the role of NO in osmoregulation may also include the regulation of blood flow through the gill.

As we begin to accumulate evidence of the role of NO in fish, it is important to relate this information to other model species with different life histories to further elucidate basic mechanisms. One potentially important model is found in anadromous salmonids that undergo a pre-adaptation to ocean life, called parr-smolt transformation or smoltification, which involves preparatory behavioral, physiological and morphological changes (Boeuf, 1993; Dickhoff, 1993; Hoar, 1988). Sequential hormone surges (thyroid hormones, growth hormone and cortisol) are central to this transformation and instrumental in the structural and chemical changes in the gill in preparation for the subsequent transition to a hyper-osmotic environment (McCormick, 2001). Osmoregulatory changes in the gill during smoltification include increases in chloride cell number and Na^+,K^+-ATPase activity, and are regulated by the changes in the endocrine system (D'Cotta et al., 2000; McCormick, 2001; Prunet et al., 1994; Seidelin et al., 2001). It is widely accepted that the increased abundance of gill Na^+,K^+-ATPase enzymes is a preparatory development prior to seawater entry; however, the functional state of this pool of enzymes while the fish is still in fresh water is uncertain. To learn more about the possible regulatory role of NO in this system, we investigated the presence of NO-producing cells in the gill of Atlantic salmon smolts and determined possible influence of NO on Na^+,K^+-ATPase activity.

Materials and methods

Fish and rearing conditions

Atlantic salmon *Salmo salar* L. parr from the anadromous population of the river Vosso (Southwestern Norway) were brought to the Aquatic Laboratory of the Bergen High Technology Center in late November 2000. The fish had been hatched from eggs collected from wild broodstock, and first fed late in May 2000. Upon arrival in Bergen, the fish were reared in 1 m^2 indoor tanks supplied with flow-through freshwater (FW; pH 6.9–7.1) at 8°C, increasing to 9°C in May, and exposed to simulated natural photoperiod (60°25′N) between December 2000 and June 2001. The fish were fed in excess on a commercial dry diet (T. Skretting A/S, Stavanger, Norway) for 8–12 h during the photo-phase. The fish were starved for 15 h before sampling.

Sampling procedures and analysis

In May, when the salmon reached peak smolt status (for details, see Nilsen et al., 2003), six fish (mean mass 29.5±0.9 g) were deeply anesthetized with tricaine methanesulphonate (MS222; Sigma, St Louis, MO, USA) and fixed by vascular perfusion with 4% paraformaldehyde (PF) in 0.1 mol l^{-1} phosphate buffer (PB; in mmol l^{-1}: 28 NaH_2PO_4, 71 Na_2HPO_4; pH 7.2). The two central gill arches on either side were removed and postfixed in the same fixative overnight at 4°C. Post-fixation was followed by a 2 h rinse in PB after which the gill arches were placed in 25% sucrose overnight. The samples were then embedded in Tissue Tek (Miles Inc., Eikhart, IN, USA) and stored at –80°C until sectioning. Eight adjacent, serial, 10 μm-thick cryostat sections were collected on frozen Superfrost Plus glass slides (Merck, Germany), dried at 60°C for 10·min then stored –80°C in airtight boxes. *In situ* hybridization, immunocytochemistry and NADPHd histochemistry were performed on parallel cryosections. Adjacent sections were used for hybridization with the antisense and sense probes, for NOS immunocytochemistry, including specificity tests in which the primary antibody was excluded, or for NADPHd histochemistry. For comparison with smolts, parr gills were sampled in March as described above and sections from both stages placed on the same slide for NADPHd histochemistry.

Immunocytochemistry

The sections were rehydrated in phosphate-buffered saline (PBS; in mmol l^{-1}: 3.2 NaH_2PO_4, 7.8 Na_2HPO_4; pH 7.2) for at least 10 min followed by 30 min incubation with 3% H_2O_2 to remove endogenous peroxidase activity and then rinsed 2×15 min each, in PBS. The sections were then rinsed in PBS containing 0.25% Triton X-100 (PBS-TX) for 10 min and incubated in either rabbit anti-rat nNOS diluted 1:1500 (a gift from Prof. Fahrenkrug, Bispebjerg Hospital Copenhagen, Denmark), rabbit anti-mouse iNOS from Affinity Bioreagents Inc. (Golden, CO, USA) diluted 1:2000, rabbit anti-mouse eNOS from Affinity Bioreagents Inc. diluted 1:2000, or Na^+,K^+-ATPase α-subunit diluted 1:600 (a gift from Dr S. Adachi, Hokkaido University, Hakodate, Japan) overnight at room temperature. The secondary antibody, anti-rabbit IgG diluted 1:50 (Dako, Glostrup, Denmark), was applied for 30 min, rinsed 2×10 min each in PBS-TX followed by incubation with rabbit PAP complex (Dako, Glostrup, Denmark) diluted 1:50 for 30 min and rinsed for 10 min in PBS-TX and 10 min in 0.05 mol l^{-1} Tris-HCl, pH 7.6. The sections were then reacted in 50 ml Tris-HCl with 25 mg diaminobenzidine (Sigma, St Louis, MO, USA) and 250 μl 3% H_2O_2 for 10 min, followed by dehydration, and mounted with Permount.

NADPHd histochemistry

For NADPHd histochemistry, sections were rinsed in PBS for 10 min followed by a rinse in Tris-HCl (0.01 mol l^{-1}, pH 7.6) for 10 min. Sections were incubated for 90 min at 37°C in

Tris-HCl (0.01 mol l^{-1}, pH 7.6) containing 0.125 mmol l^{-1} Nitroblue Tetrazolium and 1 mmol l^{-1} β-NADPH. Sections were then rinsed for 5 min in Tris-HCl followed by three rinses for 10 min in PBS. Sections were mounted in Kaisers glycerol gelatin (Merck, Darmstadt, Germany).

In situ *hybridization*

Expression of salmon Na^+, K^+-ATPase α-subunit mRNA (GenBank accession number AJ250809; Seidelin et al., 2001) in the gills was visualized by *in situ* hybridization with digoxigenin (DIG)-labeled cRNA probes. The Na^+,K^+-ATPase-α subunit inserts of approximately 890 base pairs in the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA) were transformed, amplified and isolated from overnight cultures using QIAGEN Maxi Plasmid Kit. The inserts were digested with the appropriate restriction enzymes (*Not*I and *Spe*I; Promega, Madison, WI, USA). Antisense and sense cRNA probes were synthesized with T7 RNA polymerase and SP6 RNA polymerase, respectively, using DIG RNA labeling Kit (Roche Diagnostics, Mannheim, Germany). DIG incorporation and the concentration of the probes were estimated by spot tests (Roche Diagnostics).

Prior to *in situ* hybridization, sections were rehydrated for 10 min in potassium phosphate-buffered saline (KPBS; in mmol l^{-1}: 137 NaCl, 1.4 KH_2PO_4, 2.7 KCl, 4.3 Na_2HPO_4; pH 7.3), post-fixed in 4% PF in KPBS for 10 min and permeabilized with proteinase K (5 μg ml^{-1}) in 50 mmol l^{-1} Tris-HCl (pH 7.5) for 5 min. After a 5 min rinse in Tris-HCl, sections were post-fixed in 4% PF and rinsed for 10 min in KPBS before being treated for 10 min with 0.1 mol l^{-1} triethanolamine, pH 8.0 and then for 10 min with 0.25% acetic anhydride in triethanolamine, followed by three 5 min rinses in KPBS. Then, after 2 h prehybridization at room temperature (RT) in hybridization buffer (10% dextran sulphate, 50% formamide, 5× SSC, 5× Denhardt's solution, 500 μg ml^{-1} salmon sperm DNA (Sigma) and 250 μg ml^{-1} tRNA (Promega) the buffer was removed, and 300-800 ng ml^{-1} probe in fresh hybridization buffer were applied to the slides. Hybridization was performed for 16 h at 67°C followed by post-hybridization rinses in 5× SSC for 30 min at RT, 30% formamide in 5× SSC for 15 min at 67°C, 0.2× SSC for 2×15 min at 67°C and 0.2× SSC at RT. After 5 min in wash solution (0.1 mol l^{-1} Tris-HCl, 0.15 mol l^{-1} NaCl, pH 7.5) the sections were incubated for 1 h in 1% blocking solution (Roche Diagnostics) and then incubated overnight at RT with an alkaline phosphatase-conjugated sheep anti-DIG goat antibody (1:2000; Roche Diagnostics). Sections were then rinsed 3×5 min with wash solution at RT, followed by 5 min in detection solution (0.1 mol l^{-1} Tris-HCl, 0.1 mol l^{-1} NaCl, 50 mmol l^{-1} $MgCl_2$, pH 9.5) at RT, before being incubated with reaction solution (3.4 μl Nitroblue Tetrazolium, 3.5 μl 5-Bromo-4-chloro-3-indoylphosphate and 0.24 mg ml^{-1} levamisole in detection solution), in darkness at RT. The phosphatase reaction was terminated after 1-3 h with stop solution (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 0.9% NaCl, pH 8.0) and sections mounted in 50% glycerol (in stop solution).

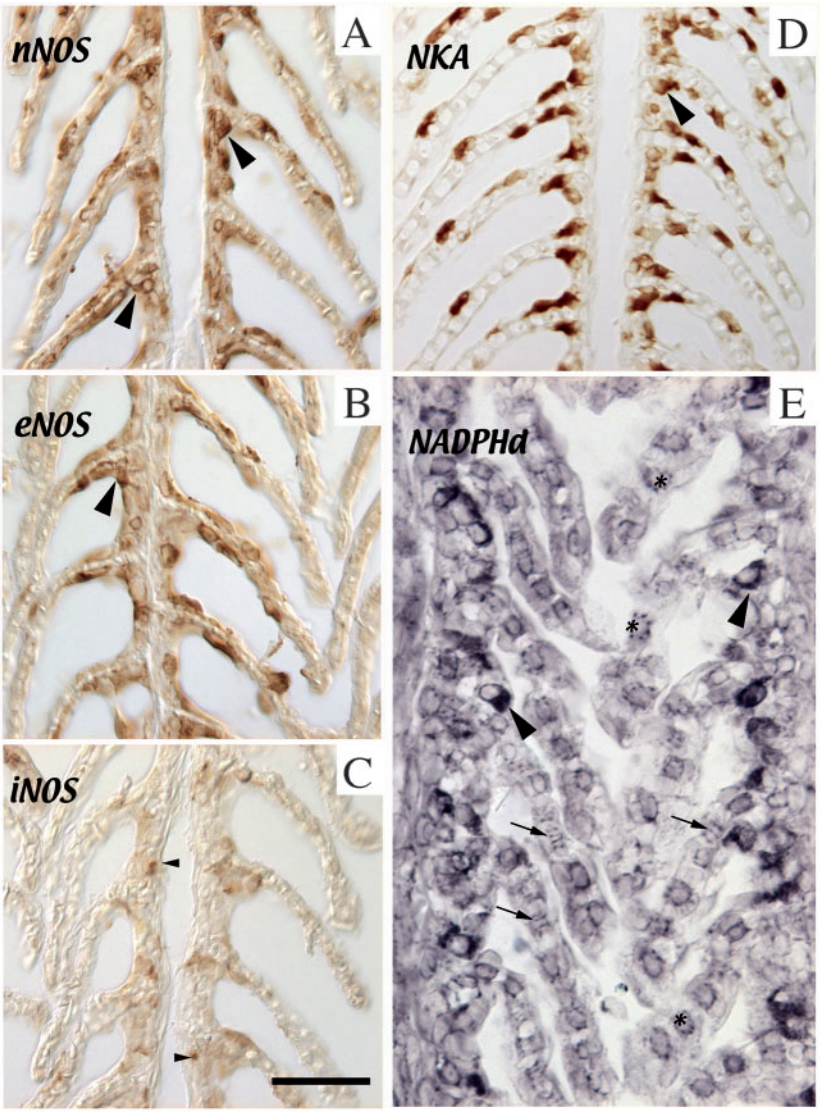

Fig. 1. (A–D) The presence of NO-producing cells (A–C) was compared with that of Na^+,K^+-ATPase (NKA) α-subunit distribution (D) in the gill of Atlantic salmon. nNOS (A) and eNOS (B) immunoreactive cells was mainly restricted to large cells at the base of and along the secondary lamellae (large arrowheads) and in the inter-lamellar spaces of the primary filaments. Only small cells located deep in the filament were iNOS immunoreactive (C, small arrowheads). (E) NADPHd histochemistry revealed strong staining of large cells similar to the staining with nNOS and eNOS. In addition, a distinct NADPHd punctuate staining (arrows) and band staining (*) were observed on the lamellae (E). Scale bar, 100 μm.

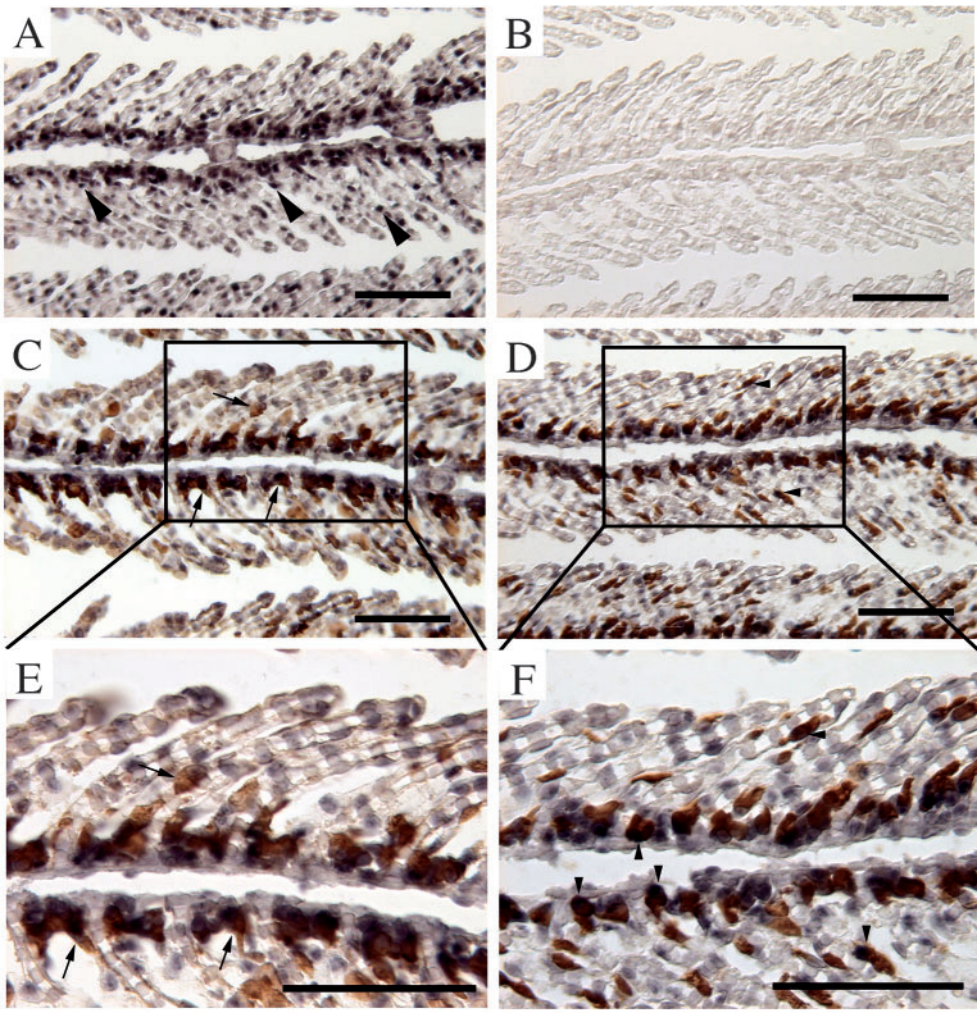

Fig. 2. (A) The expression of Na^+,K^+-ATPase (NKA) mRNA in the gill was determined using the antisense probe and was found in distinct cells on the primary filament and lamellae (large arrowheads). (B) The sense Na^+,K^+-ATPase mRNA probe revealed no staining. (C,E) The Na^+,K^+-ATPase mRNA and protein were colocalized within the same cells (small arrows). (D,F) Double labeling for Na^+,K^+-ATPase mRNA and nNOS revealed colocalization of these enzymes (arrowheads). Scale bar, 100 μm.

Colocalization of Na^+,K^+-ATPase α-subunit mRNA and nNOS

The distribution of NADPHd-positive labeling, nNOS, eNOS and Na^+,K^+-ATPase immunoreactivity, and the Na^+,K^+-ATPase α-subunit mRNA on adjacent sections indicated similar localization of NOS and NKA in chloride cells. The co-localization within the same cells was confirmed via double labeling for simultaneous visualization of nNOS and NKA immunolabeled structures and NKA α-subunit mRNA. This was achieved by first processing sections for *in situ* hybridization of the NKA α-subunit mRNA as described in the previous section, but after the stop solution, the sections were processed for nNOS immunocytochemistry as described above. The sections where then mounted in 50% glycerol in stop solution.

In vitro *Na^+,K^+-ATPase activity*

To test the effects of NO on gill Na^+,K^+-ATPase activity in Atlantic salmon, an *in vitro* study was performed by administration of NO donors to isolated gill samples and recordings of changes in rubidium ion uptake. The fish used in this *in vitro* study were Atlantic salmon smolts (20–40 g), acclimated to seawater (SW; 35 p.p.t.) at the Odense University Campus (15°C, 12 h:12 h light:dark artificial photoperiod) for at least 2 months. They were fed a maintenance diet of commercial trout pellets (2% body mass every second day).

Tissue preparations and assay procedure was done according to Tipsmark and Madsen (2001) with only minor modifications. Briefly, gill arches were excised of the cartilage and rinsed in salmon Ringer's solution equilibrated with 99% O_2/1% CO_2 (in mmol l^{-1}: 140 NaCl, 15 $NaHCO_3$, 3.5 KCl, 1.5 $CaCl_2$, 1.0 NaH_2PO_4, 0.8 $MgSO_4$, 5.0 D-glucose and 5.0 *N*-2 Hepes; pH 7.8) and then cut into small samples (5–10 mg). The samples were equilibrated for 30–60 min in Ringer's prior to experimentation. To measure Rb^+ uptake, the Ringer's was replaced with Rb^+-Ringer's solution containing 3.5 mmol l^{-1} RbCl instead of KCl. The gill samples were incubated in Rb^+-Ringer's for 10 min. Ouabain-sensitive Rb^+ uptake (*in situ* NKA activity) was calculated as the difference between total uptake and uptake in samples both pre-incubated (10 min in Ringer's) and incubated (in Rb^+-Ringer's) with 1 mmol l^{-1} ouabain. After incubation the samples were washed in Tris-sucrose buffer (4×15 min at 0°C; mmol l^{-1}: 10 Tris, 260 sucrose, pH 7.8), blotted onto filter paper and weighed. Ions were extracted overnight at 4°C in 5% trichloroacetic acid (TCA). Potassium chloride (20 mmol l^{-1}) was added and Rb^+ was determined in the extracts by atomic absorption spectrophotometry at 780.8 nm (Perkin Elmer 2380 Mountain View, CA, USA). Rubidium uptake was expressed as nmol mg wet $weight^{-1}$ h^{-1}. The effects of SNP (1 mmol l^{-1}; Sigma) and PAPA-NONOate (NOC-15; 0.5 mmol l^{-1}; Sigma) on NKA activity were measured as ouabain-sensitive Rb^+ uptake in gill tissue from SW-acclimated Atlantic salmon. The samples were pre-incubated 10 min with the NO donors before the assay.

Results

Distribution of NO-producing cells in the gill

The distribution of nNOS immunoreactive (ir) cells (Fig. 1A) was mainly restricted to large cells, putative chloride cells as identified by NKA immunoreactivity (Fig. 1D), localized at the base and along the secondary lamellae and in the interlamellar spaces of the primary filaments. There was a similar pattern of eNOS labeling but it was less intensive (Fig. 1B). iNOS immunoreactivity was only found in small cells located deep in the filament (Fig. 1C). NADPHd histochemistry revealed strong staining of large cells similar to the staining with nNOS and eNOS (Fig. 1E). In addition, a distinct punctate staining was found surrounding cells along the center of the secondary lamellae, and NADPHd-positive bands were observed crossing the lamellae. No such staining was, however, seen with any of the NOS antibodies. Adjacent sections incubated without the primary antibody showed no staining (not shown).

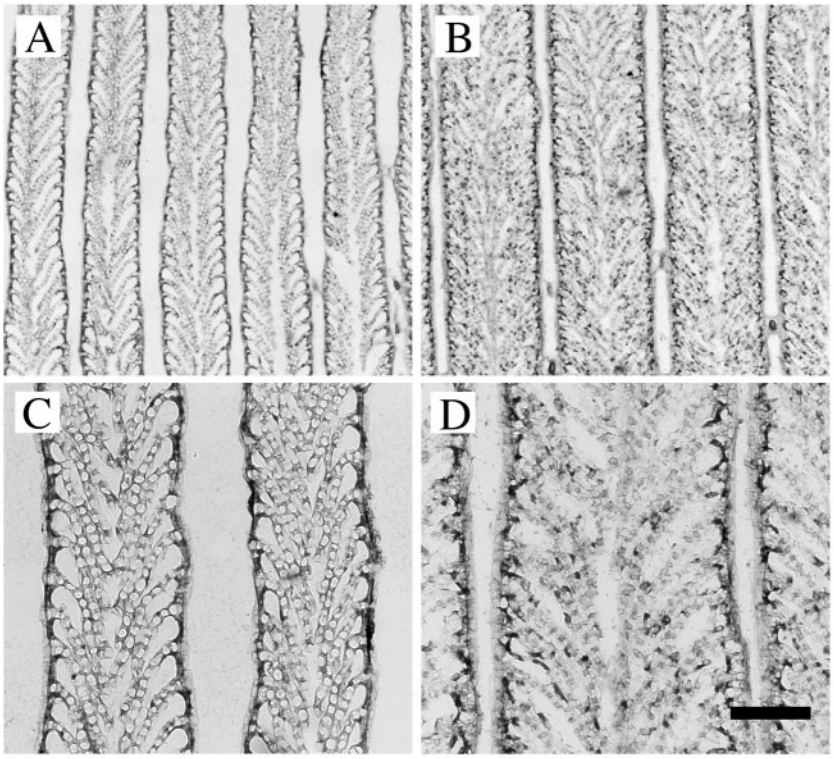

Fig. 3. NADPHd histochemical staining in the gill of salmon parr (A,C) and smolt (B,D). Scale bar, 200 μm (A,B), 100 μm (C,D).

Using the antisense probe for *in situ* hybridization, expression of the Na^+,K^+-ATPase α-subunit mRNA was found in cells on the primary filament and lamellae (Fig. 2A) and the sense NKA mRNA probe revealed no staining (Fig. 2B). The distribution of the NKA α-subunit mRNA is consistent with the distribution of the NKA α-subunit protein, by immunocytochemistry (Fig. 1D). Moreover, the NKA mRNA and protein were found to be colocalized within the same cells by double labeling (Fig. 2C,E). Double labeling with the NKA mRNA and nNOS revealed localization of these enzymes within the same cells (Fig. 2D,F).

NADPHd histochemical staining increased in intensity and distribution from parr (Fig. 3A,C) to smolt (Fig. 3B,D) in particular cells on the secondary lamellae.

In vitro *Na^+,K^+-ATPase activity*

The *in vitro* effects of both NO donors, SNP (1 mmol l^{-1}) and NOC-15 (0.5 $mmol^{-1}$), revealed a significant 30% inhibition of Na^+,K^+-ATPase activity (measured as ouabain-sensitive Rb^+ uptake) in gill tissue from SW-acclimated Atlantic salmon (Table 1).

Discussion

Here we provide the first demonstration of colocalization of NOS activity and Na^+,K^+-ATPase expression in any osmoregulatory tissue in fish. NO-producing cells in the Atlantic salmon gill were visualized by immunocytochemistry using three heterologous antibodies directed at different nitric oxide synthase (NOS) isoforms, namely nNOS, eNOS and iNOS. nNOS and eNOS antibodies both resulted in strong staining in large cells primarily at the base of the lamellae and in the interlamellar space. A few cells on the lamellae also gave positive staining with both antibodies. The immunoreaction was further confirmed with NADPHd histochemistry, a technique used to detect the catalytic enzyme activity of NOS, which can be used as an indicator of NOS activity, i.e. NO production. In addition, we demonstrated an increase of NADPHd staining between parr and smolt.

Previously, the localization of NOS and/or NADPHd in the gills of fish has only been reported in the nitrergic nerve fibers coursing along the efferent branchial artery in the puffer fish

Table 1. In vitro *inhibition of Na^+,K^+-ATPase activity by NO in gill tissue from seawater-acclimated Atlantic salmon*

Treatment	Na^+,K^+-ATPase activity (nmol Rb^+ mg^{-1} wet mass h^{-1})*
Control	2.95±0.17[a]
SNP† (1 mmol l^{-1})	2.03±0.16[b]
NOC-15† (0.5 mmol l^{-1})	2.09±0.17[b]

*Measured as ouabain-sensitive Rb^+ uptake.

†The samples were pre-incubated for 10 min with the NO donors before the assay.

Values are means ± S.E.M. (N=10). Different letters indicate significant difference (one-way block ANOVA followed by Tukey's HSD test; P<0.01).

Takifugu niphobles (Funakoshi et al., 1999). Also, NADPHd activity has been localized to parasympathetic postganglionic neurons in branchial nerves nIX and nX in the cod *Gadus morhua* (Gibbins et al., 1995). Judging by the location and shape of the present NO-producing cells, they appear identical to chloride cells. To investigate this further, gill sections were probed for the presence of NKA α-subunit mRNA and protein, which are particularly abundant in chloride cells in the gill of Atlantic salmon smolts (Karnaky et al., 1976; Pelis et al., 2001; Wilson and Laurent, 2002). The protein and messenger encoding for the NKA α-subunit were coexpressed primarily in cells at the base of the lamellae and interlamellar space. In addition, α-subunit protein was found sporadically in cells along the lamellae, where α-subunit mRNA was not heavily expressed. These cells may represent FW-type ionocytes that are about to retreat as the salmon smolt enters SW. FW-type chloride cells in lamellar positions are known to disappear when salmonids are moved into SW (Seidelin et al., 2000; Uchida et al., 1996). Thus, there is a reduced mRNA expression in these cells even though they maintain the abundance of active protein. Direct evidence for colocalization of the enzyme with nNOS was obtained from double labeling experiments, combining nNOS immunocytochemistry with Na^+,K^+-ATPase *in situ* hybridization. These experiments clearly showed that putative SW-type chloride cells are functionally equipped with both ion-transport (Na^+,K^+-ATPase) and NO-production (NOS) capacity. In this study, FW Atlantic salmon at the peak of smolt development were used for the analyses. One of the most prominent characteristics of this developmental stage of the salmon is its readiness for hypo-osmoregulation (Björnsson et al., 1989; Seidelin et al., 2001). While still residing in FW at the smolt stage, very high levels of the Na^+,K^+-ATPase enzyme localized in typical SW-type chloride cells have been reported in numerous studies (Pelis et al., 2001; Pisam et al., 1988; Ura et al., 1997). If these fish are transferred to SW, the osmotic disturbance is minimal compared to the parr stage, with low levels of gill Na^+,K^+-ATPase, suggesting that chloride cell development and high levels of Na^+,K^+-ATPase are part of the development of hypo-osmoregulatory ability as a pre-adaptation to seawater entry. In order to maintain hydro-mineral balance while still in fresh water during the final stages of smoltification, it is, however, important to prevent excessive ion loss. Therefore, *in situ* inhibition of the Na^+,K^+-ATPase might be anticipated. The presence of NO donors *in vitro* does indeed significantly inhibit Na^+,K^+-ATPase by 30%, providing preliminary evidence that this may indeed be one of the important functions of NOS activity in the salmon smolt gill, i.e. to reduce hydrolytic Na^+,K^+-ATPase activity, thereby deactivating ion excretion until SW is encountered. This part of the study was done using gills from SW-acclimated smolts, which have roughly the same total hydrolytic capacity as measured in gills of FW smolts. The increase in NADPHd staining from parr to smolt shown here further support this role. The increase in staining in smolts may represent stimulated NOS gene expression in existing chloride cells or be related to the general increase in chloride cell abundance associated with smoltification. With regard to the stimulus for increased NOS expression we speculate that thyroid hormones may have some role during smoltification. Thyroid hormones stimulate NOS gene expression in mammals (Ueta et al., 1995) and are characteristically high during the mid to later stages of smoltification. Taken together, our data suggest an important role of NO in inhibiting the peak Na^+,K^+-ATPase activity while the smolt is still in FW, thus preventing excessive ion loss. Furthermore, these results are in agreement with the notion that NO plays an important role as an inhibitor of development once a certain competence is reached (Bishop and Brandhorst, 2003).

Within the last decade, NO has proved to influence ion regulation in several tissues. In mammals, NO has been shown to have inhibitory effects on Na^+,K^+-ATPase activity in kidney (Liang and Knox, 1999), liver (Muriel and Sandoval, 2000) and brain (Sato et al., 1997). Stimulatory effects have also been shown in the trachea (de Oliveira Elias et al., 1999) and heart (Gupta et al., 1994), as well as opposing influences depending on the kidney segment (Ortiz and Garvin, 2002). Inhibition of Na^+,K^+-ATPase activity by NO was recently found in the brown trout (*Salmo trutta* L.) kidney and gill (Tipsmark and Madsen, 2003), and there is support that the action is mediated by local production of cGMP. Juxta-localization of NOS and Na^+,K^+-ATPase has recently been reported using immunocytochemistry in the killifish opercular membrane (Evans, 2002), which differs from our findings showing them to be colocalized. The physiological significance of this observation in killerfish was supported by data showing the inhibition by NO of short-circuit current (I_{sc}) across the killifish opercular skin (Evans, 2002).

In addition to putative chloride cells, NADPHd staining was also localized to structures in the secondary lamellae. These appeared as occasional bands or punctuate structures, similar to the extracellular arrangement of collagen strands found in association with branchial pillar cells (Hughes and Weibel, 1972; Olson, 2002). This staining may be an artifact, as immunocytochemistry did not reveal similar structures. However, if real, the presence of NADPHd staining in this location of the gill may suggest a role of NO in regulating vascular resistance within the gill. Pillar cells may be contractile and respond to paracrine control (Olson, 2002), and NO is well recognized as a vasodilator in mammals, and more recently in fish (Fritsche et al., 2000; Haraldsen et al., 2002).

In conclusion, this study suggests a role of NO in regulation of branchial ion transport. Colocalization of NOS activity and the essential ion pump, Na^+,K^+-ATPase in chloride cells of FW salmon smolts suggests an important physiological inhibition by NO of salt extrusion at a critical developmental stage.

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