Effects of cortisol on chloride cells in the gill epithelium of Japanese eel, *Anguilla japonica*

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Abstract

The purpose of the present study was to determine the effects of cortisol on the development of the freshwater chloride cell (CC), using flow cytometry. Scanning electron microscopy was used to determine the corresponding modifications in CC apical structure. Simultaneously, biochemical analyses were conducted to determine the activities of transport ATPases, mitochondrial enzymes (succinate dehydrogenase (SDH) and Mg^{2+} -ATPase) and lactate dehydrogenase (Ldh). The effects of daily i.m. injection of $2\text{ }\mu\text{g/g}$ cortisol were compared with sham-injected freshwater-, control freshwater- and seawater-adapted fish. The hormone did not affect the activities of Ca^{2+} -ATPases in CCs. However, it stimulated the proliferation and differentiation of the two freshwater CC subtypes (F1, $66 \pm 2.18\%$ (S.E.M.) and F2, $34 \pm 2.18\%$), in which the relative proportion of F1 CCs was transiently reduced in the first 5 days of

treatment (F1, $53 \pm 1.83\%$; F2, $47 \pm 1.83\%$) but was then restored to a higher relative percentage on day 10 (F1, $70 \pm 1.42\%$; F2, $30 \pm 1.42\%$). Biochemically, it induced the activities of Na^+/K^+ -ATPase, Mg^{2+} -ATPase, SDH and Ldh, suggesting an increase in ion pumping and its associated metabolic activities. CCs from cortisol-treated fish demonstrated recessed apical morphology, accompanied by an increase in cell density ($2012 \pm 2413/\text{mm}^2$). Nevertheless, the extent of cell proliferation and differentiation and the biochemical changes were significantly lower than those of seawater fish. Our results indicate that cortisol alone cannot stimulate a complete differentiation of freshwater CCs to seawater CCs. However, the respective roles of the two CC subtypes in freshwater and seawater environments are indicated.

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Introduction

Cortisol, a major corticosteroid factor secreted by the interrenal gland, has been proven to be important in hypo-osmoregulation in teleost fish (Mayer *et al.* 1967, Mommsen 1984, Madsen 1990). Its hormonal action may affect water and electrolyte transport in tissues of the gill, gut, kidney, urinary bladder and muscle, thereby influencing the concentration of electrolytes in blood and urine (Mayer *et al.* 1967, Henderson & Garland 1980, Veillette *et al.* 1995). In the initial phase (first 5 days) of seawater acclimation, a marked transitory increase in plasma cortisol levels has been detected (Ball *et al.* 1971, Assem & Hanke 1981). The duration of the elevated plasma cortisol level is correlated with morphological changes in the gill epithelium, whereby a drastic cellular and functional reorganization occurs to adjust to this short-term osmotic crisis (Chan & Woo 1978, Chan *et al.* 1978, Chan & Wong 1997). Within the first 4 h of acclimation, gill Na^+ excretion activity is recruited, followed by an emergence of new (transitory) subtypes of chloride cell (CC) (Chan 1967, Wong & Chan 1999a). In the next 2–4 days, there

is a profound increase in the number and size of CCs, together with a marked increase in Na^+/K^+ -ATPase activities and mitochondrial contents (Wong & Chan 1999b). An enlargement of the CC apical pit was observed where Cl^- was excreted. Accompanying the maturation of seawater CCs, normonatremia and normokalemia were restored.

Considerable numbers of studies have demonstrated that exogenous cortisol treatment stimulates Na^+/K^+ -ATPase activities, mitochondrial content and cellular differentiation of both freshwater and seawater CCs (Laurent & Perry 1990, Madsen 1990, McCormick 1990, Seidelin *et al.* 1999). They indicated that exogenous cortisol treatment can simulate biochemical and cellular modification as in the case of seawater acclimation. Recently the specific action of cortisol was addressed by the demonstration of cortisol receptor mRNA and immunity in gill CCs (Uchida *et al.* 1998). However, information on its possible effect in the regulation of different CC subtypes is limited. Our recently developed technique for CC subtype identification provides an excellent tool to study the cytological changes of these cells upon cortisol

stimulation. It also provides good statistics by analysis of high numbers of cells. The freshwater (F1, F2), transitory (T1, T2) and seawater (S1, S2) CC subtypes defined in our previous study appeared sequentially during seawater acclimation. The F1 cell was the main subtype in freshwater gill. The cells were bigger in size, but contained lower levels of mitochondrial autofluorescence than F2. The T1 and T2 subtypes, which were of bigger cell size and more intense mitochondrial autofluorescence, appeared within 2–4 days of acclimation. The S1 and S2 were the largest subtypes with highest mitochondrial autofluorescence that emerged in seawater fish (Wong & Chan 1999a). Hence they could be used as a reference to compare the corresponding CC subtypes induced by cortisol treatment, shedding light on the complex changes in gill CCs which are not accessible with conventional techniques. However, the main drawback of this technique is the lack of structural and biochemical information on the tissues. This limitation can partly be overcome by scanning electron microscopy (SEM) and biochemical analysis. The use of these techniques together could provide a better understanding on the effects of cortisol on CC differentiation.

Materials and Methods

Animals

Japanese eels (*Anguilla japonica*) weighing between 500 and 600 g, were reared in fiberglass tanks supplied with charcoal-filtering aerated tap-water at 18–20 °C under a 12 h light:12 h darkness photoperiod for at least 3 weeks. Since the fish were sexually immature (gonadosomatic index $\leq 0.18\%$), mixed sexes were used to prepare gill cells for this study. Freshwater-adapted eels ($n=32$) received daily i.m. injections of cortisol (hydrocortisone 21-hemisuccinate sodium salt; 2 µg/g body weight) dissolved in 0.1 ml 0.9% (w/v) NaCl. The fish were anesthetized in 0.05% tricaine methanesulfonate and killed at predetermined intervals (day 2, 5 and 10) by spinosectomy according to the animal care regulations of the University. Three control groups were used including one negative control group and one sham (NaCl-injected) group maintained in freshwater, while one positive control group was transferred from freshwater to seawater for 10 days.

Cell isolation and flow cytometric analysis

Gill cells were isolated using a three-step discontinuous percoll gradient system described previously (Wong & Chan 1999b). Flow cytometric analysis was performed on a Coulter (Hong Kong) EPICS Elite Flow Cytometer (FCM), equipped with an Argon laser (15 mW) emitting at 488 nm. The instrument had a standard optical filter configuration with band pass 530/30 nm and 585/42 nm

filters for FL1 and FL2 respectively, and a 610 nm band pass filter for FL3. The FCM was calibrated before use with standard DNA beads.

Isolated gill cells (about 10^6 cells/ml in Hanks' balanced salts solution (HBSS)) were analyzed directly by FCM. The change in cell size (forward light scatter (FLS)), and autofluorescence at 525 nm (FL1) were recorded. Individual cell population was sorted to reconfirm the cell type identification, using mitochondrial staining. Briefly, the sorted cells were stained by incubation with 1 µM rhodamine-123 for about 45 min. After washing in 10 mM PBS, the preparation was examined (excitation 488 nm, emission 512 nm) in a laser confocal microscope using an Argon light source (MRC-600; BioRad). The cells showed intense mitochondrial staining, indicating their identity as CCs.

Cell sizing and counting

Freshwater-adapted eels ($n=28$) received daily i.m. injections of cortisol (2 µg/g body weight) and were killed at predetermined intervals (day 2, 5 and 10). Isolated CCs were counted and sized using a Coulter Multisizer II, with an orifice tube of 70 µm in diameter and with isoton II as electrolyte. The cell count signal was the change of conductance of the electrolyte induced by particle resistance. Isoton II was used as blank and calibration was carried out with monodiameter particles (PDVB latex 5.06 µm; Coulter). The aperture coincidence correction was below 2%.

SEM and morphometric measurements

The excised gill arch was fixed overnight in 2.5% glutaraldehyde in HBSS at room temperature. After dehydration in ethanol and critical point drying with CO₂, the tissue was mounted onto aluminum stubs and coated with gold-palladium. The preparation was then examined under a Leica Stereoscan-440 SEM. The area of the filament epithelium on the trailing edge of the filaments was focused and photographed at a magnification of $1000\times$. At least five non-contiguous fields were randomly photographed from each fish for morphometric analysis. Measurement of the apical surface areas of CCs was accomplished using SEM and morphometry software (Quantimet 500C; Leica, Germany).

Biochemical analysis

Freshwater-adapted eels were pretreated with daily 2 µg/g cortisol injection for 10 days. Na⁺/K⁺-ATPase, Mg²⁺-ATPase, alkaline Ca²⁺-ATPase, low- and high-affinity Ca²⁺-ATPases, succinate dehydrogenase (SDH) and lactate dehydrogenase (Ldh) activities in isolated CCs were

determined as described before (Chan & Wong 1997, Wong & Chan 1999b).

Statistical analysis

All data are represented as means $\pm$ S.E.M. Statistical significance was tested by ANOVA followed by Duncan's multiple range test. The level of significance was set at a *P* value of <0.05 .

Results

FCM and cell-sizing analysis

In freshwater control fish, two CC subtypes, F1 and F2, were identified (Fig. 1A). The F1 CC was bigger in size but lower in autofluorescence than the F2 CC. The relative proportions of these two cell subtypes were 66 ± 2.18 and $34 \pm 2.18\%$ respectively. Chronic daily injection of $2 \mu\text{g/g}$ cortisol stimulated the proliferation and differentiation of freshwater CCs. On day 10 of cortisol treatment, the yield of CCs was increased by about 2-fold ($2.96 \pm 0.22 \times 10^5$ to $6.37 \pm 0.43 \times 10^5$) (Table 1). The mean cell size and mitochondrial autofluorescence of both F1 and F2 CCs were also increased (Fig. 1B–D). In addition to the cytological changes, an adjustment in the relative proportion of F1 and F2 CCs was observed. In the first 5 days of treatment, there was a transient reduction and induction of the relative percentage of F1 (decrease from 66 ± 2.18 to $53 \pm 1.83\%$) and F2 CCs (increase from $34 \pm 2.18\%$ to $47 \pm 1.83\%$) respectively. However, on day 10, the F1 CCs increased back to a higher percentage of about $70 \pm 1.42\%$ whereas F2 CCs decreased to $30 \pm 1.42\%$. The size and mitochondrial autofluorescence of the F1 and F2 CCs from cortisol-treated fish were considerably lower compared with the S1 ($50 \pm 2.01\%$) and S2 ($49 \pm 2.01\%$) CCs in seawater fish (Fig. 1F). There were no noticeable cytological changes in the F1 ($67 \pm 1.72\%$) and F2 ($33 \pm 1.72\%$) CCs in the sham group (Fig. 1E), indicating that saline injection had no prominent stimulatory effect on the differentiation of freshwater CCs.

SEM

Figure 2 shows representative SEMs illustrating the external surfaces of the primary gill lamellae. They were covered by the most abundant cell type, pavement cells (PVCs) which showed concentrically arranged micro-ridges on their surfaces, while CCs were relatively scarce with characteristic apical area extrusions extending out between PVCs. In freshwater fish (Fig. 2A), CCs displayed characteristic convex apical surfaces with abundant microvilli. In seawater-adapted fish (Fig. 2B), the apical surface of CCs contained less microvilli and was recessed below the surface of neighboring PVCs. In 10-day

cortisol-treated freshwater-adapted eels, the exposed apical surface of CCs showed abundant microvilli; however, it demonstrated recessed apical morphology compared with the convex appearance in freshwater control fish. Quantitatively, there was a significant increase in the density of CCs from the cortisol-treated group (Table 2), resulting in an increase in the total CC fractional area (CCFA: individual CC apical membrane area $\times$ the density of CCs per unit mm^2). The increase may be due to the retraction of neighboring PVCs or swelling of the CCs themselves (Fig. 2C–F).

Biochemical analyses

To quantify the changes in transport ATPase, mitochondrial and metabolic enzymes in association with the cytological changes of CCs, the activities of Na^+/K^+ -ATPase, alkaline Ca^{2+} -ATPase, low- and high-affinity Ca^{2+} -ATPases, Mg^{2+} -ATPase, SDH and Ldh were determined (Table 3). The Na^+/K^+ -ATPase, Mg^{2+} -ATPase, SDH and Ldh activities increased significantly in the cortisol-treated group. No significant changes in the activities of Ca^{2+} -ATPases were detected.

Discussion

FCM analysis showed the effect of exogenous cortisol in exerting a stimulatory effect on both CC subtypes, inducing cell proliferation and differentiation. There were approximately 2.3- and 1.9-fold increases of F1 and F2 CC numbers respectively, after 10 days of cortisol treatment. The newly synthesized CCs were possibly derived from the stem cells in the gill interlamellar region (Uchida *et al.* 1998, Wong & Chan 1999a). Accompanying cell proliferation, a two-phase adaptational change in the two freshwater CC subtypes was observed. The first phase of cortisol injection (day 1–5) activated the hypo-osmotic responses of gill epithelia in which the relative percentage of F1 CCs decreased from 66 to 53% while F2 CCs increased from 34 to 47%. The change may be similar to the initial phase of seawater adaptation as described in our previous study in which a reduction of F1 (67.5 to 29%) and an induction of F2 (32.5 to 35.5%) CCs were observed (Wong & Chan 1999a). Other studies have also reported a decrease of β , A or lamellar CCs and an increase in α , B or filamental CCs during seawater acclimation (Shirai & Utida 1970, Pisam & Rambourg 1991, Uchida *et al.* 1996, Shikano & Fujio 1998). The F1 and β cells were suggested to be specifically involved in freshwater adaptation while the F2 and α cells were postulated to be differentiated into seawater CCs (Pisam *et al.* 1993, Shikano & Fujio 1998, Wong & Chan 1999a). Likewise, it was hypothesized that the lamellar CC was active in ion-absorption in freshwater while the filamental CC

Figure 1 Representative FLS vs FL1 dot plots of CC subtypes isolated from gill epithelia of (A) freshwater control fish and (B) 2nd day, (C) 5th day, (D) 10th day of cortisol- and sham- (E) injected fish. It was obtained by analyzing isolated gill CCs ($\sim 10^6$ cells/ml in HBSS) with a Coulter EPICS Elite flow cytometer. (F) Cells isolated from seawater fish. F1 and F2, freshwater, S1 and S2, seawater CC subtypes.

Table 1 The effects of 2 µg/g cortisol treatment on number and mean size (± S.E.M.) of CCs isolated from gill epithelia

	Cortisol treated					Seawater
	Freshwater	Sham	Day 2	Day 5	Day 10	
Number of fish	4	4	5	5	6	4
CC number (× 10 ⁵)	2.96 ± 0.22	3.21 ± 0.28	4.22 ± 0.37*	5.82 ± 0.44*	6.37 ± 0.43*	32.5 ± 0.24*
Mean cell size (µm)	11.6 ± 0.64	11.7 ± 0.72	12.5 ± 0.59	13.2 ± 1.02*	13.6 ± 0.94*	15.3 ± 0.48*

**P* < 0.05 compared with corresponding value in freshwater eels.

participated in ion-secreting activities in seawater (Uchida *et al.* 1996, Seidelin & Madsen 1999). Therefore in the present study, the results suggested that the initial phase of cortisol treatment stimulated the proliferation and differentiation of stem cells and F2 CCs, in promoting the hypo-osmoregulatory function of the gill epithelium. This might result in a negative regulatory effect on the F1 CC. In the second phase of cortisol treatment, however, a restoration of the F1 CC to a higher relative percentage was observed. The discrepancy compared with the first phase of treatment could possibly be a result of the long-term cortisol administration that may cause secondary regulatory mechanisms or functions which nullify or change the effect of the hormone (Chan & Woo 1978, Flik & Perry 1989, Laurent & Perry 1990, Perry *et al.* 1992). The opposite responses of the two freshwater CC subtypes in the two phases of treatment implied their different functions in freshwater and seawater conditions (Pisam *et al.* 1993, Shikano & Fujio 1998, Uchida *et al.* 1998, Seidelin & Madsen 1999, Wong & Chan 1999a).

The FCM analysis resolved the two freshwater CC subtypes and characterized statistically the effects of cortisol on their development. However, no information on the functional activities of CCs was obtained. This limitation could be partly overcome by a study of the CC apical surface, a primary site of ionic transport, to give a better understanding of the specific action of the hormone. Therefore in the second part of the study, SEM and morphometric quantification were conducted to determine the changes in CC density as well as its apical surface area on gill epithelium. Our results indicated that cortisol stimulated a ~26% increase in total CCFA, resulting from a ~20% increase in CC density compared with the seawater fish in which a ~39% increase in CCFA was contributed by a ~47% increase in CC density. An increase in total CCFA upon cortisol treatment implied the possibility of an activation of CC ion transport activities (i.e. Na⁺ or Ca²⁺ uptake) (Flik & Perry 1989, Laurent & Perry 1990, Perry *et al.* 1992). Laurent & Perry (1990) reported that long-term cortisol treatment could stimulate the abundance of lamellar CCs in promoting ion-absorbing activities. A similar observation was reported in which 10-day cortisol (4 µg/g) injection stimulated an

increase of CC apical surface area in gill epithelia of the American eel in facilitating gill Ca²⁺-uptake (Perry *et al.* 1992).

The different responses of the two freshwater CC subtypes and the upregulation of transport ATPases were demonstrated; however, biochemical aspects of the mechanisms supplying the required ATP for the former processes have been largely neglected. Chan & Woo (1978) demonstrated that transfer of eels to seawater or under cortisol (1 µg/g) treatment caused a marked but transient increase in blood glucose concentration, suggesting stimulation of the utilization of carbohydrate substrates. Analysis of metabolite concentrations across gill epithelia revealed that gill cells had a preference for using lactate and glucose as oxidative substrates (Mommensen 1984). It suggested that Ldh was important in mediating energy balance, in particular of transepithelial ion transport. In fact, the induction of Na⁺/K⁺-ATPase activities and CCFA in cortisol-treated fish was accompanied by an upregulation of the mitochondrial enzymes (SDH and Mg²⁺-ATPase) as well as the metabolic enzyme, Ldh. Our previous finding showed that CCs expressed predominantly Ldh-B₄ isozyme, indicating that the process depended on aerobic metabolism (Wong 1996).

In summary, it is obvious that cortisol exerted a stimulatory effect on CCs, along with the upregulation of transport ATPases, Ldh and mitochondrial enzymes. However, the extent of cellular differentiation and proliferation were considerably less than that in seawater, in which a drastic increase in CC number, mitochondrial content and cellular complexity was observed (Wong & Chan 1999b). In addition transitory CC subtypes, which appeared on the first 3 days of seawater adaptation, were not found after cortisol treatment (Wong & Chan 1999a). The disparities indicated that exogenous cortisol treatment alone could not evoke the complete differentiation and maturation of seawater CCs. In fact during seawater acclimation, a surge in growth hormone level has been reported, which could induce the expression of cortisol receptors in gill CCs (Sakamoto & Hirano 1993, Shrimpton *et al.* 1995, Uchida *et al.* 1998). This, together with an elevated plasma cortisol in the initial phase of seawater adaptation, might synchronously stimulate the

Figure 2 Representative SEMs of the filamentous epithelia of (A) freshwater- and (B) seawater-adapted eels. PVCs and CCs are characterized by the microridge (single arrow) and microvilli (double arrows) appearance, respectively. Note the freshwater CCs displayed a convex apical surface with abundant microvilli whereas seawater CCs contained less microvilli and were recessed below the surface of neighboring PVCs. Apical surface of CCs from 10-day cortisol-treated fish (C) and (E) Sham-treated fish illustrate abundant microvilli as well as recessed apical morphology. Note the retraction of neighboring PVCs or swelling of the CC itself (open arrowheads). Panels (D) and (F) are higher magnification of (C) and (E) respectively. The scale bars in (A–C, E) represent 5 μm; scale bars in (D, F) represent 1 μm.

Table 2 The effects of 10 days of 2 µg/g cortisol treatment on CC density, apical area and fractional area (means ± S.E.M.) of gill CCs. Apical area refers to mean apical area of individual CCs

	<i>n</i>	CC density (per mm ²)	Apparent exposed CC apical area (µm ²)	CCFA (%)
Freshwater	4	2012 ± 182 ^c	10.28 ± 1.13	2.07 ± 0.18 ^b
Sham-treated	5	2053 ± 165 ^c	10.31 ± 1.23	2.12 ± 0.20 ^b
Cortisol-treated	6	2413 ± 201 ^b	10.83 ± 1.18	2.61 ± 0.27 ^a
Seawater	4	2964 ± 264 ^a	9.68 ± 0.95	2.87 ± 0.25 ^a

Data with the same letter superscript in the same column are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$).

Table 3 The effects of 10 days of 2 µg/g cortisol treatment on transport ATPase, SDH and Ldh (means ± S.E.M.) in isolated gill CCs

	Freshwater	Sham	Cortisol	Seawater
Number of fish	7	6	8	5
Na ⁺ -K ⁺ -ATPase nmol . mg protein ⁻¹ . min ⁻¹	3.26 ± 0.25 ^c	3.05 ± 0.24 ^c	17.51 ± 0.56 ^b	242.6 ± 8.67 ^a
Mg ²⁺ -ATPase nmol . mg protein ⁻¹ . min ⁻¹	1.87 ± 0.12 ^c	1.83 ± 0.10 ^c	2.26 ± 0.11 ^b	67.27 ± 1.54 ^a
SDH µmol . mg protein ⁻¹ . min ⁻¹	217 ± 9.11 ^c	205 ± 9.94 ^c	251 ± 3.60 ^b	1688 ± 25.21 ^a
Alkaline Ca ²⁺ -ATPase nmol . mg protein ⁻¹ . min ⁻¹	98.32 ± 4.95 ^a	97.80 ± 2.37 ^a	95.34 ± 1.04 ^a	31.20 ± 2.33 ^b
Low-affinity Ca ²⁺ -ATPase nmol . mg protein ⁻¹ . min ⁻¹	52.42 ± 2.34 ^a	51.00 ± 1.39 ^a	50.98 ± 0.85 ^a	21.42 ± 0.71 ^b
High-affinity Ca ²⁺ -ATPase nmol . mg protein ⁻¹ . min ⁻¹	11.93 ± 0.91 ^a	11.72 ± 0.66 ^a	11.69 ± 0.50 ^a	2.21 ± 0.15 ^b
Ldh µmol . mg protein ⁻¹ . min ⁻¹	0.15 ± 0.01 ^c	0.15 ± 0.01 ^c	0.24 ± 0.01 ^b	1.67 ± 0.10 ^a

Data with the same letter superscript in the same row are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$).

complete differentiation and maturation of seawater CCs (Ball *et al.* 1971, Assem & Hanke 1981, Seidelin *et al.* 1999). However, in the present study, the cortisol-treated fish were maintained in a freshwater environment, therefore CCs were not necessarily equipped with substantially high levels of mitochondrial and transport ATPases for Na⁺ and Cl⁻ excretion. As demonstrated by Foskett & Hubbard (1981), cortisol injection increased the density of CCs in the opercular membrane of freshwater fish; however, no chloride secretion was activated. In addition, long-term cortisol injection alone may cause a downregulation of cortisol receptors in the gill CC (Shrimpton & Randall 1994). In the absence of a growth hormone surge to limit the decline in cortisol receptors (Shrimpton *et al.* 1994), the extent of CC proliferation and differentiation were attenuated. Despite the interpretation stated above, the limitation in the present study of utilizing three separate analytical approaches including (i) FCM, (ii) biochemical methods to analyze the isolated CC, and (iii) SEM to examine the trailing edge CCs, should be addressed. The difficulty in integrating results from these approaches is the inability to relate the particular CC subtypes to their respective locations in gill epithelia. It is possible that our interpretation would be at variance with the results of such an investigation.

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