Skin ultrastructure in relation to prolactin and MSH function in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental acidification

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Abstract. The present study describes the effects of 14 days exposure to acidified (pH 4.0) soft water in the absence of aluminium, on the ultrastructure of the skin in rainbow trout (*Oncorhynchus mykiss*). Compared to control fish, there was a moderate increase in the incidence of necrosis in the filament cells of the fish exposed to pH 4.0, although these changes appeared to be maintained, most of the ultrastructural changes observed may be considered to be adaptive. There was an increase in epidermal thickness, a higher frequency of electron-dense vesicles in filament cells, an increase in the undulation of the basal lamina, and the penetration of the epidermis by cytoplasmatic processes of melanocytes in acid-exposed specimens. An infiltration of leucocytes into the epidermis, and the appearance of serous mucous cells, was also evident. Whether these changes were under control of prolactin and/or α-MSH, was also investigated, but no indication for activation or inhibition of either prolactin or α-MSH producing cells was obtained. Since a previous study (Balm and Pottinger 1993) had demonstrated that plasma cortisol levels were also identical in control and low pH treated trout throughout a 14 day experimental period, it is concluded that under conditions of environmental acidification, the integument autonomically maintains the adjustments necessary for successful acclimation, presumably via paracrine regulatory circuits.

Key words: Skin – Acidification – Prolactin – α-MSH – Melanocytes – *Oncorhynchus mykiss* (Teleostei)

Introduction

The detrimental effects of environmental acidification on fish populations have been well documented. Around pH 5, the toxic effects of acidification are most likely due to synergistic effects of protons and toxic metals, in particular aluminium (Wood et al. 1988). At pH levels below 4.5, most authors have proposed that the protons are the major toxicant. This is based on observations that at these pH levels, fish display major distress both in the presence and absence of aluminium. Consequently, it has been postulated that protons and aluminium ions compete for branchial binding sites (Neville and Campbell 1988). In a recent study, we were able to demonstrate that rainbow trout (*Oncorhynchus mykiss*), the most acid-sensitive salmonid species (Grande et al. 1988), under soft water conditions could acclimate remarkably well to pH 4.0 in the absence of aluminium (Balm and Pottinger 1993), without any apparent rise in plasma cortisol or any other sign of activation of the pituitary-interrenal axis. This raises the question as to whether acid water in the absence of aluminium, has any negative effects on these fish, and, if so, how acclimation is achieved.

It is essential for fish to maintain the integrity of the skin. Skin plays a major role in the animal’s defense, mainly via the secretion of mucus, which possesses bacteriocidal properties (Fletcher 1981; Shepard 1993), and has recently been demonstrated to be highly responsive to environmental changes (Whitear 1986), including water acidification (Notter et al. 1976, Zuchelkowski et al. 1985; Iger and Wendelaar Bonga 1994). Water of pH 5 evokes changes in carp skin, such as increased mucus secretion, cell proliferation, leucocyte infiltration, and pigment migration (Iger and Wendelaar Bonga 1994). Prolactin and α-MSH have respectively been implicated in mucus secretion and pigmentation of piscine skin (Wendelaar Bonga and Meis 1982; van Eys and Peters 1981). Moreover, enhanced secretion of prolactin (Wendelaar Bonga et al. 1984) and α-MSH (Balm et al. 1987) occurs in tilapia (*Oreochromis mossambicus*) exposed to acid water. Prolactin is also commonly referred to as one of the pivotal hormones regulating hydromineral balance in teleosts (Hirano et al. 1987).

The present investigation examines the effects of environmental acidification on the ultrastructure of rainbow trout skin, and on prolactin- and MSH-cell function.
Materials and methods

Immature rainbow trout were gradually exposed under flow through conditions to pH 4.0 in soft Windermere lake water, the decline from ambient pH to pH 4.0 occurring over a 6 h period (see Balm and Pottinger 1993). Fish were sampled after 14 days exposure to pH 4.0, both in 1989 (experiment 1; n=28), and in 1991 (experiment 2; n=16). Control groups were maintained at ambient pH (7.1) throughout (Balm and Pottinger 1993). After anaesthetizing the fish in 2-phenoxyethanol (1:2000, Sigma), blood was collected from the caudal vessels and treated with EDTA/aprotinin (1.5 mg/3000 KIU per ml blood, Sigma) and the plasma stored at -20°C. Pituitaries from fish taken at random, were either fixed for light microscopy (Bouin's; n=4 per group), or superfused in vitro (n=6 per group) as described by Balm and Pottinger (1993) for analysis of prolactin and α-MSH production. Skin samples were removed from the dorsal part of the head (5 fish per group), and were prepared for electron microscopy.

Electron microscopy

Tissues were fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.3), washed in buffer and post-fixed in osmium tetroxide (1%) in the same buffer. Ethanol-dehydrated tissues were embedded in Spurr's resin, with the tissue oriented tangentially to the mold. Thin sections were cut perpendicularly to the skin surface, stained with uranyl acetate and lead citrate, and examined in a Jeol CX11 transmission electron microscope. The following parameters were quantified: (1) epidermal thickness; (2) number of electron-dense vesicles (v hed) per pavement cell; and (3), the undulation of the basal lamina (i.e. the number of infoldings of the lamina -'peaks'were counted for fixed intervals). For each fish, 5 measurements per parameter were collected at constant intervals (distance between 7 cells).

Prolactin

Plasma and superfusion fractions were assayed directly for prolactin by homologous radioimmunoassay as described by Prunet et al. (1985). The in vitro data represent the release during the first fraction (30 min; "initial" release), and during three 20 min fractions collected after 3 h ("basal" release). The total volume of the pituitary occupied by the prolactin cells was determined as described by Balm (1986).

α-MSH

Plasma samples and superfusion fractions were assayed directly for α-MSH by radioimmunoassay, using an antisemum (L9) characterized by van Zoest et al. (1989). For the plasma measurements, a second antibody precipitation procedure was employed.

Statistics

Data are presented as means ±SE (n=1). Differences between groups were analyzed by the Mann-Whitney U test (significance was accepted if P<0.05).

Results

A macroscopic survey of the skin showed no differences between control and acidified fish, and both groups appeared free of ectoparasites, as judged by examination of...
The epidermal layer of control animals was 105±8 µm thick (Fig. 1a), and was mainly composed of filament cells, pavement cells (the upper layer of filament cells with an apical membrane folded into microridges; Fig. 2), and mucous cells. In the dermis, loosely arranged collagen fibers, and different types of pigment cells (particularly melanocytes) were observed. In acid-exposed trout, epidermal thickness had increased significantly (Fig. 1a; P>0.012). This was not due to an expansion of the intercellular spaces, but rather, was the result of an increase in the number of cell layers (not shown); mitotic figures were observed in the filament cells throughout the epidermis (Fig. 3), a phenomenon that was rarely observed in controls. In acid-exposed trout necrotic pavement cells were common. These latter were rounded, devoid of microridges, contained swollen mitochondria (Fig. 4), and, in later stages of necrosis, demonstrated swelling of cellular spaces, but rather, was the result of an increase in nuclear thickness, which had increased significantly (Fig. 1a; P>0.012). This was not due to an expansion of the intercellular spaces, but rather, was the result of an increase in the number of cell layers (not shown); mitotic figures were observed in the filament cells throughout the epidermis (Fig. 3), a phenomenon that was rarely observed in controls. In acid-exposed trout necrotic pavement cells were common. These latter were rounded, devoid of microridges, contained swollen mitochondria (Fig. 4), and, in later stages of necrosis, demonstrated swelling of the other cell compartments. Necrotic pavement cells were largely absent in the controls. In acid-exposed fish, many of the filament cells and pavement cells contained numerous vesicles of high electron density (vesicles; Fig. 5), particularly in the 3 to 4 outermost layers of the epidermis, whereas in controls, such vesicles were markedly fewer in number (Fig. 1b; P=0.004). The incidence of multilobed nuclei, Golgi vesicles and rER, were also much less in these latter fish.

In control skin, the epidermis contained mucocytes with electron-transparent mucosomes (Fig. 6). After 14 days at pH 4.0, most mucous cells contained a mixture of electron-transparent and electron-dense mucosomes, extensive rER, and vesicles of high electron density, resembling serous mucous cells (Figs. 7, 8).

The skin of acid-exposed trout did not differ from the control tissue with respect to the sensory cells and chloride cells, which were rare in both groups. Occasional rodlet cells (Fig. 9) were observed in 4 out of 5 acid-exposed fish, but not in the controls. Another striking difference between the epidermis of control and acid-exposed trout was the increase in number of leucocytes in the latter group. In control tissue, only occasional lymphocytes were observed, whereas in the acid-exposed trout the epidermis was infiltrated by numerous leucocytes, macrophages (Fig. 10), lymphocytes (Fig. 11), and granulocytes. The macrophages often showed phagosome- and lysosome-like bodies. No apoptotic leukocytes were observed.

The basal lamina (Figs. 12, 13) was more undulating in the trout kept at pH 4.0 (Fig. 1c; P<0.008). Moreover, bundles of collagen fibers ran perpendicular to the basal lamina, particularly in the "peak"-areas of the basal lamina (Fig. 13). Such bundles were not observed in controls. The melanosomes were more concentrated in the melanocyte cell bodies of acid-exposed fish than in those of controls. Despite this, only in acid trout cellular extensions of these melanocytes, containing melanosomes, could be observed in the epidermis (Fig. 14). Some of these protrusions appeared as apoptotic bodies in epidermal macrophages (Fig. 15).
Data are from experiment 1. See legends of Fig. 16.

Figure 16 depicts the prolactin data obtained from the series of experiments. There were no statistical differences between controls and acid-exposed trout with regard to circulating prolactin levels, in vitro hormone release, and total pituitary volume occupied by prolactin producing cells. Similarly, plasma α-MSH levels and in vitro α-MSH release rates were similar in all groups (Fig. 17).

Discussion

A previous study had demonstrated a remarkably successful acclimation of rainbow trout to pH 4.0 in soft water (Balm and Pottinger 1993), as judged by an absence of appreciable ionoregulatory disturbances, or activation of the pituitary-interrenal axis. The present observations, however, indicate that such treatment nevertheless results in profound alterations in skin ultrastructure. It is unclear what triggers these changes, but irritation of the skin by the high ambient proton concentration may be a major initiating factor. In the long term, any response has to be carefully controlled, since the integrity of the skin is a prerequisite for successful acclimation to any environmental challenge.

The increase in epidermal thickness in acid-exposed trout, coincident with a higher incidence of mitotic figures, indicates that an increase in cell recruitment is a major factor contributing to the increase in epidermal thickness. The greater incidence of filament cell apoptosis and necrosis noted in these fish, suggests also that a new balance between cell proliferation and cell loss has been attained. Increased necrosis (accidental cell death, Wyllie 1981) may reflect a direct damaging effect on the outer cell layer of the skin, whereas the increase in apoptosis (physiologically controlled cell death, Wyllie 1981; Schwartzman and Cidlowski 1993) may be an indirect effect, perhaps caused by increased activity and, consequently, reduced life span of these cells. The higher degree of undulation of the basal lamina, leading to the formation of deep infoldings of the dermis, may maintain rigidity, and thereby the structural integrity of the thickened epidermis of acid-exposed fish.

Several of the ultrastructural changes noted in the skin of the acid-exposed fish, indicate an activation of externally orientated defense mechanisms. First, there was an increase in number of electron-dense vesicles in the pavement cells. These vesicles contain peroxidase activity (Iger and Wendelaar Bonga 1994), which is released to the cell surface, and the covering of an electron-dense deposit over the integument of several of the acid-exposed fish in the present study supports this interpretation. The second event is an increase in electron density of the mucosomes, reflecting an alteration in the nature of the mucus being secreted (Blackstock and Pickering 1980; Pickering and Fletcher 1987), and may indicate an enhanced protective capacity against microorganisms (Fletcher and White 1973; Fletcher 1981; Kraehenbuhl and Neutra 1992). This phenomenon has been reported previously in other species challenged by environmental influences (Ferguson et al. 1992; Iger and Wendelaar Bonga 1994). For instance, the effects of ectoparasitic infestation on the mucous cells of the brown trout (Pickering and Fletcher 1987), bear a striking resemblance to the change in mucosome electron density brought about by environmental acidification in the present study. In this respect, it is worth noting that the so-called epidermal rodlet cells, observed in acid-exposed trout, are considered by most authors to represent parasites, although they have also been interpreted as secretory cells (Cenini 1984).

Two further phenomena in acid-exposed fish might indicate a mobilization of defense mechanisms against tissue infiltration by microorganisms. One is the infiltration of the epidermis by leucocytes. This has also been observed in the branchial epithelium of these animals (Balm and Pottinger 1993), and might be the underlying cause of the transient decline in circulating white blood cells noted in these fish. In the present study, some of the macrophages observed in the epidermis of the low pH treated trout contained pigment granules. Melanin-containing macrophages in teleosts are commonly organized in melano-macrophage centres, which in salmonids are randomly distributed throughout the tissues (Agius 1980). Among their functions, they serve in defense mechanisms (Wolke 1992). Peleteiro and Richards (1990), working on rainbow trout, have suggested that such macrophages had engulfed melanin granules in the dermis, prior to moving through the basal lamina. We did not observe this process in our fish, and therefore suggest that under low pH conditions most of the epidermal melanin has penetrated the epidermis in the cytoplasmatic extensions of the melanocytes, which subsequently become phagocytized by macrophages. This process might also serve as a defense mechanism, since the pigmented system in mammals has been shown to play a modulatory role during (local) inflammatory reactions in the epidermis (Norlund 1992), possi-
by scavenging free radicals (Bustamante et al. 1993). There were no indications that this epidermal melanocyte infiltration was caused by an increase in circulating α-MSH. This hormone is known to regulate dermal melanophore dispersion in fish (van Eys and Peters 1981).

Several of the changes in the structure of the skin reported here (e.g. increased necrosis and apoptosis, secretion of vesicles of high electron density by pavement cells, formation of dense mucosomes, and leucocyte infiltration) have been described also in carp exposed to various challenges, including exposure to water containing copper (Iger et al. 1994a), and acid water (Iger and Wendelaar Bonga 1994). In contrast to the fish used in the present study, in many of these cases the fish displayed an increase in circulating cortisol levels (Iger et al. 1994a; Iger et al., unpublished), and this has been interpreted as evidence for an important regulatory role for this hormone in the skin changes (Marshall 1979; Iger et al. 1994a, b). However, on the basis of the present data this seems unlikely, since many of the effects allegedly controlled by cortisol also occurred in the absence of elevated cortisol levels (see also Balm and Pottinger 1993). It could therefore be argued that the rise in cortisol observed in other studies in combination with changes in skin structure, merely reflects a response to a disturbed homeostasis (Iger et al., 1994a). Under these conditions cortisol could even be detrimental to the adaptive processes in the skin. In particular the stimulating effects of corticosteroids on apoptosis in (mammalian) lymphocytes have been documented (Schwartzman and Cidlow 1993). Accordingly, in the epidermis of our acid treated fish we did not observe apoptotic leucocytes, in contrast to a recent laboratory study on the effects of acidification on trout, in which a rise in plasma cortisol was measured (Iger et al. 1994b). In the latter study also enlarged intercellular spaces were observed between the epidermal cells of the acidified trout, a phenomenon that was absent in the present study.

The skin ultrastructure in fish kept under pH 4.0 conditions provides some evidence of a damaging effect, e.g. an increase in the incidence of necrosis, but this is very moderate compared with the results of a laboratory study by Iger et al. 1994b), performed at pH 5.0. Necrosis was much more extensive in the latter study, which illustrates the impact that experimental conditions may have on adaptive mechanisms (Balm and Pottinger 1993). All other parameters studied, however, demonstrate that our fish have been able to maintain the integrity of the tissue. This is in accordance with a previous study on the gills (Balm and Pottinger 1993), which indicate that they acclimate successfully to these adverse conditions, both in structure and ionoregulatory performance. The same phenomena observed in the skin can also be seen in gill epithelium (leucocyte infiltration, increased rate of apoptosis), thus suggesting they are adaptive events, rather than signs of damage or malfunction. A noticeable difference between skin and gill responses to low environmental pH, however, is that the former tissue shows an increased incidence of necrosis. It may be that gills are better protected against the direct damaging actions of low ambient pH, possibly because the pH at the gill surface is higher than the water pH, as a consequence of the metabolic processes taking place at this site (in particular NH₃ excretion; Neville 1985).

The present results demonstrate that prolactin cell function in the acidified trout was unaffected after 14 d at pH 4.0. Prolactin is considered one of the major endocrine factors regulating hydromineral balance in teleosts, particularly in freshwater (Prunet et al. 1990). Part of this action is achieved through the regulation of mucus secretion (Notter et al. 1976; Wendelaar Bonga and Meis 1981), which in part serves to minimize ion losses via the integument (Fromm 1980; Shepard 1993). In contrast to the present results, environmental acidification has been shown in two other teleost species to activate (Oreochromis mossambicus; Wendelaar Bonga et al. 1984), or to transiently inhibit (Salvelinus fontinalis; Notter et al. 1976; Fryer et al. 1988), the prolactin cells. Interestingly, the activation occurred in O. mossambicus after initially disturbed plasma ion levels, whereas in the case of S. fontinalis, the animals displayed declining plasma ion levels throughout the studies, which might point to a key function for this hormone in the acclimation to low environmental pH. The present data, however, suggest that, at least for rainbow trout, a sustained activation of the prolactin cells is not a prerequisite for successful acclimation to pH 4.0, nor for the responses of the skin. Since these events also occur in the absence of changes in circulating cortisol, and α-MSH, it seems possible that they are regulated via neural mechanisms (Lenke 1991), or locally, possibly through paracrine regulatory circuits involving growth factors, histamines and cytokines released by the leucocytes infiltrating the tissue, as described for the mammalian epidermis (Kupper 1990). Recently, several of these factors have been reported to regulate skin function in teleosts also (Lenke 1991; Vallejo and Ellis 1989), thereby demonstrating the fundamental importance of these regulatory mechanisms.

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