Cortisol Induces Stress-Related Changes in the Skin of Rainbow Trout (*Oncorhynchus mykiss*)

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The ultrastructure of the skin of rainbow trout, *Oncorhynchus mykiss*, was studied over a 7-day period after a single meal containing cortisol. The fish experienced increased plasma cortisol levels for 1 day. Pavement cells contained significantly more vesicles of high electron density, which were also numerous in deeper filament cells, and displayed peroxidase activity. Mitotic cells were common after 4 and 7 days. Increased apoptosis, taken to indicate accelerated ageing, was detected in both pavement and mucous cells. Newly differentiated mucous cells were found close to skin surface, and many mucous cells contained mucosomes of high electron density. The basal lamina became highly folded. The low numbers of leukocytes present in the skin did not change noticeably, but substantially more lymphocytes were apoptotic. The melanosomes in the pigment initially dispersed and subsequently reaggregated in the cell bodies of these cells. The reaggregation was accompanied by apoptosis of melanocyte extensions. The results demonstrate the ability of the hormone to regulate several of the effects observed in the skin of fish challenged by stressors. Other phenomena generally observed in stressed fish, such as pavement cell necrosis and massive leucocyte infiltration, were not found after cortisol treatment. The latter observation indicates that regulatory factors in addition to cortisol must be operative during stress. © 1995 Academic Press, Inc.

The aquatic environment, in particular freshwater, is increasingly subject to chemical and physical changes adverse to life. Such changes may stress fish and other organisms. In fish, stressors such as water acidification in the presence of aluminum, pollution with heavy metals or pesticides, oxygen depletion, or increased water temperature stimulate the activity of neuroendocrine tissues, in particular those belonging to the hypothalamus–pituitary–interrenal (HPI) axis (Tomasso et al., 1981; Fu et al., 1990; Lamers et al., 1992).

Activation of the HPI axis, resulting in transiently or chronically increased plasma cortisol levels, is considered a primary response to stressors. Cortisol promotes processes essential for adaptation to stressors, e.g., energy mobilization, osmoregulation, or synthesis of metallothioneins (Fu et al., 1990; Barton and Iwama, 1991; Redding et al., 1991). Consequently, cortisol levels have been considered a valuable stress index.

Another approach for evaluating the effects of environmental stressors is based on the changes induced by the stressors on the epithelial tissues, which are interfaces with the environment: the epithelia of the intestine, gills, and skin. Marked changes have been described for these tissues in fish exposed to stressors (Whitear, 1986; Lee and Cossins, 1988; Wendelaar Bonga et al., 1990). The effects on the skin induced by different types of stressors (water acidification; water fouling; pollution with lead, cadmium, or copper; or temperature elevation) were remarkably similar (Iger et al., 1988, 1992, 1994a,b; Iger and Wendelaar Bonga, 1994) and may therefore represent
general stressor effects. The changes in the skin were tentatively classified as indirect effects of the stressor (e.g., necrosis) and regulated responses of the tissue to the stressors, such as increased mitosis and apoptosis (Iger, 1992). Furthermore, a distinction was made between locally controlled integumentary adjustments (e.g., phagocytosis of damaged cells and cell remnants) and activities (mitosis, apoptosis) possibly mediated by cortisol (Iger, 1992). However, several of the effects ascribed to cortisol have also been observed in the skin and gills of trout exposed to acid water, in the absence of a stimulation of the HPI axis (Balm and Pottinger, 1993; Balm et al., 1994a). Mechanisms regulating other responses, such as leukocyte infiltration or appearance of serous mucous cells, remain to be identified.

The present study attempts to identify epidermal changes which are potentially controlled by cortisol. Previous studies have demonstrated effects of cortisol administration on the skin of salmonids (McBride and van Overbeeke, 1971). Therefore, the ultrastructure and peroxidase activity of the skin of trout, at various periods after feeding the fish a single meal containing cortisol, have been examined.

MATERIALS AND METHODS

Fifty-six immature male and female trout, *Oncorhynchus mykiss*, weighing 69 ± 19 g (mean ± SD), were kept in two groups for an acclimation period of 25 days in running tap water (pH 7.1–7.4, temperature 10.5–11.5°C). Each group was kept in 400-liter green plastic tanks containing well aerated tap water, with a flow rate of 9–10 liters min⁻¹. The fish were fed once daily (food pellets at a daily ration of 1.25% of their body weight) at 12.00 hr. After the acclimation period, fish of one group were fed a single meal containing cortisol (200 mg kg⁻¹ food) that was added to the food by immersion in ethanol containing dissolved cortisol (Maule and Schreck, 1991). Food for control fish was immersed in ethanol only. The solvent was subsequently evaporated from the food at room temperature.

Blood and tissue samples were taken from fish anesthetized in neutralized MS-222. Four fish from each group were sampled 3.5 hr, 7 hr, and 1, 2, 4, and 7 days after the cortisol meal. For the determination of plasma cortisol levels, blood was taken by puncturing the caudal artery with a heparinized syringe. Plasma cortisol levels were determined by RIA (Balm et al., 1994b). Skin samples were taken from the dorsal part of the heads. The tissues were prepared for conventional electron microscopy, or for the cytochemical detection of peroxidase activity at the ultrastructural level (Iger and Wendelaar Bonga, 1994). Ethanol-dehydrated tissues were embedded in Spurr’s resin. Ultrathin sections, collected on 150 mesh copper grids, were contrasted with uranyl acetate and lead citrate and were examined in a Jeol 100 CXII transmission electron microscope.

Morphometric parameters of the skin were obtained from thin sections (Iger and Wendelaar Bonga, 1994; Iger et al., 1994b). Briefly, the following were assessed:

1. Thickness of the epidermis—three photographs at low magnification (×100) were taken from each fish; negatives were magnified four times, and the thickness of the epidermis was measured; areas with taste buds were excluded from this analysis.

2. Number of vesicles of high electron density—vesicles were counted from seven pavement cells per fish, with a five-cell interval between measured cells; necrotic as well as apoptotic cells were excluded from this analysis.

3. Folding of the basal lamina—peaks higher than the thickness of the lamina were counted from seven basically located filament cells per fish, with a five-cell interval between measured cells.

No mortality occurred. For both skin and cortisol parameters, differences between experimental and control fish were tested using the Mann-Whitney U test; data are presented as means ± SD. P < 0.05 was accepted as statistically significant.

RESULTS

**Epidermis**

**Pavement and filament cells.** The ultrastructure of the skin of control rainbow trout has been described before (Iger et al., 1994b). The epidermis of the controls was about 110–135 μm thick and composed of filament-containing epithelial cells (filament cells) with typical pavement cells, characterized by apical ridges, at the interface with the water (Fig. 1). A slightly folded basal lamina separated the epidermis from the dermis (Fig. 2a). After cortisol treatment the epidermis became slightly,
but not significantly, thicker (125–150 μm). Whereas in the controls the pavement cells were flattened and had few secretory vesicles of high electron density, after cortisol treatment these cells became more oval and developed larger rER and Golgi systems. The number of secretory vesicles increased significantly throughout the study, apart from Day 4 (Fig. 17). From 7 hr on, many of these vesicles were also found in the upper layers of filament cells of the cortisol-treated fish (Fig. 3). The membranes of these vesicles possessed peroxidase activity in both groups (Fig. 4). Apoptotic pavement cells, characterized by densification of nucleus and cytoplasm (Iger et al., 1994a,b), were very rare in the controls, but were common up to 1 day after cortisol treatment (Fig. 5). Mitotic figures, not observed in controls, were seen from Day 4, mainly in filament cells of the outer epithelial layers (Fig. 6). Throughout the experiment the basal lamina was highly folded (Fig. 2b), significantly more than in the controls (Fig. 18).

Mucous cells. In the controls newly differentiated mucous cells occurred in the two to three innermost epidermal layers. Apoptosis of mucous cells was rare. Mucous cells in the outer layer were oval in shape and contained electron-transparent mucosomes. Occasionally these displayed peroxidase activity. Several changes were observed in mucous cells after cortisol intake. Many newly differentiating and mature mucous cells were found close to the skin surface, in particular at Days 1 and 2. Apoptotic features were found, in particular after 7 hr and 1 day, in both mature and newly differentiated mucous cells (Fig. 7). The latter were mostly located one to two layers deep to the pavement cells (Fig. 8). Serous mucous cells, i.e., mucous cells with mucosomes of high electron density, appeared from Day 1 onwards (Fig. 9). From this time on, most mucous cells exhibited peroxidase activity at their mucosomal membranes (Fig. 10).

Leukocytes. In controls, lymphocytes were occasionally observed in the epidermis. After cortisol intake, apoptotic lymphocytes, very scarce in the controls, were observed from Day 1 on (Fig. 11). They were found throughout the epidermis. The numbers of leukocytes were not noticeably different from those of the controls, apart from the appearance of several macrophages, which occasionally contained phagosomes (Fig. 12).

Dermis

Pigment cells. In the dermis of controls different types of pigment cells were observed: melanocytes, xanthocytes, and iridocytes. Pigment cells were restricted to the dermis. Most melanosomes were concentrated in the cellular bodies of the melanocytes. Shortly after cortisol intake, many melanosomes were dispersed in the
cytoplasmic extensions of the melanocytes and the skin became slightly darker. The extensions penetrated into the basal layers of the epidermis (Fig. 13) already after 3.5 hr. At 7 hr, 1 day, and 2 day they occurred throughout the epidermis, with exception of the layer of pavement cells. At Days 4 and 7, most melanosomes had reaggregated in the melanocyte cell bodies and were rare in the epidermis. At this time, many extensions of melanocytes, in both dermis and epidermis, became apoptotic (Fig. 14). Such extensions were mostly engulfed by macrophages (Fig. 15). At Days 4 and 7, xanthocytes also appeared active, with well-developed rER and Golgi systems and many glycogen particles (Fig. 16), while iridocytes were found less frequently.

**Leukocytes.** In the dermis an occasional lymphocyte, granulocyte, or macrophage was found. Neither their numbers nor their morphology was influenced by cortisol.

**Cortisol Levels**

The plasma cortisol levels of the control fish remained below 10 ng·ml⁻¹ throughout the experiment (Fig. 19), except for two fish sampled at Day 4, which exhibited relatively high cortisol levels (21 and 37 ng·ml⁻¹). After 3.5 hr, 7 hr, and 1 day, the plasma cortisol levels of the cortisol-fed trout were significantly higher than those in control fish. At subsequent sample points, the cortisol values of the two groups were similar (Fig. 19).

**DISCUSSION**

Feeding cortisol to rainbow trout transiently increased plasma cortisol levels. The hormone was administered by a stress-free method, so that the structural changes observed in the skin may be considered as effects triggered by the elevated cortisol levels. It has been argued that many of the effects of sublethal stressors on trout can be attributed to (chronically) elevated blood cortisol (Pickering, 1989). Accordingly, the present results on the skin indicate that some of the cellular responses found in this tissue after exposure to different types of stressors (Iger *et al.*, 1988, 1992, 1994a,b; Iger and Wendelaar Bonga, 1994) represent responses that can be induced by elevated plasma cortisol levels. However, other factors may be implicated, because (1) in studies on trout exposed to acid water similar effects were observed without elevation of the plasma cortisol level (Balm and Pottinger, 1993; Balm *et al.*, 1994a) and (2) cortisol administration did not completely reproduce the pattern of effects observed in fish exposed to environmental challenges.

**Filament cells and pavement cells.** The increased rate of mitosis of filament cells, as well as the stimulated apoptosis and shedding of pavement cells in the present experiment, indicate that cortisol increases the turnover rate of the filament cells, including the pavement cells. Increased synthesis and apoptosis have been also re-

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**Fig. 7.** Apoptotic mucous cell (amc) with several remnants of mucosomes (asterisks); mc, mucous cell; 7 hr after cortisol intake, ×10,800.

**Fig. 8.** Newly differentiated mucous cell (dmc) close to skin surface. The cell contains several electron-transparent mucosomes (asterisk) and electron dense vesicles (arrows); open arrows, vesicles of high electron density in adjacent pavement cell; 4 days after cortisol intake, ×5200.

**Fig. 9.** Superficial mucous cell containing electron-dense (arrows) as well as electron-transparent (asterisks) mucosomes; 1 day after cortisol intake, ×10,800.

**Fig. 10.** Peroxidase activity (arrows) at mucosome membranes of mature mucous cell; diamino-benzidine reaction, otherwise unstained; 4 days after cortisol intake, ×4400.

**Fig. 11.** Epidermal lymphocyte (arrows) at early stages of apoptosis; n, nucleus showing condensation of heterochromatin; 2 days after cortisol intake, ×10,800.

**Fig. 12.** Part of epidermal macrophage (ph) with several phagosomes (arrows); 7 days after cortisol intake, ×8400.
ported for filament and pavement cells of the common carp, *Cyprinus carpio*, injected with ACTH (Iger, 1992), which probably stimulated cortisol secretion. The electron dense vesicles produced by these cells contain peroxidase activity, as was confirmed in the present study, and the content of these vesicles, after release, contributes to the formation of the glycocalyx (Iger and Wendelaar Bonga, 1994). While bound to the glycocalyx, it may be a nonspecific protector against pathogens. The stimulated turnover of the pavement cells may be related to the high metabolic activity of the pavement cells, as is reflected by the increase of their rER, Golgi areas and vesicle content, and the enlargement of the cells. Increased apoptosis, the physiologically controlled cell death (Wyllie, 1981), has been commonly reported for the epithelia of the skin and the gills of fish exposed to stressors (Wendelaar Bonga et al., 1990; Iger et al., 1994a,b).

**Mucous cells.** The cortisol supplement appeared to stimulate apoptosis of mucous cells. Together with the disappearance of mucous cells as a consequence of stimulated release, an effect detected in external epithelia of many species exposed to stressors (Zuchelkowski et al., 1985; Benedetti et al., 1989; Wendelaar Bonga et al., 1990), apoptosis of mucous cells might contribute to the initial mucocytopenia reported for fish exposed to environmental stressors (Iger et al. 1988; Urawa, 1992). Apoptosis of mucous cells has also been observed after elevation of the water temperature and during water acidification and heavy metal pollution (Iger and Wendelaar Bonga, 1994; Iger et al., 1994a,b).

Cortisol administration also initiated the synthesis of electron dense, probably serous (Whitear, 1986), mucosomes. They have been also described as the effect of stressors and ectoparasitic infection (Blackstock and Pickering, 1980) and of social interactions (Iger et al., 1992). Serous mucous cells contain a more basic, PAS-positive, content of mucus than that present in normal, electron-transparent, mucosomes (Blackstock and Pickering, 1980). The appearance of newly differentiated as well as apoptotic mucous cells in the present study suggests an increased turnover of such cells in the cortisol-treated fish. Differentiation of mucous cells can also be triggered by hormones other than cortisol, such as prolactin (Wendelaar Bonga and Meis, 1981; Iger, 1992). It is possible, however, that the effect of prolactin is mediated by cortisol, because prolactin administration to tilapia increases the ACTH responsiveness of the interrenal cortisol producing cells (Balm, 1986).

**Leukocytes.** Invasion of the epidermis by many leukocytes, as reported for Gill, intestine, or skin epithelium of fish exposed to stressors (Burkhardt-Holm et al., 1990; Iger, 1992; Wendelaar Bonga and Lock, 1992; Balm and Pottinger, 1993), was not observed in the present study. Apparently, leukocyte infiltration is not a direct effect of elevated cortisol levels. In the present study cortisol initiated apoptotic processes in the lymphocytes residing in the epidermis, which suggests that the stimulatory effect of cortisol on apoptosis of lymphocytes, well established for mammals (Schwarzmann and Cidlowski, 1993), also occurs in fish. Apoptosis of lymphocytes has been also reported in fish exposed to temperature elevation or heavy metal (Iger, 1992; Iger et al., 1994a,b). It might contribute to the increased susceptibility to disease in stressed fish (Pickering, 1989).

**Pigment cells.** The observed dispersion of melanosomes into the cytoplasmic extensions of the melanocytes and the resulting darkening of the skin in the cortisol-treated fish has been reported for Atlantic salmon and carp after administration of ACTH (Langdon et al., 1984; Iger, 1992) and in trout and carp exposed to stressors (Iger et al., 1994a,b). The penetration of melanocyte extensions in the epidermis, the subsequent apoptosis of these exten-
sions, and the disappearance of iridocytes have been previously reported for carp exposed to Cd and trout exposed to temperature elevation (Iger et al., 1994a,b). Elevated cortisol levels are not a prerequisite for these effects, because they were also observed in the study by Balm et al. (1994a).

In conclusion, the present study demonstrates that cortisol in the diet transiently elevates plasma cortisol levels alongside profound and prolonged changes in the skin: increased mitosis, secretory activity and apoptosis of the filament cells, stimulated differentiation of normal and serous mucous cells, apoptosis of mucous cells.
and lymphocytes, and pigment dispersion. These changes also occur in the skin of fish exposed to a variety of stressors, and may represent a cortisol-mediated response in these animals. This response is more prolonged than the rise in plasma cortisol levels and because it also does not rely exclusively on elevated plasma cortisol levels it may be a more appropriate index for the effects of environmental stressors. This may be important because not all stressors lead to chronically elevated cortisol levels (Fu et al., 1990; Balm et al., 1994a). Additional changes, not observed after cortisol administration (in particular necrosis of pavement cells and profound infiltration of the skin by leukocytes), do occur in fish acclimating to environmental challenges and may represent direct effects of stressors or locally controlled processes, respectively.

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